Programa de Doctorado en Recursos y Tecnologías Agrarias, Agroambientales y Alimentarias

Universidad Miguel Hernández de Elche

– 2024 –

Tesis Doctoral

Valorización de ingredientes bioactivos del azafrán y sus subproductos florales para aplicarlos en la elaboración de alimentos funcionales

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La presente Tesis Doctoral, titulada **"Valorización de ingredientes bioactivos del azafrán y sus subproductos florales para aplicarlos en la elaboración de alimentos funcionales"**, se presenta bajo la modalidad de **tesis por compendio** de las siguientes **publicaciones**:

1. **Cerdá-Bernad, D.**, Valero-Cases, E., Pastor, J.-J., & Frutos, M. J. (2022). Saffron bioactives crocin, crocetin and safranal: effect on oxidative stress and mechanisms of action. *Critical Reviews in Food Science and Nutrition*, *62*(12), 3232-3249. https://doi.org/10.1080/10408398.2020.1864279

2. **Cerdá-Bernad, D.**, Valero-Cases, E., Pérez-Llamas, F., Pastor, J. J., & Frutos, M. J. (2023). Underutilized *Crocus sativus* L. flowers: A hidden source of sustainable high value-added ingredients. *Plant Foods for Human Nutritio*n, *78*(2), 458-466. https://doi.org/10.1007/s11130-023-01065-7

3. **Cerdá-Bernad, D.,** Clemente-Villalba, J., Valero-Cases, E., Pastor, J.-J., & Frutos, M.-J. (2022). Novel insight into the volatile profile and antioxidant properties of *Crocus sativus* L. flowers. *Antioxidants*, 11(9), 1650. https://doi.org/10.3390/antiox11091650

4. **Cerdá-Bernad, D.**, Baixinho, J. P., Fernández, N., & Frutos, M. J. (2022). Evaluation of microwave-assisted extraction as a potential green technology for the isolation of bioactive compounds from saffron (*Crocus sativus* L.) floral by-products. *Foods*, *11*(15), 2335. https://doi.org/10.3390/foods11152335

5. **Cerdá-Bernad, D.**, Pitterou, I., Tzani, A., Detsi, A., & Frutos, M. J. (2023). Novel chitosan/alginate hydrogels as carriers of phenolic-enriched extracts from saffron floral by-products using natural deep eutectic solvents as green extraction media. *Current Research in Food Science*, *6*, 100469. https://doi.org/10.1016/j.crfs.2023.100469

6. **Cerdá-Bernad, D.**, Valero-Cases, E., Pastor, J. J., & Frutos, M.J. (2023). Microencapsulated saffron floral waste extracts as functional ingredients for antioxidant fortification of yogurt: Stability during the storage. *LWT*, *184*, 114976. https://doi.org/10.1016/j.lwt.2023.114976

7. **Cerdá-Bernad, D.**, & Frutos, M. J. (2023). Saffron floral by-products as novel sustainable vegan ingredients for the functional and nutritional improvement of traditional wheat and spelt breads. *Foods*, *12*(12), 2380. https://doi.org/10.3390/foods12122380

Otros artículos incluidos en la Tesis Doctoral:

Cerdá-Bernad, D., D'costa, A.S., Moreno, D.A., Bordenave, N. & Frutos, M.J. (2024). Unveiling the interaction between food thickeners and flavonoids on model beverages of saffron floral by-products. *Enviado a publicar.*

Dra. Dña. María José Frutos Fernández, Catedrática de Universidad y Directora de la Tesis Doctoral y la **Dra. Dña**. **Estefanía Valero Cases**, Profesora Ayudante Doctor y Codirectora de la Tesis Doctoral,

INFORMAN:

Que **Dña. Débora Cerdá Bernad** ha realizado bajo nuestra supervisión la Tesis Doctoral titulada **"Valorización de ingredientes bioactivos del azafrán y sus subproductos florales para aplicarlos en la elaboración de alimentos funcionales"**, conforme a los términos y condiciones definidos en su Plan de Investigación y de acuerdo al Código de Buenas Prácticas de la Universidad Miguel Hernández de Elche, cumpliendo los objetivos previstos de forma satisfactoria para su defensa pública.

Lo que firmamos para los efectos oportunos, en Orihuela a 8 de marzo de 2024

Directora	Codirectora
Dra. Dña. María José Frutos Fernández	Dra. Dña. Estefanía Valero Cases

Dra. Dña. Juana Fernández López, Catedrática de Universidad y Coordinadora del Programa de Doctorado en Recursos y Tecnologías Agrarias, Agroambientales y Alimentarias (ReTos-AAA) de la Universidad Miguel Hernández de Elche (UMH),

CERTIFICA:

Que la Tesis Doctoral titulada **"Valorización de ingredientes bioactivos del azafrán y sus subproductos florales para aplicarlos en la elaboración de alimentos funcionales"** de la que es autora la graduada en Biotecnología, y Máster Universitario en Tecnología y Calidad Agroalimentaria **Dña. Débora Cerdá Bernad**, ha sido realizada bajo la dirección de la **Dra. Dña. María José Frutos Fernández** y la codirección de la **Dra. Dña**. **Estefanía Valero Cases**, actuando como tutor de la misma el Dr. D. Joaquín Julián Pastor, considero que la Tesis es conforme, en cuanto a forma y contenido, a los requerimientos del Programa de Doctorado ReTos-AAA, siendo por tanto apta para su exposición y defensa pública.

Y para que conste a los efectos oportunos firmo el presente certificado en Orihuela a 8 de marzo de 2024

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UNIVERSITAS Miguel Hernández

Esta Tesis Doctoral se ha realizado dentro del programa de Ayudas para la Formación de Profesorado Universitario (FPU) del Ministerio de Ciencia, Innovación y Universidades (FPU18/02225). Esta Tesis Doctoral se ha desarrollado dentro del proyecto europeo PRIMA - Partnership for Research and Innovation in the Mediterranean Area ("SAFFROMFOOD", *Valorization of saffron and its floral by-products as sustainable innovative sources for the development of high added-value food products*), financiado por la Unión Europea y la Diputación de Alicante.

ÍNDICE DE CALIDAD DE LAS PUBLICACIONES

Publicación 1

Saffron bioactives crocin, crocetin and safranal: effect on oxidative stress and mechanisms of action

Autores	Débora Cerdá-Bernad, Estefanía Valero Cases, Joaquín Julián Pastor & María José Frutos
Revista	Critical Reviews in Food Science and Nutrition
DOI	10.1080/10408398.2020.1864279
Editorial	Taylor & Francis
Fecha de publicación	24 de Diciembre de 2020
Categoría JCR®	Food Science and Technology
Cuartil	Q1
Rango	4/139
Factor de impacto	7,862

Publicación 2

Underutilized *Crocus sativus* L. flowers: A hidden source of sustainable high value-added ingredients

Autores	Débora Cerdá-Bernad, Estefanía Valero Cases, Francisca Pérez Llamas, Joaquín Julián Pastor & María José Frutos
Revista	Plant Foods for Human Nutrition
DOI	10.1007/s11130-023-01065-7
Editorial	Springer
Fecha de publicación	30 de Junio de 2023
Categoría JCR®	Plant Science
Cuartil	Q1
Rango	51/239
Factor de impacto	4,000

Publicación 3

Novel insight into the volatile profile and antioxidant properties of *Crocus sativus* L. flowers

Autores	Débora Cerdá-Bernad, Jesús Clemente Villalba, Estefanía Valero Cases, Joaquín Julián Pastor & María José Frutos
Revista	Antioxidants
DOI	10.3390/antiox11091650
Editorial	MDPI
Fecha de publicación	25 de Agosto de 2022
Categoría JCR®	Food Science and Technology
Cuartil	Q1
Rango	13/142
Factor de impacto	7,000

Publicación 4

Evaluation of microwave-assisted extraction as a potential green technology for the isolation of bioactive compounds from saffron (*Crocus sativus* L.) floral by-products

Autores	Débora Cerdá-Bernad, João Paulo Baixinho, Naiara Fernández & María José Frutos
Revista	Foods
DOI	10.3390/foods11152335
Editorial	MDPI
Fecha de publicación	5 de Agosto de 2022
Categoría JCR®	Food Science and Technology
Cuartil	Q1
Rango	34/142
Factor de impacto	5,200

Publicación 5

Novel chitosan/alginate hydrogels as carriers of phenolic-enriched extracts from saffron floral by-products using natural deep eutectic solvents as green extraction media

Autores	Débora Cerdá-Bernad, Ioanna Pitterou, Andromachi Tzani, Anastasia Detsi & María José Frutos
Revista	Current Research in Food Science
DOI	10.1016/j.crfs.2023.100469
Editorial	Elsevier
Fecha de publicación	26 de Febrero de 2023
Categoría JCR®	Food Science and Technology
Cuartil	Q1
Rango	21/142
Factor de impacto	6,300

Publicación 6

Microencapsulated saffron floral waste extracts as functional ingredients for antioxidant fortification of yogurt: Stability during the storage

Autores	Débora Cerdá-Bernad, Estefanía Valero Cases, Joaquín Julián Pastor & María José Frutos
Revista	LWT – Food Science and Technology
DOI	10.1016/j.lwt.2023.114976
Editorial	Elsevier
Fecha de publicación	12 de Junio de 2023
Categoría JCR®	Food Science and Technology
Cuartil	Q1
Rango	24/142
Factor de impacto	6,000

Publicación 7

Saffron floral by-products as novel sustainable vegan ingredients for the functional and nutritional improvement of traditional wheat and spelt breads

Autores	Débora Cerdá-Bernad & María José Frutos
Revista	Foods
DOI	10.3390/foods12122380
Editorial	MDPI
Fecha de publicación	15 de Junio de 2023
Categoría JCR®	Food Science and Technology
Cuartil	Q1
Rango	34/142
Factor de impacto	5,200

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RESUMEN

El azafrán (*Crocus sativus* L.) se utiliza principalmente como condimento alimentario por sus características organolépticas relacionadas con sus propiedades colorantes, saborizantes y aromatizantes. Tradicionalmente, el azafrán también se ha utilizado en medicina para tratar diversas enfermedades por sus efectos beneficiosos sobre la salud. La crocetina, la picrocrocina y el safranal, son los principales componentes químicos responsables de estos efectos por sus propiedades antioxidantes, anticancerígenas, antiinflamatorias y antidegenerativas entre otras.

Para la producción del azafrán, sólo se utilizan los estigmas florales que, tras un proceso de secado, dan lugar a la especia. El resto de las partes florales no se utilizan y se desechan como residuos. Así, el sistema actual está generando varios cientos de toneladas de residuos florales, ya que para obtener 1 Kg de especia de azafrán se necesitan alrededor de 230.000 flores (≈350 Kg de tépalos), representando una enorme falta de rentabilidad y sostenibilidad. Sin embargo, estudios recientes han demostrado que los subproductos florales del azafrán, como los tépalos, son ricos en compuestos bioactivos.

Por tanto, el objetivo principal de la Tesis Doctoral es valorizar tanto el azafrán como sus subproductos florales mediante su uso como fuente de extractos ricos en compuestos bioactivos, además de su aplicación como ingredientes de alto valor añadido en el desarrollo de nuevos alimentos funcionales, convirtiéndose en un recurso agronómico más rentable y sostenible. Para ello, se han caracterizado a nivel físico-químico y funcional, presentando tanto los estigmas como los subproductos florales elevados contenidos en fibra, glucosa, fructosa, ácidos láctico y málico, minerales, principalmente K, Ca y Mg, y ácidos grasos poliinsaturados, siendo el ácido linoleico el más abundante. También mostraron un alto contenido en polifenoles y propiedades antioxidantes. En los estigmas de azafrán se encontraron 25 compuestos volátiles, siendo el safranal el más relevante y en los subproductos florales 55 compuestos volátiles, destacando el ácido acético y la 2(5H)-furanona.

Para minimizar el impacto medioambiental de los disolventes orgánicos comúnmente utilizados y mejorar la eficiencia de la extracción, se aplicaron tecnologías respetuosas con el medio ambiente para la extracción de compuestos bioactivos, como son la extracción asistida por microondas, y la extracción asistida por ultrasonidos combinada con disolventes eutécticos profundos naturales (NaDES). Los extractos se caracterizaron, mostrando un elevado contenido en polifenoles y flavonoides totales y una alta actividad antioxidante tanto en los estigmas como en los subproductos florales

de azafrán. Los extractos con NaDES se estabilizaron mediante su incorporación en hidrogeles de quitosano/alginato, permaneciendo estable el contenido en polifenoles totales tras la digestión oral y gastrointestinal *in vitro*.

Para su aplicación en alimentación, tanto la harina deshidratada de subproductos florales de azafrán como los extractos acuosos de los estigmas y subproductos florales de azafrán encapsulados con alginato de sodio, se utilizaron para enriquecer diferentes matrices alimentarias (yogures, pan, bebidas). Los resultados mostraron que las formulaciones de yogur enriquecidas con extractos de azafrán encapsulados permitieron una fermentación adecuada, mejorando las propiedades funcionales y antioxidantes que se mantuvieron estables durante 21 días de almacenamiento en refrigeración. Los resultados de la adición de subproductos florales de azafrán deshidratados al 10% en la formulación de panes tradicionales de trigo y espelta aumentó el contenido de fibra dietética, su contenido en minerales y mejoró significativamente el contenido fenólico y la capacidad antioxidante, que se mantuvo estable durante los procesos de digestión oral y gastrointestinal *in vitro*. Desde el punto de vista sensorial, la adición de los subproductos florales modificó las propiedades organolépticas de los panes, afectando a su cohesión al interrumpir la red del gluten. En cuanto a las bebidas modelo a base de subproductos florales deshidratados, presentaron un alto contenido en compuestos fenólicos, principalmente flavonoles derivados de kaempferol, quercetina e isorhamnetina.

En conclusión, esta Tesis Doctoral supone un avance importante aportando información novedosa para el aprovechamiento del azafrán y sus subproductos florales como fuentes naturales prometedoras para el desarrollo de ingredientes funcionales, con nuevas aplicaciones en la industria alimentaria. Tanto su valorización mediante el desarrollo de ingredientes sostenibles para ser utilizados en el desarrollo de nuevos alimentos, como la aplicación de tecnologías de extracción verdes en su producción, generarán impactos económicos positivos para la industria del azafrán y contribuirán a la minimización del impacto medioambiental, haciendo que la producción de azafrán sea más rentable y sostenible.

ABSTRACT

Saffron (*Crocus sativus* L.) is used as a spice for its organoleptic characteristics related to its coloring and flavoring properties. Traditionally, saffron has also been used in medicine to treat several diseases for its beneficial effects on health. Crocetin, picrocrocin and safranal are the main chemical components responsible for these effects due to their antioxidant, anticarcinogenic, anti-inflammatory and antidegenerative properties, among others.

For the saffron production, only the floral stigmas are used which, after a drying process, give rise to the spice. The rest of the flower is discarded as waste. Thus, the current system is generating several hundred tons of floral waste, since to obtain 1 Kg of saffron spice there are needed about 230,000 flowers (≈350 Kg of tepals), representing a huge lack of profitability and sustainability. However, recent studies have shown that saffron floral by-products, such as tepals, are rich in bioactive compounds.

Therefore, the main objective of this Doctoral Thesis is to value both saffron and its floral by-products through their use as a source of bioactive extracts, in addition to their application as high-added value ingredients to develop new functional food products, so that they become a more profitable and sustainable agronomic resource. In order to achieve this aim, saffron and its floral by-products have been characterized at the physical-chemical and functional level, presenting a high content in fiber, glucose, fructose, lactic and malic acids, minerals, mainly K, Ca and Mg, and polyunsaturated fatty acids, being the linoleic acid the major one. They also showed a high concentration in polyphenols and high antioxidant properties. In saffron stigmas, 25 volatile compounds were found, being safranal the most relevant and 55 volatile compounds in saffron floral by-products, highlighting the content of acetic acid and 2(5H)-furanone.

To minimize the environmental impact of commonly used organic solvents and to improve extraction efficiency, environmentally friendly technologies were applied for the extraction of bioactive compounds, such as microwave-assisted extraction, and ultrasound-assisted extraction combined with natural deep eutectic solvents (NaDES). The extracts were characterized, showing saffron and its floral by-products a high concentration of total phenolic and flavonoids compounds and a high antioxidant activity. The NaDES-extracts were stabilized by their incorporation into chitosan/alginate hydrogels, remaining stable the total phenolic content after the oral and gastrointestinal *in vitro* digestion.

For their application in food, both dried saffron floral by-products and the encapsulated aqueous extracts of saffron and its floral by-products with sodium alginate,

were used to enrich different food matrices (yogurts, bread, beverages). The results showed that yogurt formulations enriched with encapsulated saffron extracts allowed the proper fermentation, improving their antioxidant and functional properties that remained stable during 21 days of refrigerated storage. The results of the addition of dried saffron floral by-products at 10% on the formulation of traditional wheat and spelt breads increased their dietary fiber and mineral composition, and significantly improved their phenolic content and antioxidant capacity, that remained stable during the oral and gastrointestinal *in vitro* digestion process. From the sensory point of view, the incorporation of saffron floral by-products modified the organoleptic properties of breads, affecting their cohesiveness, interrupting the gluten network. As for the model beverages based on dried saffron floral by-products, they presented a rich content in phenolic compounds, mainly flavonols such as kaempferol, quercetin and isorhamnetin derivatives.

In conclusion, this Doctoral Thesis represents an important advance providing novel information for the use of saffron and its floral by-products as promising natural sources for the development of functional ingredients with new applications in the food industry. Both their valorization through the development of sustainable ingredients to be used in the development of new foods, and the application of green extraction technologies in their production, will generate positive economic impacts for the saffron industry and will contribute to the minimization of the environmental impact, making saffron production more profitable and sustainable.


1.INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. El azafrán

1.1.1. Historia y producción

Crocus sativus L., comúnmente conocida como azafrán, es una hierba perenne que pertenece a la familia Iridaceae con más de 85 especies distribuidas por el centro y sur de Europa, oeste de Asia y norte de África. Las zonas templadas, semiáridas y áridas son algunas de las condiciones climáticas ideales que favorecen su cultivo (Kumar et al., 2008).

C. sativus L. es una especie adaptada a las condiciones mediterráneas, por lo que se cultiva ampliamente en diferentes zonas, como Irán, India, Grecia, Afganistán, Marruecos, España, Italia, Azerbaiyán, Argelia y Turquía, entre otros (Karabagias, Koutsoumpou, Liakou, Kontakos, & Kontominas, 2017). Corresponde a uno de los cultivos más antiguos de la humanidad, alrededor de 3.000 años de historia que abarcan diferentes culturas, continentes y civilizaciones. El origen del azafrán parece provenir de Asia occidental viajando desde allí a la India, e introducido en España por los árabes entre los siglos VIII y X. En las últimas décadas, el cultivo se ha ido extendiendo a América y Oceanía, aunque Irán sigue siendo el mayor productor de azafrán, representando más del 90% de toda la producción total a nivel mundial (430 toneladas en 2019, Figura 1) (FAO, 2018; Statista, 2020).

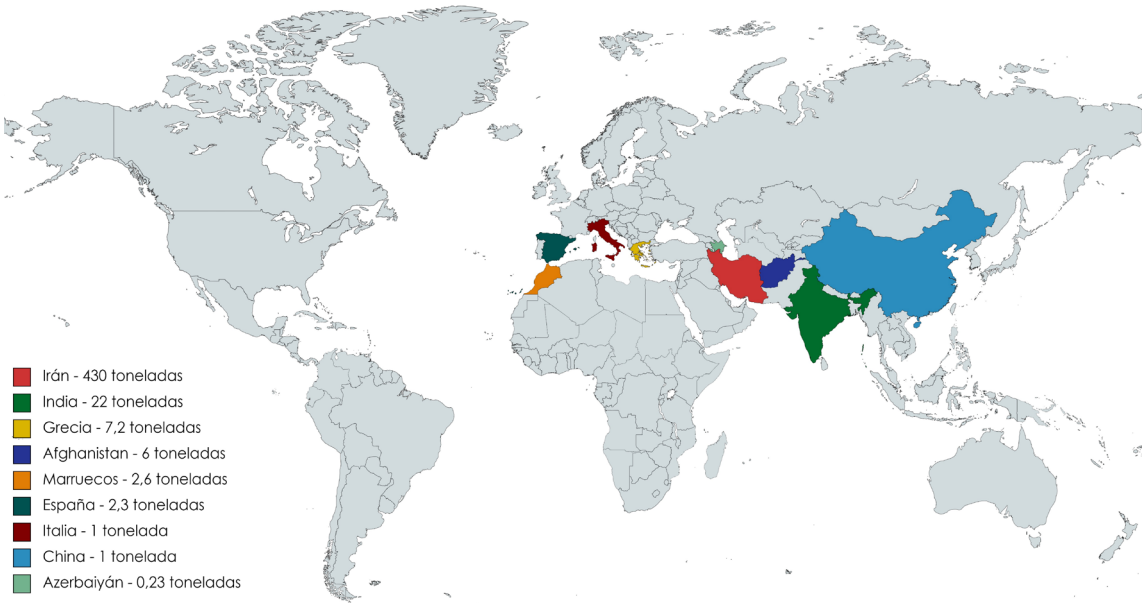

Figura 1. Producción mundial de azafrán en 2019. Elaboración propia. Fuente: Statista (2020).

El segundo país productor de azafrán es la India con una producción anual de 22 toneladas. Grecia es el tercero, seguido de Afganistán, Marruecos y España. Además, Italia, China y Azerbaiyán también se encuentran dentro de los 10 países con mayor producción de la especia del azafrán en 2019 (Statista, 2020) (Figura 1).

Respecto al cultivo de azafrán en España, han ido creciendo a lo largo de los años las superficies plantadas, donde en 2007 eran de 112 hectáreas frente a 178 en 2017. Sin embargo, este aumento de la superficie contrasta con una disminución del rendimiento, que ha sufrido un descenso a partir de 2010 con valores desde 14,13 Kg/ha hasta 8,80 Kg/ha en 2017, causado por una reducción en la producción (Figura 2) (Ministerio de Agricultura Pesca y Alimentación, 2018).

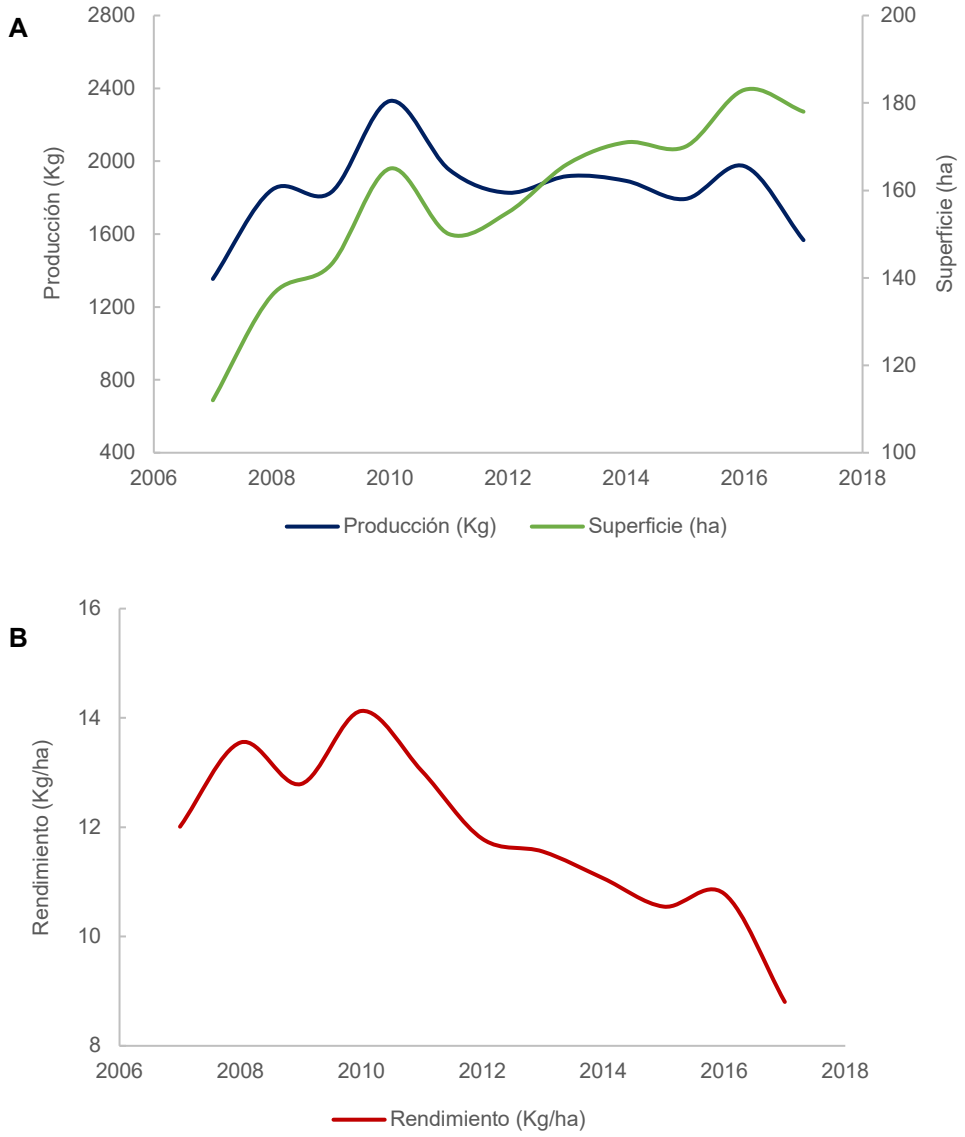

Figura 2. (A) Superficie, producción y (B) rendimiento de cultivos industriales de azafrán (estigmas tostados) en España. Elaboración propia. Fuente: Ministerio de Agricultura Pesca y Alimentación (2018).

Una de las principales causas de la gran reducción en la producción y rendimiento en los cultivos de azafrán es el incremento de las temperaturas en los últimos años. Así, el cambio climático sería uno de los principales motivos, ya que el gran incremento de las temperaturas durante el otoño puede limitar la floración, y con ello la producción de la especia.

Del total de la superficie nacional, un 80,3% se encuentra en Castilla-La Mancha, seguida de Aragón (8,4%), Cataluña (6,7%) y Canarias (2,8%). En cuanto a Navarra, Baleares y la Región de Murcia, presentan el valor más bajo de hectáreas, siendo del 0,55% cada una de ellas. Dentro de la comunidad autónoma de Castilla-La Mancha, Albacete es la provincia con mayor superficie (62,2%), seguida de Toledo y Cuenca con un 19,6% y 14%, respectivamente. En cuanto a la producción de azafrán en 2017 en España, Castilla-La Mancha lideraba las cifras con 1418 Kg, seguida de Cataluña (87 Kg), Canarias (27 Kg) y Aragón (21 Kg). La menor producción, relacionada con la menor superficie de cultivo, fueron en Baleares (6 Kg), Navarra (4 Kg) y la Región de Murcia (4 Kg) (Tabla 1) (Ministerio de Agricultura Pesca y Alimentación, 2018).

Tabla 1. Superficie, producción y rendimiento de cultivos industriales de azafrán en España por Comunidad Autónoma y Provincia. Fuente: Ministerio de Agricultura Pesca y Alimentación (2018).

	Superficie (ha)			**Rendimiento (Kg/ha)**		**Producción (Kg)**
	Secano	**Regadío**	**Total**	**Secano**	**Regadío**	
Navarra	-	1	1	-	4	4
Aragón	12	3	15	1	3	21
Cataluña	11	1	12	7	11	87
Lleida	9	1	10	8	11	83
Tarragona	2	-	2	2	-	4
Baleares	1	-	1	6	-	6
Castilla-La Mancha	65	78	143	6	13	1418
Albacete	29	60	89	6	14	1014
Ciudad real	1	5	6	4	10	54
Cuenca	20	-	20	8	16	160
Toledo	15	13	28	4	10	190
Región de Murcia	1	-	1	4	-	4
Canarias	1	4	5	3	6	27

1.1.2. Denominación de Origen Protegida

La Denominación de Origen Protegida (DOP) es uno de los regímenes de calidad en la Unión Europea. Los productos DOP son aquellos cuya calidad o características se deben al medio geográfico con sus factores naturales y humanos, y cuya producción, transformación y elaboración se realizan siempre en esa zona geográfica delimitada de la que toman el nombre (Unión Europea, 2023). Así, la existencia de figuras de calidad

alimentaria facilita la confianza del consumidor, garantizando el cumplimiento de los requisitos normativos.

En el año 1999, se creó la DOP Azafrán de La Mancha, siendo la única especia de esta naturaleza que dispone a nivel nacional de este reconocimiento de calidad. En ese mismo año, se creó La Fundación "Consejo Regulador de la Denominación de Origen Azafrán de La Mancha", que tiene como fin la representación, defensa, garantía, investigación, desarrollo y promoción del Azafrán de La Mancha DOP (DOCM, 1999).

Los requisitos que deben cumplir los azafranes DOP se recogen en el pliego de condiciones, siendo algunos de éstos los siguientes (DOCM, 1999): los azafranes deben proceder de la última cosecha obtenida; desde el punto de vista físico, la especia se presentará al consumidor únicamente en hebras, no molido; el contenido en restos florales no superará el 0,5% en peso; además, también se especifican las características organolépticas y químicas, tales como la humedad, cenizas, material volátil, poder colorante, aromático y amargo, entre otros. Por tanto, existen grandes diferencias entre el azafrán de La Mancha DOP al del resto del mundo, ya que el proceso tradicional de monda y tostado de manera natural hace que el estigma del azafrán no se mezcle con otras partes de la flor, y que el poder colorante y aromático sea de un 50% superior. Todos estos factores hacen que el azafrán de Castilla-La Mancha tenga un precio muy superior, y la creación de la DOP La Mancha para el azafrán refuerza los parámetros de calidad, implicando un lugar y unos parámetros de elaboración de la especia.

Con lo que respecta al envasado, se debe asegurar que el producto reúna todas las condiciones requeridas, controlando todas las etapas de la vida comercial del producto. En el etiquetado, para reconocer los azafranes DOP, es obligatorio incluir el símbolo de la Unión Europea, así como algún otro logotipo que haga referencia a Azafrán de La Mancha (Figura 3).

Figura 3. Logotipos representativos de la DOP Azafrán de La Mancha. Fuente: DOP Azafrán de La Mancha.

1.1.3. Recolección, tostado y envasado

C. sativus L. es una planta estéril, de poca altura, entre 10 y 25 cm, que se desarrolla y propaga a través de bulbos. Esta planta no contiene semillas y florece en otoño. La flor está compuesta de seis tépalos de color violáceo, tres estambres de color amarillo y un pistilo, que culmina con tres estigmas (filamentos) ramificados de color rojo, que tras un proceso de secado dan lugar a la especia del azafrán (Figura 4). Por tanto, sólo los estigmas de la flor son empleados para su producción (Lotfi, Kalbasi-Ashtari, Hamedi, & Ghorbani, 2015).

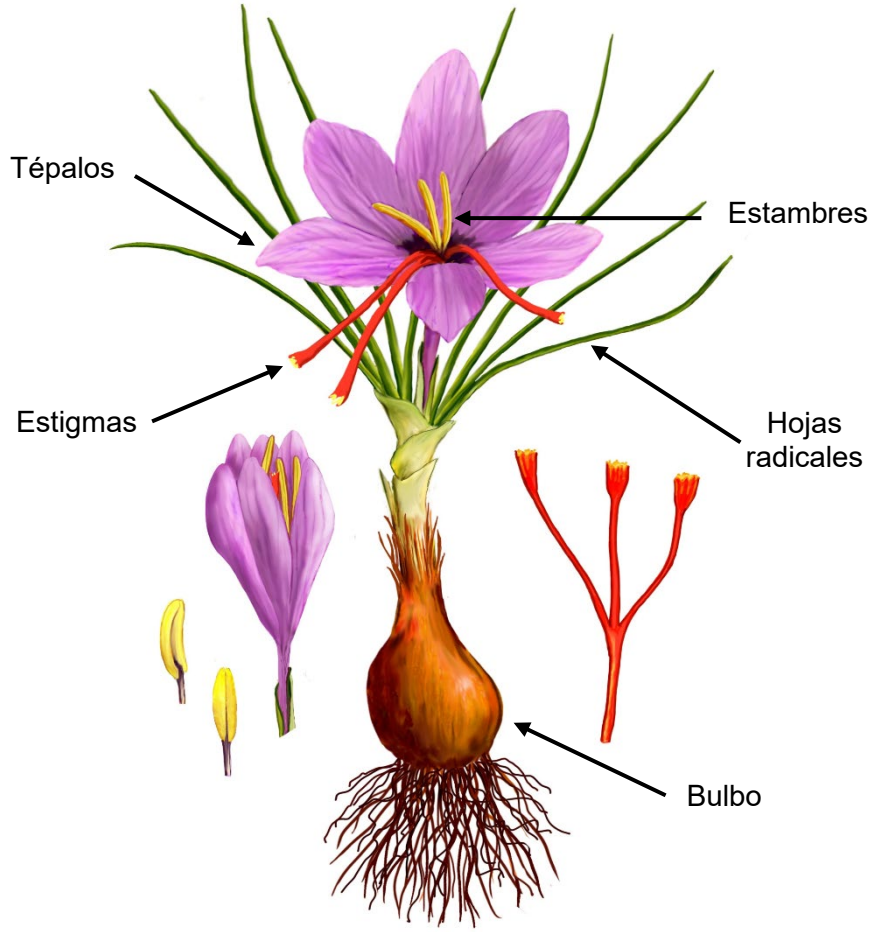

Figura 4. Partes de la planta *Crocus sativus* L. Elaboración propia. Fuente: La Boîte (2024).

La siembra de los bulbos tiene lugar entre los meses de junio y julio y la cosecha desde finales de octubre a principios de noviembre. La recolección se realiza de forma manual, y las flores se colocan en recipientes que permiten la aireación para evitar su apelmazamiento, usando tradicionalmente cestas de mimbre. Las flores, una vez recolectadas, se someten inmediatamente al proceso de desbriznado o monda, que consiste en separar manualmente los estigmas del resto de la flor, para después proceder al tostado (Figura 5) (DOCM, 1999).

Figura 5. (A) Cultivo de azafrán. (B) Recolección de las flores de azafrán. (C) Flores recolectadas en cestas tradicionales de mimbre. (D) Proceso de desbriznado o monda. (E) Tostado de los estigmas de azafrán. (F) Estigmas tostados. (G) Producto final para envasado y comercialización. (H) Subproductos florales de azafrán generados durante la producción de la especia. Elaboración propia.

El método de tostado o secado varía según las condiciones climáticas y las tradiciones de la región, pero suele realizarse en un área oscura y bien ventilada, lejos de la luz solar directa. En zonas de clima seco se suele secar de forma natural, extendiendo las hebras de azafrán sobre la superficie de secado. Este método puede tardar desde varios días hasta dos semanas, ya que depende de las condiciones ambientales. En regiones con mayor humedad o durante condiciones climáticas desfavorables, se emplean otros métodos artificiales como hornos de baja temperatura o deshidratadores diseñados específicamente para el secado del azafrán. Tras este proceso, el estigma tostado posee una intensa fragancia y constituye lo que se denomina puramente azafrán (Figura 5).

Una vez que las hebras de azafrán han alcanzado el nivel de humedad deseado, se recogen cuidadosamente y se almacenan en recipientes herméticos, protegidos de la luz y la humedad. Un almacenamiento adecuado ayuda a preservar la calidad del azafrán durante un período prolongado. El azafrán bajo la DOP Azafrán de La Mancha únicamente se comercializa en envases con contenidos netos máximos de 100 g, y siempre en un plazo máximo de un año desde la elaboración de la especia. Hasta su puesta en el mercado, el azafrán envasado se conserva protegido de la luz, de la humedad y a una temperatura no superior a 25 °C (DOCM, 1999).

Así, debido a los elevados costes de producción, el azafrán es la especia más cara del mundo.

1.1.4. Composición química y efectos beneficiosos

El azafrán (*Crocus sativus* L.) se utiliza principalmente en la industria agroalimentaria y cosmética por sus características organolépticas relacionadas con sus propiedades colorantes, saborizantes y aromatizantes, utilizándose desde hace siglos como parte de la dieta mediterránea.

La composición química del azafrán se compone principalmente de hidratos de carbono (almidón, gomas, pentosanos, azúcares reductores, pectina, dextrinas) (67%), aminoácidos y proteínas (12%), humedad (10%), grasas (6%), cenizas (5%), fibra (14%), minerales, (7%, mayoritariamente K, P, Mg, Ca, Fe, Na), e incluso vitaminas, especialmente vitamina B1 (tiamina) y vitamina B2 (riboflavina). Además, los estigmas del azafrán contienen cientos de metabolitos secundarios volátiles y no volátiles, algunos de los cuales se clasifican como compuestos bioactivos debido a sus propiedades químicas y biológicas, como los carotenoides, terpenos, antocianinas y flavonoides (Scuto et al., 2022; Serrano-Díaz, Sánchez, Martínez-Tomé, Winterhalter, & Alonso, 2013) (Tabla 2).

Tabla 2. Composición físico-química del azafrán.

Compuestos	Azafrán (Estigmas)
Constituyentes primarios	
Nutrientes principales	Hidratos de carbono, proteínas, grasa, fibra, azúcares reductores
Vitaminas	Vitamina B1 (tiamina), vitamina B2 (riboflavina)
Minerales	K, P, Mg, Ca, Fe, Na
Otros	Aminoácidos, ácidos orgánicos
Metabolitos secundarios y compuestos bioactivos	
Carotenoides	Crocina (*trans*-4-GG, *trans*-3-Gg), crocetina
Terpenoides	Safranal, picrocrocina
Otros compuestos volátiles	Aldehídos, ésteres, ácido acético
Flavonoides	Kaempferol, quercetina, epicatequina
Ácidos fenólicos	Ácido gálico, ácido vanílico, ácido rosmarínico, ácido clorogénico

Tradicionalmente, el azafrán también se ha utilizado como tinte para teñir la ropa, así como en medicina para tratar diversas enfermedades por sus efectos beneficiosos sobre la salud, siendo considerada como planta medicinal por diversas culturas. Los principales componentes químicos responsables de estas propiedades son las crocinas, la crocetina, la picrocrocina y el safranal, considerados compuestos bioactivos.

Así, la calidad y actividad funcional de esta valiosa especia se ha atribuido a estos compuestos que se pueden encontrar en niveles muy variables dependiendo de factores ambientales y agronómicos. Se ha demostrado que estos compuestos tienen

un amplio espectro de actividades biológicas, incluyendo propiedades antitumorales, anticancerígenas, antioxidantes, antiinflamatorias, y antidegenerativas, entre otras (Figura 6) (Abdullaev & Espinosa-Aguirre, 2004; Amin & Hosseinzadeh, 2012; Festuccia et al., 2014; Karimi, Oskoueian, Hendra, & Jaafar, 2010; Lee, Lee, Baek, & Kim, 2005; Sheng et al., 2008; Soeda, Aritake, Urade, Sato, & Shoyama, 2016).

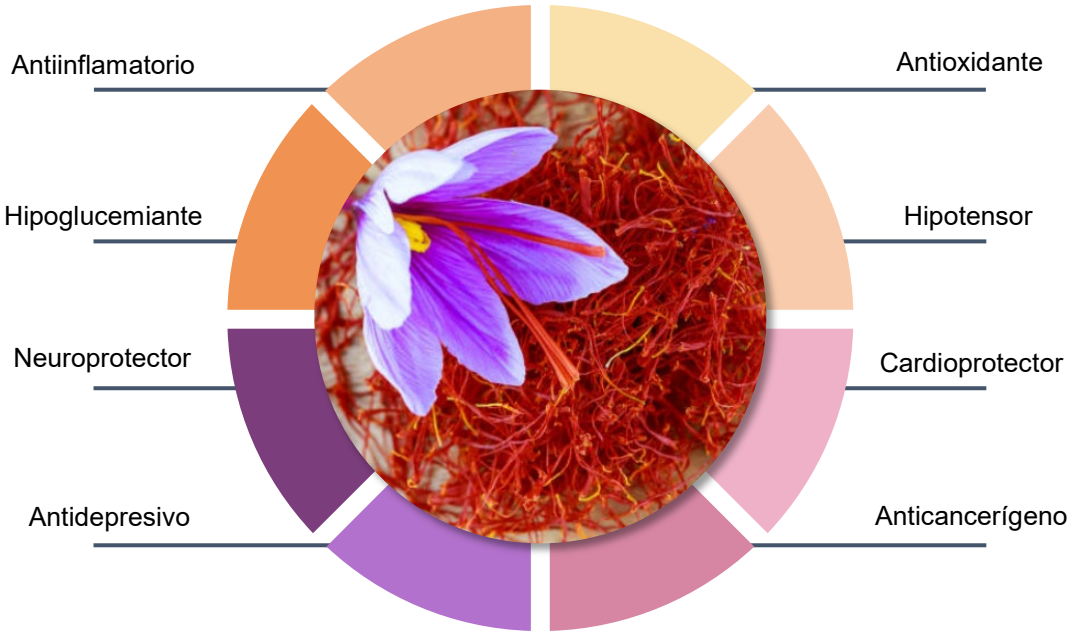

Figura 6. Propiedades beneficiosas del azafrán. Elaboración propia.

Los precursores de la crocetina y la crocina son el β-caroteno y la zeaxantina que se encuentran en los estigmas de *C. sativus* (Rubio Moraga, Ahrazem, Rambla, Granell, & Gómez Gómez, 2013). Las crocinas son los carotenoides solubles en agua responsables del color rojo-anaranjado del azafrán y están compuestos por crocetina en el que ambos grupos carboxílicos han sido esterificados con un resto glucósido. La crocetina ($C_{20}H_{24}O_4$, ácido 8,8'-diapocarotenodioico) es un ácido dicarboxílico carotenoide natural hidrofóbico, precursor de las crocinas (Figura 7) (Carmona, Zalacain, Sanchez, Novella, & Alonso, 2006).

Las crocinas son ésteres glicosilados de crocetina, formados por esterificación con diferentes glucósidos, siendo los isómeros geométricos *trans* los mayoritarios y los isómeros *cis* los minoritarios (Carmona et al., 2006). El glucósido carotenoide más abundante en el azafrán es la α-crocina o crocina-1 ($C_{44}H_{64}O_{24}$, *trans*-crocetin di-(β-d-gentiobiosyl) éster), de los cuales los más abundantes son el éster de *trans*-crocetina di-(β-D-gentiobiosil (*trans*-crocina-1 o *trans*-4-GG), y *trans*-crocetina (β-D-glucosil)-(β-D-gentiobiosil) (*trans*-3-Gg). Los niveles de crocinas pueden variar desde un 5% hasta un

40% del peso seco de los estigmas procesados (Figura 7) (Carmona et al., 2006; Moratalla-López, Bagur, Lorenzo, Salinas, & Alonso, 2019).

Los principales compuestos volátiles del azafrán son los terpenoides, principalmente alcoholes terpénicos y sus ésteres. La picrocrocina es un glucósido monoterpénico responsable del sabor amargo del azafrán (Figura 7) (Moratalla-López et al., 2019).

El safranal ($C_{10}H_{14}O$, 1,3-ciclohexadieno-1-carboxaldehído,2,6,6-trimetil-) es un aldehído monoterpénico, que representa alrededor del 60-70% de la fracción volátil del azafrán y es responsable de su aroma característico (Figura 7). El safranal, inicialmente no está presente en los estigmas frescos, sino que se forma durante su proceso de secado y almacenamiento por degradación oxidativa y/o enzimática de la picrocrocina (Carmona, Zalacain, Salinas, & Alonso, 2007).

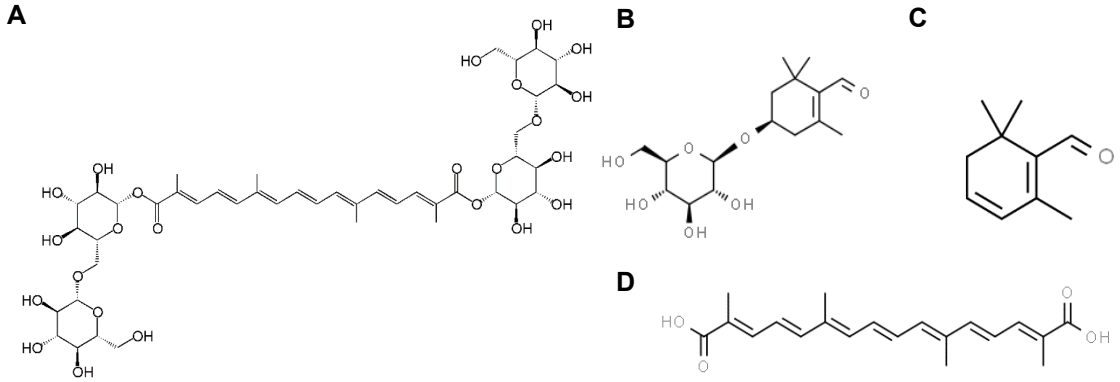

Figura 7. Estructuras químicas: (A) crocina, (B) picrocrocina, (C) safranal, (D) crocetina. Fuente: PubChem Compound Database.

Por tanto, la crocina es responsable del poder colorante, la picrocrocina de su sabor amargo y el safranal, que surge del proceso de secado de la picrocrocina, da aroma a la especia.

Otro grupo importante de compuestos bioactivos identificados y cuantificados en los estigmas del azafrán son los flavonoides, es decir, flavonoles (principalmente kaempferol y quercetina), flavanoles (epicatequina), así como pequeñas cantidades de diferentes compuestos fenólicos (ácido gálico, ácido vanílico, ácido rosmarínico, ácido clorogénico, etc.) (Tabla 2) (Belyagoubi et al., 2021).

1.2. Valorización de los subproductos florales de azafrán

Actualmente, la valorización de subproductos de la industria alimentaria es una prioridad, así como la necesidad de reducir la generación de residuos. Además, los

subproductos alimentarios contienen cantidades importantes de compuestos biológicamente activos que podrían utilizarse en diversos sectores, como en alimentación para el desarrollo de nuevos productos funcionales, suplementos, así como en el sector cosmético o farmacéutico, entre otros (Caponio, Piga, & Poiana, 2022).

Los subproductos alimentarios proceden de diferentes fuentes, entre ellas vegetales, como los subproductos florales de azafrán. El sistema de producción actual de azafrán está generando varios cientos de toneladas de desechos, ya que para la producción de ≈1 Kg de especia de azafrán se necesita un gran número de flores (≈165.000 - 230.000), generando una gran cantidad de residuos florales, constituidos en su mayoría por tépalos (≈78%, ≈350 Kg) (Lotfi et al., 2015).

Teniendo en cuenta que cada año llegan al mercado cientos de toneladas de azafrán (≈205 t/año) y los rendimientos de producción oscilan entre 0,02 y 0,03 Kg de estigmas secos por hectárea, el sistema de producción actual está generando miles de toneladas de residuos de tépalos (≈9.500 - 12.700 t/año) (de Juan, López Córcoles, Muñoz, & Picornell, 2009).

Por tanto, este proceso de producción de azafrán representa una enorme falta de rentabilidad y sostenibilidad. Sin embargo, estudios recientes han demostrado que los subproductos florales del azafrán, como los tépalos, son ricos en compuestos bioactivos como flavonoles, glucósidos flavonoides y antocianinas, que tienen efectos biológicos positivos, como sus actividades antioxidantes y antimicrobianas (Stelluti, Caser, Demasi, & Scariot, 2021; Zara et al., 2021). Además, la aplicación de técnicas analíticas más avanzadas continúa identificando nuevos metabolitos en azafrán. En un informe muy reciente que utilizó metabolómica dirigida, se identificaron y clasificaron hasta 800 moléculas en los estigmas y tépalos del azafrán (Zhou et al., 2022). En general, las cantidades de crocinas, picrocrocina, safranal y crocetina están presentes principalmente en los estigmas, pero los subproductos florales (principalmente tépalos) también constituyen una fuente de una gran variedad de otros compuestos bioactivos como flavonoides y ácidos fenólicos.

Para la valorización de los subproductos alimentarios se deben desarrollar diversas estrategias, tanto para la gestión del subproducto inmediatamente después de su producción y hasta su utilización, como para su transformación o extracción de las fracciones activas y su tratamiento. Cuando los extractos se utilizan como ingredientes en alimentación, es importante tener en cuenta las interacciones entre los extractos y la

matriz alimentaria, así como también la elección del disolvente y el proceso de extracción (Caponio et al., 2022).

1.3. Métodos de extracción de compuestos bioactivos

El proceso de extracción de compuestos bioactivos a partir de subproductos florales de azafrán se puede realizar mediante diferentes métodos de extracción convencionales (maceración, destilación, extracción Soxhlet) (Ozkan, Bayram, Karasu, Karadag, & Sagdic, 2021). Sin embargo, para minimizar el impacto medioambiental de los disolventes orgánicos comúnmente utilizados y mejorar la eficiencia de la extracción, se necesitan nuevas tecnologías respetuosas con el medio ambiente, que puedan transferirse a escala industrial.

En este campo, una alternativa prometedora a los disolventes orgánicos convencionales son los disolventes eutécticos profundos naturales (NaDES, Natural Deep Eutectic Solvents). Los NaDES, una categoría novedosa de disolventes ecológicos y sostenibles, son mezclas de dos o más compuestos naturales formadas a partir de compuestos dadores y aceptores de enlaces de hidrógeno (ácidos orgánicos, aminoácidos, azúcares, etc.), cuyas principales características son poseer un punto de fusión inferior al de los componentes puros, ya que estas mezclas pueden formar enlaces de hidrógeno intermoleculares e interacciones de Van der Waals (Choi et al., 2011). Los NaDES tienen propiedades excepcionales (Figura 8): biodegradabilidad, una muy baja volatilidad, nula o baja toxicidad, fácil, rápida y económica preparación, biocompatibles, y también pueden actuar como medios de extracción eficaces y medios protectores frente a la degradación de los compuestos bioactivos extraídos (Koutsoukos, Tsiaka, Tzani, Zoumpoulakis, & Detsi, 2019).

Las extracciones con NaDES generalmente se combinan con tecnologías de extracción verde no convencionales, como la extracción asistida por ultrasonidos (UAE, Ultrasound-Assisted Extraction), para mejorar el rendimiento de la extracción, reducir el tiempo de extracción, y evitar la degradación de los compuestos bioactivos.

Respecto a la UAE, consiste en la aplicación de energía ultrasónica que provoca la formación y colapso de burbujas de cavitación, junto con la generación de alta presión y temperatura que acelera la rotura de las paredes celulares de vegetales, promoviendo la liberación de su contenido bioactivo y mejorando la transferencia de masa (Wen et al., 2018).

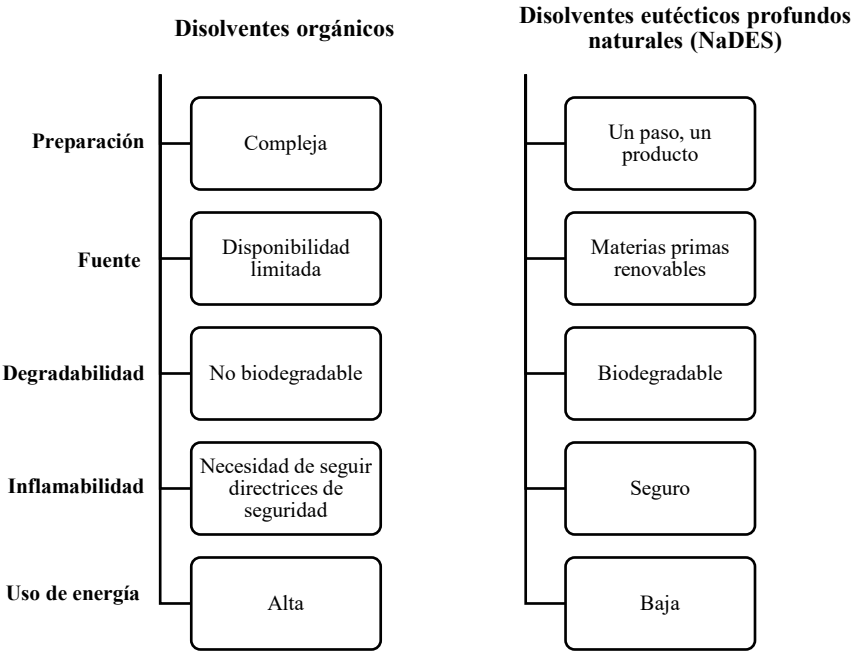

Figura 8. Comparación de disolventes orgánicos vs NaDES. Elaboración propia.

Otra alternativa de tecnología de extracción verde es la extracción asistida por microondas (MAE, Microwave-Assisted Extraction). Ésta consiste en la combinación del calentamiento por microondas con la extracción tradicional sólido-líquido. Esta técnica se basa en mecanismos de transferencia de energía como la rotación dipolar y la conducción iónica. La radiación provoca la ruptura de las células que permiten la penetración del disolvente a través de la matriz vegetal. Por otro lado, el material vegetal fluye fuera de las células hacia la solución (Ozkan et al., 2021). Algunos de los principales factores que podrían afectar el proceso MAE son el tipo de solvente, la temperatura, el tiempo de extracción y la potencia. Así, MAE se considera una tecnología verde para ahorrar costes, tiempo y energía, presentando varias ventajas en comparación con las tecnologías convencionales: aumento de la cinética de extracción, un tiempo de extracción más corto, un rápido aumento de la temperatura, una mayor eficiencia y rendimiento de extracción, así como un menor consumo y costo de energía (Álvarez, Terreros, Cocero, & Mato, 2021; Marić et al., 2018).

Estos tipos de extractos suelen degradarse con facilidad, por lo tanto, para proteger a estos extractos bioactivos de la oxidación y degradación en la matriz alimentaria y garantizar su estabilidad, las tecnologías de encapsulación pueden ser una estrategia adecuada (de Oliveira, Fraceto, Bravo, & Polanczyk, 2021). Numerosos polímeros se emplean como materiales de recubrimiento para proteger los compuestos bioactivos, como el alginato de sodio, quitosano, gomas, almidón y celulosa, entre otros (da S. Pereira, Souza, Moraes, Fontes-Sant'Ana, & Amaral, 2021). Sin embargo, uno de

los biopolímeros más utilizados para aplicaciones alimentarias es el alginato de sodio, debido a sus excelentes propiedades, como su bajo coste económico, que es biocompatible, biodegradable y Generalmente Reconocido como Seguro (GRAS) por la FDA (Cattelan et al., 2020). Además, el proceso de gelificación iónica es simple y puede procesarse fácilmente en solventes hidrófilos como el agua.

Otras alternativas para mejorar la estabilización de los ingredientes bioactivos, son su incorporación en estructuras tipo-gel. Los hidrogeles son estructuras de red tridimensionales obtenidas por polímeros que pueden absorber grandes cantidades de agua (Ćorković, Pichler, Šimunović, & Kopjar, 2021). El quitosano es un biopolímero catiónico, considerado GRAS con muy buenas propiedades como baja toxicidad, biocompatibilidad, biodegradabilidad y tiene la capacidad, en condiciones ácidas, de utilizarse en diversas formas como hidrogeles, films y emulsiones, entre otros (Detsi et al., 2020). Así, mediante la combinación de quitosano con alginato de sodio, utilizando $CaCl_2$ como reticulante iónico, se pueden desarrollar hidrogeles de quitosano/alginato, como candidatos prometedores para retener y preservar compuestos de alto valor añadido (Figura 9).

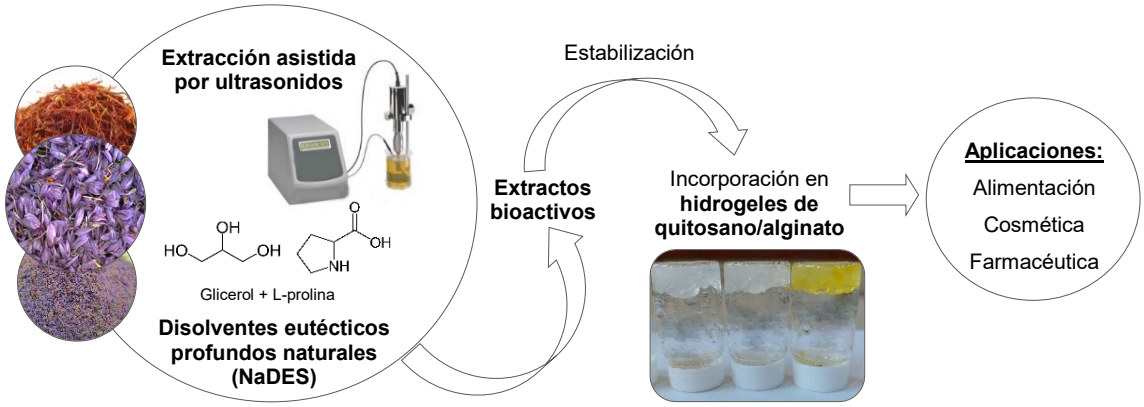

Figura 9. Ejemplo de extracción sostenible de compuestos bioactivos en azafrán y estabilización de los extractos en hidrogeles. Elaboración propia.

Actualmente, diversos estudios se centran en el desarrollo de hidrogeles como herramienta para la liberación de compuestos fenólicos de origen vegetal, ya que presentan propiedades biocompatibles, baja toxicidad y propiedades biológicas excepcionales (Micale et al., 2020).

1.4. Desarrollo e innovación de alimentos

Las tendencias actuales abren grandes oportunidades para nuevos mercados alternativos, por ello la industria alimentaria está constantemente diseñando nuevos alimentos y reformulando los alimentos tradicionales para cumplir con las demandas del

consumidor, ya que, en los últimos años, los consumidores son más conscientes de la relación entre alimentación y salud, y por lo tanto, buscan alimentos con mejor calidad nutricional y funcional, que puedan prevenir o reducir el riesgo de determinadas enfermedades (Tabanelli et al., 2018).

Sin embargo, las industrias alimentarias generan una gran cantidad de subproductos alimentarios, que podrían reutilizarse, ya que diversos estudios indican que los residuos, como los de origen vegetal, contienen moléculas de interés (antioxidantes, fibras dietéticas, proteínas, colorantes naturales, compuestos aromáticos, etc.) que podrían extraerse, purificarse, concentrarse y reutilizarse como ingredientes funcionales en alimentos, productos farmacéuticos y cosméticos (Reguengo, Salgaço, Sivieri, & Maróstica Júnior, 2022).

Así, la valorización de subproductos agroalimentarios de origen vegetal ha despertado un gran interés en la industria alimentaria, dado que estos subproductos son considerados como fuentes de ingredientes de alto valor añadido cuya función dentro de la matriz alimentaria es aportar compuestos bioactivos promotores de la salud. La selección de matrices alimentarias portadoras de estos compuestos bioactivos es un factor esencial para asegurar la estabilidad de sus propiedades funcionales en el producto final (Ravindran & Jaiswal, 2016).

Por tanto, el primer paso en el desarrollo de nuevos alimentos funcionales a base de los subproductos agroalimentarios es descubrir las propiedades funcionales de los productos tradicionales, así como encontrar matrices adecuadas para su fortificación y diseñar nuevos productos (Šeregelj et al., 2021).

El pan, un alimento básico natural y equilibrado, puede considerarse como una matriz adecuada para su enriquecimiento con ingredientes funcionales aportando compuestos bioactivos en la dieta con efectos positivos para la salud. El pan ha sido considerado el alimento por excelencia por muchas civilizaciones desde la antigüedad, siendo el resultado de hornear una mezcla de harina, agua, levadura y sal. Además, el alto consumo y disponibilidad de este producto tradicional, junto con su rápida y sencilla elaboración, lo convierten en una matriz portadora muy prometedora. También contiene una gran cantidad de nutrientes esenciales: carbohidratos, proteínas, vitaminas A, B1, B2, niacina, ácido fólico y minerales (Dewettinck et al., 2008).

Por otro lado, los yogures son una de las matrices lácteas tradicionales más adecuadas para la fortificación y desarrollo de nuevos productos funcionales, ya que se encuentran entre los alimentos más consumidos en el mundo por su versatilidad y propiedades beneficiosas (Šeregelj et al., 2021). Los yogures, fermentados por

Lactobacillus delbrueckii spp. *bulgaricus* y *Streptococcus thermophilus*, son una excelente fuente de proteínas, minerales y vitaminas, mejoran el sistema inmunológico y su consumo reduce el pH en el estómago, disminuyendo el riesgo de tránsito de patógenos. Por tanto, los productos lácteos fermentados podrían utilizarse como vehículos para aportar compuestos beneficiosos a la dieta humana, actuando como matriz portadora de ingredientes bioactivos, siendo la fortificación del yogur con subproductos agroalimentarios un buen enfoque para mejorar sus propiedades funcionales (Hashemi Gahruie, Eskandari, Mesbahi, & Hanifpour, 2015).

Además, dado que estudios recientes han demostrado que los subproductos florales de azafrán son ricos en compuestos bioactivos, la fracción fenólica soluble en agua podría aprovecharse para el desarrollo de bebidas funcionales capaces de modular la respuesta glucémica. De hecho, se ha demostrado que los compuestos fenólicos pueden inhibir la actividad de las enzimas digestivas, ralentizar la digestión del almidón y disminuir la tasa de absorción intestinal de glucosa *in vitro* (Moser et al., 2018; Moser et al., 2020; Nyambe-Silavwe et al., 2015).

1.5. Impactos económicos, sociales y ambientales esperados

Actualmente, la gestión de los subproductos agroalimentarios a través de alternativas sostenibles es uno de los principales retos de las industrias agroalimentarias que buscan continuamente soluciones innovadoras para lograr residuos cero.

Como se mencionó anteriormente, en la industria del azafrán, se generan varias toneladas de subproductos florales por cada Kg de especia, siendo esta biomasa floral una fuente de compuestos de alta calidad nutricional y funcional. Por tanto, la valorización de estos subproductos florales del azafrán conllevará a impactos económicos, sociales y ambientales positivos (Torres-León et al., 2018):

(1) beneficios sociales, ya que el uso de esta nueva fuente de alimentación puede contribuir a reducir el hambre y la desnutrición en los países en vías de desarrollo, así como contribuir a la demanda de nuevos alimentos debido a la creciente población mundial;
(2) beneficios económicos, ya que el valor añadido generado en los subproductos florales del azafrán podría crear nuevas empresas, generando oportunidades de empleo y podría suponer una nueva fuente de ingresos para los productores y la industria del azafrán;
(3) minimización del impacto medioambiental, ya que su valorización contribuye a la reducción de la acumulación de residuos, aprovechando una biomasa

que se encuentra sin explotar. Al mismo tiempo, la producción de azafrán sería más sostenible y rentable.

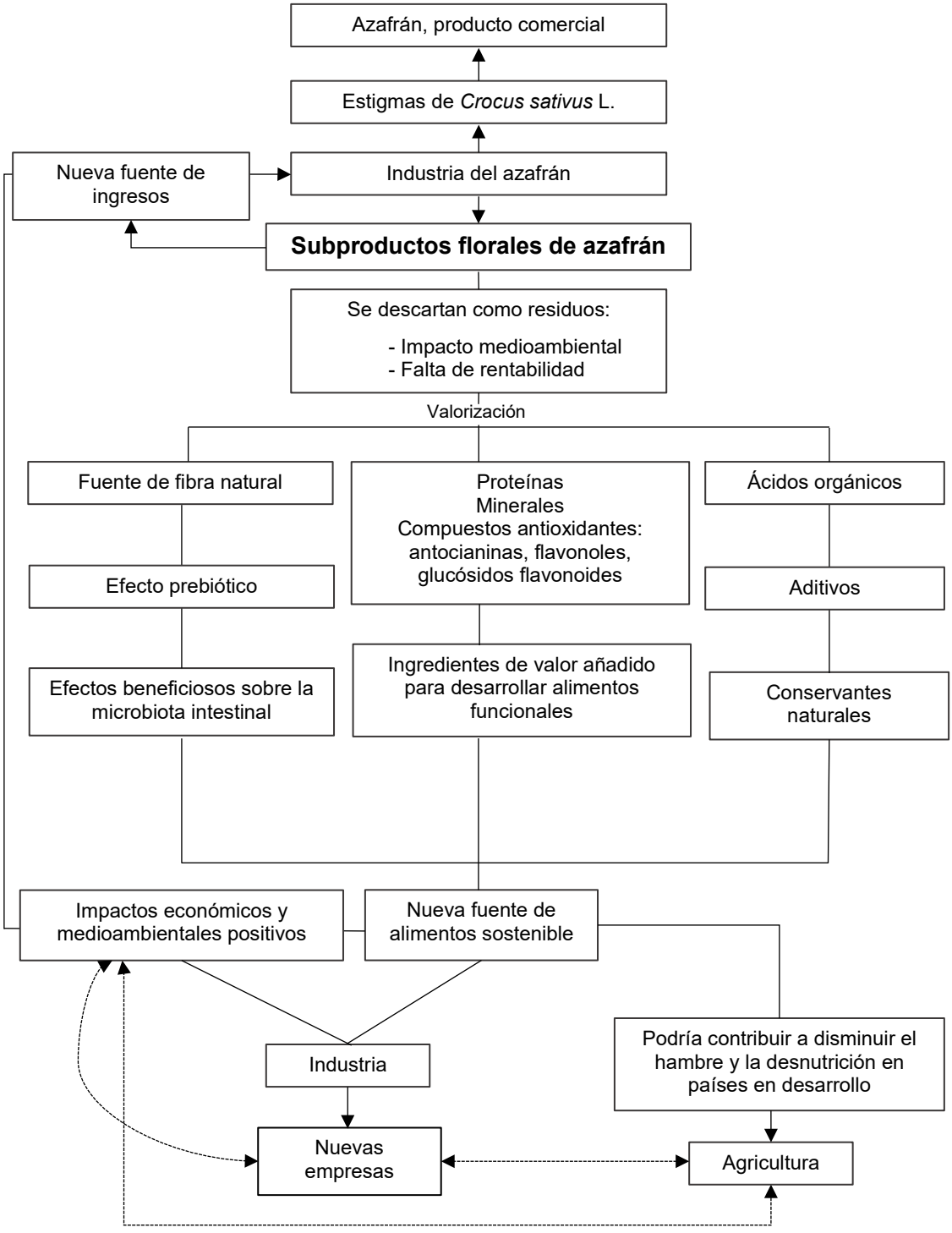

Figura 10. Impactos económicos, sociales y medioambientales esperados tras la valorización de los subproductos florales de azafrán. Elaboración propia.


2.OBJETIVOS

2. OBJETIVOS

2.1. Objetivo general

El objetivo principal de esta Tesis Doctoral es valorizar tanto el azafrán como sus subproductos florales mediante su uso como fuente de extractos bioactivos, que permitan el desarrollo de nuevos alimentos funcionales con propiedades beneficiosas para la salud del consumidor. De este modo, se contribuye a la mejora de la sostenibilidad en la producción de la especia de azafrán convirtiéndose en un recurso agronómico más rentable y sostenible, reduciendo su impacto medioambiental.

2.2. Objetivos específicos

Para alcanzar el objetivo principal, los objetivos específicos de la Tesis son:

1. Caracterización físico-química y funcional del azafrán y sus subproductos florales.
2. Optimización de la extracción de compuestos bioactivos del azafrán y de sus subproductos florales mediante tecnologías de extracción sostenibles para el desarrollo de extractos e ingredientes bioactivos.
3. Estudio de la composición y bioactividad de los extractos e ingredientes bioactivos.
4. Mejora de la estabilidad de los extractos mediante estrategias de encapsulación y estudio de la estabilidad de los ingredientes al procesado y digestión *in vitro*.
5. Desarrollo de alimentos funcionales de alta calidad mediante la incorporación de los ingredientes bioactivos derivados del azafrán y/o sus subproductos florales en la formulación de alimentos tradicionales.
6. Caracterización de las propiedades nutricionales, funcionales y sensoriales de los alimentos enriquecidos con azafrán y/o sus subproductos florales.

3.MATERIALES Y MÉTODOS

3. MATERIALES Y MÉTODOS

3.1. Material vegetal

Los subproductos florales de azafrán (SFL1, SFL2) se obtuvieron de dos productores diferentes de la provincia de Toledo (Castilla-La Mancha, España), durante las campañas de recolección de 2020 y 2021, y se cultivaron siguiendo los requisitos establecidos por la DOP Azafrán de La Mancha (DOCM, 1999). Los subproductos florales de azafrán estaban compuestos por todas las partes de la flor (tépalos, estambres y estilos), excepto los estigmas que se separaron manualmente siguiendo los procedimientos tradicionales (DOCM, 1999). Todas las flores frescas se congelaron en nitrógeno líquido y se mantuvieron a -80 °C hasta su liofilización en un liofilizador Christ Alpha 2-4 (B. Braun Biotech International, Melsungen, Alemania) durante 48 h hasta peso constante. Las condiciones del liofilizador fueron: temperatura inicial -25 ± 2 °C y presión 0,220 mbar. Tras la liofilización, se trituraron y tamizaron a través de una malla de 500 µm y se almacenaron en bolsas de polietileno a vacío a -20 °C hasta su análisis.

Los estigmas de azafrán fueron suministrados deshidratados por la empresa española Verdú Cantó Saffron Spain, S.L y procedían de cultivos españoles (Castilla-La Mancha), griegos (Kozani) e iraníes (Torbat). La humedad de todas las muestras fue inferior al 11%, y las hebras de azafrán se trituraron, tamizaron a través de una malla de 500 µm y se almacenaron en bolsas de polietileno a vacío a 4 °C hasta su posterior análisis.

3.2. Caracterización físico-química

3.2.1. Composición proximal y fibra dietética

El contenido de humedad y cenizas se determinó según la norma ISO 3632 (2011). El contenido de proteínas (método Kjeldahl utilizando un factor de conversión de 6,25), el contenido de grasa y la fibra dietética total, la fibra dietética insoluble y la fibra dietética soluble se analizaron de acuerdo con la AOAC (1995). Los carbohidratos disponibles se calcularon por diferencia (es decir, 100 menos la suma de humedad, cenizas, proteínas y grasas), y la energía (Kcal), se determinó sobre la base de una porción de 100 g utilizando valores de proteínas (×4 Kcal/g), carbohidratos (×4 Kcal/g) y grasas (×9 Kcal/g) (Serrano-Díaz et al., 2013).

3.2.2. Ácidos orgánicos y azúcares

3.2.2.1. Extracción de ácidos orgánicos y azúcares

Las extracciones se prepararon utilizando agua ultrapura y una relación muestra/agua de 1:20 (p/v) para los subproductos florales liofilizados y una relación de 1:50 (p/v) para los estigmas de azafrán. Los extractos se agitaron durante 1 h en oscuridad a 400 rpm en un agitador magnético a temperatura ambiente (Ovan, mod. MultiMix Heat D-MMH30E, Barcelona, España). Tras ello, se sonicaron durante 15 min y se centrifugaron a 11200 x g durante 10 min a 4 °C. Finalmente, los sobrenadantes se filtraron (filtro de PTFE de 0,45 µm) y se almacenaron a -20 °C.

3.2.2.2. Análisis de ácidos orgánicos, azúcares e inulina

La identificación y cuantificación de azúcares, ácidos orgánicos e inulina se realizó mediante cromatografía líquida de alta resolución utilizando un equipo HPLC Hewlett-Packard serie 1100 (Woldbronn, Alemania) equipado con una columna Supelcogel C-610H (30 cm x 7,8 mm) y una precolumna Supelcoguard C-610H (5 cm x 4,6 mm) (Supelco, Sigma Aldrich, St. Louis, MO, EE. UU.). Los ácidos orgánicos se analizaron a 210 nm en UV-Vis con un detector de matriz de diodos (DAD G1315A). Para los azúcares e inulina, se utilizó un detector de índice de refracción (G1362A RID). Como fase móvil se utilizó ácido ortofosfórico al 0,1% (v/v) con un volumen de inyección de 20 µL y un flujo de 0,5 mL/min en condiciones isocráticas según el método descrito por Cerdá-Bernad, Valero-Cases, Pastor, Frutos and Perez-Llamas (2021). Las concentraciones se calcularon mediante curvas de calibración con los estándares de azúcares, inulina y ácidos orgánicos (Sigma Aldrich, St. Louis, MO, EE. UU.). Los resultados se expresaron como mg/g de peso seco de muestra.

3.2.3. Minerales

La composición de minerales se determinó según Serrano-Díaz et al. (2013), con alguna modificación. Primeramente, se llevó a cabo la digestión de los subproductos florales liofilizados (0,5 g en 10 mL de HNO_3 al 65% (v/v)) y de los estigmas de azafrán (0,1 g en 5 mL de HNO_3 al 65% (v/v)) utilizando el sistema de digestión acelerada por microondas (CEM Mars one, Matthews, NC, EE. UU.). El proceso de digestión fue de 30 min mediante una rampa de temperatura, siendo la temperatura final de 200 °C. Todas las muestras se filtraron (papel de filtro cualitativo Whatman de 90 mm), se diluyeron con agua desionizada ultrapura 1:50 (v/v) y se almacenaron a 4 °C. Las concentraciones totales de macronutrientes (Ca, Mg, Na y K) y micronutrientes (Zn, Cu, Mn y Fe) en las muestras previamente mineralizadas se cuantificaron con un

espectrómetro de masas de plasma acoplado inductivamente (ICPMS-2030, Shimadzu, Kyoto, Japón). Los estándares internos incluyeron calcio (^{44}Ca), magnesio (^{26}Mg), sodio (^{23}Na), potasio (^{39}K), zinc (^{66}Zn), cobre (^{65}Cu), manganeso (^{55}Mn) y hierro (^{56}Fe).

3.2.4. Perfil de ácidos grasos

Los ácidos grasos se extrajeron utilizando 0,5 g de subproductos florales de azafrán liofilizados y 0,5 g de estigmas de azafrán, homogeneizándolos en un vórtex con 20 mL de cloroformo/metanol (2:1 v/v). Los lípidos totales se extrajeron según Folch, Lees and Sloane Stanley (1957) y las impurezas no lipídicas se eliminaron lavando con KCl al 0,88% (p/v). Los ésteres metílicos de ácidos grasos (Fatty Acid Methyl Esters, FAME) se prepararon mediante transesterificación catalizada por ácidos según el método descrito por Christie (2003). Los FAME se separaron y cuantificaron mediante cromatografía gas-líquido utilizando una columna capilar de sílice fundida flexible SP™ 2560 (100 m de largo, diámetro interno de 0,25 mm y espesor de 0,20 mm) (Supelco 2560 SPTM, Bellefone, PA, EE. UU.) en el cromatógrafo de gases Hewlett Packard 5890 (Bellefone, PA, EE. UU.).

3.3. Propiedades tecnofuncionales de los subproductos florales de azafrán

3.3.1. Capacidad de retención de agua y solubilidad en agua

La capacidad de retención de agua (CRA) se determinó mezclando 0,25 g de subproductos florales de azafrán liofilizados en 10 mL de agua destilada durante 1 min en vórtex y manteniendo las muestras a hidratar, a temperatura ambiente durante 30 min, antes de la centrifugación a 2000 x g durante 30 min. Se decantó el exceso de sobrenadante y se expresó la CRA como g de agua/g de muestra seca.

La solubilidad se midió siguiendo la metodología de Garau, Simal, Rosselló and Femenia (2007), como % de pérdida en el peso inicial de la muestra liofilizada, utilizada previamente para determinar la CRA, después de la recuperación del material insoluble (precipitado) tras la centrifugación.

3.3.2. Capacidad de retención de aceite

La capacidad de retención de aceite (CRO) se realizó según Mallek-Ayadi, Bahloul and Kechaou (2017), mezclando 0,25 g de subproductos florales de azafrán liofilizados en 10 mL de aceite de girasol durante 1 min en vórtex, antes de la centrifugación a 2000 x g durante 30 min. Se decantó el exceso de sobrenadante y se expresó la CRO como g de aceite/g de muestra seca.

3.3.3. Capacidad de hinchamiento

Para estudiar la capacidad de hinchamiento, se añadieron 0,1 g de subproductos florales de azafrán liofilizados a 10 mL de agua destilada y se calentaron al baño maría a 60 °C durante 30 min con agitación, siguiendo la metodología de Kusumayanti, Handayani and Santosa (2015). A continuación, las muestras se centrifugaron a 1000 x g durante 15 min y se pesó la fracción de precipitado, calculando la capacidad de hinchamiento mediante la Ecuación 1:

$$Capacidad\ de\ hinchamiento\ (g/g) = \frac{\text{peso de precipitado}}{\text{peso inicial de la muestra}} \quad (1)$$

3.4. Caracterización funcional

3.4.1. Extracción convencional de compuestos bioactivos

3.4.1.1. Compuestos fenólicos

Las extracciones se prepararon utilizando como solvente metanol y una relación muestra/solvente de 1:20 (p/v) para los subproductos florales y una relación de 1:50 (p/v) para los estigmas de azafrán. Las soluciones se agitaron durante 1 h en la oscuridad a 400 rpm con un agitador magnético (Ovan, mod. MultiMix Heat D-MMH30E), se sonicaron durante 15 min y se centrifugaron a 11200 x g durante 10 min a 4 °C. Tras la centrifugación, los sobrenadantes se filtraron (filtro de PTFE de 0,45 µm) y se almacenaron a -20 °C hasta su análisis.

3.4.1.2. Betacaroteno

Las extracciones se prepararon utilizando una solución de n-hexano:acetona:etanol (50:25:25, v/v/v) y una relación muestra/solvente de 1:20 (p/v) para los subproductos florales y una relación de 1 :50 (p/v) para los estigmas de azafrán. Los extractos se agitaron durante 10 min a 400 rpm con un agitador magnético (Ovan, mod. MultiMix Heat D-MMH30E), manteniéndolos en hielo, y se centrifugaron a 11200 x g durante 20 min a 4 °C. Finalmente, los sobrenadantes se filtraron (0,45 µm) y se almacenaron a -20 °C hasta su análisis.

3.4.1.3. Antocianinas

Las extracciones se prepararon utilizando una solución de etanol al 50% (HCl al 0,1%) y una relación muestra/disolvente de 1:20 (p/v) para las flores liofilizadas. Los

extractos se sonicaron durante 30 min y se centrifugaron a 11200 x g durante 15 min. Los sobrenadantes se filtraron (0,45 µm).

3.4.2. Propiedades antioxidantes in vitro

Para el estudio de las propiedades antioxidantes se utilizaron los extractos obtenidos de la extracción de compuestos fenólicos (apartado 3.4.1.1.).

3.4.2.1. Método DPPH (2,2-difenil-1-picrilhidrazilo)

La actividad antioxidante mediante el método DPPH se determinó siguiendo la metodología de Brand-Williams, Cuvelier and Berset (1995), con algunas modificaciones. El radical DPPH se preparó disolviendo 0,0035 g en 10 mL de metanol. La mezcla se agitó y se mantuvo en oscuridad durante 30 min. Para los ensayos, se utilizaron 100 µL de los diferentes extractos y se agregaron 900 µL de solución de DPPH. La disminución de la absorbancia se midió a 515 nm (espectrofotómetro UV-Vis T80; PG Instruments Limited, Reino Unido). Se utilizó Trolox (10 mM) como estándar de referencia en diferentes concentraciones (0,50 - 4 mmol/L). Los resultados se expresaron como mmol de equivalentes de Trolox (TE, Trolox Equivalents) por 100 g de peso seco de muestra.

3.4.2.2. Método ABTS (ácido 2,2'azino-bis (3-etilbenzotiazolina-6-sulfónico))

El método de radicales catiónicos ABTS para medir la capacidad antioxidante se realizó siguiendo la metodología de Re et al. (1999), con algunas modificaciones. El radical ABTS se preparó mezclando ABTS (7 mM) con $K_2S_2O_8$ (2,45 mM), y dejando reaccionar durante 16 h en oscuridad a temperatura ambiente. Posteriormente, la solución se diluyó con agua ultrapura hasta ajustar su absorbancia a 0,70 ± 0,02 a 734 nm. Para los ensayos se utilizaron 100 µL de los diferentes extractos y se agregaron 900 µL de solución de ABTS, dejando reaccionar en oscuridad 6 min y midiendo la absorbancia a 734 nm. Se utilizó Trolox (10 mM) como estándar de referencia en diferentes concentraciones (0,20 - 3 mmol/L). Los resultados se expresaron como mmol de TE por 100 g de peso seco de muestra.

3.4.2.3. Método FRAP (Ferric Reducing Antioxidant Power)

Se utilizó el método FRAP modificado de Benzie and Strain (1996). Brevemente, el reactivo FRAP se preparó mezclando 300 mmol/L de tampón acetato (pH 3,6), 10 mmol/L de solución de TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) en 40 mmol/L de HCl y 20

mmol/L de solución de $FeCl_3 \cdot 6H_2O$ en una proporción de volumen de 10:1:1, respectivamente. La absorbancia se midió a 593 nm. Se utilizó Trolox (10 mM) como solución estándar en el rango de 0,01 a 5 mmol/L. Los resultados se expresaron como mmol de TE por 100 g de peso seco de muestra.

3.4.3. Contenido en compuestos bioactivos

3.4.3.1. Contenido en polifenoles totales

El contenido en polifenoles totales se determinó siguiendo la metodología de Folin-Ciocalteu descrita por Singleton, Orthofer and Lamuela-Raventós (1999). En primer lugar, el reactivo de Folin-Ciocalteu se mezcló con agua ultrapura 1:10 (v/v). Se utilizó ácido gálico (1 mM) como estándar de referencia en el rango de 0 a 4,72 mg/L. Para los ensayos, se mezclaron 100 µL de los diferentes extractos con 400 µL de tampón fosfato (50 mM) a pH 7,8 y se agregaron 2,5 mL de reactivo de Folin-Ciocalteu. Después de 2 min, se agregaron 2 mL de Na_2CO_3 (75 g/L) y se mantuvieron a 50 °C durante 10 min. La absorbancia se midió a 760 nm y los resultados se expresaron como mg de equivalentes de ácido gálico (GAE, Gallic Acid Equivalents) por g de peso seco de muestra.

3.4.3.2. Contenido en flavonoides totales

El contenido en flavonoides totales se determinó según Çam and Hışıl (2010). Para ello, 1 mL de muestra se mezcló con 4 mL de agua y 300 µL de solución de nitrito de sodio (5% p/v). Tras 5 min, se añadieron 300 µL de solución de tricloruro de aluminio (10% p/v). Después de 6 min, se añadieron 2 mL de hidróxido de sodio (1 M) y el volumen se ajustó a 10 mL con agua. Se usó catequina (1000 mg/L) para la cuantificación (20 - 100 mg/L) y se midió la absorbancia a 510 nm. Los resultados se expresaron como mg de equivalentes de catequina (CE, Catechin Equivalents) por g de peso seco de muestra.

3.4.3.3. Contenido en antocianinas totales

El contenido en antocianinas totales se determinó mediante el método de Figueira et al. (2014), con algunas modificaciones. Se utilizó delfinidina como patrón y el contenido total de antocianinas se calculó mediante una curva de calibración (10 - 150 mg/L). La absorbancia se midió a 520 nm y los resultados se expresaron en mg de equivalentes de delfinidina (DE, Delphinidin Equivalents) por g de peso seco de muestra.

3.4.3.4. Contenido en betacaroteno

El contenido en betacaroteno se determinó mediante la ley de Beer-Lambert. La concentración se calculó utilizando el coeficiente de extinción del betacaroteno en hexano (2,505 $M^{-1}cm^{-1}$). El cambio de absorbancia se midió usando un espectrofotómetro UV-Vis a 450 nm. Los resultados se expresaron como mg de betacaroteno por 100 g de peso seco de muestra (% p/p).

3.4.4. Actividad quelante del hierro (II)

Para la actividad quelante de metales, las extracciones se llevaron a cabo siguiendo el mismo procedimiento explicado anteriormente para la extracción de compuestos fenólicos (apartado 3.4.1.1.), pero utilizando agua ultrapura como solvente de extracción.

La quelación de iones de hierro (II) se realizó según Carter (1971). Para ello, se utilizó una concentración final de 0,02 g/mL de extracto y se añadieron 100 µL de $FeCl_2$ acuoso (2 mM) y 900 µL de metanol. Tras 5 min, se inició la reacción añadiendo 400 µL de ferrozina (5 mM). Tras dejar reaccionar 10 min, se midió la absorbancia a 562 nm. Las actividades de quelación del hierro se calcularon a partir de la absorbancia del control (A_c) y de la muestra (A_m) usando la Ecuación 2 y se expresaron como % de inhibición:

$$\%\ Inhibición = \frac{A_c - A_m}{A_c} \cdot 100 \quad (2)$$

3.4.5. Determinación de crocinas mediante HPLC-PDA

3.4.5.1. Preparación del extracto

Los extractos acuosos tanto de los subproductos florales como de los estigmas de azafrán se prepararon según la norma ISO 3632 (2011). Brevemente, se mezclaron 0,5 g de muestra con 900 mL de agua ultrapura en un matraz volumétrico de 1 L. Las soluciones se agitaron durante 1 h en oscuridad a 1000 rpm en un agitador magnético. Se ajustó el volumen hasta 1 L y se homogeneizó mediante agitación. A continuación, se transfirieron 20 mL de la solución a un matraz aforado de 200 mL con agua ultrapura. Las extracciones se filtraron (filtro de PTFE de 0,45 µm) y se transfirieron a un vial para su posterior análisis.

3.4.5.2. HPLC-PDA

La identificación y cuantificación de crocinas se llevó a cabo mediante cromatografía líquida de alta resolución utilizando el equipo HPLC AltusTM 10 PerkinElmer (Waltham, MA, EE. UU.) equipado con una columna C18 KromaPhase (150 × 4,6 mm de diámetro interior, 3,5 µm) (Scharlab, Barcelona, España) que se equilibró a 30 °C. Los análisis se llevaron a cabo siguiendo el método descrito por Valle García-Rodríguez et al. (2014), con algunas modificaciones. Los eluyentes utilizados fueron acetonitrilo (A) y agua (B), con una proporción de 20% A y 80% B. El flujo fue de 0,8 mL/min y el volumen de inyección de los extractos fue de 25 µL. Los ésteres de crocetina se midieron a 440 nm en UV-Vis (espectrofotómetro de doble haz UV-6300PC) con el detector de fotodiodos en serie (PDA, Photodiode Array Detector) (PerkinElmer, Waltham, MA, EE. UU.).

Las identificaciones de ésteres de crocetina, como el éster de *trans*-crocetina di-(β-D-gentiobiosil (*trans*-crocina-1 o *trans*-4-GG), y *trans*-crocetina (β-D-glucosil)-(β-D-gentiobiosil) (*trans*-3-Gg), se determinaron mediante el espectro UV-Vis y el tiempo de retención mediante el método HPLC-PDA a 440 nm. Su cuantificación se realizó mediante curvas de calibración con los estándares *trans*-4-GG (0,8 - 50 mg/L), y *trans*-3-Gg (0,8 - 25 mg/L).

3.4.6. Perfil volátil

La composición volátil se determinó mediante microextracción en fase sólida en espacio de cabeza (HS-SPME, Head Space-Solid Phase Microextraction). Se pesaron entre 20 y 40 mg de estigmas de azafrán, y entre 120 y 140 mg de subproductos florales de azafrán frescos, deshidratados por liofilización y estufa por aire. Además de las muestras frescas y liofilizadas, se estudiaron los subproductos florales de azafrán deshidratados en estufa, los cuales se secaron durante 24 h a 60 °C en una estufa de aire.

Las muestras se añadieron a un vial de 40 mL con tapas de polipropileno y septos de PTFE/silicona, con acetato de isoamilo (1000 mg/L, patrón interno para semicuantificación de compuestos). El vial se colocó en un muestreador automático AOC-6000 Plus (Shimadzu Corporation, Kyoto, Japón), y tras 5 min de tiempo de equilibrio, se expuso a la muestra una fibra DVB/CAR/PDMS de 50/30 µm (1 cm) en espacio de cabeza durante 45 min a 40 °C (con agitación, 250 rpm).

La separación e identificación de compuestos se realizó mediante GC2030 (Shimadzu Scientific Instruments, Inc., Columbia, MD, EE. UU.) en una columna Sapiens X5MS (Teknokroma, Barcelona, España), 30 m × 0,25 mm d.i., 0,25 µm ft. y acoplado

con un detector de espectrómetro de masas (espectrómetro de masas de triple cuadrupolo TQ8040 NX; Shimadzu Scientific Instruments, Inc., Columbia, MD, EE. UU.). Solo se utilizó el modo de adquisición de cuadrupolo único en el TQ8040 NX (escaneo Q3; velocidad de escaneo 5000 uma/s; rango de masa 40–400 m/z; tiempo 0,100 s). El programa de temperatura del horno fue el siguiente: (i) temperatura inicial de 35 °C y mantenimiento de 5 min; (ii), incremento de 5 °C/min hasta 150 °C/min y mantenimiento durante 1 min; (iii) incremento de 10 °C/min hasta 280 °C y mantenimiento durante 5 min. La presión en la cabeza de la columna de helio fue de 47,6 kPa (modo de velocidad lineal constante de 36 cm/s). El inyector, la fuente de iones y la interfaz estaban a 250, 230 y 280 °C, respectivamente. Se utilizó helio como gas portador, flujo de columna de 1 mL/min, con relación de división 1:50 y flujo de purga de 6 mL/min.

Se utilizaron índices de retención de una mezcla estándar de alcanos comercial para identificar los compuestos, así como las 17 bibliotecas de índices de retención y espectro de masas del Instituto Nacional de Estándares y Tecnología (NIST). La identificación se consideró tentativa cuando se basó únicamente en datos espectrales de masas, y solo los compuestos con una similitud espectral > 90% se consideraron aciertos correctos. El filtro de similitud de retención lineal se configuró en ± 10 unidades. Este método de extracción de compuestos volátiles ha sido utilizado previamente para el análisis de diferentes matrices alimentarias (Clemente-Villalba et al., 2020).

3.5. Extracción de compuestos bioactivos mediante tecnologías sostenibles

3.5.1. Extracción asistida por microondas

Como material vegetal se han utilizado los subproductos florales de azafrán liofilizados (SF1) como se ha descrito en el apartado 3.1.

3.5.1.1. Diseño experimental

Se utilizó la Metodología de Superficie de Respuesta (RSM, Response Surface Methodology), una colección de técnicas estadísticas y matemáticas para mejorar y optimizar procesos, como el proceso de extracción, y predecir las respuestas que dependen de variables experimentales. Utilizando el software MODDE® versión 12.1 (Sartorius Stedim Biotech, Suecia), se aplicó un diseño Ortogonal Compuesto Central (CCO, Central Composite Orthogonal) con tres variables independientes para evaluar el efecto del tiempo (X_1), la temperatura (X_2) y la concentración de etanol (X_3) en el

rendimiento de extracción, contenido de compuestos bioactivos y actividad antioxidante. Los niveles de las variables codificadas se resumen en la Tabla 3.

Tabla 3. Matriz CCO del diseño experimental.

	Variables independientes: condiciones MAE		
Experimentos	**Tiempo (min) (X_1)**	**Temperatura (°C) (X_2)**	**Concentración etanol (% v/v) (X_3)**
1	0,5 (-1)	25 (-1)	0 (-1)
2	5 (+1)	25 (-1)	0 (-1)
3	0,5 (-1)	100 (+1)	0 (-1)
4	5 (+1)	100 (+1)	0 (-1)
5	0,5 (-1)	25 (-1)	100 (+1)
6	5 (+1)	25 (-1)	100 (+1)
7	0,5 (-1)	100 (+1)	100 (+1)
8	5 (+1)	100 (+1)	100 (+1)
9	6,2 (+1,35)	62,5 (0)	50 (0)
10	3,15 (0)	11, 75 (-1,35)	50 (0)
11	3,15 (0)	113, 24 (+1,35)	50 (0)
12	3,15 (0)	62,5 (0)	50 (0)
13	3,15 (0)	62,5 (0)	50 (0)
14	3,15 (0)	62,5 (0)	50 (0)

3.5.1.2. Procedimiento de extracción

La extracción MAE se realizó utilizando el sistema Discover SP-CEM MW (CEM Co., Matthews, NC, EE. UU.), funcionando a 500 PSI con una potencia de salida máxima de 300 W. Los parámetros variables incluyeron el tiempo de radiación (0,5 a 5 min), la temperatura (25 a 100 °C) y la concentración de etanol (0 a 100% v/v). Se fijó la relación masa:solvente (1:10, p/v) y el ensayo se realizó con 2 g de muestra disueltos en 20 mL de solvente. Se realizaron catorce extracciones, siguiendo las condiciones indicadas en la Tabla 3. Una vez completada la extracción, los extractos se secaron mediante un concentrador CentriVap® (Labconco, Kansas City, MO, EE. UU.) a 37 °C durante 24 h. El rendimiento de extracción se determinó utilizando la Ecuación 3:

$$\%\ Rendimiento = \frac{peso\ de\ extracto\ seco}{peso\ inicial\ de\ extracto} \cdot 100 \quad (3)$$

3.5.1.3. Caracterización de los extractos

Para los experimentos de caracterización, los extractos secos se reconstituyeron en etanol al 50% (p/v). Para determinar el contenido de polifenoles totales y flavonoides totales se siguieron los métodos descritos en los apartados 3.4.3.1. y 3.4.3.2., respectivamente.

3.5.1.3.1. Ensayo capacidad de absorción de radicales de oxígeno (ORAC, Oxygen Radical Absorbance Capacity)

El ensayo ORAC se llevó a cabo siguiendo el método descrito por Serra, Duarte, Bronze and Duarte (2011). Este ensayo midió la capacidad de inhibir la oxidación de fluoresceína ($3 \cdot 10^{-4}$ mM) catalizada por radicales peroxilo generados a partir de AAPH (2,2-azobis(2-methylpropionamidine) dihydrochloride) utilizando un lector fluorescente de microplacas (FL800 Bio-Tek Instruments, Winooski, VT, EE.UU.). Se utilizó Trolox (1 mM) como estándar de referencia (5 - 30 µM) y los resultados se expresaron como micromoles de TE por g de peso seco de muestra.

3.5.1.3.2. Ensayo de capacidad de eliminación de radicales hidroxilo (HOSC, Hydroxyl radical scavenging capacity assay)

El ensayo se realizó utilizando el método descrito por Moore, Yin and Yu (2006). Brevemente, en un lector fluorescente de microplacas, se utilizó fluoresceína ($9{,}96 \cdot 10^{-8}$ M) como sonda y la reacción de cloruro férrico (3,42 mM) y peróxido de hidrógeno (0,20 M) como fuente de radicales hidroxilo. Se utilizó Trolox (1 mM) como estándar de referencia (5 - 30 µM) y los resultados se expresaron como micromoles de TE por g de peso seco de muestra.

3.5.2. Extracción asistida por ultrasonidos con NaDES

Como material vegetal se han utilizado los subproductos florales de azafrán liofilizados (SF1), así como los estigmas de azafrán de origen griego, como se ha descrito en el apartado 3.1.

La síntesis de los disolventes eutécticos profundos naturales se realizó siguiendo la metodología descrita por Tzani et al. (2021), mezclando cantidades apropiadas de L-prolina y glicerol (ratio molar 1:2) con agitación continua y vigorosa durante 3 h a temperaturas entre 65-70 °C, bajo atmósfera inerte. Las mezclas homogéneas se transfirieron a viales de vidrio, se cerraron herméticamente y se almacenaron en oscuridad a temperatura ambiente hasta su uso posterior.

3.5.2.1 Diseño experimental

Se utilizó RSM para optimizar el proceso de extracción y predecir las respuestas que dependen de variables experimentales. Utilizando el software Design-Expert® versión 12.0 (Stat-Ease, Inc., Minneapolis, MN, EE. UU.), se aplicó un diseño Box-Behnken (BBD) con tres variables independientes para evaluar el efecto del tiempo (X_1), potencia (X_2) y % NaDES (p/p) en la mezcla NaDES:H_2O (X_3) sobre el contenido bioactivo (contenido en polifenoles y flavonoides totales) y la actividad antioxidante de

las flores de azafrán. Los niveles de las variables codificadas se resumen en la Tabla 4. Los datos experimentales se ajustaron a un polinomio de tercer orden, que correlacionaba cada respuesta con los factores.

Tabla 4. Matriz BBD del diseño experimental.

	Variables independientes: condiciones UAE		
Experimentos	**Tiempo (min) (X_1)**	**Potencia (W) (X_2)**	**%NaDES (p/p) (X_3)**
1	35 (+1)	180 (+1)	60 (0)
2	20 (0)	60 (-1)	30 (-1)
3	20 (0)	120 (0)	60 (0)
4	20 (0)	120 (0)	60 (0)
5	35 (+1)	60 (-1)	60 (0)
6	20 (0)	180 (+1)	30 (-1)
7	5 (-1)	60 (-1)	60 (0)
8	20 (0)	60 (-1)	90 (+1)
9	20 (0)	180 (+1)	90 (+1)
10	35 (+1)	120 (0)	90 (+1)
11	20 (0)	120 (0)	60 (0)
12	20 (0)	120 (0)	60 (0)
13	35 (+1)	120 (0)	30 (-1)
14	5 (-1)	120 (0)	90 (+1)
15	5 (-1)	180 (+1)	60 (0)
16	5 (-1)	120 (0)	30 (-1)

3.5.2.2. Procedimiento de extracción

Las extracciones de los subproductos florales de azafrán se llevaron a cabo utilizando el ultrasonido de alta intensidad Vibra-Cell VCX 400 (400 W) (Sonics and Materials Inc., Newtown, CT, EE. UU.), equipado con un convertidor piezoeléctrico y una aleación de titanio de 13 mm de diámetro (Ti-6Al-4V). Los parámetros variables incluyeron tiempo (5-35 min), potencia (60-180 W) y concentración de NaDES (30-90%). La relación masa:solvente se fijó en 1:20 (p/v) y las muestras se mantuvieron en un baño de hielo durante la extracción para evitar el sobrecalentamiento. Se realizaron 16 extracciones, siguiendo las condiciones que se presentan en la Tabla 4. Tras la extracción, mediante centrifugación a 11200 x g, 10 min y filtrado a vacío, se recuperó el sobrenadante. Todos los extractos fueron almacenados en oscuridad a 4 °C hasta su análisis.

Una vez que los extractos se caracterizaron en términos de contenido de compuestos bioactivos, se utilizaron las condiciones óptimas (20 min, 180 W, 90% NaDES) para la extracción de compuestos bioactivos de los estigmas griegos, siguiendo el mismo procedimiento indicado.

3.5.2.3. Caracterización de los extractos

Para determinar el contenido de polifenoles totales y flavonoides totales se siguieron los métodos descritos en los apartados 3.4.3.1. y 3.4.3.2., respectivamente.

3.5.2.3.1. Método DPPH

La capacidad antioxidante mediante el método DPPH, se realizó en los extractos óptimos seleccionados según su contenido bioactivo, siguiendo el método descrito por Boly, Lamkami, Lompo, Dubois and Guissou (2016). Para ello, en una placa de 96 pocillos se añadieron 100 µL de solución DPPH a 100 µL de extractos de azafrán diluidos al 2% (v/v) en etanol (concentración inicial C) y se siguió el mismo procedimiento para las muestras diluidas en etanol, con concentraciones 0,8C, 0,6C, 0,4C y 0,2C. Las muestras se incubaron durante 30 min en oscuridad a temperatura ambiente y se midió la absorbancia a 515 nm utilizando un lector UV-Vis de microplacas SpectraMax 250 de Molecular Devices. El porcentaje de inhibición se calculó utilizando la Ecuación 4:

$$\%\ Inhibición = \frac{A_b - A_m}{A_b} \cdot 100 \quad (4),$$

donde A_b es la absorbancia de las muestras que contienen etanol/DPPH y A_m es la absorbancia de cada muestra que contiene extracto. Se trazó una curva de % de inhibición y se determinó la concentración de muestra que se requiere para una inhibición del 50% de los radicales DPPH (IC_{50}).

3.6. Estabilización de los extractos mediante hidrogeles

Los extractos óptimos de los subproductos florales de azafrán y los extractos de los estigmas de origen griego obtenidos mediante extracción asistida por ultrasonidos, utilizando NaDES como solvente, se incorporaron en hidrogeles de quitosano/alginato.

3.6.1. Síntesis de hidrogeles de quitosano/alginato

En primer lugar, se preparó una solución de quitosano (0,2% p/v) en ácido láctico (3% v/v). A esta solución se le añadió el extracto de subproductos florales o estigmas de azafrán para preparar una solución de concentración final del 2% (v/v) (solución A). A continuación, se preparó una solución acuosa al 3% (p/v) de alginato de sodio, agitando a 70 °C hasta que la mezcla se volvió homogénea (solución B). A continuación, se mezclaron 2 mL de solución A y 5 mL de solución B y se añadió gota a gota a la mezcla una solución de cloruro de calcio (1% p/v), utilizada para reticular el alginato y el quitosano. Tras 24 h, se drenó el exceso de $CaCl_2$ y se obtuvieron los hidrogeles. Estos hidrogeles se lavaron 3 veces con agua destilada y se liofilizaron durante 48 h.

3.6.1.1. Capacidad de absorción y retención de agua

La capacidad de absorción de agua del hidrogel liofilizado se midió mediante un método gravimétrico convencional. Se pesó la muestra inicial y la muestra final tras un tiempo *t* sumergida en tampón fosfato salino a 37 °C, utilizando la Ecuación 5:

$$Capacidad\ de\ absorción\ de\ agua\ (\%) = \frac{m_1 - m_0}{m_0} \cdot\ 100 \quad (5),$$

donde m_0 es el peso inicial de la muestra seca y m_1 es el peso del hidrogel en el tiempo *t*. Después de los tiempos predeterminados, las muestras se extrajeron de la solución y se pesaron rápidamente.

Los hidrogeles, tras alcanzar su máxima capacidad de absorción de agua (equilibrio), se utilizaron en la determinación de retención de agua. Cuando la capacidad de absorción de agua (%) es máxima, asumimos que la retención de agua (%) del hidrogel es del 100%. Así, calculamos la retención de agua con la Ecuación 6:

$$Capacidad\ de\ retención\ de\ agua\ (\%) = \frac{m_1 - m_0}{m_2 - m_0} \cdot\ 100 \quad (6),$$

donde m_0 es el peso inicial de la muestra seca, m_1 es el peso del hidrogel en el momento *t* y m_2 es el peso del hidrogel en el momento de máxima capacidad de absorción de agua (equilibrio).

3.6.1.2. Digestión in vitro

La digestión *in vitro* se realizó según Gawlik-Dziki, Dziki, Baraniak and Lin (2009) y Cerdá-Bernad et al. (2021), con algunas modificaciones. Los hidrogeles se homogeneizaron (Stomacher®) durante 30 s para simular la masticación con la presencia de 5 mL de tampón fosfato salino (hidrogel:tampón 1:50 p/v). La solución se ajustó a pH 6,75 y se añadió α-amilasa (E.C. 3.2.1.1.) para obtener 100 U por mL de actividad enzimática. A continuación, las muestras se sometieron a digestión gástrica simulada durante 60 min a 37 °C bajo agitación, ajustando la solución a pH 3 con HCl 1 M, y se añadió pepsina hasta alcanzar una concentración de 3 g/L. Para la simulación intestinal, se aumentó el pH a 7 con $NaHCO_3$ 1 M y se añadieron 4,5 g/L de sales biliares y 1 g/L de pancreatina. Las muestras se incubaron durante 120 min a 37 °C. Se tomaron muestras en las diferentes etapas de la digestión *in vitro* y se filtraron (0,45 µm) para el análisis del contenido en polifenoles totales: tras la digestión oral, tras 60 min de digestión gástrica y tras 60 min y 120 min de digestión intestinal.

3.7. Incorporación en matrices alimentarias

3.7.1. Yogures

3.7.1.1. Extracción y encapsulación

Como material vegetal se han utilizado los estigmas de azafrán de origen español y los subproductos florales (SF1) descritos en el apartado 3.1. pero deshidratados en una estufa a vacío (VACIOTEM, JP SELECTA®, Barcelona, España) a 50 ± 3 °C, 28 mbar, durante 36 h, triturados, tamizados (malla de 500 µm) y almacenados a -20 °C hasta su posterior uso.

Los extractos se prepararon utilizando agua ultrapura, en una proporción muestra/agua de 1:50 (p/v). Los extractos se agitaron durante 30 min en oscuridad a 400 rpm, se sonicaron en un baño de 15 min y se centrifugaron a 11200 x g durante 10 min a 4 °C. Los sobrenadantes se filtraron (0,45 µm) y se almacenaron a -20 °C.

La encapsulación de los extractos acuosos de los subproductos florales y estigmas de azafrán se preparó siguiendo la metodología descrita por Vinceković, Jurić, Đermić and Topolovec-Pintarić (2017), con algunas modificaciones. Se disolvieron los diferentes extractos en una solución de alginato de sodio al 1,5% (p/v) y se homogeneizaron en un agitador magnético a temperatura ambiente durante 60 min. También se prepararon muestras control utilizando agua ultrapura. Las mezclas se agregaron a través de una aguja de 21G x 5/8" (0,8 x 16 mm) en una solución de $CaCl_2$ 1 M bajo agitación magnética constante. Para permitir que las microcápsulas se endurecieran, se mantuvieron a temperatura ambiente durante 30 min. Las microcápsulas se lavaron tres veces con agua destilada para eliminar el exceso de $CaCl_2$ y se almacenaron a 4 °C. Parte de las microcápsulas se almacenaron a -20 °C durante 24 h y otra parte se liofilizaron durante 24 h (temperatura inicial -25 ± 2 °C y presión 0,220 mbar).

3.7.1.2. Caracterización de los extractos y microcápsulas

Para determinar el contenido de polifenoles totales de los extractos se siguió el método descrito en el apartado 3.4.3.1.

La eficiencia de encapsulación (EE) se realizó según Pasukamonset, Kwon and Adisakwattana (2016). Para ello, se disolvieron alícuotas de 50 mg de microcápsulas en 2 mL de citrato de sodio (5% p/v), se sonicaron en un baño de ultrasonidos 30 min y se

centrifugaron a 11200 x g durante 10 min a 4 °C. La eficiencia de encapsulación se calculó utilizando la Ecuación 7:

$$EE\ (\%) = \frac{CPT_e}{CPT_i} \cdot 100 \quad (7),$$

donde CPT_e era el contenido de polifenoles totales encapsulado en las microcápsulas, mientras que CPT_i era el contenido de polifenoles totales en el extracto acuoso utilizado inicialmente para el proceso de encapsulación.

La capacidad de absorción de agua de las microcápsulas se determinó mediante el método descrito en el apartado 3.6.1.1.

3.7.1.3. Preparación de las formulaciones de yogur

Los yogures se elaboraron con leche entera de vaca UHT (Ultra High Temperature) y se utilizó un 4% de leche desnatada en polvo. Para preparar las diferentes formulaciones se agregaron extractos encapsulados en diferentes concentraciones y se prepararon siete lotes de yogures: yogur control sin ingredientes adicionales (YC), yogur con adición de 0,5% (p/p) (YB0,5) y 1% (p/p) (YB1) de extractos encapsulados control (con agua), yogur con la adición de 0,5% (p/p) (YSF0,5) y 1% (p/p) (YSF1) de extractos encapsulados de subproductos florales de azafrán, yogur con la adición de 0,5% (p/p) de extractos encapsulados de subproductos florales de azafrán combinado con 0,05% (p/p) de extractos encapsulados de estigmas de azafrán (YSF0,5SS0,05), y yogur con la adición de un 1% (p/p) de extractos encapsulados de subproductos florales de azafrán combinado con un 0,1% (p/p) de extractos encapsulados de estigmas de azafrán (YSF1SS0,1).

Las diferentes formulaciones se pasteurizaron a 80 °C durante 30 min y se enfriaron en un baño de hielo a 43 °C para inocular asépticamente el cultivo iniciador de *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis* y *Lactobacillus delbrueckii* subsp. *bulgaricus* (CHOOZIT™ MY800 LYO 5 DCU, Danisco, París, Francia), siguiendo las indicaciones del proveedor. La fermentación se realizó en recipientes de polipropileno estériles y en una incubadora a 43 °C hasta alcanzar un pH de 4,6. Tras ello, se almacenaron a 4°C durante 21 días.

3.7.1.4. Caracterización de las formulaciones de yogur

3.7.1.4.1. Propiedades físico-químicas: pH, acidez, sólidos solubles totales, actividad de agua, color y sinéresis

El pH y la acidez titulable (expresada como % de ácido láctico) se midieron utilizando un titrador automático (TitroMatic Crison pH-Matic 23, Barcelona, España). La determinación de sólidos solubles totales (SST) se realizó con un refractómetro digital (Hanna® HI 96801, Bedfordshire, Reino Unido) y se expresó como °Brix.

La actividad de agua (aw) de las diferentes muestras se determinó utilizando un medidor de actividad de agua (Novasina AW Sprint TH 500, Pfäffikon, Suiza) a temperatura ambiente.

El color se midió con un colorímetro Minolta CR-300 Chroma Meter (Konica Minolta, Tokio, Japón), usando la escala L*, a*, b* (sistema CIELAB). Los resultados se expresaron como luminosidad L*, a* (verdoso/rojizo), b* (azul/amarillento), diferencia de color total (ΔE), tono (h°), croma (C*) e índice de pardeamiento (IP), que se calcularon con las Ecuaciones 8, 9, 10 y 11, respectivamente:

$\Delta E = \sqrt{(L*_0 - L*_1)^2 + (a*_0 - a*_1)^2 + (b*_0 - b*_1)^2}$ (8), donde $L*_0$, $a*_0$, $b*_0$ son los valores de la muestra control (YC) a tiempo *t* de almacenamiento, y $L*_1$, $a*_1$ and $b*_1$ son los valores de cada muestra de yogur a tiempo *t* de almacenamiento;

$$h° = \left(\arctan\left(\frac{b^*}{a^*}\right)\right) \cdot \frac{180}{\pi} \quad (9)$$

$$C^* = (a^{*2} + b^{*2})^{1/2} \quad (10)$$

$$IP = \frac{[100(x - 0{,}31)]}{0{,}17}, \text{ siendo } \boldsymbol{x} = \frac{(\boldsymbol{a*} + \mathbf{1{,}75}\boldsymbol{L*})}{(\mathbf{5{,}645}\boldsymbol{L*} + \boldsymbol{a*} - \mathbf{3{,}012}\boldsymbol{b*})} \quad (11)$$

La sinéresis de los yogures se determinó según Lima et al. (2021), centrifugando 10 g de yogur a 176 x g durante 20 min a 4 °C. La sinéresis (%) se calculó como el peso del sobrenadante liberado sobre el peso del yogur inicial x 100.

3.7.1.4.2. Propiedades antioxidantes y contenido en ácidos orgánicos y azúcares

Para estudiar las propiedades antioxidantes, el contenido de polifenoles totales y la composición de ácidos orgánicos y azúcares solubles, se prepararon los extractos de yogur siguiendo el método descrito por Tizghadam, Roufegari-nejad, Asefi and Jafarian Asl (2021). Se mezclaron 10 g de cada formulación de yogur con 2,5 mL de agua destilada y se usó HCl 0,1 N para ajustar el pH a 4. Durante 45 min, la mezcla se mantuvo en un baño de agua (45 °C) y se centrifugó a 11200 x g durante 10 min a 4 °C.

Los sobrenadantes se ajustaron a pH 7 usando NaOH 0,1 N, se filtraron (0,45 µm) y se almacenaron a -20 °C para su análisis posterior.

Para el estudio de la capacidad antioxidante (métodos DPPH, ABTS, FRAP) se siguió la misma metodología explicada en los apartados 3.4.2.1., 3.4.2.2. y 3.4.2.3., respectivamente.

Para el análisis del contenido en polifenoles totales de las diferentes formulaciones de yogures se siguió el método descrito en el apartado 3.4.3.1.

La identificación y cuantificación de azúcares, inulina y ácidos orgánicos se realizó mediante HPLC, siguiendo la metodología descrita en el apartado 3.2.2.2.

3.7.1.4.3. Análisis microbiológicos

Para la evaluación microbiológica de los yogures, se pesó 1 g de cada formulación, se transfirió a bolsas de polietileno estériles con 9 mL de peptona (Oxoid, Unipath Ltd., Basingstoke, Reino Unido) y se homogeneizó durante 1 min (Stomacher®) a temperatura ambiente. A continuación, se prepararon diluciones apropiadas para las siguientes determinaciones bacteriológicas: (i) *Streptococcus thermophilus*, en placas de agar M17 y (ii) *L. delbrueckii* subsp. *lactis* y *L. delbrueckii* subsp. *bulgaricus* en placas de MRS Agar, ambas incubadas a 42 °C y 37 °C, respectivamente, durante 48 h en un frasco anaeróbico con el sistema AnaeroGen (Oxoid S.A., Madrid, España). Los resultados se expresaron como Log UFC (Unidades Formadoras de Colonias) por g de muestra.

También se determinaron mohos y levaduras en placas de recuento de levaduras y mohos 3M™ Petrifilm™ (3 M, Minnesota, MN, EE. UU.). Las placas se incubaron en condiciones aeróbicas a 25 °C durante 48-72 h para levaduras y 72-140 h para mohos.

Todos los análisis se realizaron el día de la preparación de los yogures (tiempo 0) y después de 7, 14, 21 días de almacenamiento a 4 °C.

3.7.2. Panes de trigo y espelta

3.7.2.1. Preparación de las formulaciones de pan

Como material vegetal se utilizaron los subproductos florales de azafrán (SF1) descritos en el apartado 3.1. pero deshidratados en estufa a vacío a 50 ± 3 °C, 28 mbar, durante 36 h, triturados, tamizados (malla de 500 µm) y almacenados a -20 °C hasta su posterior uso.

Para la elaboración de las diferentes formulaciones de pan se utilizaron dos tipos de harina: harina de trigo (*Triticum aestivum*; W: 360, P: 100, L: 100, 14,5% de proteína) o harina de espelta ecológica (*Triticum spelta*; W:95, P:36, L:105, 13,9% de proteína) (El Amasadero, Málaga, España). También se utilizó levadura liofilizada de panadería (Mauripán, Barcelona, España), sal comercial común y agua.

Las formulaciones de pan se prepararon siguiendo una receta tradicional con harina de trigo o harina de espelta (100% p/p) y añadiendo, en función del peso de la harina, agua (65–70% p/p), levadura (1% p/p), sal (2% p/p) y subproductos florales de azafrán en diferentes concentraciones (0%, 2,5%, 5% y 10% p/p) (Świeca, Gawlik-Dziki, Dziki, & Baraniak, 2017). Se elaboraron ocho formulaciones diferentes, cuatro utilizando harina de trigo al 0% (WB0%), 2,5% (WB2,5%), 5% (WB5%) y 10% (WB10%) de subproductos florales de azafrán; y cuatro formulaciones utilizando harina de espelta al 0% (SB0%), 2,5% (SB2,5%), 5% (SB5%) y 10% (SB10%) de subproductos florales de azafrán.

Los ingredientes se mezclaron y amasaron durante 10 min. Se realizó una primera fermentación corta (30 min) a 30 ± 2 °C y humedad de 40 ± 5%. Los panes se bolearon manualmente y se fermentaron de nuevo (30 min) bajo las mismas condiciones controladas de temperatura y humedad. Finalmente, se hornearon a 210 °C durante 20 min en un horno doméstico, siendo el peso final de unos 40 g por pieza. Una vez enfriados, una parte de ellos se almacenaron a -20 °C hasta su posterior análisis (fisicoquímico, textura y digestión *in vitro*). Otra parte se liofilizó durante 24 h, se trituraron, tamizaron (malla de 500 µm) y se almacenaron en bolsas de polietileno a -20 °C hasta su posterior análisis (minerales, ácidos orgánicos y azúcares).

3.7.2.2. Caracterización de las formulaciones de pan

3.7.2.2.1. Humedad y cenizas

La humedad (%) de las muestras frescas se determinó manteniendo las formulaciones de pan en un horno a 105 ± 5 °C hasta alcanzar peso constante (AOAC, 1995). El contenido de cenizas (%) se llevó a cabo mediante la incineración de las muestras obtenidas tras la humedad en una mufla (Habersal PR 1300 PAD, Barcelona, España) a 550 ± 25 °C, durante 1 h (AOAC, 1995).

3.7.2.2.2. pH, acidez, SST, aw, color

Las muestras frescas se mezclaron previamente con agua destilada (1:10, p/v) con un ULTRA-TURRAX® (IKA T18, Werke GmbH & Co, Staufen, Alemania) a 5000

rpm durante 10 s. Para determinar pH, acidez, SST, aw y color, se siguió la metodología descrita en el apartado 3.7.1.4.1.

3.7.2.2.3. Textura

El análisis de textura se realizó en rebanadas de pan frescas (2,5 cm de ancho) utilizando un texturómetro TA-XTPlus (Stable Micro Systems Ltd., Godalming, Reino Unido), con una sonda cilíndrica de aluminio (SMS P100, 10 cm de diámetro), y una célula de carga de 50 Kg (García-Segovia, Igual, & Martínez-Monzó, 2020). Los parámetros del ensayo se definieron como velocidad de la cruceta 1,70 mm/s y 40% de deformación de la longitud original. Se determinaron la dureza (N), cohesividad, elasticidad (mm), gomosidad (N) y masticabilidad (N).

3.7.2.2.4. Minerales

La composición de los minerales se determinó siguiendo la metodología explicada en el apartado 3.2.3., utilizando 0,5 g de muestras de pan liofilizadas con 10 mL de HNO_3 al 65% (v/v).

3.7.2.2.5. Ácidos orgánicos, azúcares solubles y contenido de inulina

Las extracciones se prepararon utilizando agua ultrapura y una relación muestra liofilizada/agua de 1:20 (p/v). Los extractos se agitaron durante 5 min a 300 rpm a temperatura ambiente, se sonicaron durante 15 min y se centrifugaron a 11200 x g durante 10 min a 4 °C. Los sobrenadantes se filtraron (0,45 µm) y se almacenaron a -20 °C. La identificación y cuantificación de azúcares, inulina y ácidos orgánicos se realizó mediante HPLC, siguiendo la metodología descrita en el apartado 3.2.2.2.

3.7.2.2.6. Digestión in vitro

La digestión *in vitro* se llevó a cabo siguiendo el procedimiento descrito en el apartado 3.6.1.2., utilizando muestras de pan fresco (2 g). Las muestras digeridas se almacenaron a -20 °C hasta su posterior análisis (capacidad antioxidante y contenido en polifenoles totales).

Para el estudio de la capacidad antioxidante de las muestras digeridas (métodos DPPH, ABTS, FRAP) se siguió la misma metodología explicada en los apartados 3.4.2.1., 3.4.2.2. y 3.4.2.3., respectivamente, usando 100 µL de las diferentes muestras.

Para el análisis del contenido en polifenoles totales de las diferentes muestras digeridas se siguió el método descrito en el apartado 3.4.3.1.

3.7.2.2.7. Análisis sensorial

Un panel sensorial compuesto por 12 jueces entrenados según la norma ISO 8586 (2023), siendo cinco hombres y siete mujeres con edades comprendidas entre 21 y 60 años en la Universidad Miguel Hernández (España), realizó un análisis descriptivo cuantitativo (QDA) de formulaciones de pan seleccionadas, siguiendo la norma ISO 11132 (2021). Las condiciones en las que se realizó la prueba estaban normalizadas según la norma ISO 6658 (2017). Ninguno de los panelistas padecía alergias o intolerancias alimentarias y eran consumidores habituales de productos de panadería. Las muestras fueron etiquetadas con un código alfanumérico y distribuidas en orden aleatorio. Se consideraron un total de 17 descriptores sensoriales relacionados con la apariencia, el sabor y la textura, utilizando una escala de 10 puntos.

3.7.3. Bebidas

3.7.3.1. Preparación de las bebidas modelo

Las bebidas modelo se prepararon usando agua (> 90% p/p), sacarosa (7% p/p), ácido cítrico (0,20% p/p), espesante alimentario (0,25% p/p) (goma xantana, goma guar, β-glucano, pectina y sin espesante como control) y subproductos florales de azafrán (1% p/p). Como material vegetal se utilizaron los subproductos florales de azafrán liofilizados (SF1) descritos en el apartado 3.1. Los polisacáridos β-glucano, goma guar y goma xantana se adquirieron en Cedarlane (Burlington, ON, Canadá): β-glucano de cebada P-BGBM, pureza 95%; goma guar P-GGMMV, pureza 98,5%; goma xantana P-XANTH, pureza 97%, y la pectina procedente de manzana se adquirió en Sigma-Aldrich (St. Louis, MO, EE. UU.).

Para cada espesante se preparó una bebida modelo y también se elaboró una muestra control que incluía todos los ingredientes excepto los subproductos florales de azafrán. Las bebidas modelo se prepararon mezclando los espesantes alimentarios con agua, y agitando siguiendo las instrucciones de disolución de los proveedores. A continuación, se añadió el resto de ingredientes en agitación hasta su total disolución. Las muestras se centrifugaron (11200 x g, 20 min) (Eppendorf Centrifuge 5804/5804R, Sigma Aldrich, St. Louis, MO, EE. UU.) para eliminar los compuestos insolubles.

Además, para estudiar el efecto antes y después del procesamiento térmico, parte de las muestras se trataron térmicamente a 80 °C durante 10 min en un baño de agua (Unitronic 200, J.P. Selecta, Barcelona, España) y se enfriaron a 37 °C con hielo.

Se obtuvieron un total de 20 bebidas diferentes, incluidas las muestras control:

• Control (agua, sacarosa, ácido cítrico) sin espesante sin tratamiento térmico (C), bebida modelo sin espesante sin tratamiento térmico (SF), control sin espesante tratado térmicamente (C80), bebida modelo sin espesante tratada térmicamente (SF80).

• Control (agua, sacarosa, ácido cítrico) con goma xantana sin tratamiento térmico (XGC), bebida modelo con goma xantana sin tratamiento térmico (XGSF), control con goma xantana tratado térmicamente (XGC80), bebida modelo con goma xantana tratada térmicamente (XGSF80).

• Control (agua, sacarosa, ácido cítrico) con goma guar sin tratamiento térmico (GGC), bebida modelo con goma guar sin tratamiento térmico (GGSF), control con goma guar sin tratamiento térmico (GGC80), bebida modelo con goma guar tratada térmicamente (GGSF80).

• Control (agua, sacarosa, ácido cítrico) con β-glucano sin tratamiento térmico (BGC), modelo de bebida con β-glucano sin tratamiento térmico (BGSF), control con β-glucano tratado térmicamente (BGC80), bebida modelo con β-glucano tratada térmicamente (BGSF80).

• Control (agua, sacarosa, ácido cítrico) con pectina no tratada térmicamente (PC), bebida modelo con pectina no tratada térmicamente (PSF), control con pectina tratada térmicamente (PC80), bebida modelo con pectina tratada térmicamente (PSF80).

3.7.3.2. Extracción de compuestos fenólicos

Se realizó la extracción de compuestos fenólicos en los subproductos florales de azafrán liofilizados para su análisis por HPLC, pesando 100 mg del material seco y agregando 1 mL de metanol al 50% acidificado con ácido fórmico al 1%. Se mezclaron las soluciones durante 30 s, se sonicaron en un baño de ultrasonidos durante 1 h (Branson 551021 Sigma -Aldrich, St. Louis, MO, EE. UU.) y se dejaron 24 h a 4 °C. A continuación, se centrifugaron a 11200 x g durante 15 min (Hettich® EBA 21 Sigma-Aldrich, St. Louis, MO, EE. UU.). Los sobrenadantes se filtraron a través de una membrana de politetrafluoroetileno de 0,22 µm (Millipore, Bedford, MA, EE. UU.) y se almacenaron a -20 °C hasta su posterior análisis.

Las muestras de las bebidas se utilizaron directamente para el análisis por HPLC, para lo que previamente se filtraron (0,22 µm) y se almacenaron a -20 °C hasta su posterior análisis.

3.7.3.3. Identificación y cuantificación de compuestos fenólicos mediante HPLC-DAD-ESI/MS^n

La identificación de los compuestos fenólicos se realizó mediante HPLC-DAD-ESI/MS^n con un modelo Agilent HPLC serie 1200 equipado con un detector de matriz de fotodiodos (modelo G1315B), un detector de masas en serie (Agilent Technologies, Waldbronn, Alemania), una bomba binaria (modelo G1312A), un desgasificador (modelo G1322A) y un muestreador automático (modelo G1313A), basándose en el método descrito por Gonçalves et al. (2021). El detector de masas consistió en un espectrómetro de trampa de iones (modelo G2445A) equipado con una interfaz de ionización por electropulverización y estaba controlado por un software LC/MS (Esquire Control Ver. 6.1. Build No. 534.1., Bruker Daltoniks GmbH, Bremen, Alemania). Se utilizó una columna Nucleosil® 100–5 C18 (25 cm x 0,46 cm; tamaño de partícula de 5 µm; Macherey-Nagel, Düren, Alemania). La fase móvil, bombeada a un flujo de 0,8 mL/min, consistía en ácido fórmico al 1% (disolvente A) y acetonitrilo (disolvente B). El sistema de solvente comenzó con 8% de B y alcanzó 15% de B a los 25 min, 22% a los 55 min y 40% a los 60 min, con un período de lavado de 5 min. Los espectros de masas se adquirieron con un rango de escaneo de m/z de 100 a 1200 y se establecieron los parámetros de espectrometría de masas: la temperatura capilar fue de 350 °C, el voltaje capilar se estableció en 4 kV, la presión del nebulizador fue de 65 psi y el flujo de nitrógeno de 11 L/min. Los análisis de fragmentación inducida por colisión se realizaron en una trampa de iones utilizando helio como gas de colisión, con ciclos de aumento de voltaje de 0,3 a 2 V. Para las antocianinas, los datos de espectrometría de masas se adquirieron en modo de ionización positiva, mientras que, para los compuestos fenólicos no coloreados, la adquisición se realizó en un modo de ionización negativa. MS^n se llevó a cabo en modo automático en los fragmentos de iones que fueron más abundantes en MS$^{(n-1)}$. El sistema HPLC fue controlado por ChemStation para el software LC 3D Systems Rev. B.01.03-SR2 (204) (Agilent Technologies Spain S.L., Madrid, España). Las inyecciones (20 µL) de cada muestra se realizaron por triplicado.

Los datos espectrales de absorbancia de todos los picos se acumularon en un rango de 200 a 600 nm y los cromatogramas se registraron a 360 nm (flavonoles) y 520 nm (antocianinas). Los compuestos en cada muestra se identificaron tentativamente en función de los tiempos de retención y las características del espectro ultravioleta-visible y de las masas en comparación con los estándares de referencia analizados en las mismas condiciones y datos disponibles en la literatura. Los compuestos identificados se cuantificaron utilizando curvas de calibración de los compuestos de referencia

estándar, o utilizando el compuesto de referencia estructuralmente más relacionado, siendo el rango de concentración de 0,06 a 1 mmol/L.

3.7.3.4. Inhibición in vitro de α-amilasa

El ensayo de inhibición se realizó según D'Costa and Bordenave (2021) con algunas modificaciones. Consistió en medir, mediante un método colorimétrico, la cantidad de maltosa generada por la hidrólisis de amilopectina por la enzima α-amilasa. Para ello, se agregaron 200 µL de α-amilasa (páncreas porcino, tipo VI-B, 15 unidades/mg sólido, Millipore-Sigma, Oakville, ON, Canadá) de 1,25 U/mL a 50 µL de tampón fosfato (0,1 M, pH 6,9). A continuación, se agregaron 50 µL de tampón fosfato (blanco) o 50 µL de cada bebida modelo y se incubaron a 37 °C durante 5 min. Las bebidas modelo estaban previamente neutralizadas, ya que el pH del quimo se neutraliza rápidamente en el duodeno, por lo que es poco probable que las enzimas pancreáticas se vean afectadas por el pH nativo de los alimentos (Freitas & Le Feunteun, 2019). La cantidad de maltosa generada por este ensayo en la bebida control sin espesante y sin tratamiento térmico (C) se usó como referencia correspondiendo al 100% de actividad de α-amilasa. La cantidad de maltosa liberada a través del ensayo se expresó como porcentaje de esta actividad de α-amilasa de referencia, en función de la cantidad de maltosa liberada de la bebida control.

Tras el período de incubación, se agregaron 200 µL de una solución de 2,5 mg/mL de amilopectina gelatinizada en agua ultrapura y se incubó durante 10 min a 37 °C. Tras la incubación, 1 mL de una solución del reactivo DNS, preparada siguiendo la metodología de Nyambe-Silavwe et al. (2015), se añadió a la mezcla de reacción, se incubó en un baño de agua a 100 °C durante 10 min y se enfrió posteriormente en hielo. Finalmente, se transfirieron 250 µL de cada muestra a una microplaca de 96 pocillos y se midió la absorbancia a 540 nm a 25 °C (lector de microplacas multimodo Tecan Spark, Baldwin Park, CA, EE. UU.). La concentración de maltosa se calculó mediante la absorbancia, utilizando una curva estándar de maltosa, con una concentración entre 0 y 1 mg/mL.

3.7.3.5. Propiedades reológicas

La caracterización reológica de las bebidas se determinó utilizando un reómetro híbrido de la serie Discovery (HR-2) de TA Instruments (New Castle, DE, EE. UU.) con medición de geometría de cilindro concéntrico. Para cada bebida, se midieron los perfiles de tensión de corte (τ en Pa) vs velocidad de corte (γ en s^{-1}), la cual oscilaba entre 10^{-1} y 10^{3} s^{-1}.

Estos perfiles se ajustaron a la ecuación de Herschel-Bulkley, $\tau = \tau_0 + K\gamma^n$ mediante el método de mínimos cuadrados utilizando la función Solver en Microsoft Excel 365 (Microsoft, Redmond, WA, EE. UU.). El índice de consistencia del flujo (K, en $Pa \cdot s^{-n}$) y el índice de comportamiento del flujo (n, adimensional) de las bebidas se obtuvieron a partir de este método de regresión no lineal.

3.8. Análisis estadístico

Todas las determinaciones se realizaron por triplicado. Los resultados se expresaron como media ± desviación estándar. Las comparaciones de medias se llevaron a cabo mediante un análisis de varianza (ANOVA) y mediante la prueba de rangos múltiples de Tukey, utilizando el software SPSS versión 21.0 (SPSS Inc., Chicago, IL, EE.UU.). Las diferencias significativas se establecieron como ($p \leq 0,05$).

4.RESULTADOS Y DISCUSIÓN

4. RESULTADOS Y DISCUSIÓN

4.1. Caracterización físico-química

Los resultados sobre la composición nutricional de los subproductos florales y de los estigmas del azafrán se presentan en la Tabla 5.

Los carbohidratos fueron el macronutriente más abundante en todas las muestras de estigmas y subproductos florales de azafrán, seguido del contenido en proteínas, siendo el más bajo el de grasas. Las flores mostraron cantidades de carbohidratos significativamente mayores que los estigmas (76 g/100 g y 65 g/100 g, respectivamente). Sin embargo, los estigmas de azafrán mostraron un contenido de proteínas significativamente mayor (13 g/100 g), que las flores (8 g/100 g). De este modo, el azafrán presenta elevadas cantidades de proteínas, lo que supone una buena fuente de aminoácidos para funciones fisiológicas vitales.

Las flores de azafrán presentaron una composición proximal similar a la de otras flores comestibles como son los pétalos de diferentes especies (dalia, rosa, caléndula y centaurea) que contenían carbohidratos como macronutriente más abundante (81-88%) seguido de proteínas (6-7%) y el menor contenido en grasas (0,1-5%) (Pires, Dias, Barros, & Ferreira, 2017).

Tabla 5. Composición proximal y contenido de fibra dietética en azafrán y sus subproductos florales.

	SFL1	SFL2	Azafrán español	Azafrán iraní	Azafrán griego
Valor nutricional (g/100 g peso seco)					
Humedad	6,42 ± 0,68^{bc}	5,52 ± 1,63^{c}	8,90 ± 0,14^{ab}	9,01 ± 0,05^{ab}	10,74 ± 0,55^{a}
Cenizas	8,39 ± 0,00^{a}	4,89 ± 0,31^{b}	5,59 ± 0,09^{b}	5,57 ± 0,26^{b}	4,81 ± 0,14^{b}
Proteínas	8,58 ± 0,08^{d}	8,68 ± 0,02^{d}	13,15 ± 0,13^{b}	13,61 ± 0,13^{a}	12,29 ± 0,02^{c}
Grasas	5,81 ± 0,01^{ab}	4,56 ± 0,77^{b}	7,01 ± 0,29^{a}	6,08 ± 0,17^{ab}	6,22 ± 0,20^{a}
Carbohidratos	70,80 ± 0,75^{b}	76,35 ± 2,07^{a}	65,26 ± 0,25^{c}	65,73 ± 0,16^{c}	65,94 ± 0,23^{c}
Energía (Kcal/100 g peso seco)					
	369 ± 3^{b}	381 ± 1^{a}	377 ± 2^{ab}	372 ± 2^{ab}	369 ± 3^{b}
Fibra dietética (g/100 g peso seco)					
Total	26,59 ± 1,30^{a}	22,56±0,27^{ab}	17,86 ± 2,64^{b}	21,17 ± 0,02^{ab}	17,64 ± 2,51^{b}
Insoluble	19,07 ± 0,59^{a}	20,24 ± 1,11^{a}	14,61 ± 0,88^{b}	14,55 ± 0,04^{b}	15,15 ± 0,21^{b}
Soluble	7,51 ± 0,71^{a}	2,32 ± 1,38^{c}	3,25 ± 1,76^{bc}	6,61 ± 0,06^{ab}	4,26 ± 0,21^{abc}
Inulina (g/100 g peso seco)					
	1,46 ± 0,47^{a}	1,60 ± 0,51^{a}	1,60 ± 0,65^{a}	1,59 ± 0,15^{a}	1,81 ± 0,67^{a}

Media ± desviación estándar en la misma fila seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra ($n = 3$); SFL1, SFL2: Subproductos florales de azafrán de dos productores diferentes.

También se estudió la fibra dietética (FD), cuyo consumo tiene efectos positivos sobre la salud digestiva. La FD total fue mayor en las flores (22-26 g/100 g) que en los estigmas de azafrán (17-21 g/100 g). La fibra insoluble fue la fracción mayor en todas las muestras estudiadas. Estos resultados fueron similares a los encontrados en otros estudios en el que el azafrán y los subproductos florales de azafrán de origen español presentaron mayor contenido de FD insoluble que FD soluble, siendo menor el contenido de fibra en los estigmas (Serrano-Díaz et al., 2013). Las diferencias mostradas en cuanto a la composición proximal y FD entre SFL1 y SFL2 y entre los estigmas procedentes de España, Irán y Grecia pueden deberse a las condiciones de cultivo, como son temperatura, humedad y propiedades del suelo, que podrían influir notablemente en la composición química de la planta (Majolo et al., 2021).

Además, se estudió el contenido de inulina (como parte de la fibra dietética soluble). La inulina generalmente se usa como fibra dietética y como prebiótico en alimentos funcionales, ya que no es hidrolizada por las enzimas intestinales humanas. El azafrán y sus subproductos florales presentaron contenidos de inulina en cantidades similares (1,50 g/100 g), lo que sugiere que podrían usarse como una buena fuente de ingredientes prebióticos, estimulando la proliferación de la microbiota intestinal.

El contenido mineral del azafrán y sus subproductos florales se presenta en la Tabla 6. El mineral más abundante en todas las muestras de azafrán y sus subproductos florales fue el K, siendo significativamente mayor en los subproductos florales de azafrán (alrededor de 1500 mg/100 g) en comparación con los estigmas de azafrán (alrededor de 1000 mg/100 g). Estos valores representan el 31,91% y el 21,28% aproximadamente de la ingesta diaria de potasio en adultos (4700 mg por día) con un consumo de 100 g de flores o estigmas, respectivamente (U.S. Food & Drugs Administration, 2020). Además, SFL1 presentó una alta cantidad de Ca (415,20 ± 25,46 mg/100 g), mientras que los estigmas de azafrán presentaron un bajo contenido (86-120 mg/100 g). Estos valores representan el 31,94% y 9,23% aproximadamente de la ingesta diaria de calcio en adultos (1300 mg por día) con un consumo de 100 g de flores o estigmas, respectivamente (U.S. Food & Drugs Administration, 2020).

Respecto al Mg, SFL1 presentó un contenido significativamente mayor (120,30 ± 4,95 mg/100 g) respecto a SFL2 (103,30 ± 2,68 mg/100 g). Los estigmas del azafrán español e iraní mostraron valores similares a los del SFL2. Sin embargo, el azafrán griego presentó la menor cantidad (88,70 ± 4,60 mg/100 g). Estos valores representan alrededor del 28,64 y 24,40% de la ingesta diaria de magnesio en adultos (420 mg por día) con un consumo de 100 g de flores o estigmas, respectivamente (U.S. Food & Drugs

Administration, 2020). Los valores de Na fueron bajos en todas las muestras, sin mostrar diferencias estadísticamente significativas entre ellas.

Tabla 6. Contenido de minerales en azafrán y sus subproductos florales.

	SFL1	SFL2	Azafrán español	Azafrán iraní	Azafrán griego
Macrominerales (mg/100 g peso seco)					
Ca	415,20 ± 25,46^{a}	112,60 ± 1,98^{b}	118,30 ± 25,10^{b}	86,25 ± 6,01^{b}	123,30 ± 63,98^{b}
K	1530 ± 16^{a}	1450 ± 51^{a}	1114 ± 63^{bc}	1136 ± 18^{b}	971,30 ± 31,82^{c}
Mg	120,30 ± 4,95^{a}	103,30 ± 2,68^{b}	100,20 ± 1,77^{bc}	102,50 ± 1,41^{b}	88,70 ± 4,60^{c}
Na	9,00 ± 3,53^{a}	9,20 ± 1,55^{a}	19,00 ± 1,25^{a}	33,75 ± 2,89^{a}	33,25 ± 3,45^{a}
Microminerales (mg/100 g peso seco)					
Cu	0,57 ± 0,01^{b}	0,55 ± 0,04^{b}	0,35 ± 0,07^{bc}	0,25 ± 0,00^{c}	1,63 ± 0,11^{a}
Fe	46,26 ± 1,44^{a}	6,38 ± 0,03^{bc}	5,45 ± 0,57^{c}	12,28 ± 3,29^{b}	8,13 ± 1,03^{bc}
Mn	2,51 ± 0,21^{a}	0,95 ± 0,01^{b}	1,15 ± 0,01^{b}	1,30 ± 0,00^{b}	1,25 ± 0,07^{b}
Zn	3,89 ± 0,27^{a}	2,10 ± 0,08^{b}	2,65 ± 0,35^{ab}	2,13 ± 0,53^{b}	1,98 ± 0,46^{b}

Media ± desviación estándar en la misma fila seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra ($n = 3$); SFL1, SFL2: Subproductos florales de azafrán de dos productores diferentes.

En cuanto a los microminerales, la concentración en todas las muestras de Fe fue la más alta, seguida de Zn. SFL1 mostró niveles significativamente más altos de Fe (46,26 ± 1,44 mg/100 g) con respecto a SFL2 (6,38 ± 0,03 mg/100 g). Los estigmas de azafrán iraní y griego presentaron valores similares a SFL2. Sin embargo, el azafrán español presentó la concentración más baja (5,45 ± 0,57 mg/100 g). Estas diferencias podrían deberse a las diferentes condiciones de cultivo, como el contenido de minerales del suelo, el uso de algunos fertilizantes o la relativa bioacumulación de este mineral en la planta. Estos valores representan alrededor del 35,44% y 45,17% de la ingesta diaria de hierro en adultos (18 mg por día) con un consumo de 100 g de flores o estigmas, respectivamente (U.S. Food & Drugs Administration, 2020). Con respecto a Zn, SFL1 presentó un contenido significativamente mayor (3,89 ± 0,27 mg/100 g) que SFL2 (2,10 ± 0,08 mg/100 g), que mostró cantidades similares a los estigmas de azafrán. Estos valores representan el 35,36% y 24% aproximadamente de la ingesta diaria de zinc en adultos (11 mg por día) con un consumo de 100 g de flores o estigmas, respectivamente (U.S. Food & Drugs Administration, 2020). Los contenidos de Mn y Cu fueron bajos en todas las muestras.

Los resultados sobre la composición de ácidos orgánicos y azúcares solubles de los subproductos florales y los estigmas de azafrán se presentan en la Tabla 7. Los estigmas de azafrán presentaron las mayores concentraciones de ácido láctico (5,27-6,02 g/100 g) con respecto a las flores (3,59-4,0 g/100 g).

Sin embargo, los subproductos florales de azafrán tuvieron mayor contenido de ácido málico. Además, los subproductos florales del azafrán presentaron ácido propiónico (2,13-2,34 g/100 g) y un bajo contenido de ácido fumárico (0,17 g/100 g), que no se detectaron en los estigmas de azafrán; pero éstos sí presentaron ácido fórmico y ácido oxálico. Así, tanto los subproductos florales como los estigmas del azafrán mostraron altas cantidades de ácidos láctico y málico, que podrían ejercer efectos positivos en la salud humana y tener otras funcionalidades tecnológicas, ya que el ácido láctico podría actuar como conservante, y el ácido málico como acidulante (Khan & Iqbal, 2015). Además, cabe señalar que las flores de azafrán presentaron ácido propiónico que se utiliza como conservante en los alimentos por sus propiedades antimicrobianas (Khan & Iqbal, 2015).

Tabla 7. Contenido de ácidos orgánicos y azúcares solubles en azafrán y sus subproductos florales.

	SFL1	SFL2	Azafrán español	Azafrán iraní	Azafrán griego
Ácidos orgánicos (g/100 g peso seco)					
Ácido láctico	3,59 ± 0,85^{b}	4,00 ± 0,62^{bc}	6,02 ± 0,16^{a}	5,27 ± 0,43^{ac}	5,55 ± 0,77^{a}
Ácido málico	3,86 ± 0,88^{b}	5,89 ± 0,72^{a}	2,72 ± 0,31^{b}	2,57 ± 0,65^{b}	2,03 ± 0,62^{bc}
Ácido fumárico	0,17 ± 0,03^{a}	0,17 ± 0,01^{a}	nd	nd	nd
Ácido propiónico	2,13 ± 0,85^{a}	2,34 ± 0,60^{a}	nd	nd	nd
Ácido oxálico	nd	nd	0,32 ± 0,02^{a}	0,28 ± 0,02^{a}	0,29 ± 0,04^{a}
Ácido fórmico	nd	nd	4,42 ± 0,45^{b}	4,65 ± 0,27^{b}	7,18 ± 0,39^{a}
Azúcares solubles (g/100 g peso seco)					
Glucosa	12,81 ± 1,23^{a}	13,55 ± 1,49^{a}	6,50 ± 0,06^{b}	6,32 ± 0,14^{b}	6,63 ± 0,28^{b}
Fructosa	28,67 ± 3,19^{b}	35,33 ± 4,29^{a}	2,82 ± 0,25^{c}	1,58 ± 0,31^{c}	0,63 ± 0,14^{c}

Media ± desviación estándar en la misma fila seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra ($n = 3$); nd: no detectado; SFL1, SFL2: Subproductos florales de azafrán de dos productores diferentes.

En cuanto a la composición de azúcares solubles, todas las muestras presentaron como azúcares reductores los monosacáridos glucosa y fructosa. Los subproductos florales de azafrán tuvieron un contenido elevado de glucosa (12,81-13,55 g/100 g) y de fructosa, especialmente SFL2 (35,33 ± 4,29 g/100 g). En las muestras de estigmas de azafrán, la concentración de glucosa fue mayor que la de fructosa (6,32-6,63 y 0,63-2,82 g/100 g, respectivamente). Estos resultados son comparables a los de otras plantas comestibles, como la dalia y los pétalos de rosa, en las que la fructosa fue el azúcar soluble encontrado en mayor cantidad (~4-5 g/100 g), seguido de la glucosa (3,23 g/100 g) (Pires et al., 2017).

El perfil de ácidos grasos de los estigmas de azafrán y sus subproductos florales se muestra en la Tabla 8. El estudio de la composición de ácidos grasos en los alimentos es muy importante ya que los ácidos grasos esenciales, que deben provenir de la dieta, tienen

un papel funcional en la salud humana (Calder, 2015). Respecto a los ácidos grasos saturados, el ácido palmítico (C16:0) se encontró en mayor concentración en ambas muestras de subproductos florales de azafrán (20,19 g/100 g) y en los estigmas de azafrán, con mayor proporción en los estigmas griegos (15,18 ± 0,21 g/100 g).

Tabla 8. Perfil de ácidos grasos (principales grupos y ratios) en azafrán y sus subproductos florales.

Ácidos grasos (g/100 g ácidos grasos totales)	**SFL1**	**SFL2**	**Azafrán español**	**Azafrán iraní**	**Azafrán griego**
Ácido mirístico (C14:0)	1,41 ± 0,03	3,63 ± 2,19	1,51 ± 0,03	2,45 ± 1,38	2,82 ± 1,13
Ácido pentadecanoico (C15:0)	0,18 ± 0,00	0,37 ± 0,24	0,12 ± 0,00	0,22 ± 0,11	0,21 ± 0,02
Ácido palmítico (C16:0)	20,19 ± 0,08	20,19 ± 2,09	12,40 ± 0,25	14,19 ± 2,62	15,18 ± 0,21
Ácido esteárico (C18:0)	2,56 ± 0,04	3,15 ± 0,85	1,57 ± 0,03	0,85 ± 0,17	0,89 ± 0,02
Ácido behénico (C22:0)	1,64 ± 0,06	1,12 ± 0,03	0,49 ± 0,01	0,73 ± 0,13	0,93 ± 0,01
Ácido lignocérico (C24:0)	2,26 ± 0,06	1,01 ± 0,02	0,18 ± 0,00	0,54 ± 0,12	0,56 ± 0,00
∑ AGS	**28,55 ± 0,19^{ab}**	**29,64 ± 5,15^{a}**	**16,27 ± 0,33^{c}**	**18,98 ± 4,53^{bc}**	**20,70 ± 1,03^{ac}**
Ácido hipogeo (C16:1n-9)	0,61 ± 0,00	1,27 ± 0,87	0,35 ± 0,01	0,85 ± 0,24	0,87 ± 0,16
Ácido palmitoleico (C16:1n-7)	1,69 ± 0,01	2,02 ± 0,55	0,30 ± 0,01	0,40 ± 0,06	0,40 ± 0,05
Ácido oleico (C18:1n-9)	2,57 ± 0,05	2,13 ± 0,25	4,39 ± 0,09	5,51 ± 0,94	7,66 ± 0,17
Ácido vaccénico (C18:1n-7)	1,52 ± 0,02	1,35 ± 0,13	2,88 ± 0,06	2,93 ± 0,52	4,00 ± 0,08
Ácido eicosanoico (C20:1n-9)	11,04 ± 0,38	5,56 ± 0,17	5,23 ± 0,10	6,37 ± 1,31	7,92 ± 0,26
Ácido cis-14-tricosenoico (C23:1n-9)	5,59 ± 0,03	2,24 ± 0,45	0,38 ± 0,01	0,91 ± 0,18	0,96 ± 0,01
Ácido erúcico (C22:1n-9)	1,26 ± 0,02	0,55 ± 0,20	0,28 ± 0,00	0,35 ± 0,08	0,29 ± 0,00
Ácido nervónico (C24:1n-9)	0,84 ± 0,02	0,63 ± 0,01	0,47 ± 0,01	0,77 ± 0,16	0,59 ± 0,01
∑ AGMI	**25,13 ± 0,37^{a}**	**15,76 ± 2,22^{c}**	**14,28 ± 0,29^{c}**	**18,16 ± 3,39^{bc}**	**22,79 ± 0,18^{ab}**
Ácido linoleico (C18:2n-6)	35,12 ± 0,26	39,62 ± 2,83	35,76 ± 0,72	40,24 ± 7,20	45,43 ± 1,08
Ácido γ-linolénico (C18:3n-6)	nd	7,16 ± 0,82	25,95 ± 0,52	12,94 ± 1,30	nd
Ácido eicosadienoico (C20:2n-6)	0,25 ± 0,03	0,10 ± 0,03	0,36 ± 0,01	0,19 ± 0,07	0,43 ± 0,00
Ácido araquidónico (C20:4n-6)	nd	nd	0,26 ± 0,00	nd	0,35 ± 0,06
Ácido docosadienoico (C22:2n-6)	0,15 ± 0,00	nd	0,93 ± 0,02	0,59 ± 0,03	1,12 ± 0,04
∑ AGPI omega-6	**35,94 ± 0,23^{b}**	**47,12 ± 7,77^{ab}**	**63,26 ± 1,27^{a}**	**54,05 ± 10,13^{ab}**	**47,33 ± 1,19^{ab}**
Ácido α-linolénico (C18:3n-3)	9,28 ± 0,40	6,87 ± 0,68	5,77 ± 0,12	7,76 ± 1,38	8,50 ± 0,10
Ácido eicosatrienoico (C20:3n-3)	0,28 ± 0,04	0,33 ± 0,11	0,23 ± 0,01	0,50 ± 0,08	0,39 ± 0,03
∑ AGPI omega-3	**10,38 ± 0,41^{a}**	**7,48 ± 0,39^{ab}**	**6,19 ± 0,12^{b}**	**8,80 ± 2,21^{ab}**	**9,18 ± 0,35^{ab}**
∑ AGPI	**46,31 ± 0,18^{b}**	**54,60 ± 7,37^{ab}**	**69,45 ± 1,39^{a}**	**62,85 ± 7,92^{ab}**	**56,52 ± 0,85^{ab}**

Media ± desviación estándar en la misma fila seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra ($n = 3$); nd: no detectado; SFL1, SFL2: Subproductos florales de azafrán de dos productores diferentes; AGS: ácidos grasos saturados; AGMI: ácidos grasos monoinsaturados; AGPI: ácidos grasos poliinsaturados.

Con respecto a los ácidos grasos monoinsaturados, los más importantes cuantitativamente fueron el ácido eicosanoico (C20:1n-9) y el ácido oleico (C18:1n-9) tanto en los estigmas del azafrán, como en los subproductos florales. La proporción de ácidos grasos poliinsaturados fue predominante en todas las muestras, siendo el ácido linoleico

(C18:2n6) el de mayor concentración. El ácido linoleico, un ácido graso esencial producido en las plantas, es el ácido graso omega-6 más frecuente en la dieta humana. La muestra SFL2 presentó una cantidad de ácido linoleico (39,62 ± 2,83 g/100 g) superior a SFL1 (35,12 ± 0,26 g/100 g). Además, los estigmas del azafrán griego (45,43 ± 1,08 g/100 g) mostraron una elevada concentración de ácido linoleico, que fue mayor en comparación a los estigmas españoles (35,76 ± 0,72 g/100 g) e iraníes (40,24 ± 7,20 g/100 g). Las fuentes tradicionales de ácido linoleico son los aceites vegetales, como el aceite de girasol, que contiene alrededor del 65% de este ácido graso, cuyo consumo puede reducir el riesgo de enfermedades cardiovasculares. Según las recomendaciones de la Autoridad Europea de Seguridad Alimentaria, la ingesta diaria adecuada de ácido linoleico debe ser del 4% de la ingesta energética total (European Food Safety Authority, 2017).

En cuanto a los ácidos grasos poliinsaturados omega-3, el ácido esencial α-linolénico (C18:3n-3), que se encuentra principalmente en alimentos vegetales como la linaza, las nueces y los aceites vegetales, fue predominante en todas las muestras. Estos resultados concordaron con otros estudios, mostrando la misma tendencia en el contenido de ácidos grasos de algunas plantas comestibles, siendo el grupo de ácidos grasos poliinsaturados el predominante (46-57%) en pétalos de diferentes especies (dalia, rosa, caléndula y centaurea) y fueron similares al contenido de aceites vegetales como el aceite de soja (6%) (Pires et al., 2017). Por tanto, debido al alto contenido encontrado en ácidos grasos omega-6 y omega-3 y un menor contenido en ácidos grasos saturados, la ingesta de azafrán y sus subproductos florales puede aportar beneficios adicionales para la salud cardiovascular.

En resumen, estos resultados obtenidos revelan el potencial del azafrán y sus subproductos florales como ingredientes prometedores para incorporarse a diferentes matrices alimentarias, debido a su rico contenido en fibra dietética, ácidos orgánicos y azúcares solubles, macrominerales y ácidos grasos omega-6 y omega-3.

4.2. Propiedades tecnofuncionales

Las propiedades tecnofuncionales de los subproductos florales de azafrán liofilizados se presentan en la Figura 11.

Se evaluó la CRA, donde las muestras mostraron diferencias estadísticamente significativas, 15 g agua/g para SFL1 y 10 g agua/g para SFL2. Estas diferencias podrían atribuirse a diferentes concentraciones de compuestos hidrófilos entre SFL1 y SFL2. En cuanto a la solubilidad en agua, que está relacionada con la digestibilidad de los alimentos, los subproductos florales del azafrán presentaron una alta solubilidad, siendo ingredientes

apropiados para desarrollar nuevos productos. Los valores oscilaron entre un 34% y 37% para SFL2 y SFL1.

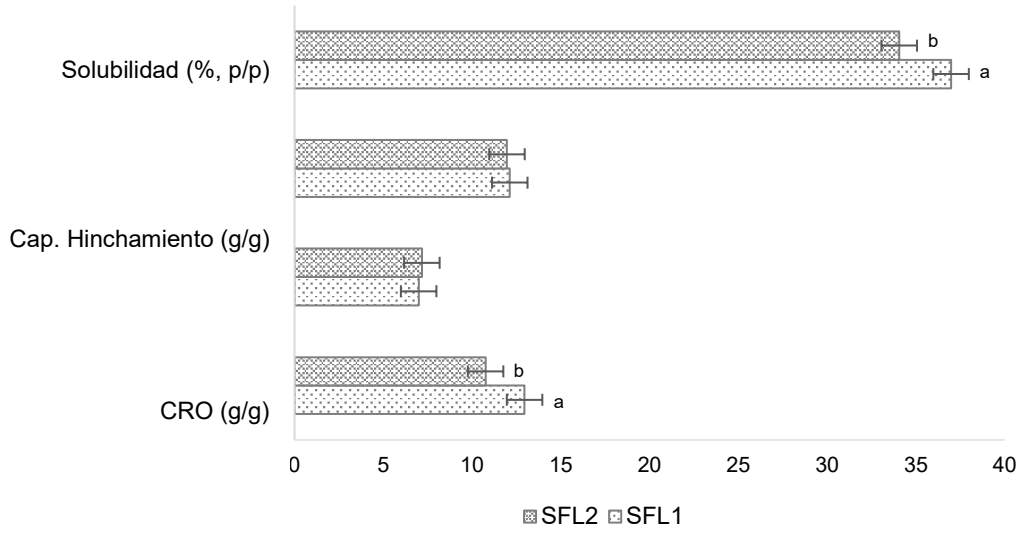

Figura 11. Propiedades tecnofuncionales de los subproductos florales de azafrán. Las barras de error representan la desviación estándar y las diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra ($n = 3$); SFL1, SFL2: subproductos florales de azafrán de dos productores diferentes; CRO: capacidad de retención de aceite; CRA: capacidad de retención de agua.

Los valores de CRO fueron similares en SFL1 y SFL2 (7 g de aceite/g). Es fundamental evaluar la CRO para utilizar estos ingredientes en la formulación de alimentos con alto contenido de grasa y emulsiones para mejorar su palatabilidad y retención de sabor (productos cárnicos o de panadería).

Para desarrollar productos de panadería, la capacidad de hinchamiento también es un parámetro de calidad relevante que podría verse influenciado por el tamaño de las partículas y el contenido de almidón de las muestras. Debido a su alta concentración en carbohidratos disponibles, los subproductos florales de azafrán mostraron valores en torno a 12 g/g.

Así, además del valor nutricional, las flores de azafrán presentaron propiedades tecnofuncionales adecuadas para utilizarlas como ingredientes sostenibles y desarrollar nuevos alimentos funcionales.

4.3. Caracterización funcional

4.3.1. Propiedades antioxidantes, contenido bioactivo y actividad quelante de hierro (II)

Los resultados de la actividad antioxidante y el contenido bioactivo en los estigmas y subproductos florales de azafrán se indican en la Tabla 9.

Los subproductos florales y los estigmas de azafrán presentaron una alta concentración de polifenoles totales, entre 32-36 mg GAE/g, excepto el azafrán español, que presentó la concentración más alta (44,80 ± 2,30 mg GAE/g). En cuanto al contenido total de flavonoides, los estigmas de azafrán mostraron un alto contenido (15-18 mg CE/g). Sin embargo, en las flores de azafrán el contenido fue más bajo (4–5 mg CE/g).

Estos resultados obtenidos para los subproductos florales de azafrán fueron similares a los descritos por Sun et al. (2020), indicando valores alrededor de 30 mg GAE/g y valores inferiores a 10 mg CE/g en extractos de tépalos de azafrán. Dentro del grupo de los flavonoides, las antocianinas también presentan propiedades antioxidantes. Los subproductos florales de azafrán contenían altos niveles de antocianinas totales, mostrando SFL2 una concentración significativamente mayor que SFL1. En el estudio de Serrano-Díaz et al. (2013), también se encontró un alto contenido de antocianinas en tépalos de azafrán de origen español.

Tabla 9. Capacidad antioxidante y contenido bioactivo en azafrán y sus subproductos florales.

Capacidad antioxidante (µmol TE/100 g peso seco)	**SFL1**	**SFL2**	**Azafrán español**	**Azafrán iraní**	**Azafrán griego**
DPPH	98,82 ± 4,79^{d}	107,40 ± 1,95^{d}	201,27 ± 7,71^{b}	278,92 ± 8,37^{a}	145,58 ± 21,98^{c}
ABTS	50,99 ± 2,51^{c}	48,41 ± 4,67^{c}	110,88 ± 9,99^{b}	142,22 ± 5,23^{a}	124,95 ± 27,44^{ab}
FRAP	1250 ± 88^{b}	1181 ± 26^{b}	3667 ± 319^{a}	3471 ± 123^{a}	3445 ± 274^{a}
Compuestos bioactivos					
Polifenoles totales (mg GAE/g peso seco)	32,42 ± 6,90^{b}	32,82 ± 2,23^{b}	44,80 ± 2,30^{a}	36,35 ± 3,47^{b}	34,00 ± 3,22^{b}
Flavonoides totales (mg CE/g peso seco)	3,99 ± 0,79^{b}	5,37 ± 0,19^{b}	18,74 ± 1,22^{a}	15,32 ± 1,73^{a}	17,39 ± 4,14^{a}
Antocianinas totales (mg DE/g peso seco)	39,17 ± 2,98^{b}	69,02 ± 4,34^{a}	nd	nd	nd
β-caroteno total (% p/p)	28,39 ± 2,17^{c}	39,59 ± 1,47^{c}	77,51 ± 14,93^{ab}	71,06 ± 6,47^{b}	90,82 ± 8,37^{a}

Media ± desviación estándar en la misma fila seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra ($n = 3$); nd: no determinado; SFL1, SFL2: Subproductos florales de azafrán de dos productores diferentes.

Respecto a los carotenoides, pigmentos orgánicos del grupo de los isoprenoides que se encuentran de forma natural en las plantas, ejercen varias funciones beneficiosas en el organismo debido a sus propiedades antioxidantes, entre otras. Según su estructura, los carotenos son una de las principales subclases de carotenoides, siendo el β-caroteno y el α-caroteno los dos tipos principales. El compuesto β-caroteno es el precursor de las crocinas, que son los principales componentes químicos del azafrán responsables de muchas de sus propiedades farmacológicas. Los estigmas de azafrán mostraron una alta proporción de β-caroteno total superior al 70% (Tabla 9). Respecto al contenido de ésteres de crocetina, se estudiaron los isómeros *trans*, que son los mayoritarios. Los resultados se presentan en la Tabla 10. La crocina *trans*-4-GG se encontró en concentraciones más altas que la crocina *trans*-3-Gg en todas las muestras de estigmas de azafrán. Los estigmas

españoles presentaron la proporción significativamente mayor de crocinas totales (17,04 ± 0,18%) y de crocina *trans*-4-GG (11,91 ± 0,13%). Estos resultados coincidieron con el contenido de ésteres de crocetina reportado por Moratalla-López et al. (2019), en el que estos compuestos representaban entre el 16% y el 28% de la composición del azafrán. Así, las diferencias encontradas entre los estigmas de diferente origen se deben a que el nivel de apocarotenoides puede variar debido al origen geográfico, condiciones de procesamiento y almacenamiento.

Tabla 10. Contenido de crocinas en azafrán y sus subproductos florales.

	***trans*-4-GG (% p/p)**	***trans*-3-Gg (% p/p)**	**Crocinas totales (% p/p)**
SFL1	0,009 ± 0,002^{d}	0,034 ± 0,005^{b}	0,042 ± 0,007^{c}
SFL2	nd	nd	-
Azafrán español	11,91 ± 0,13^{a}	5,13 ± 0,05^{a}	17,04 ± 0,18^{a}
Azafrán iraní	9,22 ± 0,30^{c}	3,98 ± 0,48^{a}	13,19 ± 0,78^{b}
Azafrán griego	10,74 ± 0,24^{b}	3,93 ± 0,53^{a}	14,67 ± 0,77^{b}

Media ± desviación estándar en la misma columna seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra ($n = 3$); nd: no detectado; SFL1, SFL2: Subproductos florales de azafrán de dos productores diferentes.

Los subproductos florales de azafrán presentaron un bajo contenido de β-caroteno total (28-39%) que se localiza principalmente en los estambres amarillos (Tabla 9). Además, las crocinas solo se detectaron en SFL1 pero en cantidades muy bajas (0,042%) debido a la presencia de trazas de estigmas durante el proceso manual de monda (Tabla 10).

Los resultados de las diferentes actividades antioxidantes del azafrán y sus subproductos florales mediante DPPH, ABTS y FRAP se muestran en la Tabla 9.

Los estigmas de azafrán presentaron una capacidad antioxidante mayor que los subproductos florales, lo que podría estar relacionado con su contenido más alto en flavonoides totales y crocinas, ya que diversos estudios han demostrado la alta actividad antioxidante de estos compuestos bioactivos (Chen et al., 2008). Sin embargo, los subproductos florales, SFL1 y SFL2, también mostraron una buena capacidad antioxidante que podría estar relacionada con su alto contenido en polifenoles totales. Estos resultados son similares a otros estudios que informaron del alto poder antioxidante tanto de los estigmas como de los tépalos de azafrán (Karimi et al., 2010; Muzaffar, Rather, Khan, & Akhter, 2016; Sun et al., 2020).

Además del estudio de la actividad antioxidante por diferentes métodos, también se determinó la actividad quelante del hierro (II) (Figura 12).

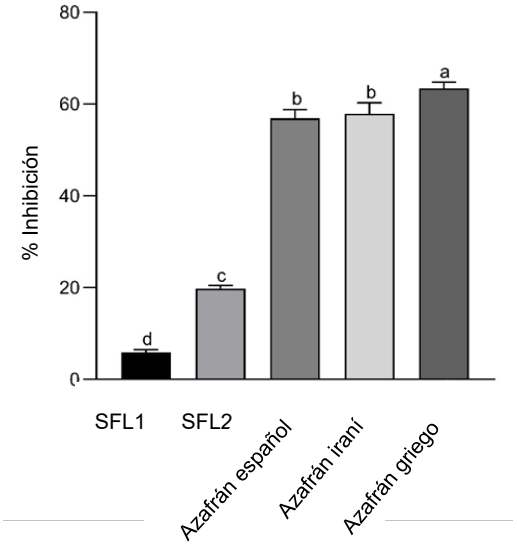

Figura 12. Actividad quelante de hierro (II) en azafrán y sus subproductos florales. Las barras de error representan la desviación estándar y las diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra ($n = 3$); SFL1, SFL2: Subproductos florales de azafrán de dos productores diferentes.

Los iones de metales de transición bivalentes, como el Fe^{2+}, juegan un papel importante como catalizadores de procesos oxidativos, participando en la generación de radicales hidroxilo mediante la reacción de Fenton (Cherrak et al., 2016). Así, el exceso de iones metálicos podría conducir a la formación de radicales libres generando altos niveles de estrés oxidativo, por lo que la quelación del hierro podría retrasar estos procesos oxidativos.

Los estigmas de azafrán presentaron una buena actividad quelante (60% de inhibición), siendo la más alta para el azafrán griego. Esta actividad podría deberse a su contenido en flavonoides, ya que estudios previos demostraron que estos compuestos bioactivos tienen la capacidad de neutralizar radicales libres y secuestrar iones metálicos, suprimiendo las reacciones de Fenton (Kejík et al., 2021). Sin embargo, los subproductos florales de azafrán indicaron una baja actividad quelante del hierro (menos del 20% de inhibición), siendo estos resultados similares a los de Sánchez-Vioque et al. (2012).

Por tanto, la actividad quelante de Fe^{2+} y la capacidad antioxidante que presenta el azafrán y sus subproductos florales podrían prevenir el daño oxidativo, y proteger contra el estrés oxidativo. Estas actividades podrían estar relacionadas con su contenido en compuestos bioactivos como β-caroteno, ésteres de crocetina y flavonoides en los estigmas de azafrán, y con las antocianinas y otros compuestos fenólicos en los subproductos florales.

4.3.2. Perfil volátil

Los metabolitos secundarios volátiles, además de contribuir al aroma de los alimentos, también presentan importantes propiedades bioactivas, como actividades antioxidantes, antimicrobianas y anticancerígenas (Aguiar, Gonçalves, Alves, & Câmara, 2021).

Los resultados del perfil volátil en los estigmas de azafrán se muestran en la Tabla 11. En todas las muestras de estigmas de azafrán, se identificaron y cuantificaron un total de 25 compuestos volátiles diferentes, siendo los grupos químicos más representativos cetonas (n=8), terpenos (n=8), aldehídos (n=3), ésteres (n=3), alcoholes (n=2) y ácidos (n=1). En cuanto a la concentración de los diferentes compuestos volátiles, el safranal (1,3-ciclohexadieno-1-carboxaldehído,2,6,6-trimetil-, V16) fue el compuesto encontrado en una cantidad superior en todas las muestras independientemente de su origen (7429-7656 µg/g). Estas concentraciones de safranal fueron superiores a las obtenidas por otros estudios (Culleré, San-Juan, & Cacho, 2011; Jalali-Heravi, Parastar, & Ebrahimi-Najafabadi, 2009; Kanakis, Daferera, Tarantilis, & Polissiou, 2004). Estas diferencias podrían deberse a distintas condiciones del procesamiento postcosecha, como el proceso de secado, ya que el safranal se forma a partir de la picrocrocina cuando se utilizan temperaturas superiores a los 50-55 °C.

Otros compuestos volátiles presentes en los estigmas del azafrán pertenecen a las cetonas (n=3) y los alcoholes (n=1), como la isoforona (V11); 1,4-ciclohexanodiona, 2,2,6-trimetil-(dihidrooxoforona, V14); 1-butanol, 3-metil- (alcohol isoamílico, V2) y 2,6,6-trimetil-2-ciclohexeno-1,4-diona (4-ceitoisoforona, V12). Otros estudios también indicaron que el safranal, la isoforona, la 4-cetoisoforona y la dihidrooxoforona fueron los principales volátiles encontrados en los estigmas de azafrán de origen italiano (Urbani, Blasi, Chiesi, Maurizi, & Cossignani, 2015).

Respecto a las muestras de subproductos florales de azafrán frescos y deshidratados por dos métodos diferentes (liofilización y estufa de aire), se identificaron y cuantificaron 55 compuestos volátiles (Tabla 12).

Los grupos químicos más representativos en todas las muestras fueron: ésteres (n=13), aldehídos (n=12) y ácidos y alcoholes (n=5). Todas las muestras presentaron ácido acético (V1), 2(5H)-furanona (V14) y alcohol feniletílico (V31). SFL2 (secado en estufa de aire) tuvo la mayor concentración de ácido acético (161,55 µg/g), seguido por SFL1 fresco (145,21 µg/g). SFL2 (secado en estufa de aire) presentó un contenido más alto de 2(5H)-furanona (V14) (452 µg/g), mientras que SFL1 (secado en estufa de aire) mostró la concentración más baja (4,11 µg/g). Sin embargo, la generación de furanona, como la

2(5H)-furanona, puede deberse a diversos procesos: mediante reacciones de oxidación, por microorganismos o mediante la reacción de Maillard (Badovskaya, Poskonin, Tyukhteneva, & Kozhina, 2021).

El alcohol feniletílico estaba presente en SFL2 (secado en estufa de aire) en altas concentraciones (35,63 µg/g). Sin embargo, se encontraron concentraciones mínimas en SFL1 (secado en estufa de aire) (0,49 µg/g). Este volátil también se encontró en otros extractos florales (rosa, jacinto, geranio), presentando aroma a rosa y propiedades antimicrobianas y antifúngicas, y es ampliamente utilizado en alimentos y cosméticos (de Melo Pereira et al., 2019).

Cabe señalar que el safranal (V39) solo se encontró en SFL2 (fresco y secado en estufa de aire), pero en cantidades muy bajas (0,11 y 7,53 µg/g, respectivamente). Esto podría deberse a la presencia de trazas de estigmas de azafrán en los subproductos florales, siendo la cantidad de safranal mayor en SFL2 (secado en estufa de aire) ya que se genera a las temperaturas utilizadas (50-55 °C) en el proceso de secado. Además, la isoforona (V35) también estaba presente en SFL2 (22,28 µg/g), que puede producirse a partir de safranal y/o de otros precursores generados durante el proceso de secado.

Otros compuestos a destacar por su alta concentración fueron 3-hidroxi 2-butanona (acetoína, V4) y ácido butanoico, 3-metilo (ácido isovalérico, V9). Estos volátiles se encontraron en SFL1 fresco en 299,42 µg/g y 177,52 µg/g, respectivamente. Las acetoínas, que existen ampliamente en la naturaleza, destacan por sus acciones antimicrobianas (Xiao & Lu, 2014). El ácido isovalérico es un compuesto presente de forma natural que puede formarse a partir del metabolismo secundario de las plantas (Dillon, 2014). Cabe destacar, que estos volátiles sólo se encontraron en muestras frescas, lo que significa que podrían ser susceptibles de degradarse con el calor durante el proceso de secado.

Algunos volátiles presentes en las flores frescas aumentaron en las flores deshidratadas debido al proceso de secado en estufa de aire como consecuencia del tratamiento térmico. Estos resultados se justifican por la generación de volátiles a partir de otros precursores no volátiles mediante el procesamiento térmico. Así, los procedimientos de preparación postcosecha, como el proceso de secado, son muy importantes ya que influyen notablemente en la composición volátil del producto final.

En resumen, el azafrán estudiado presentó altos niveles de safranal, lo que contribuye a la bioactividad y aroma de esta especia. La composición volátil de los subproductos florales de las muestras frescas y las deshidratadas por liofilización o estufa de aire estuvo altamente influenciada por el método de secado, presentando algunos compuestos con propiedades antioxidantes y antimicrobianas. Por lo tanto, estos

resultados sugieren que el azafrán y sus subproductos florales son fuentes naturales de compuestos antioxidantes que podrían usarse como ingredientes sostenibles e innovadores para su aplicación en el desarrollo de nuevos productos funcionales o para otras aplicaciones en salud humana.

Tabla 11. Identificación y concentración de la composición volátil en azafrán.

Código	Compuesto	TR (min)	IK (EXP)	IK (LIT)	Azafrán español (µg/g)	Azafrán iraní (µg/g)	Azafrán griego (µg/g)
V1	Ácido acético	2,422	645	646	18,30^{c}	40,40^{a}	35,80^{b}
V2	1-Butanol, 3-metil- (alcohol isoamílico)	5,445	744	750	311^{c}	770^{b}	1018^{a}
V3	Ciclopentanona	6,750	774	767	2,01^{a}	0,63^{c}	1,65^{b}
V4	2(5H)-Furanona	12,27	903	913	173^{a}	123^{b}	63,50^{c}
V5	Acetato de hexilo	16,070	1012	1012	3,62^{c}	10,10^{a}	9,36^{b}
V6	3-Ciclohexen-1-ona, 3,5,5-trimetil- (beta isoforona)	17,041	1040	1044	5,40^{c}	7,60^{b}	8,92^{c}
V7	Linalol	19,026	1098	1098	5,50^{a}	3,97^{b}	5,26^{a}
V8	Nonanal	19,159	1101	1101	7,40^{a}	1,71^{c}	6,08^{b}
V9	Ciclohexeno, 1-metil-4-(1-metiletilideno)- (terpinoleno)	19,256	1105	1098	49,00^{b}	49,20^{b}	59,50^{a}
V10	Alcohol feniletílico	19,389	1109	1110	22,80^{c}	39,80^{b}	110^{a}
V11	Isoforona	19,687	1119	1118	1477^{b}	1531^{b}	1936^{a}
V12	2,6,6-Trimetil-2-ciclohexeno-1,4-diona (4-cetoisoforona)	20,409	1142	1139	273^{c}	690^{b}	789^{a}
V13	2-Hidroxi-3,5,5-trimetil-ciclohex-2-enona	20,504	1145	1149	20,10^{b}	39,70^{a}	14,80^{c}
V14	1,4-Ciclohexanodiona, 2,2,6-trimetil- (dihidrooxoforona)	21,181	1166	1168	748^{c}	848^{a}	792^{c}
V15	Benzaldehído, 2,4-dimetil-	21,693	1183	1180	17,30^{a}	13,00^{b}	12,90^{b}
V16	1,3-Ciclohexadieno-1-carboxaldehído, 2,6,6-trimetil- (safranal)	22,191	1198	1197	7443^{a}	7656^{a}	7429^{a}
V17	2,4-Cicloheptadien-1-ona, 2,6,6-trimetil- (eucarvona)	22,817	1220	1222	54,00^{c}	120^{b}	127^{a}
V18	Ácido acético, 2-feniletil éster	23,757	1252	1250	8,30^{c}	31,60^{b}	72,90^{a}
V19	4-Hidroxi-3,5,5-trimetilciclohex-2-enona	25,339	1307	1317	16,60^{c}	54,10^{a}	35,40^{b}
V20	Benzaldehído, 2,4,6-trimetil-	25,563	1315	1323	4,40^{c}	6,89^{a}	5,01^{b}
V21	α-Cubebeno	27,321	1379	1372	4,00^{a}	1,36^{c}	3,20^{b}
V22	4-Hidroxi-2,6,6-trimetil-3-oxociclohexa-1-eno-1-carbaldehído	27,429	1383	1396	289^{b}	286^{b}	311^{a}
V23	4-Hidroxi-2,6,6-trimetilciclohex-1-enocarbaldehído (HTTC)	28,380	1418	1431	140^{c}	172^{b}	211^{a}
V24	2-Butanona, 4-(2,6,6-trimetil-1-ciclohexen-1-il)-	28,802	1434	1433	7,90^{a}	3,80^{c}	5,68^{b}
V25	3-Buten-2-ona, 4-(2,6,6-trimetil-1-ciclohexen-1-il)-	30,048	1480	1486	3,30^{c}	4,00^{b}	4,43^{c}
	Total				11104,03	12503,86	13067,39

Media ± desviación estándar en la misma fila seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra ($n = 3$);
TR: tiempo de retención; IK: índices de Kovats; EXP: experimental; LIT: literatura.

Tabla 12. Identificación y concentración de la composición volátil en subproductos florales de azafrán.

Código	Compuesto	TR (min)	IK (EXP)	IK (LIT)	SFL1 Fresco (µg/g)	SFL1 Liofilización (µg/g)	SFL1 Estufa de aire (µg/g)	SFL2 Fresco (µg/g)	SFL2 Liofilización (µg/g)	SFL2 Estufa de aire (µg/g)
V1	Ácido acético	2,441	646	645	145,21^{b}	5,09c^{d}	0,26^{d}	8,20^{c}	0,83^{d}	161,55^{a}
V2	Butanal, 3-metil- (isovaleraldehído)	3,370	686	690	13,66^{b}	nd	nd	nd	nd	28,30^{a}
V3	Butanal, 2-metil-	3,531	688	699	nd	nd	nd	nd	nd	50,93
V4	3-Hidroxi-2-butanona (acetoína)	4,531	721	722	299,42	nd	nd	nd	nd	nd
V5	1-Butanol, 2-metil-	5,617	748	748	5,84	nd	nd	nd	nd	nd
V6	Ácido propanoico, 2-metil-	6,163	757	760	3,13	nd	nd	nd	nd	nd
V7	2,3-Butanodiol	7,405	786	789	2,61	nd	nd	nd	nd	nd
V8	Hexanal	7,888	800	800	nd	1,52	nd	nd	nd	nd
V9	Ácido butanoico, 3-metil- (ácido isovalérico)	10,222	858	858	177,52	nd	nd	nd	nd	nd
V10	Ácido butanoico, 2-metil-	10,364	861	862	8,26	nd	nd	nd	nd	nd
V11	1,2-Propanodiol, 2-acetato	11,489	891	889	2,51	nd	nd	nd	nd	nd
V12	Heptanal	11,941	893	894	nd	3,96	nd	nd	nd	nd
V13	Acetato de 4-penten-1-ilo	11,962	890	895	nd	nd	3,67	nd	nd	nd
V14	2(5H)-Furanona	12,104	913	906	4,42^{b}	38,01^{b}	4,11^{b}	14,64^{b}	4,95^{b}	452^{a}
V15	Butirolactona	12,127	908	906	nd	24,47	nd	nd	nd	nd
V16	Ácido acético, éster pentílico	12,449	912	914	3,40^{e}	3,95^{d}	11,12^{a}	8,85^{b}	5,81^{c}	nd
V17	2-Furancarboxaldehído, 5-metil-	14,186	961	960	nd	nd	nd	nd	nd	6,92
V18	1-Butanol, 3-metil-, propanoato	14,551	969	970	nd	0,56^{d}	3,16^{a}	2,20^{b}	0,84^{c}	nd
V19	Ácido carbólico	14,816	976	977	7,60	nd	nd	nd	nd	nd
V20	Éter diisoamil	15,746	1002	1002	nd	nd	1,13	nd	nd	nd
V21	Ácido acético, éster hexílico	16,080	1011	1012	nd	1,13^{c}	4,22^{a}	2,86^{b}	0,90^{d}	nd
V22	1-Hexanol, 2-etil-	16,653	1029	1029	nd	nd	nd	nd	13,38	nd
V23	D-Limoneno	16,657	1029	1029	nd	nd	nd	0,59	nd	nd
V24	Bencenoacetaldehído	17,099	1043	1042	nd	3,86^{c}	nd	4,27^{b}	1,11^{d}	13,39^{a}
V25	Ácido butanoico, éster pentílico	17,596	1059	1056	nd	nd	0,65^{a}	0,58^{b}	nd	nd
V26	Etanona, 1-(1H-pirrol-2-il)-	17,717	1060	1060	nd	nd	nd	nd	nd	25,28
V27	Benzaldehído, 4-metil-	18,448	1080	1081	nd	0,53	nd	nd	nd	nd
V28	2-Nonanona	18,734	1089	1089	3,44	nd	nd	nd	nd	nd
V29	Linalol	19,031	1098	1098	2,46^{a}	nd	nd	nd	0,77^{b}	nd
V30	Nonanal	19,193	1102	1103	nd	12,40^{b}	0,62^{c}	nd	0,94^{c}	14,98^{a}
V31	Alcohol feniletílico	19,404	1110	1110	7,37^{d}	10,03^{c}	0,49^{f}	16,64^{b}	3,89^{e}	35,63^{a}

V32	4H-Piran-4-ona, 2,3-dihidro-3,5-dihidroxi-6-metil- (DDMP)	20,326	1140	1139	nd	nd	nd	nd	nd	62,83
V33	Éster 2-etilhexílico del ácido acético	20,559	1157	1147	nd	nd	nd	0,52	nd	nd
V34	2(3H)-Furanona, dihidro-4-hidroxi-	20,609	1153	1148	nd	109,82^{a}	nd	nd	9,01^{b}	nd
V35	Isoforona	20,675	1146	1150	nd	nd	nd	nd	nd	22,28
V36	Ciclohexanona, 5-metil-2-(1-metiletil)-, trans-	20,859	1152	1156	nd	nd	nd	nd	0,60	nd
V37	Ácido acético, fenilmetil éster	21,000	1161	1161	nd	nd	nd	0,30^{b}	0,84^{a}	nd
V38	Ciclohexanol, 5-metil-2-(1-metiletil)-, (1α,2β,5α)- (mentol)	21,529	1171	1177	nd	nd	nd	nd	0,98	nd
V39	1,3-Ciclohexadieno-1-carboxaldehído, 2,6,6-trimetil- (safranal)	22,180	1201	1198	nd	nd	nd	0,11^{b}	nd	7,53^{a}
V40	Benzofurano, 2,3-dihidro-	22,630	1219	1213	2,59	nd	nd	nd	nd	nd
V41	2-Dodeceno, (Z)-	22,375	1213	1205	nd	nd	nd	nd	1,20	nd
V42	3-Dodeceno, (Z)-	22,634	1222	1214	nd	nd	nd	nd	1,14	nd
V43	Benzaldehído, 2,4-dimetil-	22,659	1208	1214	nd	nd	0,32	nd	nd	nd
V44	2-Ciclohexen-1-ona, 2-metil-5-(1-metiletil)-, (S)-	23,656	1250	1249	nd	nd	nd	nd	nd	14,98
V45	Benceno, 1,3-bis(1,1-dimetiletil)-	23,668	1249	1249	nd	3,75^{a}	0,47^{b}	0,55^{b}	nd	nd
V46	Ácido acético, 2-feniletil éster	23,773	1254	1252	9,96^{b}	2,34^{d}	nd	32,42^{a}	3,72^{c}	nd
V47	Bencenoacetaldehído, α-etiliden-	24,200	1273	1267	nd	nd	nd	0,97	nd	nd
V48	2-Propenal, 3-fenil-	24,337	1278	1272	nd	nd	nd	0,50	nd	nd
V49	Dodecano, 4,6-dimetil-	24,450	1285	1276	nd	nd	0,34^{c}	0,67^{bc}	0,92^{b}	16,12^{a}
V50	2-Undecanona	24,903	1291	1291	0,73	nd	nd	nd	nd	nd
V51	α-Cubebeno	27,334	1380	1379	nd	nd	nd	1,00^{a}	0,53^{b}	nd
V52	Dodecanal	28,113	1409	1408	nd	nd	nd	nd	1,79	nd
V53	1-Dodecanol	29,900	1474	1475	nd	nd	nd	nd	4,63	nd
V54	Acetato de laurilo	32,679	1605	1606	nd	nd	0,48	nd	nd	nd
V55	Ácido hexadecanoico, metil éster	37,034	1921	1921	0,93	nd	nd	nd	nd	nd
	Total				700,13	217,46	27,37	93,90	58,78	833,49

Media ± desviación estándar en la misma fila seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra ($n = 3$); TR: tiempo de retención; IK: índices de Kovats; EXP: experimental; LIT: literatura; nd: no detectado; SFL1, SFL2: Subproductos florales de azafrán de dos productores diferentes.

4.4. Optimización del proceso de extracción de compuestos bioactivos

4.4.1. Extracción asistida por microondas (MAE)

Para optimizar el procedimiento de extracción de compuestos bioactivos por MAE de los subproductos florales del azafrán mediante un diseño de experimentos, se evaluó el efecto del tiempo de extracción, temperatura y concentración del solvente etanol sobre el rendimiento, el contenido en polifenoles y flavonoides totales, y la capacidad antioxidante (ORAC, HOSC) de los extractos.

Los resultados del rendimiento, el contenido en polifenoles y flavonoides totales, y la capacidad antioxidante (ORAC, HOSC) de los extractos obtenidos por MAE se muestran en la Tabla 13.

Tabla 13. Resultados del contenido en polifenoles y flavonoides totales, el rendimiento y la capacidad antioxidante (ORAC, HOSC) de los extractos de subproductos florales de azafrán obtenidos mediante extracción asistida por microondas.

Extracción	Tiempo (min)	Temperatura (°C)	Etanol (% v/v)	Polifenoles totales (mg GAE/g peso seco)	Flavonoides totales (mg CE/g peso seco)	Rendimiento (% p/p)	ORAC (µmol TEAC/g peso seco)	HOSC (µmol TEAC/g peso seco)
1	0,5	25	0	93,87 ± 3,33^{cd}	3,56 ± 0,23^{efg}	20,65	4777 ± 352^{bcd}	2286 ± 583^{abc}
2	5	25	0	80,54 ± 1,56^{ef}	3,33 ± 0,039^{fg}	25,15	2679 ± 504^{fg}	2034 ± 253^{cd}
3	0,5	100	0	54,82 ± 4,33^{gh}	3,26 ± 0,25^{fg}	36,40	2170 ± 304^{g}	1281 ± 230^{d}
4	5	100	0	58,62 ± 4,44^{g}	3,13 ± 0,03^{g}	32,35	2175 ± 268^{g}	2117 ± 127^{bcd}
5	0,5	25	100	52,12 ± 1,75^{gh}	5,62 ± 0,02^{c}	23,65	2019 ± 439^{g}	1716 ± 44^{cd}
6	5	25	100	126,20 ± 2,99^{a}	6,80 ± 0,33^{b}	17,10	5128 ± 303^{bc}	3131 ± 205^{a}
7	0,5	100	100	49,19 ± 1,67^{h}	7,41 ± 0,35^{ab}	29,70	3451 ± 443^{ef}	1623 ± 277^{cd}
8	5	100	100	75,47 ± 1,02^{f}	8,05 ± 0,11^{a}	27,00	4026 ± 84de	2124 ± 383^{bcd}
9	6,2	62,5	50	105,50 ± 3,05^{b}	4,23 ± 0,14^{de}	36,75	5027 ± 351^{bc}	2444 ± 112^{abc}
10	3,15	11,75	50	85,13 ± 2,13^{e}	4,15 ± 0,42^{de}	31,80	5641 ± 384^{ab}	2407 ± 315^{abc}
11	3,15	113,24	50	120,70 ± 3,69^{a}	3,67 ± 0,15^{defg}	27,75	4556 ± 390^{cd}	1779 ± 272^{cd}
12	3,15	62,5	50	86,97 ± 1,38^{de}	4,34 ± 0,38^{d}	23,60	4637 ± 37^{cd}	1995 ± 131^{cd}
13	3,15	62,5	50	96,77 ± 3,46^{bc}	3,99 ± 0,22^{def}	34,15	3278 ± 121^{ef}	2240 ± 265^{bc}
14	3,15	62,5	50	79,28 ± 3,14^{ef}	4,14 ± 0,31^{de}	25,70	6219 ± 246^{a}	2944 ± 407^{ab}

Media ± desviación estándar en la misma columna seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra ($n = 3$).

Los valores de polifenoles totales obtenidos en el experimento 6 (25 °C, 5 min, 100% etanol) fueron los óptimos (126,20 ± 2,99 mg GAE/g), siendo superiores a los indicados en estudios anteriores que utilizaron MAE en subproductos florales de azafrán, obteniendo valores de 40-50 mg GAE/g (Álvarez et al., 2021). Sin embargo, los valores de polifenoles totales fueron más bajos en condiciones de alta temperatura y mayor tiempo de extracción. Este hecho podría deberse a una degradación térmica inicial de los compuestos fenólicos libres en los extractos florales a los 5 min, y/o a la

disminución de la constante dieléctrica y la polaridad del agua y el etanol a altas temperaturas, disminuyendo su capacidad para disolver compuestos polares (Hassas-Roudsari, Chang, Pegg, & Tyler, 2009; Sólyom, Solá, Cocero, & Mato, 2014). Por tanto, al aumentar el tiempo de extracción mientras se disminuye la temperatura y se mantiene constante la proporción de etanol en 50% o 100%, se aumenta el rendimiento de extracción de estos compuestos bioactivos.

Con respecto al contenido en flavonoides totales, se observa que los resultados óptimos se encontraron en las extracciones 5, 6, 7 y 8, donde la concentración de etanol utilizada fue del 100% (5,62–8,05 mg CE/g). Estos resultados concuerdan con investigaciones previas donde la proporción de solventes fue el factor que tuvo el efecto más significativo en la extracción de antocianinas (Jafari, Mahdavee Khazaei, & Assadpour, 2019). Estos valores obtenidos fueron similares a los descritos por Sun et al. (2020), obteniendo valores inferiores a 10 mg/g en extractos de tépalos de azafrán.

Además, el rendimiento de extracción aumentó con el incremento de la temperatura de extracción (62,5 °C y 100 °C), siendo el experimento 9 (62,5 °C y 50% etanol) el que presentó las condiciones óptimas conduciendo a un 36,75% de rendimiento. Los rendimientos de extracción obtenidos fueron superiores a los de Hashemi Gahruie et al. (2020) en el que los valores de rendimiento, utilizando MAE para extraer compuestos bioactivos de tépalos de azafrán iraníes, fueron entre 8,07% y 19,42%.

Respecto a la capacidad antioxidante mediante ORAC y HOSC, las condiciones de extracción del experimento 6 fueron las óptimas, alcanzando valores de 5128 ± 303 µmol TEAC/g y 3131 ± 205 µmol TEAC/g, para ORAC y HOSC, respectivamente. En todos estos experimentos, la temperatura estuvo por debajo de 100 °C y la proporción de etanol superior al 50%. Las reacciones involucradas en estos métodos analíticos están asociadas a reacciones de oxidación enzimáticas y no enzimáticas, por lo que estos resultados pueden deberse a la oxidación de compuestos fenólicos a altas temperaturas que conducen a la pérdida de su actividad antioxidante (Larrauri, Sánchez-Moreno, & Saura-Calixto, 1998). Los resultados de ORAC fueron comparables con los obtenidos por Sun et al. (2020), en los que los tépalos de azafrán mostraron una fuerte capacidad de eliminación de radicales de oxígeno.

En este estudio, la actividad antioxidante mostró correlación con los valores del contenido en polifenoles totales, ya que en los experimentos 6 y 9 los extractos presentaron una alta concentración de compuestos fenólicos y una alta capacidad antioxidante. En cuanto a los valores de flavonoides totales, los resultados mostraron

que el experimento óptimo también fue el 6, utilizando 100% de etanol como solvente de extracción durante 5 min a baja temperatura (25 °C).

Así, los resultados de ORAC y HOSC revelaron las actividades antioxidantes de los subproductos florales del azafrán. Este poder antioxidante podría estar relacionado con su contenido bioactivo, como los polifenoles, ya que son compuestos naturales antioxidantes (Yashin, Yashin, Xia, & Nemzer, 2017).

En resumen, a escala de laboratorio, los resultados mostraron que las condiciones óptimas de MAE para la extracción de compuestos bioactivos son el uso de etanol como solvente primario (50% o 100%) y bajas temperaturas. Por tanto, esta información podría ayudar a seleccionar el método y las condiciones de extracción más adecuadas para obtener compuestos de interés a partir de fuentes vegetales naturales, incluido su escalado a nivel industrial.

4.4.2. Extracción asistida por ultrasonidos (UAE)

Para optimizar el procedimiento de extracción de compuestos bioactivos por UAE de los subproductos florales del azafrán mediante un diseño de experimentos, se evaluó el efecto del tiempo de extracción, la potencia y concentración de NaDES sobre el contenido en polifenoles y flavonoides totales, y la capacidad antioxidante (DPPH) de los extractos.

Los resultados del contenido en polifenoles y flavonoides totales, y la capacidad antioxidante (DPPH) de los extractos obtenidos por UAE se presentan en la Tabla 14.

Los valores del contenido en polifenoles totales fueron menores utilizando como condiciones de extracción un tiempo más corto, una proporción de NaDES del 30% y 60 W o 120 W de potencia. Sin embargo, los valores fueron mayores usando 20 o 35 min, 60 o 90% de NaDES y 180 W de potencia. La extracción óptima fue utilizando 20 min, 180 W y 90% de NaDES (88,96 ± 1,08 mg GAE/g dw), lo que indica que aumentar el tiempo de extracción y la proporción de NaDES, manteniendo constante la potencia a 180 W, conduce a un aumento del rendimiento de extracción de estos compuestos bioactivos. Este hecho podría deberse al efecto significativo de la energía en la extracción acelerando la degradación de las células vegetales, debido a un rápido aumento de temperatura y presión, aumentando la permeabilidad y liberación de compuestos intracelulares y mejorando la transferencia y difusión de masa (González-Centeno, Comas-Serra, Femenia, Rosselló, & Simal, 2015). Además, una proporción mayor de NaDES podría mejorar la solubilización de metabolitos no solubles en agua.

Respecto al contenido en flavonoides totales, la concentración en el extracto estuvo afectada positivamente por la temperatura y la proporción de NaDES, siendo la extracción óptima en condiciones de 20 min de extracción, 60% de solvente NaDES, y 120 W (5,49 ± 0,05 mg CE/g). Estos valores fueron más altos que los de estudios previos que mostraron valores de 130 mg/100 g en extractos de tépalos de azafrán obtenidos por UAE (Hashemi Gahruie et al., 2020) . Además, estos resultados fueron similares a los obtenidos por MAE.

Tabla 14. Resultados del contenido en polifenoles y flavonoides totales y la capacidad antioxidante (DPPH) de los extractos de subproductos florales de azafrán y estigmas griegos obtenidos mediante extracción asistida por ultrasonidos.

	Extracción	Tiempo (min)	Potencia (W)	NaDES (% p/p)	Polifenoles totales (mg GAE/g peso seco)	Flavonoides totales (mg CE/g peso seco)	DPPH (IC_{50} mg extracto/mL)
Subproductos florales	1	35	180	60	42,27 ± 2,55^{b}	3,60 ± 0,32^{cde}	6,64 ± 0,24^{b}
	2	20	60	30	27,89 ± 0,61^{cde}	3,10 ± 0,07^{e}	-
	3	20	120	60	27,10 ± 0,83^{cde}	3,67 ± 0,08^{cde}	-
	4	20	120	60	28,21 ± 2,41^{cd}	4,60 ± 0,04^{abc}	-
	5	35	60	60	29,38 ± 2,13^{c}	4,93 ± 0,25^{ab}	-
	6	20	180	30	22,15 ± 0,02^{efg}	3,15 ± 0,08^{e}	-
	7	5	60	60	26,66 ± 0,45^{cdef}	4,54 ± 0,03^{abcd}	-
	8	20	60	90	5,99 ± 0,15^{h}	1,30 ± 0,06^{f}	-
	9	20	180	90	88,96 ± 1,08^{a}	4,36 ± 0,48^{bcd}	2,06 ± 0,15^{a}
	10	35	120	90	20,17 ± 0,67^{g}	0,60 ± 0,11^{f}	-
	11	20	120	60	29,26 ± 0,58^{c}	4,77 ± 0,14^{ab}	-
	12	20	120	60	27,49 ± 2,23^{cde}	5,49 ± 0,05^{a}	-
	13	35	120	30	26,01 ± 2,57^{cdefg}	3,02 ± 0,13^{e}	-
	14	5	120	90	31,56 ± 0,09^{c}	2,73 ± 0,24^{e}	6,36 ± 0,51^{b}
	15	5	180	60	23,24 ± 1,68^{defg}	3,59 ± 0,42^{de}	-
	16	5	120	30	21,17 ± 0,78^{fg}	2,75 ± 0,56^{e}	-
Azafrán griego		20	180	90	95,66 ± 9,34	9,56 ± 0,60	2,74 ± 0,47

Media ± desviación estándar en la misma columna seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra ($n = 3$).

En cuanto a los estigmas de azafrán griego, se llevó a cabo la extracción utilizando las condiciones de la extracción 9, ya que fue la extracción óptima considerando la maximización simultánea de los valores de polifenoles y flavonoides totales para los subproductos florales de azafrán (20 min, 90% de NaDES y 180 W). Los resultados mostraron que los extractos de estigmas de azafrán poseen un alto contenido de polifenoles (95,66 ± 9,34 mg GAE/g) y flavonoides totales (9,56 ± 0,60 mg CE/g) (Tabla 14).

Como se muestra en la Tabla 14, la actividad antioxidante se determinó en los extractos con mayor contenido bioactivo. Todos los extractos estudiados mostraron un poder antioxidante notablemente alto mediante el ensayo DPPH. La mayor capacidad de eliminación de radicales DPPH la presentó el extracto 9 de los subproductos florales

de azafrán (IC_{50} = 2,06 ± 0,15 mg/ml). Además, los extractos de estigmas de azafrán griego también mostraron una alta actividad antioxidante (IC_{50} = 2,74 ± 0,47 mg/mL).

Por tanto, MAE y UAE son técnicas eficientes y sostenibles para extraer compuestos de alto valor añadido a partir de subproductos florales de azafrán. Además, con ambas tecnologías se mejora la extracción del contenido en polifenoles totales obtenidos mediante sonicación, tanto en los subproductos florales como en los estigmas de azafrán griego (32,82 ± 2,23 y 34,00 ± 3,22 mg GAE/g, respectivamente). Así, estos extractos ricos en compuestos antioxidantes naturales podrían considerarse como una fuente de ingredientes bioactivos prometedores para enriquecer alimentos y mejorar sus propiedades funcionales.

4.5. Estabilización de los extractos mediante hidrogeles

Los extractos con NaDES óptimos obtenidos por UAE (extracto 9 de los subproductos florales de azafrán y el extracto de azafrán griego) se incorporaron en hidrogeles de quitosano/alginato para mejorar su estabilidad, con el fin de estudiar su potencial uso como formulación para el desarrollo de nuevos alimentos.

La capacidad de absorción de agua y la estabilidad estructural de los hidrogeles es un factor importante para su uso práctico, ya que podría existir una relación directa entre las propiedades de hinchamiento de los hidrogeles y la permeabilidad del soluto (Gehrke, Fisher, Palasis, & Lund, 2006). Los resultados de la capacidad de absorción de agua mostraron que todos los hidrogeles de quitosano/alginato tuvieron una absorción máxima a los 20 min (Figura 13A). Con respecto a los datos de la capacidad de retención de agua (Figura 13B), los hidrogeles que contenían los extractos de estigmas de azafrán presentaron la mayor capacidad de retención de agua, alrededor del 80% tras 1 h, en comparación con los hidrogeles con extractos de subproductos florales (20%), cuya capacidad de retención de agua disminuyó con el tiempo siendo inferior al 20% a las 3 h. Sin embargo, la capacidad de retención de agua de los hidrogeles con azafrán se mantuvo estable a partir de las 2 h (60%), mostrando propiedades adecuadas para retener grandes cantidades de agua.

Estos hidrogeles de quitosano/alginato podrían ser candidatos prometedores para retener y preservar compuestos de alto valor añadido, por ello se evaluó el impacto de la digestión *in vitro* en la estabilidad de los compuestos fenólicos de los extractos incorporados.

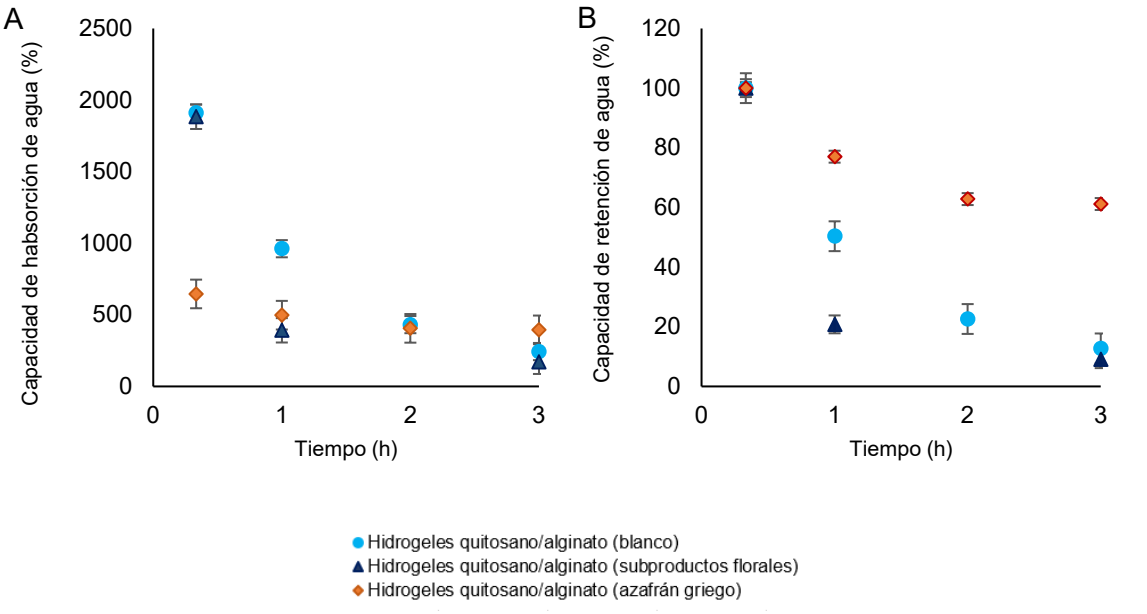

Figura 13. Capacidad de absorción de agua (A) y capacidad de retención de agua (B) de los hidrogeles desarrollados. Las barras de error representan la desviación estándar (*n* = 3).

En la Figura 14 se muestra el contenido de polifenoles totales de los diferentes hidrogeles durante la digestión oral, gástrica e intestinal *in vitro*. Los resultados mostraron que en condiciones gástricas solo se detectó el contenido de polifenoles totales en los hidrogeles de quitosano/alginato con extractos de azafrán, siendo el valor muy bajo, aunque superior al determinado en la fase oral. Este aumento paulatino puede deberse a la liberación de algunos compuestos fenólicos unidos a carbohidratos en condiciones de bajo pH y por la acción de la pepsina (Cunha et al., 2018). Sin embargo, hubo un aumento en el contenido de polifenoles totales tras 1 h de digestión intestinal en todos los hidrogeles, permaneciendo prácticamente sin cambios en los hidrogeles con extractos de azafrán tras 2 h de digestión intestinal. Este hecho podría deberse a las condiciones intestinales (pH neutro, presencia de enzimas pancreáticas y sales biliares) que conducirían a una mejora en la liberación de compuestos bioactivos (Ketnawa, Reginio Jr, Thuengtung, & Ogawa, 2022). Así, tras el proceso de digestión gastrointestinal *in vitro*, los valores de polifenoles totales fueron muy altos, permaneciendo estables en la matriz del hidrogel.

Por tanto, estos novedosos hidrogeles de quitosano/alginato mostraron propiedades favorables como matrices adecuadas para incorporar extractos bioactivos que pueden usarse como candidatos prometedores para diversas aplicaciones tanto en alimentación como en cosmética, aunque es necesario realizar más investigaciones para identificar los compuestos fenólicos y estudiar si estos compuestos bioactivos son absorbidos en el colon para ejercer sus efectos beneficiosos.

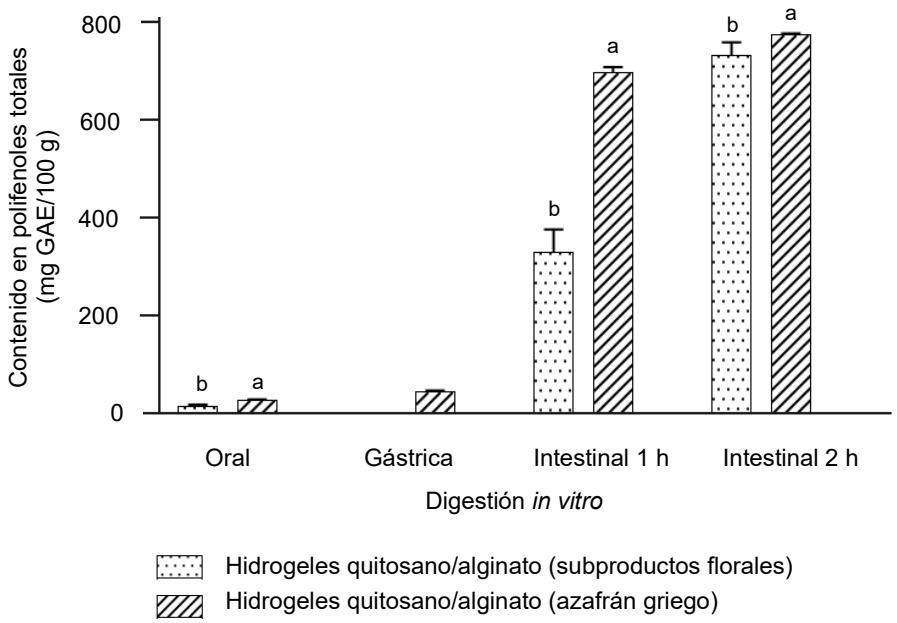

Figura 14. Contenido de polifenoles totales en los hidrogeles de quitosano/alginato desarrollados durante la digestión oral y gastrointestinal *in vitro*. Las barras de error representan la desviación estándar y las diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra ($n = 3$).

4.6. Incorporación en matrices alimentarias

4.6.1. Fortificación del yogur con extractos encapsulados de azafrán y sus subproductos florales: estabilidad durante el almacenamiento

En cuanto a la caracterización de las microcápsulas desarrolladas, el contenido de polifenoles totales en los encapsulados con extractos de subproductos florales de azafrán y de estigmas de azafrán de origen español fue de 1,243 mg de GAE/g y 1,065 mg de GAE/g, siendo la EE del 55,66% y 67,55%, respectivamente. Además, los resultados de la capacidad de absorción de agua indicaron que el contenido de agua aumentó en un 3050% y 2672%, después de 3 h, en los encapsulados con extractos de subproductos florales y de estigmas de azafrán, respectivamente, presentando la misma tendencia (Figura 15). Sin embargo, tras 4 h, las microcápsulas se desintegraron. Este hecho podría deberse a la presencia de iones de sodio en el tampón fosfato que ingresan en la matriz y se produce un intercambio iónico con iones de Ca^{2+}, lo que conlleva a un aumento de la capacidad de absorción de agua por relajación de las cadenas de alginato. Así, estas interacciones iónicas influyen en la posterior desintegración, ya que las cadenas de alginato comienzan a degradarse y disolverse en el medio (Bajpai & Tankhiwale, 2006).

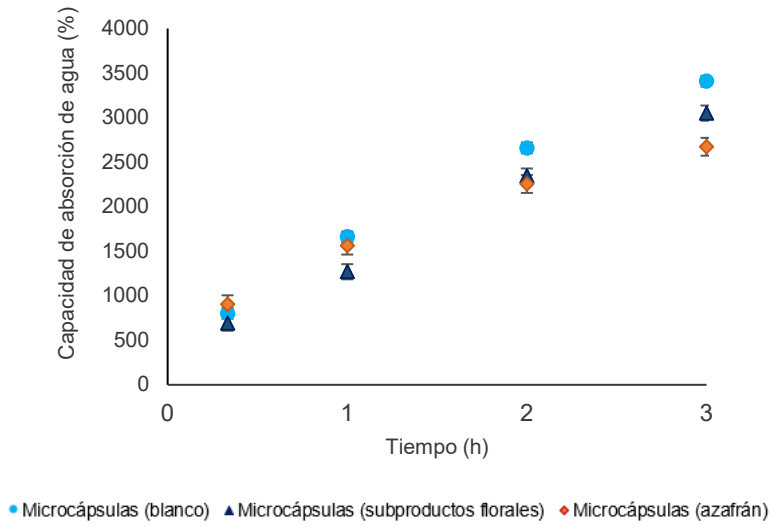

Figura 15. Capacidad de absorción de agua de las microcápsulas desarrolladas. Las barras de error representan la desviación estándar (*n* = 3).

Respecto al metabolismo de ácidos orgánicos, azúcares y las propiedades físico-químicas de las diferentes formulaciones de yogur durante el almacenamiento, los resultados se muestran en la Tabla 15.

Como resultado del metabolismo de las bacterias lácticas, los yogures presentaron ácido láctico, fórmico y propiónico. El ácido láctico fue el principal compuesto producido y se encontró en la concentración más alta en todas las muestras (1,51–2,73 mg/g). Además, en algunas formulaciones de yogur (YC, YB0,5, YB1, YSF0,5) hubo un aumento de este ácido orgánico con el tiempo, durante los 21 días de almacenamiento. Este aumento de ácido láctico se debió a la fermentación por parte de las bacterias lácticas, a partir de la lactosa. Por tanto, los extractos de azafrán no afectaron al proceso de fermentación natural en las nuevas formulaciones de yogur desarrolladas. El ácido fórmico fue el segundo ácido orgánico predominante en diversas formulaciones de yogur (0,74 – 1,96 mg/g de yogur). Su contenido fluctuó durante el período de almacenamiento en todas las muestras, lo que sugiere la producción y consumo de este ácido orgánico por el metabolismo de las bacterias lácticas (da Costa, Frasao, Lima, Rodrigues, & Junior, 2016). El ácido propiónico y cítrico también se cuantificaron, pero en cantidades más bajas.

Estos resultados concuerdan con Ndhlala, Kavaz Yüksel and Yüksel (2022) en el que yogures enriquecidos con alcachofa presentaron ácido láctico, cítrico y propiónico, cuya concentración fluctuó durante el tiempo de almacenamiento.

Por consiguiente, estos cambios en el contenido de ácidos orgánicos conllevaron a cambios de pH durante el período de almacenamiento a 4 °C. En la Tabla 16 se muestran los parámetros fisicoquímicos de las diferentes formulaciones de yogur

durante 21 días de almacenamiento a 4 °C. Los resultados indicaron que hubo una disminución del pH durante los primeros siete días en todas las muestras. Sin embargo, tras 7 días de almacenamiento no se observaron diferencias significativas hasta el final del período de almacenamiento. Esta disminución del pH está relacionada con la fermentación de la lactosa, y la producción de ácidos orgánicos. Sin embargo, la acidez titulable permaneció constante durante todo el período de almacenamiento para las diferentes formulaciones con valores entre 0,26% en YSF1SS0,1 y 0,41% en YSF0,5SS0,05. Este hecho puede deberse a la presencia de algunos ácidos orgánicos predominantes respecto al ácido láctico durante el período de almacenamiento evaluado.

Como se muestra en la Tabla 15, durante el proceso de fermentación láctica, la concentración de lactosa disminuyó significativamente durante el tiempo de almacenamiento debido a su conversión en ácido láctico por la fermentación bacteriana, en las muestras YSF0,5, YSF1, YSF0,5SS0,05 y YSF1SS0,1. Además de la lactosa, que fue predominante en todas las muestras de yogur (12,57-18,85 mg/g), la glucosa y la galactosa también se encontraron, aunque en concentraciones más bajas (0,26-0,91 mg/g y 1,67-3,25 mg/g, respectivamente), como resultado de la hidrólisis de la lactosa por la actividad metabólica bacteriana (Vénica, Perotti, & Bergamini, 2014). La concentración de galactosa fue mayor que la de glucosa, dado que esta última fue metabolizada por las bacterias lácticas.

Además, se estudió el contenido de inulina como parte de la fibra dietética soluble. La concentración de inulina no cambió en ninguna de las formulaciones de yogur durante el almacenamiento ya que las bacterias lácticas metabolizaron los azúcares disponibles (galactosa, glucosa y lactosa) como principales fuentes de energía. Las muestras con extractos de subproductos florales de azafrán presentaron el mayor contenido de inulina (1,93-2,54 mg/g) que se vieron reflejadas en el alto contenido en fibra encontrado en las flores de azafrán. Al mismo tiempo, la inulina contribuyó a un aumento en los SST, ya que los yogures con mayor concentración de inulina presentaron valores de SST más altos (9 °Brix) (Tabla 16). Este aumento de SST podría deberse a la presencia de fructosa en la composición de la inulina. En cuanto a la actividad del agua, no se vio afectada ni por el período de almacenamiento ni por la formulación del yogur (0,89-0,98). Respecto al índice de sinéresis, fue mayor durante los primeros 7 días de almacenamiento debido a la reducción del pH que conllevó a una mayor liberación de suero debido al efecto de contracción en la matriz micelar de caseína (Akgün et al., 2020).

Tabla 15. Contenido de ácidos orgánicos, azúcares solubles e inulina (mg/g de yogur) de las diferentes formulaciones de yogur durante 21 días de almacenamiento a 4 °C.

	Tiempo (días)	YC	YB0,5	YB1	YSF0,5	YSF1	YSF0,5SS0,05	YSF1SS0,1
Ácido cítrico	0	0,17 ± 0,01^{b}	0,18 ± 0,00^{b}	0,16 ± 0,01^{b}	0,18 ± 0,00^{b}	0,18 ± 0,02^{b}	0,24 ± 0,00^{Aa}	0,18 ± 0,00^{Bb}
	7	nd	0,18 ± 0,03^{bc}	0,18 ± 0,00^{bc}	0,19 ± 0,00^{b}	0,17 ± 0,00^{c}	0,19 ± 0,00^{Bb}	0,30 ± 0,00^{Aa}
	14	nd	nd	nd	nd	nd	nd	nd
	21	nd	nd	nd	nd	nd	nd	nd
Ácido láctico	0	1,76 ± 0,08^{Cb}	1,84 ± 0,02^{Cb}	1,51 ± 0,00^{Cb}	1,81 ± 0,00^{Db}	1,82 ± 0,35^{ab}	2,36 ± 0,04^{a}	1,90 ± 0,14^{Bab}
	7	2,71 ± 0,02^{Aa}	2,52 ± 0,07^{Bab}	2,13 ± 0,06^{Bde}	2,44 ± 0,01^{Abc}	2,08 ± 0,00^{e}	2,30 ± 0,01^{cd}	2,69 ± 0,07^{Aa}
	14	2,51 ± 0,11^{Aa}	2,58 ± 0,05^{Ba}	1,98 ± 0,00^{Bc}	2,07 ± 0,00^{Cbc}	1,92 ± 0,00^{c}	2,15 ± 0,00^{b}	1,72 ± 0,00^{B}
	21	2,23 ± 0,06^{Bbc}	3,19 ± 0,02^{Aa}	2,73 ± 0,07^{Aab}	2,20 ± 0,00^{Bbc}	1,96 ± 0,00^{c}	2,27 ± 0,00^{bc}	1,78 ± 0,00^{Bc}
Ácido fórmico	0	1,48 ± 0,00^{Bb}	1,21 ± 0,03^{Bc}	0,74 ± 0,00^{Bd}	1,15 ± 0,02^{Bc}	0,40 ± 0,08^{Bf}	1,87 ± 0,03^{Aa}	0,56 ± 0,05^{e}
	7	1,96 ± 0,01^{Aa}	1,52 ± 0,03^{ABb}	0,92 ± 0,05^{Ad}	1,16 ± 0,02^{Ac}	0,35 ± 0,06^{Be}	1,48 ± 0,05^{Bb}	0,76 ± 0,10^{d}
	14	1,44 ± 0,05^{Ba}	1,37 ± 0,01^{ABa}	0,87 ± 0,03^{ABb}	0,99 ± 0,00^{Cb}	0,60 ± 0,04^{Ac}	1,46 ± 0,08^{Ba}	0,54 ± 0,04^{c}
	21	1,20 ± 0,00^{Cc}	1,62 ± 0,01^{Aa}	0,94 ± 0,03^{Ad}	0,82 ± 0,00^{De}	0,46 ± 0,01^{Bg}	1,42 ± 0,00^{Bb}	0,61 ± 0,05^{f}
Ácido propiónico	0	0,51 ± 0,12^{Ab}	0,58 ± 0,21^{b}	0,62 ± 0,07^{b}	0,54 ± 0,05^{ABb}	0,57 ± 0,04^{ABb}	0,62 ± 0,03^{Aab}	0,92 ± 0,06^{Aa}
	7	0,77 ± 0,18^{A}	0,78 ± 0,19	0,60 ± 0,02	0,86 ± 0,23^{A}	0,67 ± 0,09^{A}	0,55 ± 0,08^{AB}	0,87 ± 0,05^{A}
	14	0,55 ± 0,11^{Aab}	0,70 ± 0,20^{a}	0,39 ± 0,19^{b}	0,29 ± 0,00^{Bb}	0,39 ± 0,04^{BCb}	0,36 ± 0,00^{Cb}	0,43 ± 0,00^{Bab}
	21	0,14 ± 0,08^{Bb}	nd	nd	0,21 ± 0,00^{Bb}	0,31 ± 0,04^{Cab}	0,47 ± 0,06B^{Ca}	0,42 ± 0,06^{Ba}
Lactosa	0	12,86 ± 0,02^{Bb}	12,99 ± 0,00^{Cb}	13,51 ± 0,01^{Cb}	13,44 ± 0,00^{Bb}	16,08 ± 0,28^{Aa}	17,96 ± 0,08^{Aa}	16,39 ± 0,37^{Aa}
	7	18,15 ± 0,11^{Aa}	16,82 ± 0,28^{Bb}	17,18 ± 0,14^{Bb}	16,61 ± 0,04^{Ab}	16,32 ± 0,19^{Ab}	16,36 ± 0,09^{Bb}	16,46 ± 0,54^{Ab}
	14	17,08 ± 0,66^{Aa}	16,72 ± 0,17^{Ba}	13,53 ± 0,04^{Cb}	12,57 ± 0,01^{Cb}	13,58 ± 0,06^{Bb}	12,96 ± 0,22^{Cb}	14,10 ± 0,01^{Bb}
	21	12,79 ± 0,06^{Bb}	19,12 ± 0,88^{Aa}	18,85 ± 0,58^{Aa}	12,95 ± 0,06^{Cb}	13,40 ± 0,02^{Bb}	13,20 ± 0,03^{Cb}	13,60 ± 0,00^{Bb}
Glucosa	0	0,28 ± 0,00^{B}	0,26 ± 0,01^{C}	0,30 ± 0,00^{D}	0,27 ± 0,00^{C}	0,34 ± 0,03	0,32 ± 0,00^{A}	0,36 ± 0,10^{B}
	7	0,33 ± 0,00^{Ad}	0,30 ± 0,00^{Be}	0,41 ± 0,00^{Bb}	0,31 ± 0,00^{Ade}	0,38 ± 0,00^{c}	0,31 ± 0,00^{Ade}	0,91 ± 0,01^{Aa}
	14	0,33 ± 0,00^{Ab}	0,30 ± 0,00^{Bd}	0,36 ± 0,01^{Cb}	0,28 ± 0,00^{Cd}	0,36 ± 0,00^{b}	0,29 ± 0,00^{Bd}	0,52 ± 0,00^{Ba}
	21	0,30 ± 0,00^{Bc}	0,35 ± 0,01^{Ac}	0,44 ± 0,01^{Aab}	0,29 ± 0,00^{Bc}	0,36 ± 0,00^{bc}	0,30 ± 0,00^{Bc}	0,50 ± 0,00^{Ba}
Galactosa	0	1,95 ± 0,01^{Cb}	1,93 ± 0,00^{Cb}	1,67 ± 0,00^{Db}	1,98 ± 0,00^{Db}	2,08 ± 0,47^{ab}	2,76 ± 0,04^{Aa}	2,15 ± 0,27^{Bab}
	7	3,25 ± 0,05^{Aa}	2,90 ± 0,07^{Bb}	2,57 ± 0,04^{Bcd}	2,81 ± 0,02^{Ab}	2,46 ± 0,04^{d}	2,69 ± 0,00^{Abc}	3,25 ± 0,08^{Aa}
	14	3,02 ± 0,15^{aA}	2,94 ± 0,02^{Ba}	2,14 ± 0,02^{Cbc}	2,21 ± 0,00^{Cbc}	2,14 ± 0,00^{bc}	2,29 ± 0,00^{Cb}	2,52 ± 0,00^{Bc}
	21	2,42 ± 0,02^{Bb}	3,67 ± 0,23^{Aa}	3,20 ± 0,10^{Aa}	2,35 ± 0,00^{Bb}	2,17 ± 0,00^{bc}	2,39 ± 0,00^{Bb}	2,10 ± 0,00^{Bc}
Inulina	0	0,68 ± 0,00^{Bf}	1,14 ± 0,00^{Be}	1,64 ± 0,00^{Cbc}	1,17 ± 0,00^{Ade}	1,93 ± 0,30^{b}	1,54 ± 0,04^{Acd}	2,54 ± 0,05^{Ba}
	7	0,84 ± 0,02^{Ae}	1,24 ± 0,04^{ABd}	1,95 ± 0,03^{Bb}	1,17 ± 0,00^{Ad}	1,80 ± 0,03^{c}	1,24 ± 0,00^{Bd}	2,70 ± 0,04^{Aa}
	14	0,78 ± 0,03^{Ae}	1,21 ± 0,00^{ABc}	1,85 ± 0,03^{Bb}	1,09 ± 0,00^{Cd}	1,80 ± 0,00^{b}	1,16 ± 0,00^{Cc}	2,01 ± 0,00^{Ca}
	21	0,68 ± 0,00^{Bf}	1,29 ± 0,09^{Ad}	2,20 ± 0,05^{Aa}	1,15 ± 0,00^{Be}	1,80 ± 0,00^{c}	1,21 ± 0,01B^{Cde}	1,97 ± 0,01^{Cb}

Media ± desviación estándar en la misma columna seguida de diferentes letras mayúsculas indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra de yogur a diferente tiempo de almacenamiento ($n = 3$). Media ± desviación estándar en la misma fila seguida de diferentes letras minúsculas indican diferencias estadísticamente significativas ($p \leq 0,05$) entre las muestras de yogur al mismo tiempo de almacenamiento ($n = 3$). YC: yogur control sin ingredientes adicionales; YB0,5 y YB1: yogures con adición de 0,5 g/100 g y 1 g/100 g de extractos encapsulados control (con agua), respectivamente; YSF0,5 y YSF1: yogures con la adición de 0,5 g/100 g y 1 g/100 g de extractos encapsulados de subproductos florales de azafrán, respectivamente; YSF0,5SS0,05: yogur con la adición de 0,5 g/100 g de extractos encapsulados de subproductos florales de azafrán combinado con 0,05 g/100 g de extractos encapsulados de estigmas de azafrán; YSF1SS0,1:

yogur con la adición de un 1 g/100 g de extractos encapsulados de subproductos florales de azafrán combinado con un 0,10 g/100 g de extractos encapsulados de estigmas de azafrán; nd: no detectado.

Tabla 16. Parámetros fisicoquímicos de las diferentes formulaciones de yogur durante 21 días de almacenamiento a 4 °C.

	Tiempo (días)	YC	YB0,5	YB1	YSF0,5	YSF1	YSF0,5SS0,05	YSF1SS0,1
pH	0	4,51 ± 0,06^{Ab}	4,50 ± 0,06^{Ab}	4,58 ± 0,07^{Aa}	4,59 ± 0,07^{Aa}	4,59 ± 0,07^{Aa}	4,59 ± 0,06^{Aa}	4,61 ± 0,07^{Aa}
	7	4,15 ± 0,06^{Bb}	4,14 ± 0,00^{Bb}	4,39 ± 0,01^{Ba}	4,16 ± 0,01^{Bb}	4,38 ± 0,01^{Ba}	4,18 ± 0,02^{Bb}	4,46 ± 0,03^{Ba}
	14	4,17 ± 0,08^{Bb}	4,17 ± 0,04^{Bb}	4,35± 0,03^{Ba}	4,20 ± 0,03^{Bb}	4,39 ± 0,01^{Ba}	4,22 ± 0,02^{Bb}	4,42 ± 0,10^{Ba}
	21	4,07 ± 0,04^{Bb}	4,02 ± 0,03^{Bb}	4,24 ± 0,01^{Ba}	4,08 ± 0,02^{Bb}	4,28 ± 0,04^{Ba}	4,06 ± 0,03^{Bb}	4,26 ± 0,07^{Ba}
Acidez (% ácido láctico)	0	0,30 ± 0,00^{c}	0,41 ± 0,01^{a}	0,35 ± 0,00^{b}	0,31 ± 0,00^{c}	0,30 ± 0,00^{c}	0,35 ± 0,00^{b}	0,26 ± 0,00^{d}
	7	0,31 ± 0,05	0,39 ± 0,05	0,37 ± 0,07	0,32 ± 0,07	0,34 ± 0,03	0,41 ± 0,07	0,31 ± 0,03
	14	0,33 ± 0,04	0,33 ± 0,09	0,35 ± 0,07	0,37 ± 0,07	0,32 ± 0,03	0,40 ± 0,09	0,29 ± 0,05
	21	0,31 ± 0,01	0,38 ± 0,03	0,34 ± 0,00	0,38 ± 0,04	0,37 ± 0,07	0,37 ± 0,05	0,31 ± 0,07
Actividad de agua (aw)	0	0,89 ± 0,00	0,89 ± 0,02	0,89 ± 0,04^{B}	0,89 ± 0,05^{B}	0,89 ± 0,00	0,89 ± 0,04^{B}	0,89 ± 0,03^{B}
	7	0,90 ± 0,01^{b}	0,90 ± 0,01^{b}	0,98 ± 0,03^{Aa}	0,97 ± 0,01^{Aa}	0,90 ± 0,00^{b}	0,96 ± 0,01^{Aa}	0,97 ± 0,01^{Aa}
	14	0,89 ± 0,00	0,89 ± 0,00	0,90 ± 0,01^{B}	0,90 ± 0,02^{B}	0,89 ± 0,01	0,89 ± 0,02^{B}	0,89 ± 0,04^{B}
	21	0,89 ± 0,02	0,89 ± 0,01	0,90 ± 0,02^{B}	0,90 ± 0,01^{B}	0,89 ± 0,01	0,89 ± 0,05^{B}	0,89 ± 0,07^{B}
SST (°Brix)	0	8,08 ± 0,11^{d}	8,38 ± 0,11^{Ad}	9,39 ± 0,13^{Ab}	8,89 ± 0,10^{Ac}	9,09 ± 0,23^{bc}	7,98 ± 0,33^{d}	9,90 ± 0,15^{Aa}
	7	8,05 ± 0,09^{c}	8,38 ± 0,11^{Abc}	8,35 ± 0,12^{Bbc}	8,01 ± 0,22^{Bc}	8,72 ± 0,05^{b}	8,35 ± 0,13^{bc}	9,26 ± 0,17^{Ba}
	14	8,05 ± 0,10^{c}	8,08 ± 0,09^{Bc}	8,38 ± 0,12^{Babc}	8,18 ± 0,06^{Bbc}	8,75 ± 0,08^{a}	8,21 ± 0,08^{bc}	8,59 ± 0,33^{Cab}
	21	8,01 ± 0,11^{b}	8,21 ± 0,10^{Ab}	8,82 ± 0,20^{Ba}	8,28 ± 0,08^{Bb}	8,85 ± 0,15^{a}	8,21 ± 0,11^{b}	9,02 ± 0,14^{Ca}
Sinéresis (%)	0	17,27 ± 0,14^{Bc}	19,06 ± 0,27^{Ab}	20,93 ± 0,19^{Aa}	19,89 ± 0,18^{Aab}	20,69 ± 0,29^{Aa}	20,20 ± 0,43^{Aa}	20,76 ± 0,34^{Aa}
	7	18,83 ± 0,26^{Ab}	18,77 ± 0,26^{Ab}	19,08 ± 0,27^{Bb}	19,83 ± 0,15^{Aab}	19,58 ± 0,18^{Bab}	19,59 ± 0,13^{Aab}	20,47 ± 0,21^{Aa}
	14	19,02 ± 0,37^{A}	18,64 ± 0,26^{A}	18,52 ± 0,46^{B}	19,03 ± 0,20^{B}	18,93 ± 0,09^{C}	19,33 ± 0,23^{A}	19,32 ± 0,13^{B}
	21	18,43 ± 0,21^{Aa}	17,15 ± 0,24^{Bb}	17,80 ± 0,25^{Cab}	18,40 ± 0,26^{Ba}	18,22 ± 0,12^{Ca}	17,73 ± 0,07^{Bab}	18,71 ± 0,38^{Ba}

Media ± desviación estándar en la misma columna seguida de diferentes letras mayúsculas indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra de yogur a diferente tiempo de almacenamiento ($n = 3$). Media ± desviación estándar en la misma fila seguida de diferentes letras minúsculas indican diferencias estadísticamente significativas ($p \leq 0,05$) entre las muestras de yogur al mismo tiempo de almacenamiento ($n = 3$). YC: yogur control sin ingredientes adicionales; YB0,5 y YB1: yogures con adición de 0,5% (p/p) y 1% (p/p) de extractos encapsulados control (con agua), respectivamente; YSF0,5 y YSF1: yogures con la adición de 0,5% (p/p) y 1% (p/p) de extractos encapsulados de subproductos florales de azafrán, respectivamente; YSF0,5SS0,05: yogur con la adición de 0,5% (p/p) de extractos encapsulados de subproductos florales de azafrán combinado con 0,05% (p/p) de extractos encapsulados de estigmas de azafrán; YSF1SS0,1: yogur con la adición de un 1% (p/p) de extractos encapsulados de subproductos florales de azafrán combinado con un 0,10% (p/p) de extractos encapsulados de estigmas de azafrán; SST: sólidos solubles totales.

También se evaluó si las nuevas formulaciones de yogur mantenían concentraciones adecuadas de los cultivos iniciadores de *Lactobacillus* y *Streptococcus*. Estos resultados sobre la viabilidad de los microorganismos se indican en la Figura 16.

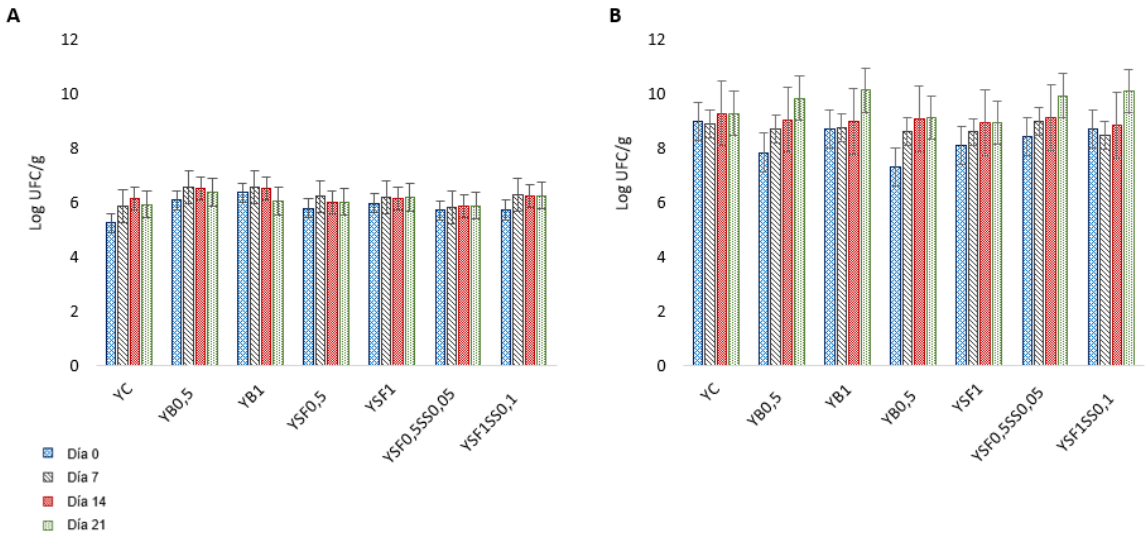

Figura 16. Viabilidad de *Lactobacillus* sp. (A) y B *Streptococcus* sp. (B) (Log UFC/g) en las diferentes formulaciones de yogur durante 21 días de almacenamiento a 4 °C. Las barras de error representan la desviación estándar (n = 3). La falta de etiquetado con letras diferentes indica diferencias estadísticamente no significativas (p > 0,05) para cada muestra de yogur a diferentes tiempos de almacenamiento y entre las muestras de yogur al mismo tiempo de almacenamiento. YC: yogur control sin ingredientes adicionales; YB0,5 y YB1: yogures con adición de 0,5% (p/p) y 1% (p/p) de extractos encapsulados control (con agua), respectivamente; YSF0,5 y YSF1: yogures con la adición de 0,5% (p/p) y 1% (p/p) de extractos encapsulados de subproductos florales de azafrán, respectivamente; YSF0,5SS0,05: yogur con la adición de 0,5% (p/p) de extractos encapsulados de subproductos florales de azafrán combinado con 0,05% (p/p) de extractos encapsulados de estigmas de azafrán; YSF1SS0,1: yogur con la adición de un 1% (p/p) de extractos encapsulados de subproductos florales de azafrán combinado con un 0,10% (p/p) de extractos encapsulados de estigmas de azafrán.

Los datos mostraron que, tras 21 días de almacenamiento en refrigeración, no se observaron diferencias significativas en la viabilidad de *Lactobacillus* sp. y *Streptococcus* sp., manteniéndose las concentraciones estables (5-6 Log UFC/g y 8-10 Log UFC/g, respectivamente) en todas las formulaciones estudiadas. Por tanto, las nuevas formulaciones de yogures fortificadas con azafrán son buenas matrices para alcanzar una alta concentración de cultivos iniciadores de bacterias lácticas. Respecto al análisis microbiológico de mohos y levaduras, no se detectaron en ninguna muestra (< 10 UFC/g) durante todo el período de almacenamiento, mostrando una buena calidad higiénico-sanitaria durante el almacenamiento durante al menos 21 días a 4 °C.

El color también es un atributo importante que evaluar en los yogures, ya que es la primera característica percibida por los consumidores. La Tabla 17 muestra los cambios en los parámetros de color (L*, a*, b*, ΔE, croma, tono, IP) de las diferentes formulaciones de yogur durante 21 días de almacenamiento a 4 °C. La adición de

extractos de azafrán no influyó en la luminosidad de las muestras de yogur, pero las formulaciones con extractos de estigmas tendieron a mostrar valores más altos de b* durante el almacenamiento que implicó un cambio en la intensidad del color. Este hecho está relacionado con el color amarillo anaranjado de los estigmas de azafrán y con la liberación paulatina de los extractos de azafrán de las microcápsulas (Carmona, Robert, Vergara, & Sáenz, 2021). El índice de pardeamiento disminuyó significativamente durante el período de almacenamiento en todas las formulaciones de yogur, excepto en el control (YC). Este hecho podría estar relacionado con las propiedades antioxidantes de los encapsulados que redujeron el IP y extendieron la vida útil del producto final.

La Tabla 18 muestra las actividades antioxidantes y el contenido de polifenoles totales en las formulaciones de yogur desarrolladas durante los 21 días de almacenamiento en refrigeración.

En cuanto al contenido de polifenoles totales, los valores tras 7 días de almacenamiento, en las muestras YC, YB1, YSF0,5, YSF1 y YSF1SS0,1 mostraron un aumento significativo respecto a los valores iniciales, manteniéndose estables hasta el final del almacenamiento. Este hecho puede estar relacionado con las interacciones de los compuestos fenólicos con las proteínas de la leche, siendo esta interacción proteína-polifenol máxima a pH 4,6 coincidiendo con el punto isoeléctrico de las proteínas. Durante el almacenamiento, el pH disminuyó y, por lo tanto, las interacciones se redujeron, lo que llevó a valores más altos de polifenoles totales (Akgün et al., 2020). La fortificación del yogur con 1% (p/p) de extracto de subproductos florales de azafrán aumentó su funcionalidad, ya que YSF1 y YSF1SS0,1 mostraron valores más altos de polifenoles totales (3,20 y 3,51 mg GAE/100 g, respectivamente), siendo éstos una buena fuente natural de compuestos antioxidantes. Además, la capacidad antioxidante (DPPH y ABTS) se mantuvo estable en todas las formulaciones de yogur y no se observaron diferencias significativas durante el almacenamiento, mejorando las propiedades antioxidantes respecto al control. Estos resultados son similares a los de Lima et al. (2021), donde aumentó la actividad antioxidante de los yogures enriquecidos con hidrolizado de proteína de pescado microencapsulado y no se observaron diferencias en la actividad antioxidante durante el almacenamiento.

En resumen, los resultados demostraron que la microencapsulación mejoró la estabilidad y protección de los extractos manteniendo las propiedades antioxidantes de los yogures durante 21 días en refrigeración. Además, el perfil microbiológico y los parámetros fisicoquímicos no se vieron afectados por la adición de los extractos de azafrán en las nuevas formulaciones, las cuales mostraron una buena composición de ácidos orgánicos y azúcares solubles para mejorar la vida útil del producto.

Tabla 17. Parámetros de color de las diferentes formulaciones de yogur durante 21 días de almacenamiento a 4 °C.

	Tiempo (días)	YC	YB0,5	YB1	YSF0,5	YSF1	YSF0,5SS0,05	YSF1SS0,1
L*	0	55,86 ± 0,78^{deC}	70,51 ± 0,99^{aAB}	64,25 ± 0,90^{bC}	61,93 ± 0,87^{bcC}	58,99 ± 0,83^{cdC}	57,17 ± 0,80^{dC}	53,09 ± 0,74^{eC}
	7	62,01 ± 0,87^{dB}	65,58 ± 0,92^{abC}	67,51 ± 0,95^{aB}	65,64 ± 0,92a^{bBC}	64,10 ± 0,90^{bcB}	63,08 ± 0,88^{cdB}	63,23 ± 0,89^{cdB}
	14	67,50 ± 0,95^{A}	67,10 ± 0,94^{BC}	67,98 ± 0,95^{B}	68,02 ± 0,85^{B}	69,82 ± 0,98^{A}	69,03 ± 0,97^{A}	67,87 ± 0,95^{A}
	21	68,43 ± 0,96^{bcA}	72,24 ± 1,01^{aA}	70,51 ± 0,99^{abA}	72,13 ± 1,01^{aA}	66,92 ± 0,94^{cAB}	72,39 ± 1,01^{aA}	68,36 ± 0,96^{bcA}
a*	0	-2,04 ± 0,03^{aA}	-3,38 ± 0,05^{cC}	-3,07 ± 0,04^{b}	-3,01 ± 0,04^{b}	-3,17 ± 0,04^{bB}	-5,84 ± 0,08^{dB}	-5,80 ± 0,08^{dB}
	7	-2,92 ± 0,04^{aBC}	-2,96 ± 0,04^{aA}	-3,19 ± 0,02^{b}	-3,18 ± 0,03^{b}	-3,15 ± 0,03^{bB}	-5,53 ± 0,06^{cA}	-5,87 ± 0,07^{dB}
	14	-3,03 ± 0,02^{aC}	-3,05 ± 0,03^{aAB}	-3,11 ± 0,03^{a}	-3,07 ± 0,03^{a}	-3,11 ± 0,04^{aB}	-5,38 ± 0,07^{bA}	-5,77 ± 0,08^{cB}
	21	-2,87 ± 0,04^{bB}	-3,20 ± 0,04^{cB}	-3,13 ± 0,04^{c}	-3,16 ± 0,04^{c}	-2,40 ± 0,03^{aA}	-5,42 ± 0,08^{dA}	-5,47 ± 0,05^{dA}
b*	0	3,05 ± 0,04^{eC}	5,13 ± 0,07^{dB}	5,88 ± 0,08^{cA}	4,67 ± 0,07^{dD}	5,08 ± 0,07^{dD}	14,53 ± 0,20^{bD}	16,79 ± 0,24^{aC}
	7	3,24 ± 0,05^{fC}	3,75 ± 0,05^{fC}	4,42 ± 0,06^{eC}	5,61 ± 0,08^{dC}	6,98 ± 0,10^{cB}	16,48 ± 0,23^{bC}	23,97 ± 0,34^{aB}
	14	5,57 ± 0,08^{eA}	5,09 ± 0,07^{eB}	5,17 ± 0,07^{eB}	6,89 ± 0,10^{dB}	8,15 ± 0,11^{cA}	19,43 ± 0,27^{bB}	25,79 ± 0,36^{aA}
	21	4,74 ± 0,07^{eB}	5,42 ± 0,08^{dA}	5,08 ± 0,07^{eB}	7,27 ± 0,10^{cA}	6,10 ± 0,09^{dC}	20,88 ± 0,29^{bA}	26,10 ± 0,37^{aA}
∆E	0	-	14,85 ± 0,21^{aA}	8,90 ± 0,12^{cA}	6,36 ± 0,09^{dA}	3,90 ± 0,05^{eC}	12,16 ± 0,10^{bC}	14,50 ± 0,20^{aB}
	7	-	3,70 ± 0,05^{Be}	5,62 ± 0,08^{cB}	4,33 ± 0,06^{Bd}	4,29 ± 0,06^{Ade}	13,53 ± 0,06^{bB}	20,96 ± 0,29^{aA}
	14	-	0,62 ± 0,00^{eC}	0,63 ± 0,00^{eD}	1,42 ± 0,02^{dC}	3,48 ± 0,05^{cB}	14,14 ± 0,02^{bB}	20,41 ± 0,28^{aA}
	21	-	3,88 ± 0,05^{cD}	2,12 ± 0,03^{dC}	4,50 ± 0,06^{cB}	2,08 ± 0,03^{dD}	16,81 ± 0,06^{bA}	21,51 ± 0,30^{aA}
C*	0	3,67 ± 0,05^{fA}	6,15 ± 0,09^{cdBC}	6,63 ± 0,09^{cC}	5,55 ± 0,08^{eA}	5,99 ± 0,08^{deA}	15,66 ± 0,22^{bA}	17,76 ± 0,25^{aA}
	7	4,36 ± 0,06^{eB}	4,77 ± 0,07^{eA}	5,45 ± 0,08^{dA}	6,45 ± 0,09^{dB}	7,66 ± 0,11^{cC}	17,39 ± 0,24^{bB}	24,68 ± 0,35^{aB}
	14	6,34 ± 0,09^{eD}	5,93 ± 0,08^{eB}	6,03 ± 0,08^{eB}	7,54 ± 0,11^{dC}	8,74 ± 0,12^{cD}	20,16 ± 0,28^{bC}	26,42 ± 0,37^{aC}
	21	5,54 ± 0,08^{eC}	6,30 ± 0,09^{dC}	5,97 ± 0,07^{deB}	7,93 ± 0,10^{cC}	6,56 ± 0,09^{dB}	21,57 ± 0,30^{bD}	26,67 ± 0,40^{aC}
h°	0	307 ± 4^{a}	306 ± 4^{a}	301 ± 4^{ab}	306 ± 5^{a}	305 ± 4^{a}	295 ± 4^{b}	292 ± 4^{ab}
	7	315 ± 5^{a}	311 ± 6^{a}	309 ± 6^{a}	303 ± 6^{ab}	297 ± 3^{bc}	291 ± 5^{c}	287 ± 5^{c}
	14	302 ± 3^{a}	304 ± 5^{a}	304 ± 8^{a}	297 ± 4^{ab}	294 ± 5^{ab}	288 ± 4^{b}	285 ± 6^{b}
	21	304 ± 4^{ab}	303 ± 4^{ab}	305 ± 4^{a}	296 ± 2^{b}	294 ± 6^{b}	287 ± 3^{bc}	285 ± 5^{c}
IP	0	3,15 ± 0,00^{gD}	4,14 ± 0,02^{fA}	4,32 ± 0,03^{dA}	4,21 ± 0,01^{eB}	4,68 ± 0,04^{cA}	9,77 ± 0,10^{bA}	10,90 ± 0,12^{aA}
	7	3,88 ± 0,01^{fB}	3,78 ± 0,00^{gD}	4,02 ± 0,00^{eB}	4,30 ± 0,02^{dA}	4,58 ± 0,02^{cB}	8,84 ± 0,05^{bB}	10,41 ± 0,05^{aB}
	14	4,02 ± 0,01^{eA}	4,01 ± 0,01^{eB}	4,02 ± 0,01^{eB}	4,22 ± 0,01^{dB}	4,37 ± 0,01^{cC}	8,36 ± 0,02^{bC}	9,86 ± 0,00^{aC}
	21	3,68 ± 0,00^{eC}	3,91 ± 0,03^{dC}	3,88 ± 0,02^{dC}	4,13 ± 0,00^{cC}	3,47 ± 0,10^{fD}	8,21 ± 0,00^{bD}	9,54 ± 0,07^{aD}

Media ± desviación estándar en la misma columna seguida de diferentes letras mayúsculas indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra de yogur a diferente tiempo de almacenamiento ($n = 3$). Media ± desviación estándar en la misma fila seguida de diferentes letras minúsculas indican diferencias estadísticamente significativas ($p \leq 0,05$) entre las muestras de yogur al mismo tiempo de almacenamiento ($n = 3$). YC: yogur control sin ingredientes adicionales; YB0,5 y YB1: yogures con adición de 0,5% (p/p) y 1% (p/p) de extractos encapsulados control (con agua), respectivamente; YSF0,5 y YSF1: yogures con la adición de 0,5% (p/p) y 1% (p/p) de extractos encapsulados de subproductos florales de azafrán, respectivamente; YSF0,5SS0,05: yogur con la adición de 0,5% (p/p) de extractos encapsulados de subproductos florales de azafrán combinado con 0,05% (p/p) de extractos encapsulados de estigmas de azafrán; YSF1SS0,1: yogur con la adición de un 1% (p/p) de extractos encapsulados de subproductos florales de azafrán combinado con un 0,10% (p/p) de extractos encapsulados de estigmas de azafrán. IP: índice de pardeamiento.

Tabla 18. Propiedades antioxidantes y contenido de polifenoles totales de las diferentes formulaciones de yogur durante 21 días de almacenamiento a 4 °C.

	Tiempo (días)	YC	YB0,5	YB1	YSF0,5	YSF1	YSF0,5SS0,05	YSF1SS0,1
Polifenoles totales	0	1,70 ± 0,08^{Bd}	1,99 ± 0,34^{Bcd}	1,71 ± 0,08^{Bd}	2,22 ± 0,18^{Bc}	2,88 ± 0,01^{Bab}	2,26 ± 0,14^{c}	3,20 ± 0,02^{Ca}
(mg GAE/100 g)	7	2,49 ± 0,18^{Ac}	2,52 ± 0,05^{ABc}	2,75 ± 0,19^{Ac}	2,69 ± 0,23^{Ac}	3,29 ± 0,28^{Ab}	2,47 ± 0,05^{c}	4,02 ± 0,07^{Aa}
	14	2,38 ± 0,12^{Ac}	2,53 ± 0,22^{ABc}	2,45 ± 0,16^{Ac}	2,31 ± 0,16^{ABc}	3,05 ± 0,05^{ABab}	2,34 ± 0,24^{c}	3,75 ± 0,18^{ABa}
	21	2,47 ± 0,02^{Ac}	2,80 ± 0,25^{Abc}	2,60 ± 0,14^{Ac}	2,38 ± 0,02^{ABc}	3,20 ± 0,11^{ABab}	2,64 ± 0,10^{c}	3,51 ± 0,09^{Ba}
FRAP	0	1,21 ± 0,06^{ABc}	1,65 ± 0,03^{Ab}	1,66 ± 0,09^{Ab}	1,58 ± 0,11^{b}	1,68 ± 0,09^{a}	1,56 ± 0,05^{b}	1,79 ± 0,01^{Aa}
(mmol Trolox/100 g)	7	1,36 ± 0,10^{ABb}	1,46 ± 0,06^{Bb}	1,48 ± 0,07^{ABb}	1,44 ± 0,09^{b}	1,73 ± 0,07^{a}	1,50 ± 0,10^{b}	1,76 ± 0,00^{Aa}
	14	1,09 ± 0,18^{Bd}	1,24 ± 0,06^{Ccd}	1,18 ± 0,10^{Cd}	1,29 ± 0,17^{bd}	1,61 ± 0,09^{ab}	1,51 ± 0,08^{abc}	1,67 ± 0,02^{Ba}
	21	1,39 ± 0,02^{Ae}	1,49 ± 0,05^{Bbce}	1,27 ± 0,03^{BCd}	1,46 ± 0,07^{be}	1,65 ± 0,05^{a}	1,58 ± 0,02^{ac}	1,66 ± 0,03^{Ba}
ABTS	0	0,79 ± 0,02^{ab}	0,83 ± 0,03^{ab}	0,77 ± 0,07^{b}	0,84 ± 0,04^{ab}	0,89 ± 0,05^{a}	0,84 ± 0,02^{ab}	0,89 ± 0,01^{a}
(mmol Trolox/100 g)	7	0,80 ± 0,00^{b}	0,82 ± 0,04^{ab}	0,84 ± 0,02^{ab}	0,94 ± 0,09^{a}	0,94 ± 0,07^{a}	0,80 ± 0,04^{ab}	0,92 ± 0,04^{ab}
	14	0,61 ± 0,10^{c}	0,71 ± 0,10^{bc}	0,71 ± 0,11^{bc}	0,87 ± 0,02^{ab}	0,91 ± 0,06^{ab}	0,85 ± 0,00^{ab}	0,92 ± 0,02^{a}
	21	0,81 ± 0,03^{bc}	0,79 ± 0,03^{cd}	0,76 ± 0,04^{c}	0,88 ± 0,01^{ab}	0,91 ± 0,04^{a}	0,87 ± 0,03^{abd}	0,91 ± 0,00^{a}
DPPH	0	0,27 ± 0,03^{b}	0,29 ± 0,06^{b}	0,33 ± 0,01^{ab}	0,24 ± 0,04^{b}	0,39 ± 0,08^{ab}	0,28 ± 0,09^{b}	0,48 ± 0,04^{Ba}
(mmol Trolox/100 g)	7	0,26 ± 0,05^{b}	0,27 ± 0,02^{b}	0,24 ± 0,08^{b}	0,29 ± 0,03^{b}	0,32 ± 0,00^{b}	0,24 ± 0,07^{b}	0,52 ± 0,01^{Ba}
	14	0,27 ± 0,01^{c}	0,24 ± 0,01^{c}	0,28 ± 0,04^{c}	0,25 ± 0,02^{c}	0,36 ± 0,04^{b}	0,27 ± 0,03^{c}	0,66 ± 0,03^{Aa}
	21	0,24 ± 0,06^{b}	0,27 ± 0,03^{b}	0,24 ± 0,07^{b}	0,29 ± 0,06^{b}	0,32 ± 0,02^{ab}	0,29 ± 0,03^{b}	0,46 ± 0,07^{Ba}

Media ± desviación estándar en la misma columna seguida de diferentes letras mayúsculas indican diferencias estadísticamente significativas ($p \leq 0,05$) para cada muestra de yogur a diferente tiempo de almacenamiento ($n = 3$). Media ± desviación estándar en la misma fila seguida de diferentes letras minúsculas indican diferencias estadísticamente significativas ($p \leq 0,05$) entre las muestras de yogur al mismo tiempo de almacenamiento ($n = 3$). YC: yogur control sin ingredientes adicionales; YB0,5 y YB1: yogures con adición de 0,5% (p/p) y 1% (p/p) de extractos encapsulados control (con agua), respectivamente; YSF0,5 y YSF1: yogures con la adición de 0,5% (p/p) y 1% (p/p) de extractos encapsulados de subproductos florales de azafrán, respectivamente; YSF0,5SS0,05: yogur con la adición de 0,5% (p/p) de extractos encapsulados de subproductos florales de azafrán combinado con 0,05% (p/p) de extractos encapsulados de estigmas de azafrán; YSF1SS0,1: yogur con la adición de un 1% (p/p) de extractos encapsulados de subproductos florales de azafrán combinado con un 0,10% (p/p) de extractos encapsulados de estigmas de azafrán.

4.6.2. Enriquecimiento de panes tradicionales de trigo y espelta con subproductos florales del azafrán

Se evaluaron las propiedades fisicoquímicas, funcionales, tecnológicas y sensoriales de las nuevas formulaciones de pan enriquecidas con diferentes concentraciones de subproductos florales de azafrán deshidratados en polvo.

La Tabla 19 muestra los resultados de los parámetros fisicoquímicos (humedad, cenizas, pH, acidez, aw y SST) de las formulaciones de pan de trigo y espelta con subproductos florales de azafrán.

Tabla 19. Parámetros fisicoquímicos de las diferentes formulaciones de pan de trigo y espelta enriquecidas con subproductos florales de azafrán.

	Humedad (%)	Cenizas (%)	pH	Acidez (% Ácido cítrico)	aw	SST (°Brix)
WB0%	31,12 ± 2,43^{ab}	2,75 ± 0,07^{c}	5,62 ± 0,01^{a}	0,09 ± 0,01^{d}	0,85 ± 0,03	0,77 ± 0,01^{d}
WB2,5%	25,47 ± 2,49^{b}	2,95 ± 0,17^{c}	5,50 ± 0,01^{b}	0,12 ± 0,01^{c}	0,84 ± 0,02	0,93 ± 0,02^{c}
WB5%	31,12 ± 2,40^{ab}	3,44 ± 0,10^{b}	5,31 ± 0,02^{c}	0,23 ± 0,02^{b}	0,84 ± 0,04	1,07 ± 0,02^{b}
WB10%	32,44 ± 2,44^{a}	3,79 ± 0,12^{a}	5,20 ± 0,01^{d}	0,28 ± 0,02^{a}	0,84 ± 0,02	1,30 ± 0,01^{a}
SB0%	32,71 ± 2,61	3,05 ± 0,15^{c}	5,54 ± 0,01^{a}	0,10 ± 0,02^{b}	0,86 ± 0,01	0,97 ± 0,01^{d}
SB2,5%	29,51 ± 2,46	3,37 ± 0,11^{b}	5,23 ± 0,00^{b}	0,21 ± 0,01^{a}	0,85 ± 0,00	1,03 ± 0,03^{c}
SB5%	31,89 ± 2,36	3,39 ± 0,08^{b}	5,21 ± 0,00^{c}	0,23 ± 0,02^{a}	0,85 ± 0,01	1,07 ± 0,02^{b}
SB10%	31,50 ± 2,33	3,95 ± 0,16^{a}	5,18 ± 0,01^{d}	0,24 ± 0,01^{a}	0,85 ± 0,03	1,30 ± 0,01^{a}

Media ± desviación estándar en la misma columna seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) entre las muestras de pan de trigo o entre las de espelta ($n = 3$). SST: sólidos solubles totales; WB0%, WB2,5%, WB5%, WB10%: panes de harina de trigo al 0, 2,5, 5, y 10% (p/p) de subproductos florales de azafrán, respectivamente; SB0%, SB2,5%, SB5% y SB10%: panes de harina de espelta al 0, 2,5, 5, y 10% (p/p) de subproductos florales de azafrán, respectivamente.

Respecto a los resultados, la adición de subproductos florales de azafrán no alteró la humedad ni la actividad de agua en comparación con las muestras control, pero aumentó los valores de cenizas, debido a su rico contenido en minerales. Además, como se observa en la Tabla 20, las concentraciones de macrominerales y microminerales mostraron que el enriquecimiento de los panes de trigo y espelta con subproductos florales de azafrán tuvo un efecto nutricional positivo debido a un aumento en su contenido.

El mineral encontrado en mayores concentraciones en todos los panes de trigo y espelta fue el Na debido a la sal utilizada en la formulación (600-800 mg/100 g). El K fue el macromineral que también se encontró en niveles altos, con concentraciones crecientes a medida que la cantidad de subproductos florales de azafrán agregados era mayor. Además, el Ca y el Mg también estaban presentes en cantidades elevadas en las formulaciones con un 5 o 10% de subproductos florales de azafrán.

Los microminerales Fe, Zn y Mn también estuvieron presentes en las diferentes formulaciones de pan, aunque en menor cantidad en comparación con Ca, Mg, Na y K. El más abundante fue el Fe en todas las muestras, seguido de Mn y Zn. Así, los subproductos florales del azafrán pueden proporcionar un enriquecimiento mineral a las formulaciones de pan de trigo y espelta tradicional, especialmente al 10%, mostrando valores de alrededor de 270 - 290 mg/100 g para K, 90 - 95 mg/100 g para Ca, 40 - 50 mg/100 g para Mg y 15 - 18 mg/100 g para Fe.

Tabla 20. Composición de minerales (mg/100 g peso seco) de las diferentes formulaciones de pan de trigo y espelta enriquecidas con subproductos florales de azafrán.

	WB0%	WB2,5%	WB5%	WB10%	SB0%	SB2,5%	SB5%	SB10%
Macrominerales								
Ca	39,22 ± 1,24^{b}	56,18 ± 5,15^{b}	67,92 ± 8,02^{a}	91,63 ± 10,02^{a}	33,47 ± 6,00^{c}	52,74 ± 8,10^{bc}	65,98 ± 1,33^{b}	94,56 ± 3,14^{a}
K	162 ± 3^{d}	196 ± 9^{c}	226 ± 8^{b}	277 ± 14^{a}	153 ± 10^{c}	178 ± 11^{b}	209 ± 16^{b}	289 ± 18^{a}
Mg	31,21 ± 1,54^{c}	34,72 ± 1,82^{bc}	36,97 ± 1,43^{b}	41,61 ± 1,93^{a}	33,95 ± 3,01^{b}	34,46 ± 3,11^{b}	40,81 ± 1,04^{a}	48,46 ± 8,03^{a}
Na	641 ± 18^{ab}	713 ± 32^{a}	682 ± 23^{ab}	605 ± 85^{b}	810 ± 55	713 ± 55	743 ± 13	739 ± 12
Microminerales								
Fe	2,01 ± 0,10^{c}	6,01 ± 1,15^{b}	9,15 ± 1,96^{b}	15,87 ± 1,25^{a}	3,27 ± 1,83^{c}	6,27 ± 0,35^{c}	10,63 ± 0,39^{b}	17,85 ± 0,40^{a}
Mn	1,03 ± 0,19^{b}	1,22 ± 0,03^{ab}	1,25 ± 0,05^{ab}	1,42 ± 0,21^{a}	0,74 ± 0,09^{b}	0,91 ± 0,10^{ab}	1,04 ± 0,01^{ab}	1,37 ± 0,20^{a}
Zn	0,77 ± 0,11	0,97 ± 0,00	0,97 ± 0,01	1,03 ± 0,06	0,71 ± 0,02	0,78 ± 0,06	0,81 ± 0,10	0,96 ± 0,20

Media ± desviación estándar en la misma fila seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) entre las muestras de pan de trigo o entre las de espelta ($n = 3$); WB0%, WB2,5%, WB5%, WB10%: panes de harina de trigo al 0, 2,5, 5, y 10% (p/p) de subproductos florales de azafrán, respectivamente; SB0%, SB2,5%, SB5% y SB10%: panes de harina de espelta al 0, 2,5, 5, y 10% (p/p) de subproductos florales de azafrán, respectivamente.

El valor del pH y la acidez también son factores relevantes para determinar la calidad de los panes. En la tabla 19 se muestran valores de pH entre 5,20 y 5,62 para los panes de trigo y 5,18 y 5,54 para los panes de espelta, siendo significativamente inferiores en los panes con mayor concentración de subproductos florales de azafrán, respecto a las muestras control. Al mismo tiempo, la acidez fue mayor en las formulaciones de pan con mayor cantidad de flores con respecto a las muestras control, lo cual está relacionado con la concentración de ácidos orgánicos en el ingrediente floral.

En cuanto al estudio de la composición de los ácidos orgánicos, azúcares solubles e inulina, los resultados se presentan en la Tabla 21. Se encontraron diversos ácidos orgánicos en las diferentes formulaciones de pan, no sólo debido a su presencia en las harinas de trigo y espelta o en los subproductos florales del azafrán, sino también como resultado del proceso de fermentación durante la elaboración del pan. Durante el período de fermentación, tanto la levadura *Saccharomyces cerevisiae* utilizada en la formulación, como la microbiota (bacterias y levaduras) presente de forma natural las harinas, desempeñaron un papel importante en las características del pan (Arendt, Ryan, & Dal Bello, 2007).

Los cambios bioquímicos debidos a los efectos de la fermentación sobre la degradación de carbohidratos y proteínas, principalmente por las levaduras y las bacterias lácticas, condujeron a la producción de ácidos orgánicos y otros metabolitos. El ácido láctico fue uno de los principales ácidos orgánicos presentes en todas las formulaciones de pan, siendo significativamente mayor en panes al 10% (p/p) de subproductos florales de azafrán. También se encontró un alto contenido de ácido málico y cítrico y una baja concentración de ácido oxálico y fumárico. Estos resultados fueron similares a los de otros estudios que indicaron la presencia de ácido láctico, málico y fumárico en harinas de trigo y espelta (De Luca et al., 2021). También se identificó ácido propiónico en las muestras enriquecidas que pudo haberse generado como resultado de la actividad metabólica de los microorganismos al metabolizar moléculas presentes en los subproductos florales del azafrán, generando diferentes compuestos (Shewry et al., 2022).

Tabla 21. Contenido en ácidos orgánicos, azúcares solubles e inulina (mg/100 g peso seco) de las diferentes formulaciones de pan de trigo y espelta enriquecidas con subproductos florales de azafrán.

	WB0%	**WB2,5%**	**WB5%**	**WB10%**	**SB0%**	**SB2,5%**	**SB5%**	**SB10%**
Ácidos orgánicos								
Ácido fítico	nd	nd	nd	nd	178 ± 27	165 ± 5	168 ± 11	172 ± 3
Ácido láctico	241 ± 60^{d}	384 ± 22^{c}	503 ± 19^{b}	595 ± 20^{a}	301 ± 33^{d}	385 ± 6^{c}	448 ± 5^{b}	676 ± 11^{a}
Ácido cítrico	1878 ± 7^{c}	1895 ± 6^{bc}	1922 ± 14^{b}	1957 ± 19^{a}	1600 ± 24^{c}	1885 ± 5^{b}	1910 ± 9^{b}	1934 ± 22^{a}
Ácido málico	132 ± 5^{d}	214 ± 11^{c}	335 ± 30^{b}	516 ± 30^{a}	102 ± 10^{d}	189 ± 16^{c}	292 ± 26^{b}	521 ± 32^{a}
Ácido oxálico	14,24 ± 1,90	13,91 ± 0,62	13,84 ± 0,93	13,79 ± 0,93	11,33 ± 0,76	11,75 ± 0,22	9,77 ± 3,73	10,98 ± 0,26
Ácido fumárico	0,24 ± 0,07^{c}	0,04 ± 0,00^{c}	1,05 ± 0,08^{b}	1,98 ± 0,13^{a}	nd	nd	nd	1,71 ± 0,20
Ácido propiónico	nd	835 ± 71^{b}	933 ± 33^{a}	982 ± 48^{a}	nd	845 ± 9^{c}	910 ± 16^{b}	1051 ± 48^{a}
Azúcares solubles								
Glucosa	1303 ± 12^{d}	1411 ± 16^{c}	1617 ± 13^{b}	1917 ± 34^{a}	1210 ± 62^{d}	1317 ± 25^{c}	1512 ± 82^{b}	1829 ± 35^{a}
Maltosa	37,70 ± 0,14^{a}	33,32 ± 0,10^{c}	36,75 ± 0,16^{b}	33,11 ± 0,33^{c}	33,19 ± 0,24^{a}	33,03 ± 0,41^{a}	31,19 ± 0,32^{b}	30,49 ± 0,13^{b}
Fructosa	nd	nd	480 ± 40^{b}	913 ± 82^{a}	nd	nd	307 ± 48^{b}	813 ± 12^{a}
Inulina	5011 ± 70^{c}	5377 ± 95^{b}	6504 ± 58^{a}	6511 ± 223^{a}	5464 ± 80^{c}	5682 ± 112^{c}	6231 ± 211^{b}	6846 ± 48^{a}

Media ± desviación estándar en la misma fila seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) entre las muestras de pan de trigo o entre las de espelta ($n = 3$); WB0%, WB2,5%, WB5%, WB10%: panes de harina de trigo al 0, 2,5, 5, y 10% (p/p) de subproductos florales de azafrán, respectivamente; SB0%, SB2,5%, SB5% y SB10%: panes de harina de espelta al 0, 2,5, 5, y 10% (p/p) de subproductos florales de azafrán, respectivamente; nd: no detectado.

Así, los ácidos orgánicos en las diferentes formulaciones de pan podrían actuar como conservantes naturales para mejorar su vida útil, y evitar el deterioro microbiológico.

Respecto a los azúcares, que contribuyen al color y la textura del producto final, la glucosa estaba presente en mayores concentraciones en todas las formulaciones de pan, seguida de la fructosa y maltosa. El disacárido maltosa es el primer producto liberado por la digestión del almidón de la harina por la enzima amilasa que tras ello se hidroliza para liberar glucosa. Por tanto, la maltosa se encuentra en concentraciones más bajas que la glucosa en el producto final. Estos resultados coincidieron con otros

estudios que informaron de la presencia de maltosa y glucosa en harinas de trigo y espelta (Shewry et al., 2022).

La fructosa también estaba presente en formulaciones de pan de trigo y pan de espelta al 5% y 10% de subproductos florales de azafrán. El contenido de este monosacárido podría deberse a su presencia natural en el ingrediente floral. Además, se estudió el contenido de inulina que aumentó significativamente con la concentración de subproductos florales de azafrán en el pan de trigo y de espelta. Las mayores cantidades de inulina estuvieron presentes en WB10% y SB10%, con valores alrededor de 6511 y 6846 mg/100 g, respectivamente, encontrándose las concentraciones más bajas en los panes sin subproductos florales de azafrán. La inulina también contribuyó a un aumento en los SST, ya que las formulaciones de pan con mayor concentración de inulina presentaron SST significativamente mayores que las muestras control (1,30 °Brix).

Los parámetros de textura (dureza, cohesividad, elasticidad, gomosidad, masticabilidad) también se analizaron, ya que determinan la vida útil y la aceptabilidad del consumidor, y los resultados se presentan en la Tabla 22.

Los panes control de trigo y espelta, WB0% y SB0%, se caracterizaron por tener menor dureza, mientras que el enriquecimiento del pan con subproductos florales de azafrán resultó en un aumento estadísticamente significativo en este parámetro. Todas las formulaciones de pan presentaron una disminución estadísticamente significativa en la cohesión en comparación con los panes control. Así, estos resultados indicaron que la adición de subproductos florales de azafrán afectó significativamente a la textura de los panes, lo que podría estar relacionado con la interrupción de la red de gluten que conduce a una baja cohesividad y a la desintegración de la miga (Pycia & Ivanišová, 2020). En las muestras de pan enriquecidas también hubo una disminución estadísticamente significativa en los valores de elasticidad en comparación con los controles, lo cual está relacionado con una alta fragilidad de la miga (Tóth, Kaszab, & Meretei, 2022). La masticabilidad y la gomosidad aumentaron significativamente con la incorporación de los subproductos florales de azafrán en panes de trigo y espelta en comparación con las muestras control. García-Segovia et al. (2020) encontraron resultados similares utilizando proteína de guisante o polvo de insectos en la formulación de panes. Estos valores podrían estar relacionados con el contenido de fibra dietética, como la inulina, que da como resultado una fuerte masticabilidad y una alta capacidad de absorción de agua (Tóth et al., 2022).

Tabla 22. Parámetros de textura de las diferentes formulaciones de pan de trigo y espelta enriquecidas con subproductos florales de azafrán.

	Dureza (N)	Cohesividad	Elasticidad (mm)	Masticabilidad (N)	Gomosidad (N)
WB0%	42,24 ± 2,45^{d}	0,93 ± 0,02^{a}	1,59 ± 0,01^{a}	39,47 ± 0,41^{d}	37,73 ± 0,74^{c}
WB2,5%	48,36 ± 3,32^{c}	0,88 ± 0,00^{b}	1,50 ± 0,08^{b}	42,58 ± 0,67^{c}	40,00 ± 0,49^{b}
WB5%	51,12 ± 1,12^{b}	0,87 ± 0,01^{b}	1,49 ± 0,03^{b}	44,68 ± 0,48^{b}	41,51 ± 0,23^{b}
WB10%	54,17 ± 0,53^{a}	0,86 ± 0,00^{b}	1,47 ± 0,02^{c}	46,91± 0,82^{a}	43,21 ± 0,15^{a}
SB0%	34,65 ± 1,66^{c}	0,89 ± 0,01^{a}	1,53 ± 0,04^{a}	31,14 ± 1,11^{d}	28,64 ± 0,16^{c}
SB2,5%	41,31 ± 2,24^{b}	0,84 ± 0,02^{b}	1,43 ± 0,00^{b}	34,76 ± 0,53^{c}	31,93 ± 0,27^{b}
SB5%	43,75 ± 2,35^{b}	0,82 ± 0,02^{bc}	1,40 ± 0,00^{c}	36,06 ± 0,14^{b}	32,94 ± 0,98^{b}
SB10%	52,12 ± 3,87^{a}	0,80 ± 0,00^{c}	1,37 ± 0,01^{d}	41,91 ± 1,14^{a}	38,22 ± 0,54^{a}

Media ± desviación estándar en la misma columna seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) entre las muestras de pan de trigo o entre las de espelta ($n = 3$); WB0%, WB2,5%, WB5%, WB10%: panes de harina de trigo al 0, 2,5, 5, y 10% (p/p) de subproductos florales de azafrán, respectivamente; SB0%, SB2,5%, SB5% y SB10%: panes de harina de espelta al 0, 2,5, 5, y 10% (p/p) de subproductos florales de azafrán, respectivamente.

Además, se evaluó el color de la miga y de la corteza, ya que es un factor importante en la elección del pan por parte de los consumidores (Tabla 23). El enriquecimiento del pan disminuyó los valores L* de corteza y miga que podría estar relacionado con un aumento en las reacciones de Maillard en la corteza, y por el color intrínseco de los subproductos florales del azafrán en la miga. En cuanto a los parámetros a* y b*, en la corteza los valores fueron similares al control. Sin embargo, en la miga aumentaron con la adición de los subproductos florales, por lo que este cambio estaba relacionado con el color del ingrediente floral, ya que las temperaturas en el interior de los panes durante el horneado no superaron los 100 °C (García-Segovia et al., 2020).

Tabla 23. Color de la corteza y la miga de las diferentes formulaciones de pan de trigo y espelta enriquecidas con subproductos florales de azafrán.

	Corteza			Miga		
	L*	a*	b*	L*	a*	b*
WB0%	40,25 ± 3,15	12,49 ± 0,19^{a}	22,22 ± 2,19^{b}	61,86 ± 1,69^{a}	0,28 ± 0,10^{b}	18,45 ± 0,35^{b}
WB2,5%	37,21 ± 2,36	11,35 ± 0,63^{ab}	20,18 ± 2,51^{b}	51,72 ± 3,73^{b}	−0,38 ± 0,02^{c}	27,83 ± 0,60^{a}
WB5%	41,95 ± 2,37	10,72 ± 0,47^{b}	22,99 ± 1,56^{a}	47,72 ± 0,63^{b}	0,08 ± 0,04^{b}	27,44 ± 3,54^{a}
WB10%	34,78 ± 3,56	9,21 ± 0,53^{c}	17,05 ± 2,32^{b}	41,29 ± 1,47^{c}	1,52 ± 0,32^{a}	28,35 ± 1,89^{a}
SB0%	50,58 ± 1,71^{a}	11,05 ± 0,34^{a}	25,35 ± 1,16^{a}	63,15 ± 5,68^{a}	0,02 ± 0,00^{d}	19,08 ± 1,07^{b}
SB2,5%	43,54 ± 5,48^{ab}	10,60 ± 1,15^{a}	23,30 ± 2,87^{a}	58,45 ± 1,81^{ab}	0,30 ± 0,01^{c}	28,21 ± 1,40^{a}
SB5%	40,15 ± 0,42^{bc}	11,06 ± 0,56^{a}	21,42 ± 0,57^{a}	52,50 ± 2,54^{c}	0,59 ± 0,03^{b}	30,70 ± 0,81^{a}
SB10%	33,75 ± 1,42^{c}	8,73 ± 0,53^{b}	15,22 ± 1,14^{b}	47,01 ± 3,63^{c}	1,75 ± 0,09^{a}	30,57 ± 1,70^{a}

Media ± desviación estándar en la misma columna seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) entre las muestras de pan de trigo o entre las de espelta ($n = 3$); WB0%, WB2,5%, WB5%, WB10%: panes de harina de trigo al 0, 2,5, 5, y 10% (p/p) de subproductos florales de azafrán, respectivamente; SB0%, SB2,5%, SB5% y SB10%: panes de harina de espelta al 0, 2,5, 5, y 10% (p/p) de subproductos florales de azafrán, respectivamente.

Los compuestos bioactivos son muy sensibles a diversos factores ambientales, afectando a sus propiedades biológicas, como el poder antioxidante, que podría cambiar

significativamente dependiendo de las condiciones ambientales (pH, presencia de enzimas digestivas, temperatura, etc.) (Czubaszek, Czaja, Sokół-Łętowska, Kolniak-Ostek, & Kucharska, 2021). Por ello, se estudiaron los cambios en la actividad antioxidante y en el contenido de polifenoles totales en las diferentes etapas de la digestión *in vitro* (oral, gástrica, intestinal) de las formulaciones de pan desarrolladas. Los resultados se presentan en la Tabla 24.

Según los valores obtenidos, la adición de subproductos florales de azafrán a los panes de trigo y espelta mejoró significativamente el contenido polifenólico y su capacidad antioxidante, especialmente en las formulaciones al 10% (p/p) del ingrediente floral. Los panes enriquecidos, WB10% y SB10%, mostraron una actividad antioxidante significativamente mayor (ABTS y FRAP) en comparación con el control en todas las fases de digestión *in vitro*.

Tabla 24. Propiedades antioxidantes (FRAP, ABTS) y contenido de polifenoles totales de las diferentes formulaciones de pan de trigo y espelta enriquecidas con subproductos florales de azafrán durante el proceso de digestión oral y gastrointestinal *in vitro*.

		Digestión *in vitro*			
		Oral	**Gástrica**	**Intestinal (1 h)**	**Intestinal (2 h)**
ABTS (mmol Trolox/100 g)	WB0%	3,47 ± 1,75	22,37 ± 1,99^{c}	421 ± 12^{a}	458 ± 6^{b}
	WB2,5%	3,94 ± 1,28	34,32 ± 8,47^{b}	437 ± 4^{a}	452 ± 10^{b}
	WB5%	3,61 ± 2,61	48,74 ± 2,14^{a}	435 ± 8^{a}	461 ± 5^{b}
	WB10%	4,94 ± 1,50	49,99 ± 0,98^{a}	399 ± 7^{b}	1576 ± 34^{a}
	SB0%	2,20 ± 0,16^{b}	30,74 ± 3,31^{b}	429 ± 11	437± 27^{b}
	SB2,5%	5,49 ± 0,67^{a}	43,34 ± 1,99^{a}	430 ± 12	1373 ± 59^{a}
	SB5%	4,91 ± 0,81^{a}	45,44 ± 6,40^{a}	426 ± 19	1458± 177^{a}
	SB10%	5,69 ± 0,20^{a}	50,54 ± 0,39^{a}	437 ± 27	1583 ± 83^{a}
FRAP (mmol Trolox/100 g)	WB0%	0,89 ± 0,50^{c}	8,07 ± 2,03^{c}	nd	nd
	WB2,5%	14,09 ± 4,08^{b}	17,12 ± 0,56^{bc}	1,40 ± 0,83^{b}	2,43 ± 0,73^{b}
	WB5%	28,05 ± 4,85^{a}	25,64 ± 6,04^{b}	9,83 ± 5,35^{ab}	15,00 ± 6,58^{ab}
	WB10%	38,77 ± 5,90^{a}	37,67 ± 4,67^{a}	20,71 ± 7,96^{a}	27,89 ± 6,99^{a}
	SB0%	6,99 ± 0,73^{c}	11,21 ± 1,01^{b}	nd	nd
	SB2,5%	18,08 ± 4,03^{bc}	21,56 ± 1,19^{b}	3,97 ± 2,81^{b}	7,60 ± 0,34^{c}
	SB5%	32,01 ± 9,90^{b}	26,69 ± 12,58^{ab}	14,26 ± 8,84^{b}	18,16 ± 4,89^{b}
	SB10%	61,86 ± 10,75^{a}	43,97 ± 6,96^{a}	31,79 ± 1,22^{a}	36,62 ± 3,130^{a}
Polifenoles totales (mg GAE/100 g)	WB0%	76,99 ± 6,60^{d}	118 ± 8^{d}	109 ± 7^{d}	91,31 ± 5,25^{c}
	WB2,5%	111 ± 6^{c}	149 ± 16^{c}	140 ± 16^{c}	146 ± 13^{b}
	WB5%	198 ± 8^{b}	207 ± 1^{b}	222 ± 8^{b}	166 ± 14^{b}
	WB10%	221 ± 18^{a}	231 ± 11^{a}	272 ± 17^{a}	220 ± 17^{a}
	SB0%	102 ± 16^{c}	181 ± 92	137 ± 18^{c}	105 ± 15^{c}
	SB2,5%	124 ± 32^{bc}	236 ± 129	135 ± 10^{c}	135 ± 45^{bc}
	SB5%	175 ± 1^{b}	251 ± 71	187 ± 6^{b}	186 ± 5^{b}
	SB10%	261 ± 13^{a}	313 ± 63	258 ± 25^{a}	251 ± 17^{a}

Media ± desviación estándar en la misma columna seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) entre las muestras de pan de trigo o entre las de espelta ($n = 3$); WB0%, WB2,5%, WB5%, WB10%: panes de harina de trigo al 0, 2,5, 5, y 10% (p/p) de subproductos florales de azafrán, respectivamente; SB0%, SB2,5%, SB5% y SB10%: panes de harina de espelta al 0, 2,5, 5, y 10% (p/p) de subproductos florales de azafrán, respectivamente; nd: no detectado.

Para el ensayo ABTS, la capacidad antioxidante aumentó en todas las formulaciones de pan en cada fase de digestión, alcanzando los valores más altos en

condiciones de digestión intestinal. Este incremento podría deberse a condiciones intestinales (pH neutro, presencia de enzimas pancreáticas y sales biliares) que facilitarían una mejora en la liberación de compuestos bioactivos que previamente pueden estar unidos o insolubilizados debido a su interacción con la matriz del pan (Czubaszek et al., 2021). Para el ensayo FRAP, los valores máximos se obtuvieron tras la digestión oral simulada, y se mantuvieron estables durante la digestión gástrica, pero disminuyeron en la fase intestinal en todas las formulaciones. Este hecho podría deberse a la influencia de la matriz del pan y su interacción con los compuestos bioactivos de los ingredientes florales a lo largo de la digestión oral y gastrointestinal *in vitro*.

Respecto a los valores de polifenoles totales, en todas las formulaciones las concentraciones se mantuvieron estables durante las etapas de digestión oral, gástrica e intestinal, alcanzando el mayor contenido para las formulaciones de pan, WB10% y SB10%, siendo los valores elevados tras 2 h de digestión intestinal (220 ± 17 y 251 ± 17 mg GAE/100 g, respectivamente). En los panes de espelta se observó un ligero aumento tras las condiciones gástricas, lo que podría estar relacionado con un aumento en la solubilidad de ciertos compuestos fenólicos en condiciones de pH ácido (Gião et al., 2012).

Las formulaciones de pan al 5% de subproducto floral fueron seleccionadas como las óptimas para realizar un análisis sensorial, ya que en las formulaciones WB5% y SB5% ya se mejora significativamente el valor funcional del pan en comparación con el control. La Figura 17 resume los resultados del análisis sensorial. Los descriptores de apariencia mostraron puntuaciones altas con respecto a la uniformidad en la forma del pan en todas las formulaciones, pero para la uniformidad del color de la corteza, las formulaciones enriquecidas WB5% y SB5% exhibieron valores más bajos en comparación con las muestras control, debido a la incorporación de flores de azafrán que incidieron en el color de la corteza.

En cuanto a la evaluación del sabor, WB5% y SB5% tuvieron valores ligeramente superiores en términos de dulzor, probablemente debido a la concentración de azúcar de las flores de azafrán. De la misma manera, los atributos florales-herbáceos y de identificación floral se relacionaron con las flores de *Crocus sativus* L. en las formulaciones de pan enriquecidos, WB5% y SB5%. La intensidad de la astringencia y el amargor fue mayor en las muestras formuladas con subproductos florales de azafrán debido al contenido de compuestos fenólicos. La astringencia y el amargor podrían derivarse de la precipitación de proteínas salivales ricas en prolina en la boca causada

por compuestos fenólicos (Hoye & Ross, 2011; Soares et al., 2013). Además, el regusto puede estar relacionado con la alta astringencia de las muestras.

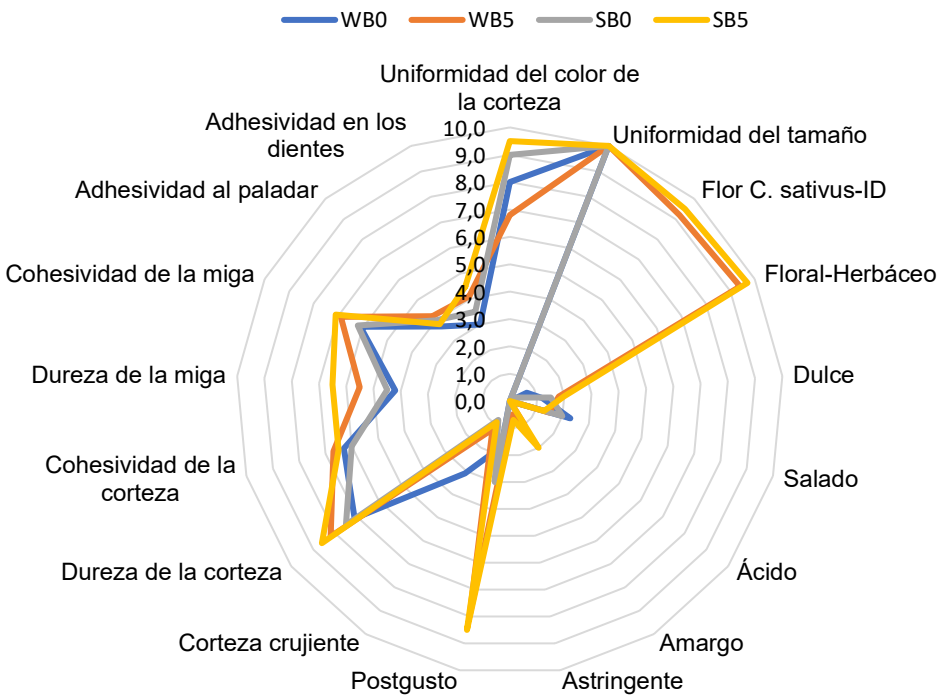

Figura 17. Resultados del análisis sensorial de las formulaciones de pan seleccionadas. Los datos se representan como Media ± desviación estándar (*n* = 3); WB0%, WB5%: panes de harina de trigo al 0, y 5% de subproductos florales de azafrán, respectivamente; SB0%, SB5%: panes de harina de espelta al 0 y 5% de subproductos florales de azafrán, respectivamente.

Además, los resultados de los atributos de textura a nivel sensorial concordaban con el análisis de textura, según el cual WB5% y SB5% tenían mayor dureza que las muestras control. La incorporación de subproductos florales del azafrán redujo la textura crujiente y afectó a la cohesividad de los panes, probablemente debido a que la adición de este ingrediente vegetal interrumpe la red de gluten, haciendo que los panes sean más frágiles (Altinok et al., 2022). Sin embargo, con respecto a la adhesividad al paladar y a los dientes, no se observaron diferencias importantes entre las formulaciones de pan estudiadas.

Como muestran los resultados, el enriquecimiento de los panes tradicionales con subproductos florales de azafrán deshidratados mejoran las propiedades nutricionales y funcionales, aumentando el contenido en fibra dietética, como la inulina, minerales y ácidos orgánicos, presentando una alta capacidad antioxidante y un alto contenido bioactivo tras el proceso de digestión *in vitro*, especialmente en las formulaciones con mayores contenidos de ingrediente floral (5% y 10%). Por tanto, los subproductos florales del azafrán son una alternativa sostenible y apropiada para el desarrollo de

nuevos productos funcionales de panadería con potenciales efectos beneficiosos para la salud.

4.6.3. Desarrollo de bebidas modelo a base de subproductos florales de azafrán

Los subproductos florales de azafrán liofilizados y las bebidas modelo sin espesante (SF, SF80) se caracterizaron mediante su perfil fenólico. Como se indica en la Tablas 25 y 26 se identificaron y cuantificaron un total de 14 y 13 compuestos fenólicos en los subproductos florales de azafrán y en las bebidas modelo, respectivamente. En cuanto a la fracción de flavonoles en los extractos de los subproductos florales de azafrán (Tabla 25), estuvo compuesta por glucósidos de kaempferol, quercetina e isorhamnetina. El compuesto kaempferol-3-*O*-soforosido fue el más abundante (1058 ± 38 mg/100 g), seguido por quercetina 3,4'-*O*-diglucósido (II) (222 ± 12 mg/100 g) y kaempferol-3-*O*-soforosido-7-*O*-glucósido (204 ± 16 mg/100 g). También se presentaron glucósidos de isorhamnetina, pero en concentraciones más bajas (42-74 mg/100 g). Además, se identificaron y cuantificaron cuatro antocianinas en los extractos de subproductos florales del azafrán. La delfinidina-3,5-*O*-diglucósido, seguida de la delfinidina-3-*O*-glucósido, fueron las principales antocianinas encontradas, con una concentración de 145 ± 13 y 80,7 ± 6,8 mg/100 g, respectivamente. La petunidina-3,5-*O*-diglucósido y la petunidina-3-*O*-glucósido estaban presentes en menores cantidades.

Tabla 25. Identificación y cuantificación de compuestos fenólicos nativos en extractos de subproductos florales de azafrán.

Compuesto	TR (min)	$[M]^+$ (m/z)	$[M-H]^-$ (m/z)	MS^n (m/z)	Concentración (mg/100 g peso seco)
Flavonoles					
Kaempferol-3-*O*-soforosido-7-*O*-glucósido	7,50		771	609, 285, 429	204 ± 16
Isorhamnetina 3,7-di*O*-hexósido (I)	13,63		639/641	315, 271	44,9 ± 4,5
Quercetina 3,4'-*O*-diglucósido (I)	16,03		625	463, 301	70,6 ± 2,7
Quercetina 3,4'-*O*-diglucósido (II)	16,45		625	301, 463, 445	222 ± 12
Isorhamnetina 3,7-di*O*-hexósido (II)	17,30		639	315, 477, 300	74,3 ± 2,7
Kaempferol-3-*O*-soforosido	18,95		609	285, 429	1058 ± 38
Quercetina 3-*O*-glucósido	21,51		463	301, 151	42,6 ± 4,2
Isorhamnetina-3-*O*-rutinósido	21,68		623	315, 459	71,5 ± 4,2
Kaempferol-3-*O*-(acetil-glucósido)-7-*O*-glucósido	24,66		651	285, 489, 471	103 ± 5
Kaempferol 3-*O*-glucósido	24,88		447	285	151 ± 8
Antocianinas					
Delfinidina-3,5-*O*-diglucósido	5,05	627		465, 303	145 ± 13
Petunidina-3,5-*O*-diglucósido	7,21	641		479, 317	43,5 ± 3,7
Delfinidina-3-*O*-glucósido	8,96	465		303	80,7 ± 6,8
Petunidina-3-*O*-glucósido	12,02	479		317	23,9 ± 1,4

Media ± desviación (*n* = 3); TR: tiempo de retención; m/z: relación masa-carga.

En cuanto a las bebidas modelo con subproductos florales de azafrán, se encontró la misma composición fenólica que en los extractos florales de azafrán, excepto para la quercetina 3-*O*-glucósido. Sin embargo, como era de esperar, las concentraciones de cada compuesto fueron más bajas que en los extractos, ya que las bebidas contenían un 1% (p/p) de subproductos florales de azafrán (Tabla 26). También se observaron diferencias estadísticamente significativas en la concentración de algunos compuestos entre las bebidas tratadas térmicamente (SF80) y las no tratadas (SF). Ambas bebidas presentaron principalmente glucósidos de kaempferol, quercetina e isorhamnetina, siendo el kaempferol-3-*O*-soforosido el de mayor concentración, mostrando SF cantidades significativamente mayores (22,9 ± 0,6 mg/100 mL), en comparación con la bebida tratada térmicamente SF80 (21,6 ± 0,4 mg/100 mL). Además de los derivados de kaempferol, también se detectó quercetina 3,4'-O-diglucósido (II) en cantidades elevadas (4,1-4,6 mg/100 mL). En cuanto a la composición de antocianinas, los derivados de petunidina y delfinidina también estuvieron presentes en las bebidas modelo, siendo la delfinidina-3,5-*O*-diglucósido y la delfinidina-3-*O*-glucósido las más abundantes. La bebida modelo no tratada térmicamente mostró cantidades más altas de antocianinas en comparación con la bebida tratada térmicamente. Estas diferencias encontradas en la concentración de fenoles en las bebidas podrían estar relacionadas con la degradación de estos compuestos bioactivos durante el procesamiento térmico (D'Archivio, Filesi, Varì, Scazzocchio, & Masella, 2010).

Tabla 26. Identificación y cuantificación de compuestos fenólicos nativos en bebidas modelo con subproductos florales de azafrán.

Compuesto	TR (min)	$[M]^+$ (m/z)	$[M-H]^-$ (m/z)	MS^n (m/z)	Concentración (mg/100 mL de bebida)	
Flavonoles					**SF**	**SF80**
Kaempferol-3-*O*-soforosido-7-*O*-glucósido	7,50		771	609, 285, 42	5,57 ± 0,11[a]	5,27 ± 0,06[b]
Isorhamnetina 3,7-di*O*-hexósido (I)	13,63		639/641	315, 271	2,71 ± 0,12	2,74 ± 0,01
Quercetina 3,4'-*O*-diglucósido (I)	16,03		625	463, 301	2,85 ± 0,02	2,82 ± 0,02
Quercetina 3,4'-*O*-diglucósido (II)	16,45		625	301, 463, 44	4,64 ± 0,10[a]	4,17 ± 0,09[b]
Isorhamnetina 3,7-di*O*-hexósido (II)	17,30		639	315, 477, 30	2,93 ± 0,04[a]	2,85 ± 0,02[b]
Kaempferol-3-*O*-soforosido	18,95		609	285, 429	22,9 ± 0,6[a]	21,6 ± 0,4[b]
Isorhamnetina-3-*O*-rutinósido	21,68		623	315, 459	2,85 ± 0,01[a]	2,81 ± 0,02[b]
Kaempferol-3-*O*-(acetil-glucósido)-7-*O*-glucósido	24,66		651	285, 489, 47	3,08 ± 0,01[a]	3,02 ± 0,02[b]
Kaempferol 3-*O*-glucósido	24,88		447	285	3,37 ± 0,03	3,33 ± 0,04
Antocianinas						
Delfinidina-3,5-*O*-diglucósido	5,05	627		465, 303	3,57 ± 0,37	3,44 ± 0,07
Petunidina-3,5-*O*-diglucósido	7,21	641		479, 317	1,29 ± 0,03[a]	1,23 ± 0,02[b]
Delfinidina-3-*O*-glucósido	8,96	465		303	1,67 ± 0,03[a]	1,60 ± 0,02[b]
Petunidina-3-*O*-glucósido	12,02	479		317	0,88 ± 0,00[a]	0,86 ± 0,00[b]

Media ± desviación estándar en la misma fila seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) entre las muestras de bebida ($n = 3$); TR: tiempo de retención; m/z: relación masa-carga; SF: bebida sin espesante y sin tratamiento térmico; SF80: bebida sin espesante con tratamiento térmico.

Debido a la composición fenólica de las bebidas desarrolladas, se estudió su capacidad para inhibir la actividad de amilasas digestivas. Los alimentos funcionales ricos en compuestos fenólicos podrían mejorar la homeostasis de la glucosa al reducir la absorción intestinal de la glucosa de la dieta mediante la inhibición de las enzimas digestivas y ralentizar la digestión del almidón (Nyambe-Silavwe et al., 2015).

El efecto inhibidor de las bebidas modelo con subproductos florales de azafrán sobre la actividad de la α-amilasa pancreática se presenta en la Figura 18. En las muestras no tratadas térmicamente, no se detectó ningún efecto inhibidor, por ello se muestran los resultados de las bebidas procesadas térmicamente.

La bebida modelo sin espesante con tratamiento térmico (SF80) mostró el mayor efecto inhibidor sobre la enzima (~37%), ya que la actividad de la α-amilasa fue de 62,46 ± 7,18%, seguida por las muestras GGSF80 y XGSF80. La bebida modelo tratada térmicamente con goma guar también presentó una capacidad inhibidora similar (~29%), siendo la actividad de la enzima del 71,42 ± 5,38%. Estas diferencias en la inhibición de la α-amilasa pancreática podrían deberse tanto a la adición del espesante alimentario como al procesamiento térmico.

Los polifenoles pueden inhibir la α-amilasa uniéndose a ella. Sin embargo, la presencia de polisacáridos, como los presentes naturalmente en las flores de azafrán y los espesantes alimentarios añadidos, podrían generar una unión competitiva y disminuir la unión polifenol-enzima y, en consecuencia, la capacidad de los polifenoles para inhibirla (Bordenave, Hamaker, & Ferruzzi, 2014; Sun, Warren, & Gidley, 2019). Este hecho se ha demostrado en estudios previos con fibras solubles como β-glucano, pectinas, goma xantana y goma guar (D'Costa, Golding, Raval, Rolland-Sabaté, & Bordenave, 2023; Sun et al., 2019). Por tanto, estos resultados concuerdan con estas observaciones previas, ya que la bebida modelo sin espesante (sin polisacárido para interferir con la unión e inhibición de enzima-polifenol) mostró la mayor inhibición.

Además, la temperatura también jugó un papel importante, ya que las muestras tratadas térmicamente no mostraron actividad inhibitoria. Esta observación sugiere que, en las muestras tratadas térmicamente, los polifenoles estaban disponibles en cantidad suficiente para unirse a la enzima inhibiendo su actividad, independientemente de la presencia de los polisacáridos a pesar de estar en el medio. El procesamiento térmico podría haber afectado a los enlaces de hidrógeno que impulsan las interacciones polisacáridos-polifenoles, y prevenir la unión polisacárido-polifenol, que es más débil que la unión polifenol-proteína (Dobson et al., 2019; Tudorache, McDonald, & Bordenave, 2020). Esto podría explicar por qué las bebidas modelo tratadas

térmicamente, como SF80, XGSF80 y GGSF80, mostraron un efecto inhibidor sobre las enzimas digestivas.

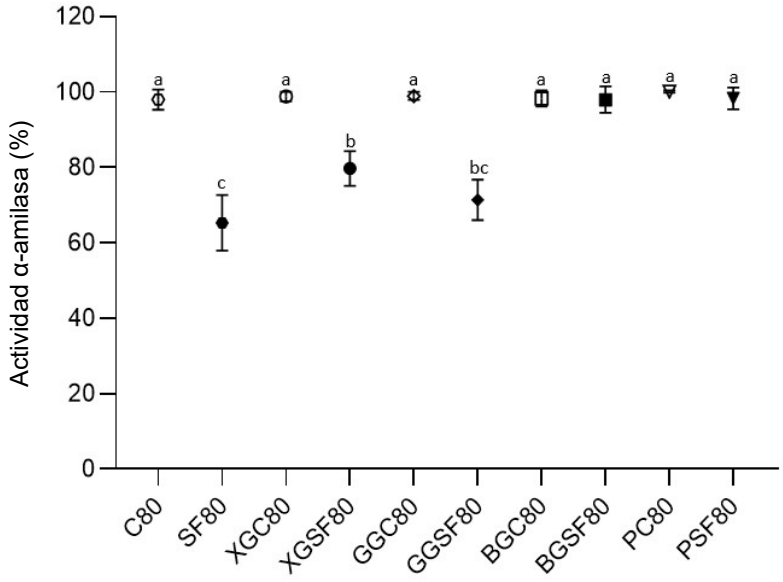

Figura 18. Inhibición de α-amilasa de las bebidas desarrolladas. Media ± desviación estándar; las diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) entre las muestras ($n = 3$); C80: control sin espesante tratado térmicamente; SF80: bebida modelo sin espesante tratada térmicamente; XGC80: control con goma xantana tratado térmicamente; XGSF80: bebida modelo con goma xantana tratada térmicamente; GGC80: control con goma guar tratado térmicamente; GGSF80: bebida modelo con goma guar goma tratada térmicamente; BGC80: control con β-glucano tratado térmicamente; BGSF80: bebida modelo con β-glucano tratada térmicamente; PC80: control con pectina tratado térmicamente; PSF80: bebida modelo con pectina tratada térmicamente.

Las propiedades reológicas también tienen un papel importante en el desarrollo de nuevos alimentos funcionales, proporcionando información sobre los cambios estructurales asociados con el procesamiento de alimentos y ayudándonos a comprender la interacción de los componentes de los alimentos.

Las propiedades reológicas de las bebidas modelo de subproductos florales de azafrán se caracterizaron a partir del ajuste de reogramas de tensión de corte vs velocidad de corte con la ecuación de Herschel-Bulkley. Los resultados se presentan en la Tabla 27.

El índice de comportamiento del flujo, n, indica un comportamiento de flujo newtoniano ($n = 1$), de adelgazamiento por cizallamiento (pseudaplástico, $n < 1$) y de espesamiento por cizallamiento (dilatante, $n > 1$). El índice de consistencia del flujo, K, refleja la viscosidad de una solución a una velocidad de corte y un índice de comportamiento del flujo comparables.

Tabla 27. Reología de las bebidas desarrolladas (modelo Herschel-Bulkley).

	τ_0 (Pa)	K (Pa.s^{-n})	n
C	0,56 ± 0,62^{a}	0,19 ± 0,03	1,01 ± 0,01
SF	1,4 ± 1,6^{ab}	0,18 ± 0,02	1,02 ± 0,01
C80	0,78 ± 0,68^{ab}	0,20 ± 0,05	1,01 ± 0,02
SF80	0,0 ± 0,0^{b}	0,22 ± 0,04	1,00 ± 0,02
XGC	750 ± 70^{a}	294 ± 7^{b}	0,39 ± 0,00^{bc}
XGSF	615 ± 71^{b}	342 ± 33^{a}	0,38 ± 0,01^{c}
XGC80	115 ± 4^{c}	167 ± 45^{c}	0,42 ± 0,02^{b}
XGSF80	206 ± 11^{c}	1,52 ± 0,11^{d}	0,84 ± 0,01^{a}
GGC	0,0 ± 0,0^{c}	95,3 ± 5,7^{a}	0,48 ± 0,00^{c}
GGSF	121 ± 4^{a}	1,54 ± 0,29^{c}	0,87 ± 0,02^{a}
GGC80	0,0 ± 0,0^{c}	77,2 ± 4,0^{b}	0,50 ± 0,00^{c}
GGSF80	90,9 ± 2,6^{b}	9,42 ± 0,80^{c}	0,70 ± 0,01^{b}
BGC	38,0 ± 1,8^{a}	0,14 ± 0,02	1,09 ± 0,01^{a}
BGSF	12,8 ± 3,5^{b}	0,17 ± 0,02	1,05 ± 0,01^{b}
BGC80	39,3 ± 5,4^{a}	0,14 ± 0,02	1,09 ± 0,02^{a}
BGSF80	33,4 ± 1,7^{a}	0,17 ± 0,01	1,07 ± 0,01^{ab}
PC	8,2 ± 1,3^{a}	0,19 ± 0,02^{b}	1,03 ± 0,01^{a}
PSF	287 ± 256^{b}	1,06 ± 0,12^{a}	0,88 ± 0,01^{b}
PC80	10,0 ± 0,9^{a}	0,18 ± 0,01^{b}	1,03 ± 0,01^{a}
PSF80	8,3 ± 7,3^{a}	0,20 ± 0,02^{b}	1,02 ± 0,02^{a}

Media ± desviación estándar en la misma columna seguida de diferentes letras indican diferencias estadísticamente significativas ($p \leq 0,05$) entre las muestras de bebida ($n = 3$); C: control sin espesante, sin tratamiento térmico; SF: bebida modelo sin espesante , sin tratamiento térmico; C80: control sin espesante tratado térmicamente; SF80: bebida modelo sin espesante tratada térmicamente;); XGC: control con goma xantana, sin tratamiento térmico; XGSF: bebida modelo con goma xantana , sin tratamiento térmico XGC80: control con goma xantana tratado térmicamente; XGSF80: bebida modelo con goma xantana tratada térmicamente;); GGC: control con goma guar, sin tratamiento térmico; GGSF: bebida modelo con goma guar , sin tratamiento térmico GGC80: control con goma guar tratado térmicamente; GGSF80: bebida modelo con goma guar goma tratada térmicamente;); BGC: control con β-glucano, sin tratamiento térmico; BGSF: bebida modelo con β-glucano, sin tratamiento térmico BGC80: control con β-glucano tratado térmicamente; BGSF80: bebida modelo con β-glucano tratada térmicamente; PC80: control con pectina tratado térmicamente; PSF80: bebida modelo con pectina tratada térmicamente.

Los resultados muestran que las bebidas sin espesante son esencialmente newtonianas, al igual que las bebidas con β-glucanos y pectinas (n~1) que también exhibieron una baja viscosidad (índice de consistencia similar, K). Por el contrario, las bebidas modelo que contienen goma xantana y goma guar exhibieron un comportamiento de adelgazamiento por cizallamiento (pseudoplástico, n~0,4-0,9) y una marcada viscosidad. La adición de subproductos florales de azafrán en las bebidas modelo espesadas con goma guar y goma xantana condujo a una disminución del índice de consistencia de flujo, K, y a un aumento del índice de comportamiento de flujo, n. Esto se ha observado previamente en otros estudios y se ha atribuido a la agregación de los espesantes con los compuestos fenólicos, lo que hace que la solución se comporte más como agua en términos de flujo y viscosidad (Nyambe-Silavwe et al.,

2015; Tudorache et al., 2020). Específicamente, en las bebidas modelo con goma xantana, el procesamiento térmico (XGC80, XGSF80) tiende a disminuir significativamente K y τ_0. El procesamiento térmico y los subproductos florales del azafrán (XGSF80) tienden a aumentar significativamente n. En bebidas modelo espesadas con goma guar, los subproductos florales de azafrán (GGSF, GGSF80) tienden a aumentar significativamente τ_0 y n, y combinados con el procesamiento térmico tienden a disminuir significativamente K (GGSF, GGC80, GGSF80). Los resultados de las bebidas modelo con β-glucanos fueron consistentes con los de la goma guar y la goma xantana, pero tuvieron una sensibilidad muy limitada a la presencia de subproductos florales del azafrán. Este hecho puede estar relacionado con una menor unión de los β-glucanos con los flavonoles, los principales compuestos fenólicos presentes en las bebidas estudiadas (Jakobek, Ištuk, Matić, & Skendrović Babojelić, 2021).

Las bebidas modelo con pectina mostraron un comportamiento opuesto en comparación con la goma xantana, la goma guar y β-glucano, ya que la pectina fue el único espesante fuertemente iónico en este estudio, lo que puede afectar a las interacciones con los compuestos fenólicos. Los subproductos florales del azafrán (PSF) tienden a aumentar significativamente K y τ_0, y disminuir n. Sin embargo, para las bebidas modelo sin espesante no se encontraron variaciones estadísticamente significativas para K y n, respecto a la presencia de subproductos florales de azafrán y el procesamiento térmico.

Desde el punto de vista del impacto de las interacciones polifenol-polisacárido en estas bebidas modelo, los resultados observados son muy importantes ya que pueden influir en el procesamiento de alimentos y en las opciones de formulación dependiendo de los requisitos de composición del espesante y el contenido fenólico de la formulación. De hecho, en estas bebidas modelo de subproductos florales del azafrán, la agregación puede producir efectos drásticamente diferentes dependiendo del tipo de espesante alimentario elegido.

Por tanto, estos resultados demuestran que la adición de espesantes alimentarios en las formulaciones de bebidas desempeña un papel clave en la bioactividad y bioaccesibilidad de los flavonoides debido a las interacciones polifenol-polisacárido.


5.CONCLUSIONES

5. CONCLUSIONES/ CONCLUSIONS

1. El azafrán y sus subproductos florales destacan por su contenido alto en fibra dietética, proteínas, ácidos orgánicos, minerales y ácidos grasos poliinsaturados, siendo además fuentes naturales de compuestos bioactivos, destacando su contenido en carotenoides y terpenos, como crocinas, crocetina, picrocrocina y safranal en los estigmas, y en compuestos fenólicos principalmente flavonoles y antocianinas en los subproductos florales.

2. Entre la composición volátil, los estigmas de azafrán presentaron altos niveles de safranal, mientras que la composición volátil de los subproductos florales de azafrán estuvo influenciada por el método de secado aplicado.

3. La extracción asistida por microondas (MAE) optimizada mediante el estudio de los efectos del tiempo, temperatura y concentración de etanol mostró las condiciones óptimas para la extracción de compuestos bioactivos de los subproductos florales del azafrán, siendo una técnica sostenible y eficiente de bajo consumo energético. Esta información permitiría seleccionar el método y los parámetros de extracción más adecuados, así como su escalado a nivel industrial.

4. La aplicación de disolventes de extracción ecológicos como los disolventes eutécticos profundos naturales (NaDES) combinados con la extracción asistida por ultrasonidos (UAE), junto con la optimización de las condiciones de extracción (tiempo, potencia y relación NaDES:agua), es una alternativa eficiente, innovadora y respetuosa con el medio ambiente para obtener compuestos de alto valor añadido a partir del azafrán y sus subproductos florales.

5. La aplicación de tecnologías verdes de extracción como MAE y UAE mejoró significativamente el contenido de compuestos bioactivos expresado en términos de contenido fenólico y de flavonoides totales, así como la actividad antioxidante de los extractos de azafrán y sus subproductos florales, respecto a los métodos convencionales por sonicación.

6. Los hidrogeles de quitosano/alginato son unas matrices adecuadas para la estabilización de los extractos de NaDES, ya que su incorporación en estos hidrogeles permitió que el contenido en polifenoles totales tras la digestión oral y gastrointestinal *in vitro* permaneciera estable.

7. Las microcápsulas a base de alginato de sodio con extractos de azafrán y de sus subproductos florales son ingredientes adecuados para su incorporación en matrices lácteas como el yogur, ya que no alteran el proceso de fermentación y mejoran sus propiedades funcionales en términos de contenido bioactivo y propiedades antioxidantes, permaneciendo estables durante 21 días de almacenamiento en refrigeración.

8. Los panes de trigo y espelta enriquecidos con subproductos florales de azafrán mejoraron sus propiedades nutricionales y funcionales, principalmente su actividad antioxidante y su contenido en compuestos bioactivos. Estas propiedades antioxidantes se mantuvieron estables tras la digestión oral y gastrointestinal *in vitro.*

9. Las bebidas desarrolladas mediante el uso de los subproductos florales de azafrán como ingredientes con diferentes espesantes alimentarios (goma xantana, goma guar, β-glucano, pectina) presentaron un alto contenido en compuestos fenólicos (derivados de kaempferol, quercetina e isorhamnetina).

10. Tanto el azafrán como sus subproductos florales, y sus respectivos extractos, obtenidos mediante tecnologías verdes de extracción, han sido utilizados exitosamente en formulaciones de pan, yogur y bebidas, convirtiéndolos en ingredientes sostenibles e innovadores para la mejora nutricional y funcional de alimentos tradicionales y para nuevas aplicaciones en la industria agroalimentaria o en otros sectores, como el cosmético o el farmacéutico.

11. Esta Tesis Doctoral supone un avance sobre el valor nutricional y funcional de la especia de azafrán y sus subproductos florales, cuya valorización generará impactos económicos positivos para la industria del azafrán y contribuirá a la minimización del impacto medioambiental, mejorando la sostenibilidad y rentabilidad en la producción de azafrán.

12. Es necesario seguir investigando mediante ensayos clínicos para reforzar la evidencia actual de los efectos beneficiosos del azafrán y de sus principales compuestos bioactivos, así como también de los subproductos florales.

1. Saffron and its floral by-products stand out for their high content of dietary fiber, proteins, organic acids, minerals and polyunsaturated fatty acids, as well as being natural sources of bioactive compounds, especially carotenoids and terpenes, such as crocins, crocetin, picrocrocin and safranal in the stigmas, and phenolic compounds, mainly flavonols and anthocyanins in the floral by-products.

2. Among the volatile composition, saffron stigmas presented high levels of safranal, and the floral by-products volatile composition from fresh to dried samples was highly influenced by the drying method.

3. Microwave-assisted extraction (MAE) optimized by studying the effects of time, temperature and ethanol concentration showed the optimal conditions for the extraction of bioactive compounds from saffron floral by-products, being a sustainable and efficient technique with low energy footprint. This information would allow the selection of the most suitable extraction method and parameters, as well as to be scaled up to the industrial level.

4. The application of environmentally friendly extraction solvents such as natural deep eutectic solvents (NaDES) combined with ultrasound-assisted extraction (UAE), together with the optimization of extraction conditions (time, power and NaDES:water ratio), is an efficient, innovative and green alternative to obtain high-added value compounds from saffron and its floral by-products.

5. The application of green extraction technologies such as MAE and UAE significantly improved the content of bioactive compounds expressed in terms of total phenolic and flavonoid content, as well as the antioxidant activity of saffron and its floral by-products extracts, with respect to conventional sonication methods.

6. Chitosan/alginate hydrogels are suitable matrices for the stabilization of NaDES-extracts, as their incorporation into these hydrogels allowed that total phenolic content remained stable after the oral and gastrointestinal *in vitro* digestion.

7. Microcapsules based on sodium alginate with saffron and its floral by-products extracts are suitable ingredients for their incorporation in dairy matrices such as yogurts, since they do not alter the fermentation process and improve their functional properties in terms of bioactive content and antioxidant properties, remaining stable for 21 days of refrigerated storage.

8. Wheat and spelt breads enriched with saffron floral by-products improved their nutritional and functional properties, mainly their antioxidant activity and bioactive content. These antioxidant properties remained stable after the oral and gastrointestinal *in vitro* digestion.

9. The developed beverages by using saffron floral by-products as ingredients with different food thickeners (xanthan gum, guar gum, β-glucan, pectin) presented a high content of phenolic compounds (kaempferol, quercetin and isorhamnetin derivatives).

10. Both saffron and its floral by-products, and their respective extracts, obtained by green extraction technologies, have been successfully used in bread, yogurt and beverage formulations, making them sustainable and innovative ingredients for the nutritional and functional improvement of traditional foods and for new applications in the agri-food industry or in other sectors, such as cosmetics or pharmaceuticals.

11. This Doctoral Thesis represents an advance on the nutritional and functional value of saffron spice and its floral by-products, whose valorization will generate positive economic impacts for the saffron industry and will contribute to the minimization of the environmental impact, improving sustainability and profitability in saffron production.

12. Further research is needed through clinical trials to reinforce the current evidence of the beneficial effects of saffron and its main bioactive compounds, as well as of the floral by-products.


6.REFERENCIAS

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7.PUBLICACIONES

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Publicación 1

(Transcripción literal)

Saffron bioactives crocin, crocetin and safranal: effect on oxidative stress and mechanisms of action

Autores	Débora Cerdá-Bernad, Estefanía Valero Cases, Joaquín Julián Pastor & María José Frutos
Revista	Critical Reviews in Food Science and Nutrition
DOI	10.1080/10408398.2020.1864279
Editorial	Taylor & Francis
Fecha de publicación	24 de Diciembre de 2020
Categoría JCR®	Food Science and Technology
Cuartil	Q1
Rango	4/139
Factor de impacto	7,862

Saffron bioactives crocin, crocetin and safranal: effect on oxidative stress and mechanisms of action

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ABSTRACT

Saffron (*Crocus sativus* L.) is used as a spice for its organoleptic characteristics related to its coloring and flavoring properties, and it has been also used in traditional medicine to treat various diseases. The main chemical components responsible for these properties are crocin, crocetin and safranal. These compounds have been shown to have a wide spectrum of biological activities, including several properties as antigenotoxic, antioxidant, anticancer, antiinflammatory, antiatherosclerotic, antidiabetic, hypotensive, hypoglycemic, antihyperlipidemic, antidegenerative and antidepressant, among others. The present review article highlights the antioxidant effects of these bioactive compounds to reduce reactive oxygen species and the mechanisms of action involved, since there are a multitude of diseases related to oxidative stress and the generation of free radicals. Recent studies have shown that the effects of crocin, crocetin and safranal against oxidative stress include the reduction in lipid peroxidation (malondialdehyde levels) and nitric oxide levels, and the increase in the levels of glutathione, antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) and thiol content. Therefore, due to the great antioxidant effects of these saffron compounds, it makes saffron a potential source of bioactive extracts for the development of bioactive ingredients, which can be used to produce functional foods.

Keywords: saffron apocarotenoids; functional ingredients; saffron supplementation; antioxidant activity; nutrients; health

Introduction

Crocus sativus L., commonly known as saffron, is a perennial herb which belongs to the *Iridaceae* family with more than 85 species. It is a traditional Mediterranean plant, and it is widely cultivated in different areas such as Iran, India, Morocco, Azerbaijan, Spain, Greece, Italy, Turkey and China, among others (Karabagias et al., 2017). *C. sativus* L. is a sterile low-rise plant developed and propagated by corms, a bulbous tuberous structure. The flower is composed of six purple tepals, three golden yellow stamens and one red pistil, which culminates with three red branched stigmas (filaments) that when dried up give the spice saffron (Lotfi et al., 2015; Mathew, 1977) (Figure 1). Therefore, only that part of the saffron flower is used to obtain the spice. It is the most expensive spice in the world because of its production costs, since to produce 1 kg of saffron are necessary around 230.000 flowers (350 kg of tepals) (Kafi et al., 2006).

Saffron is mainly used in agro-food and cosmetic industries due to its organoleptic characteristics related to its coloring and flavoring properties, and it has been used for centuries as part of a healthy Mediterranean diet. The chemical composition of saffron consists mostly on carbohydrates (starch, gums, pentosans, reducing sugars, pectin, dextrins) (63%), amino acids and proteins (12%), moisture (10%), fat (5%), minerals (5%), crude fiber (5%), and vitamins specially vitamin B1 (thiamine) and vitamin B2 (riboflavin). Other important constituents of saffron are carotenoids, monoterpenes, anthocyanins and flavonoids (Rios et al., 1996).

Traditionally, it has been also used in medicine to treat various diseases, since it is considered as a medicinal plant in many cultures. The main chemical components responsible for these properties are apocarotenoids such as crocin, crocetin and safranal considered as bioactive compounds (Melnyk, Wang, and Marcone, 2010). These compounds have been shown to have a wide spectrum of biological activities, including several properties as antigenotoxic, antitumor (Festuccia et al., 2014), anticancer (Abdullaev and Espinosa-Aguirre, 2004), antioxidant (Ghadrdoost et al., 2011; Karimi et al., 2010; Verma and Bordia, 1998), antiinflammatory (Amin and Hosseinzadeh, 2012), antidiabetic (Sheng et al., 2008), antiatherosclerotic (He et al., 2005), hypotensive, hypoglycemic, antihyperlipidemic (Lee et al., 2005), antidepressant (Schmidt, Betti, and Hensel, 2007) and antidegenerative (Soeda et al., 2016), among others.

One of the greatest interests of saffron bioactive compounds is their impact on the human health due to their high antioxidant capacity and free radical (FR) scavenging activity (Hosseinzadeh, Shamsaie, and Mehri, 2009). The generation process of reactive oxygen species (ROS) takes place in normal cells, but oxidative stress originates as a consequence of the uncontrolled generation and increase of ROS levels (Valko et al., 2007). Oxidative stress is a harmful process because ROS and FRs can attack and lead

to extensive damage to biological molecules such as DNA, lipids and proteins, causing a variety of diseases: cancer, metabolic syndrome, neurological disorders, inflammation and cardiovascular diseases, affecting almost all organs in the body (Fang, Yang, and Wu, 2002; Seifried, 2007). The oxidative systems may be disturbed by various physical, chemical and biological agents. Therefore, a wide range of factors such as psychological stress, diet (high in fat, sugar and processed foods), lifestyle, drugs, alcohol, smoking, obesity, infections, and environmental factors such as the exposure to corrosive chemicals and toxins, excessive exposure to x-rays, sunlight and radioactive materials, or extreme temperatures can contribute to the excess FR production, causing the generation of abnormally high levels of oxidative stress (Gholamnezhad, Keyhanmanesh, and Boskabady, 2015; Makhlouf et al., 2011).

In order to combat the oxidative damage, cells have evolved a complex antioxidant system including exogenous and endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). SOD is an endogenous antioxidant enzyme that constitutes an important defense system against ROS. It catalyzes the dismutation of two molecules of superoxide anion to hydrogen peroxide and oxygen, rendering the superoxide anion less hazardous (Fridovich, 1995). CAT is an antioxidant enzyme which is present almost in all living tissues that use oxygen and completes the detoxification process started by SOD, catalyzing the degradation or reduction of hydrogen peroxide to water and oxygen (Chelikani, Fita, and Loewen, 2004). GPx is an important intracellular enzyme which also plays an important role against oxidative stress. It breaks down hydrogen peroxides to water and decomposes lipid peroxides to their corresponding alcohols mainly in the mitochondria, acting against a wide range of peroxides which react with glutathione (Goth, Rass, and Pay, 2004). Glutathione, a non-protein intracellular thiol, is generated by cells as an antioxidant against oxidative stress. In normal cells, more than 90% of glutathione is in the reduced form (GSH), which is maintained by glutathione reductase (GR). Glutathione S-transferase (GST) conjugates GSH to a variety of substrates for detoxification (Hayes, Flanagan, and Jowsey, 2005). Other important scavengers of FRs and ROS are sulfhydryl (thiol, -SH) groups, highly-reactive constituents of protein molecules which play important functions in several biochemical and metabolic processes, such as the activation of antioxidant enzymes. Other interesting markers of oxidative stress are malondialdehyde (MDA) and nitric oxide (NO). MDA is one of the end products in the lipid peroxidation process, one of the reactions induced by oxidative stress, that leads to an increase in phospholipids rigidity (Gawel et al., 2004). NO is an unstable free radical which can be generated endogenously in several cells by nitric oxide synthases (NOS) and has different physiological and biochemical functions. In

oxidative stress conditions, it is produced in large excess or produced with ROS concurrently, causing tissue damage and inducing apoptotic cell death (Mayer and Hemmens, 1997).

Thus, the antioxidant properties of saffron bioactive compounds could be an effective way to fight against several disorders and to prevent diseases via modulation of oxidative stress markers, depressing ROS-derived oxidative stress. The effects of saffron extracts and its bioactive compounds have been reviewed previously (Bathaie and Mousavi, 2010; Broadhead et al., 2016; Giaccio, 2004). However, the present article is focused on reviewing the studies conducted to date about the antioxidant effects of crocin, crocetin and safranal to inhibit the oxidative stress, featuring the most recent studies with new findings, and trying to explain their possible mechanisms of action. Therefore, the present review article highlights the antioxidant effects of saffron bioactive compounds crocin, crocetin and safranal against oxidative stress to reduce ROS and promote the reduction of lipid peroxidation (MDA levels) and the increase of GSH level and antioxidant enzymes (SOD, CAT, GPx), among other possible mechanisms of action involved. Besides, this review will examine the beneficial effects of these saffron bioactive compounds on several diseases, and the future perspectives to use saffron as a potential source of bioactive extracts for the development of bioactive ingredients to produce functional foods.

Saffron bioactive compounds: crocin, crocetin and safranal and their physicochemical properties, bioavailability and toxicity

Crocin

Crocins are glycosyl esters of crocetin, formed by esterification of crocetin with different glycosides, being the geometric isomers *trans* the majority and *cis* isomers the minority (Carmona et al., 2006). Crocetin molecule is modified by the activity of glucosyltransferases, adding different numbers of glycosidic molecules to produce crocins, the major components of the stigmas of saffron and which confer solubility in water (Ahrazem et al., 2015). The content of crocetin esters in saffron represents 16%–28% (Moratalla-López et al., 2019).

The most abundant carotenoid glycoside in saffron is α-crocin or crocin-1 ($C_{44}H_{64}O_{24}$, *trans*-crocetin di-(β-d-gentiobiosyl) ester), a diester formed from the disaccharide gentiobiose and the dicarboxylic acid crocetin, with a molecular weight of 976.96 g/mol and a melting point of 186 °C (Figure 2). It is also the most important and studied due to its antioxidant activity, protecting against oxidative stress (Rahaiee et al., 2015). It easily dissolves in water, unlike most carotenoids, because of the saccharide

link with sugars, and is barely soluble in alcohol, ether and other organic solvents (Carmona et al., 2006). Crocin has a golden-yellow colour and it is responsible for the intense characteristic red colour of saffron, used as a natural food colorant (Lage and Cantrell, 2009; Pfander and Schurtenberger, 1982). Besides, the maximum absorbance showed is at 440 nm (Shahi, Assadpour, and Jafari, 2016).

Crocins lose most of their functionality after exposure to oxygen, light, heat and acidic environment due to their low stability. They also have low bioavailability, and poor absorption, being not absorbed after oral administration, until they are hydrolyzed due to enzymatic processes in the intestinal epithelial cells and by the fecal microbiome to deglycosylated *trans*-crocetin in the intestinal tract. *Trans*-crocetin is absorbed by passive diffusion through the intestinal barrier (Lautenschläger et al., 2015).

The safety of crocins has been evaluated in some studies in mice. The acute and sub-acute toxicity of α-crocin were evaluated in mice and rats. The acute toxicity during 2 days with doses administration orally and intraperitoneally up to 3 g/kg b.w. did not produce death. The sub-acute toxicity during 21 days administered intraperitoneally at doses of 15, 45, 90 and 180 mg/kg b.w. did not show toxic effects at pharmacological doses on any pathological, biochemical and hematological parameters (Hosseinzadeh et al., 2010). Regarding genotoxicity and mutagenicity, crocin did not exhibit mutagenic and toxic effects in the Ames test (Abdullaev et al., 2003; Alavizadeh and Hosseinzadeh, 2014). A summary of the toxicological effects of crocin is presented in Table 1.

Crocetin

Crocetin ($C_{20}H_{24}O_4$, 8,8'-Diapocarotenedioic acid) is a natural carotenoid dicarboxylic acid with a molecular weight of 328.40 g/mol and a melting point of 285 °C (Figure 2) (Samarghandian and Borji, 2014). It is a hydrophobic compound, insoluble in water and most organic solvents, except for pyridine and dimethylsulfoxide. In its anionic form is water soluble, easily dissolves in dilute aqueous sodium hydroxide or other aqueous alkali solutions (Escribano et al., 1996). Crocetin is the precursor of crocin, and it is obtained as the result of crocin glycosides hydrolysis (Moraga et al., 2004).

Crocetin just like crocins have low stability, but a higher bioavailability, and rapid absorption. After the oral administration of crocins which are hydrolyzed to *trans*- and *cis*-crocetin, they are incorporated into the bloodstream. Several studies have reported that levels of crocetin in blood were very high by the oral administration of crocin that could be due to the crocins hydrolysis to *trans*-crocetin in the gastrointestinal lumen and the intestinal mucosa, which is rapidly absorbed by passing to the blood stream through the portal vein (Christodoulou et al., 2019; Moratalla-López et al., 2019).

Respect to crocetin toxicity, some studies have revealed that crocetin is not teratogenic and genotoxic, but there is no data about its LD_{50} value (Martin, Goh, and Neff, 2002; Ozaki et al., 2002). Several studies showed that LC_{50} values for saffron and its constituents against normal cells could be higher than pharmacological doses (Hashemi and Hosseinzadeh, 2019). Besides, the safety of crocetin was evaluated in healthy adult volunteers (37.5 mg per day) for four weeks and the results did not show significant changes in blood biochemistry and hematology (Yamashita et al., 2018). A summary of the toxicological effects of crocetin is presented in Table 1.

Safranal

Safranal ($C_{10}H_{14}O$, 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde) is a monoterpene aldehyde with a molecular weight of 150.21 g/mol and a melting point of below 25 °C (Figure 2). Safranal represents about 60–70% of volatile fraction of saffron and it is responsible for its characteristic aroma. Safranal has a low aqueous solubility and its maximum absorbance is at 330 nm (Carmona et al., 2007; Tarantilis and Polissiou, 1997).

Safranal is a cyclic terpenic aldehyde which is produced via enzymatic and thermal degradation during saffron storage and drying process (Rezaee and Hosseinzadeh, 2013). Greater amounts of safranal are obtained at higher temperatures (80 °C) and less processing time (Himeno and Sano, 1987).

However, the biomedical and pharmacological properties of saffron are attributed mostly to the carotenoids, crocetin and crocins, and not to the aldehyde safranal, the major compound of the volatile fraction of saffron, because its bioactivity is more variable and less known than that of carotenoids (Bolhassani, Khavari, and Bathaie, 2014; Kyriakoudi et al., 2015; Moratalla-López et al., 2019). The safety of safranal has been evaluated in some studies. The acute and sub-acute toxicity of safranal was evaluated in mice and rats. The acute toxicity during 2 days with different doses of safranal administered orally and intraperitoneally, and the sub-acute toxicity during 21 days, administrating safranal orally at doses of 0.1, 0.25 or 0.5 ml/kg b.w. per day. The LD_{50} values indicated that safranal was non-toxic in acute oral administration and of low toxicity in acute intraperitoneal administration, however in sub-acute toxicity, safranal induced some changes in biochemical and hematological parameters (Hosseinzadeh et al., 2013). A summary of the toxicological effects of safranal is presented in Table 1.

Bioactive compounds, antioxidant effects and the mechanism of action of crocin, crocetin and safranal

Crocin

Crocin is a high potency antioxidant with enormous pharmacological properties. Its beneficial physiological effects against certain diseases have been tested. Bandegi et al. (2014) investigated the protective effects of saffron and crocin extract against chronic stress induced oxidative stress in adult male rats. To develop the study, the authors investigated whether saffron and crocin extract can protect the kidney, liver and brain against chronic stress. The levels of the lipid peroxidation (MDA content), and the activity of the antioxidant enzymes (SOD, GPx, GR) were measured after the end of the induced chronic stress. Stress was induced by restriction of the animals for 6 h per day for 21 days. After 6 h of restriction, the rats were injected intraperitoneally with saffron and crocin extract at doses of 30 mg/kg b.w. both the control and the stressed groups. The results showed that the administration of crocin and saffron could reverse the changes in stressed rats and had a positive effect to prevent the induced oxidative stress. Xiao et al. (2019) evaluated the antidepressant activity of crocin in male mice C57BL/6L. The depression was induced into three groups with corticosterone (20 mg/kg b.w.) by subcutaneous injection during 28 days. The control group was treated with saline solution. Depression was detected through the attenuation of depression-like behaviours such as the forced swim test, tail suspension test, and open field test. After detecting depression, two experimental groups were treated with 20 and 40 mg/kg b.w. of crocin administered orally during 2 weeks. Liver function and histomorphology, body weight, neuroinflammation, and oxidative stress were investigated. The results showed that the oral administration with 40 mg/kg b.w. of crocin acted as a good antidepressant through the reduction of oxidative stress and inflammation. However, none of the administered doses had an effect in restoring the histomorphological findings compared to those of the control group. Only when 40 mg/kg b.w. of crocin was administered, a reduction in ballooning degeneration (a histomorphologic change seen in liver pathology) was observed in the liver of the mice treated with corticosterone.

The therapeutic effect of crocin after intracerebral haemorrhage that may be related to oxidative stress was tested *in vivo* with male mice. Intracerebral haemorrhage was induced with collagenase injection. Mice were randomly divided in two groups; one group was treated with crocin (40 mg/kg b.w.) and the other with saline solution through oral gavage 6 h after intracerebral haemorrhage and then every 12 h for up to 7 days. The results showed that crocin could reduce the neurological deficit, myelin loss, neuronal degeneration and iron deposition (as the principal factor for ROS generation)

after intracerebral haemorrhage. A reduction was also observed in hemo-oxigenase expression and in ROS production during the early stage of intracerebral haemorrhage (Duan et al., 2019).

The effect of crocin has also been investigated as a potential anti-inflammatory in acute pancreatitis induced by cerulein in rats. Animals were randomized into five groups: normal control, cerulein control, crocin control (100 mg/kg b.w.), low dose of crocin with acute pancreatitis (30 mg/kg b.w.) and high dose of crocin with acute pancreatitis (100 mg/kg b.w.). The results showed that crocin could protect against acute pancreatitis, being able to observe the enormous reduction in pancreatic edema and the levels of inflammatory pancreatic cytokines (Godugu et al., 2020). Qiu et al. (2020) reported protection of renal and hypoglycemic properties with the administration of crocin in obese and type 2 diabetic (db/db) and nondiabetic (db/m) mice. The db/db and db/m were fed with a high-sucrose/hig-fat diet and with a normal diet, respectively. Mice were treated with normal saline (8 ml/kg b.w.), metformin (positive control group) (100 mg/kg b.w.), and crocin (50 mg/kg b.w.) by gavage dissolved in the physiological saline with a daily dose for 8 weeks. The administration of crocin showed that the regulation of the reduced nuclear factor-kappa B (NF-κB) signal suppressed its activation through the nuclear factor erythroid 2-related factor 2 (Nrf2) activation signal, and this effect was in relation to the modulation of oxidative stress. At the same time, the anti-inflammatory properties of crocin showed hypolipidemic, hypoglycemic and renal protective activities. In another study, the daily oral administration of crocin by gavage (25 mg/kg b.w.) for 28 days showed a potential effect as an effective therapy against bleomycin-induced idiopathic pulmonary fibrosis in rats. The idiopathic pulmonary fibrosis was induced by a single dose of bleomycin dissolved in physiological saline on day 7. Bleomycin administration produced pathological changes in the lung, which may be related to the suppression of antioxidant enzymes and the induction of inflammatory and oxidative markers. However, the crocin-treated rats showed a decrease in the damage caused by the administration of bleomycin (tumor necrosis factor-α (TNF-α), MDA levels, CAT and GPx activities), except in SOD activity and lung index, that may be related to the antioxidant capacity of crocin (Mehrabani et al., 2020). Therefore, it is necessary to continue conducting studies to know the protection mechanisms because they are still unclear.

The cardioprotective effect of crocin has been evaluated through the study of different cardiovascular diseases. Myocardial fibrosis was induced by isoproterenol in mice. The animals were randomly divided into 5 groups, the crocin groups were injected intraperitoneally with 100 mg/kg b.w. and 200 mg/kg b.w., respectively, for 14 days. Crocin administration decreased oxidative stress, apoptosis and inflammation

associated with myocardial fibrosis, which may be related to reduced NF-κB, p65 and toll like receptor (TLR-4) expression (Jin et al., 2020). The possible cardioprotective effect of crocin against doxorubicin (a common drug to treat a wide range of tumors) that induces cardiotoxicity as the main adverse reaction. Therefore, the cardioprotective effect of crocin against doxorubicin was tested in 4 groups of male rats. Each group was given an intraperitoneal injection: control group with a daily dose of normal saline solution and low, medium and high dose groups with crocin with 30 mg/kg b.w., 60 mg/kg b.w., and 120 mg/kg b.w., respectively for 6 days. Finally, the results showed that the administration of crocin protected against cardiotoxicity induced by doxorubicin by inhibiting inflammation, correcting cardiomyocyte calcium dysomeostasis, oxidative stress and mitochondria damage (Chu et al., 2020). Another study investigated the cardioprotective effect on isoprenol-induced cardiotoxicity in rats in the form of myocardial infarction. The groups of animals were treated daily with crocin at 5, 10 and 15 mg/kg b.w. using a gastric tube for 21 days. This study also demonstrated the cardioprotective potential of crocin administration through modulation of oxidative stress (Goyal et al., 2010). Wang et al. (2018) also reported the potential antioxidant effect of crocin to improve myocardial infarction in rats treated with crocin 20, 40, and 60 mg/kg b.w., intraperitoneally, for 7 days. However, further studies are necessary to know other pathways and possible mechanisms involved with cardioprotective effects.

In addition, in the animal model and *in vitro* culture of human cancer cells, administration of crocin also had promising results through suppression of tumor cell proliferation, apoptosis, and inhibition of telomerase activity in different types of cancer such as colorectal (Aung et al., 2007), skin (Sun et al., 2011), prostate (Festuccia et al., 2014), lung, liver and breast (Ashrafi et al., 2015; Khosrojerdi et al., 2012; Nasimian et al., 2020).

Although it is well established that crocin has a potential antioxidant effect to prevent oxidative stress and related diseases, the underlying mechanisms are still unclear. Therefore, more studies are needed to delve into other pathways and possible mechanisms involved in the effects of crocin in different diseases. Furthermore, most of the studies are carried out *in vitro* or with animal models. Thus, it is necessary to further investigate the effects of crocin in oxidative stress related pathologies in intervention studies. A summary of the studies conducted to date of the antioxidant effects of crocin is presented in Table 2 and the possible mechanisms of action involved are showed in Figure 3.

Crocetin

Several researchers have investigated the antioxidant activity of crocetin against oxidative stress. Regarding *in vitro* studies, Karimi et al. (2020) investigated the effects of crocetin, on an *in vitro* retinal pigment epithelium model (aged ARPE19 cells) of oxidative stress induced by tert-butyl hydroperoxide (TBHP). ARPE19 cells were incubated for 24 h with crocetin (0, 100 and 200 μM), and it was able to pass through the blood–retinal-barrier. The mechanism of action of crocetin to protect stressed cells was through the activation, in the first minutes of TBHP exposure, of the extracellular signal-regulated kinase 1/2 (ERK1/2), a member of mitogen-activated protein kinases (MAPK) signaling cascade involved in regulation of many cellular processes, and in the preservation of energy production pathways. Thus, crocetin may be a potential agent to prevent neurodegenerative diseases as age-related macular degeneration. Furthermore, Sapanidou et al. (2016) studied the antioxidant effects of crocetin on bovine spermatozoa during *in vitro* fertilization against oxidative stress. After the incubation of bovine sperm samples with crocetin (1, 2.5, and 5 μM) for 120 or 240 min, the results showed an improvement in bovine sperm quality and its fertilization ability with 2.5 μM of crocetin by modulating ROS production and lipid peroxidation, decreasing superoxide anion and hydrogen peroxide generation.

Other studies have also evaluated the antioxidant effects of crocetin against oxidative stress in cells. Zheng et al. (2015) indicated that crocetin had positive effects on angiotensin II-induced vascular cell adhesion molecule-1 (VCAM-1) and monocyte-endothelial cell adhesion in human umbilical vein endothelial cells. Crocetin (0.1, 1, and 10 μM) was able to inhibit angiotensin II-induced VCAM-1 expression and monocyteendothelial cell adhesion by blocking of NF-κB activation that could be due to its antioxidant properties by decreasing ROS generation and increasing antioxidant content. Papandreou et al. (2011) demonstrated that crocetin protected human neuroblastoma cells viability against H2O2-induced toxicity. Cells were incubated for 18 h with crocetin (1–125 μM) and this carotenoid decreased ROS production and repressed caspase-3 activation involved in apoptosis, showing its protective effects. Cai et al. (2009) showed that crocetin protected primary rat cultured cardiac myocytes against cardiac hypertrophy via modulating MAPK/ERK1/2 signaling pathway. Crocetin (1–10 μM, for 6, 12, 24 and 48 h) dose-dependently inhibited cardiac hypertrophy through blocking ROS-dependent MAPK/ERK1/2 pathway and GATA binding protein 4 activation and had anti-inflammatory effects by blocking NF-κB signaling. Tseng et al. (1995) studied the protective effects of crocetin against genotoxicity induced by paraquat in rat primary hepatocytes. Crocetin (5, 10 and 20 μM, for 1 h) prevented hepatotoxicity

through decreasing lipid peroxidation (MDA levels) and superoxide anion formation, by its FR-quenching capacity.

According to *in vivo* investigation with animal models, some studies have reported cardiovascular protective effects of crocetin. Higashino et al. (2014) studied the cardioprotective effects of the oral administration of crocetin (25 or 50 mg/kg b.w. per day) for 3 weeks in stroke-prone spontaneously hypertensive male rats. After the treatment, the levels of 8-hydroxy-2-deoxyguanosine (8-OHdG), a marker of oxidative stress (DNA damage), and NO metabolites nitrite/nitrate in urine were significantly decreased. Shen and Qian (2006) evaluated the antioxidant properties of crocetin against cardiac hypertrophy induced by norepinephrine in rats. The treated group with crocetin, administered intraperitoneally at 25 and 50 mg/kg b.w. for 15 days, showed a decrease in the lipid peroxidation and an increase in endogenous antioxidant enzymatic activities (SOD, GPx) in cardiac tissue. Therefore, these studies demonstrated the cardioprotective effect of crocetin, that might be related to the modulation of antioxidant enzymatic activities and it could restrict the effects of ROS-related mechanisms, ameliorating cardiovascular damages and being useful to treat cardiovascular diseases.

Other *in vivo* studies have focused on the neuroprotective effects of crocetin. Khan et al. (2012) studied the effects of crocetin in neurodegenerative disorders, combined with certain natural antioxidants (extracts of roots of *Nardosatchys jatamansi* and sodium selenite) against oxidative stress in male albino rats with cognitive impairment induced by streptozotocin. The treatment (crocetin 25 μg/kg b.w., orally) for 15 days showed a synergic potential effect that significantly reduced the lipid peroxidation (MDA levels), and increased GSH levels and the activity of the antioxidant enzymes, GPx, CAT and GST, in rat hippocampus and frontal cortex, compared to the control group (normal saline). Thus, this combination that includes crocetin could provide a protective effect to neurodegeneration. Ishizuka et al. (2013) researched the effect of crocetin to inhibit retinal ischemic damage which leads to neuronal cell death in male mice. The oral administration with crocetin (20 mg/kg b.w. twice a day) for 4 days showed a protective effect against retinal ischemic damage partly due to its antioxidant properties. In the crocetin treated group, oxidative stress was reduced, since the expression of 8-OHdG in the retina was significantly lower than in the control group (oral administration of 20 mg/kg b.w. sodium carboxymethyl cellulose). Furthermore, crocetin prevented the activation of MAPK and redox-sensitive transcriptional factors, which play important roles in the retinal cell death induced by oxidative stress and they also showed anti-apoptotic properties, inhibiting retinal cell death. Crocetin reduced the phosphorylations of MAPK, c-Jun N-terminal kinases (JNK), and p38, and those of the redox-sensitive transcription factors c-Jun and NF-κB in the retina. These results

indicated that this bioactive compound may be a potential preventive treatment against retinal ischemic injury. Yoshino et al. (2011) showed that crocetin reduced oxidative stress induced by ROS in the brain of stroke-prone spontaneously hypertensive male rats, a high-oxidative stress model. After the oral administration of crocetin (100 mg/kg b.w.), there was a reduction of oxidative damage in the brain of treated rats, acting crocetin as a scavenger of ROS, such as the hydroxyl radical, exhibiting its antioxidant properties, compared to the control group (administration of sodium carboxymethyl cellulose). Therefore, these findings demonstrated that crocetin could provide protective effects to neuronal disorders due to its antioxidant capacity.

Crocetin also showed protective effects on skin oxidative damage. Ohba et al. (2016) investigated its effects on skin induced oxidative damage by ultraviolet-A *in vivo* and *in vitro*. After oral crocetin administration (100 mg/kg b.w. at 2, 4, and 6 h after the initiation of UV-A irradiation) in month old male kelch-like ECH-associated protein 1 (Keap1)-dependent oxidative stress detector transgenic mice, the oxidative stress was ameliorated, since lipid peroxidation in the skin was decreased. Regarding *in vitro* effects, in human skin-derived fibroblasts cells (NB1-RGB), after the incubation with crocetin (1 μM, for 1 h), ROS production was significantly reduced, and the expression levels of cleaved caspase-3 were decreased. Therefore, this carotenoid may be useful to protect skin against oxidative damage, decreasing ROS generation and subsequent cell apoptosis.

Besides, crocetin had antioxidant properties against oxidative damage in lungs. Several studies demonstrated the antioxidant capacity of this carotenoid against oxidative stress in lungs. Venkatraman et al. (2008) investigated its effects against mitochondrial damage induced by benzo(a)pyrene. Crocetin was administered intraperitoneally (20 mg/kg b.w.) in male albino mice for 18 weeks and the levels of ROS, lipid peroxides, GSH and the activities of ATPase, and mitochondrial enzymes were assessed in the lung. After the treatment, the results revealed that crocetin modulated lung mitochondria oxidative damage, increasing ATPase and mitochondrial enzymes activities and GSH levels, decreasing ROS generation and normalizing lipid peroxides, compared to the control group (corn oil intraperitoneal injection). Magesh et al. (2006) evaluated the intraperitoneal administration of crocetin (20 mg/kg b.w. dissolved in dimethyl sulphoxide) for 4 weeks in lung cancer-bearing male albino mice. The results of the treatment showed a decrease in lipid peroxidation levels, and an increase in the activities of antioxidant enzymes (SOD, CAT, GPx,) and GSH metabolizing enzymes (GR, GST) in lung and liver tissues from the treated mice, compared to the control group (orally corn oil). Crocetin, due to its antioxidant capacity that influences in the detoxification processes, may be a potential chemotherapeutic agent and be useful to

treat lung disorders. Yang et al. (2012) examined the effect of crocetin on acute lung injury induced by lipopolysaccharide in male and female mice. Crocetin was administered intragastrically (50 and 100 mg/kg b.w. for 1, 12, 24, 36, and 48 h) and after the treatment, the results showed that this bioactive compound was able to protect against acute lung injury by reducing oxidative stress indices, compared to the control group (normal saline solution). Lung myeloperoxidase (MPO) activity was significantly reduced, and the SOD activity was significantly increased in mice lung.

Other studies examined crocetin effects to inhibit oxidative stress in intestine and colon. Zhou et al. (2015) researched its protective effects on intestinal injury burn-induced in male rats. Crocetin was administered intraperitoneally immediately after burn injury (100 and 200 mg/kg b.w.), and animals in the control group received a subcutaneous injection of normal saline without crocetin treatment. The results showed that this bioactive compound was able to inhibit oxidative stress, increasing levels of the antioxidant enzymes (SOD, CAT, GPx) and decreasing the lipid peroxidation (MDA content) in intestinal tissue. In addition to its antioxidant activities, crocetin also reduced the levels of proinflammatory response, TNF-α, interleukin-6 (IL-6), and NF-κB activation in intestinal tissue. Kazi and Qian (2009) studied the protective mechanism of crocetin on ulcerative colitis induced by 2,4,6- trinitrobenzene sulfonic acid in female BALB/c mice. The treatment with crocetin (50 mg/kg, intragastrically) for 8 days significantly reduced the lipid peroxidation (MDA levels), the MPO activity and NO levels in the inflamed colon. Therefore, crocetin might be useful as a supplement to ameliorate diarrhea and the disruption of colon, and also to treat intestinal disorders, inhibiting oxidative tissue damage and improving the antioxidant defense system.

Therefore, these *in vivo* and *in vitro* studies conducted to date demonstrated the antioxidant properties of crocetin to inhibit oxidative stress, preventing and protecting against oxidative damage, but further research is needed for a deeper understanding of its mechanisms of action and more *in vivo* intervention studies with patients, including human clinical trials. A summary of the studies conducted to date of the antioxidant effects of crocetin is presented in Table 3 and the possible mechanisms of action involved are showed in Figure 3

Safranal

Recently, a large number of studies have been focused on the antioxidant activity of safranal to inhibit the oxidative stress which is related with a multitude of diseases. Regarding several *in vitro* studies, Rahiman et al. (2018) investigated the protective effect of safranal against oxidative damage and apoptosis in endothelial cells. Bovine

aortic endothelium cell line was incubated with safranal (2, 10, 20 and 40 μM) for 24 h. After the treatment, safranal reduced intracellular ROS levels and the rate of cell apoptosis, which was mediated via MAPK signaling pathways, activating stress-activated protein kinases (SAPK)/ JNK and ERK1/2. Safranal may be a potential drug for cardiovascular diseases therapies due to its antioxidant and anti-apoptotic activities. Pan, Qiao, and Wen (2016) studied the potential effect of safranal on Parkinson's disease using an *in vitro* model induced by rotenone which increased ROS generation and cell apoptosis. Primary dopaminergic cells isolated from rat embryos were incubated with safranal (10, 15, 20, and 50 μg/ml) for 4 h. The results revealed that safranal decreased intracellular ROS level and inhibited apoptosis. Furthermore, safranal inhibited the expression of Keap1 and upregulated the nuclear translocation of Nrf2. Nrf2 regulates antioxidant genes to protect against oxidative damage, and its transcription activity is controlled by Keap1. Safranal also promoted the antioxidant capacity, since the downstream antioxidant enzyme genes of Nrf2, such as glutamate-cysteine ligase catalytic subunit, GST, heme oxygenase 1 and NADPH-quinone oxidoreductase 1 were induced by this bioactive compound in the dopaminergic neurons. Therefore, safranal could be a potential and therapeutic agent to treat neurodegenerative diseases, protecting neurotoxicity associated with the Keap1/Nrf2 signaling pathway. Bukhari et al. (2015) showed that safranal reduced oxidative damage and prevented apoptosis in normal human bronchial epithelial cells asthma-induced. Cells were incubated with different concentrations of safranal (10 and 100 ng/ml, 1μg/ml) and after the treatment, NOS levels were significantly reduced, that led to a significant suppression of NO production and a reduction of peroxynitrite ion production, which was related to epithelial cell apoptosis. Therefore, this agent decreased oxidative stress in bronchial epithelial cells via reducing NOS and preventing apoptosis in these cells. Hosseinzadeh, Shamsaie, and Mehri (2010) evaluated the antioxidant capacity of safranal under *in vitro* methods, such as deoxyribose assay, erythrocyte membrane lipid peroxidation, and liver microsomal non-enzymatic lipid peroxidation. The results indicated that safranal (0.5, 1 and 2 mM) decreased MDA levels in red blood cells and liver microsomal non-enzymatic lipid peroxidation. In addition, safranal, in deoxyribose assay, showed hydroxyl radical scavenging effect, increasing antioxidant activity in a dose-dependent manner.

According to *in vivo* studies with animal models, safranal presented neuroprotective effects due to its antioxidant properties. Samarghandian et al. (2017) investigated about the antioxidant effects of safranal against chronic stress in rat brain. Chronic stress leads to several disorders that induce oxidative damage, producing a higher amount of FRs, generating an imbalance between ROS production and the antioxidant system. Albino rats were treated with safranal (0.25, 0.50 and 0.75 mg/kg

b.w. per day, intraperitoneally) for 21 days. In the stressed animals the levels of GSH and SOD and CAT antioxidant enzymes in the brain tissues were significantly lower and MDA levels were significantly higher than non-stressed rats. The results demonstrated that safranal had a neuroprotective effect, because the serum levels of MDA in the brain were significantly lower and levels of GSH and antioxidant enzymes were significantly higher in the treated group compared to the control. These findings indicated that the antioxidant effect of safranal could be mediated via increasing antioxidant enzymatic levels and decreasing the lipid peroxidation, being a potential compound to enhance the brain oxidative response in rats subjected to chronic stress. Baluchnejadmojarad, Mohamadi-Zarch, and Roghani (2019) evaluated the antioxidant effects of safranal on neurodegenerative disorders using male rats with Alzheimer's disease (AD) induced by intrahippocampal amyloid beta. During AD progression, an increment of oxidative damage occurred. Safranal was administered orally (0.025, 0.1, and 0.2 ml/kg b.w. per day) for one week. The results showed that safranal was able to ameliorate oxidative stress, since it reduced the hippocampal level of MDA decreasing the lipid peroxidation, ROS levels and protein carbonyl content (an index of protein oxidation), and improved SOD activity with no significant and beneficial effect regarding nitrite, CAT and GSH in hippocampal tissue. Therefore, safranal may prevent learning and memory impairment at a molecular level by reducing oxidative stress. Sadeghnia et al. (2017) studied the neuroprotective effect of safranal on brain injuries in adult male rats of transient focal cerebral ischemia induced by middle cerebral artery occlusion (MCAO). In ischemia, the oxygen restoration could lead to the generation of toxic levels of FRs, which end in lipid peroxidation, inhibition of protein synthesis and cell death (Ginsberg, 2009). Safranal was administered intraperitoneally (72.5 and 145 mg/kg b.w.) at 0, 3, and 6 h after MCAO induction. Markers of oxidative stress were evaluated using left cerebral portions, and the results demonstrated that safranal inhibited the oxidative stress caused by ischemia in the brain tissues of rats treated, since the lipid peroxidation was decreased, reducing MDA levels at both doses of safranal and there was an increment in the antioxidant capacity, and a significant increase in the total -SH content, compared to the control group that received saline solution. Thus, safranal administration had protective effects on ischemic reperfusion by the modulation of oxidative stress markers and increasing the antioxidant activity in brain tissues. Sadeghnia et al. (2013) also demonstrated the protective effect of safranal to the oxidative damage induced by quinolic acid in the hippocampus of adult male rats. The intraperitoneal administration of high doses of safranal (145.5 and 291 mg/kg b.w., after the induction) decreased quinolinic acid-induced lipid peroxidation and the oxidative DNA damage (% tail DNA, comet assay), and restored thiol redox status and the antioxidant power in the hippocampus the treated

group, compared to the control group (normal saline solution), showing that safranal could be a useful therapy to prevent and treat neurodegenerative disorders. Another study also indicated the potential effect of this bioactive compound to protect the oxidative damage induced by ischemia-reperfusion injury which leads to overproduction of ROS in the hippocampus of adult male rats. After intraperitoneal safranal administration (727.5 mg/kg, 363.75 mg/kg, 145.5 mg/kg, and 72.75 mg/kg b.w. for 72 h after the induction), the treated group showed a significant increase in the antioxidant capacity and total -SH content and a significant decrease in MDA levels at higher doses in hippocampal tissue compared to the control group, in which saline solution was given intraperitoneally (Hosseinzadeh and Sadeghnia, 2005).

In addition to the neuroprotective effects of safranal in the brain, several studies have demonstrated that the antioxidant properties of safranal had a positive effect regarding oxidative stress on other disorders, such as asthma, diabetes and other pathologies that affects several organs like liver, heart or skeletal muscle. The study of Hazman and Bozkurt (2015) indicated the potential antioxidant activity of safranal and free radical scavenging activity on the oxidative stress in male albino rats with type 2 diabetes (diabetic nephropathy) induced by high-fat diet and streptozotocin. Safranal, administered intraperitoneally (30 mg/kg b.w.) for 4 weeks, decreased the levels of oxidative stress in kidney by increasing total antioxidant capacity levels and decreasing total oxidant capacity and NO levels and oxidative stress index values (calculated by using total antioxidant capacity and total oxidant capacity levels) in the diabetic group compared to the control group (composed of healthy rats), but did not have a positive effect on GSH. Therefore, safranal due to its antioxidant activity may improve kidney damage in renal tissue and could be effective to treat the diabetic nephropathy. Another study also showed that safranal might be effective in the treatment of type 2 diabetes and its related gastric disorders, since the treatment with safranal (0.25, 2, 5 ml/kg b.w., orally) inhibited gastric lesions, induced by indomethacin in male adult nondiabetic and diabetic rats. Safranal decreased the gastric ulcer index (by dividing total number of ulcer spots by the number of animals), and the lipid peroxidation in the gastric mucosa, and increased GSH levels of gastric tissue in the treated group, compared to the control group (physiological saline). Safranal, like omeprazole, may be a potential antiulcer agent to prevent the gastric mucosa damage in rats (Kianbakht and Mozaffari, 2009). Regarding antioxidant effects of safranal against oxidative damage in other pathologies, Bukhari et al. (2015) presented that safranal ameliorated asthma in BALB/c mice. During asthma, NOS levels were induced, and NO production was increased, causing bronchorelaxation and it reacted with superoxide ions, producing peroxynitrite ion that leads to epithelial cell damage. Safranal, administered orally (1 and 10 mg/kg b.w.) for 7

days, reduced the oxidative damage in inflamed lungs due to allergic reaction in mice lungs, decreasing significantly NOS levels and preventing the epithelial cell injury which was associated to peroxynitrite ion production. Farahmand et al. (2013) were focused on the safranal effects during aging in male aged rats, in which the oxidative damage was increased. Safranal was administered intraperitoneally (0.5 mg/kg b.w. per day) for one month, and after the treatment in aged rats, the hepatic antioxidant enzymes SOD, CAT and GST levels increased significantly, and MDA level in liver homogenate and NO serum content decreased significantly. This study elucidated the potential treatment of safranal to prevent oxidative damage in liver and as anti-aging compound in old rats.

In addition to these protective effects, other researchers investigated the cardioprotective effect of safranal and its anti-ischemia activity. Mehdizadeh et al. (2013) evaluated the potential effect of safranal against the oxidative damage in heart tissue induced by isoproterenol which leads to myocardial infarction followed by several biochemical alterations such as lipid peroxidation in male albino rats. Safranal was administered intraperitoneally (0.025, 0.050, 0.075 ml/kg b.w.) for 8 days, and the results revealed that MDA level in heart tissue was significantly decreased in the treated group, compared to the control group (normal saline solution for 9 days). Safranal protected myocardium from myocardial functional and structural damage through the modulation of antioxidant defense system, reducing the lipid peroxidation. Therefore, it could be effective against heart tissue oxidative damages. Hosseinzadeh, Modaghegh, and Saffari (2009) studied the protective effect of safranal against oxidative damage induced by ischemia-reperfusion injury in skeletal muscles of male rats. Intraperitoneal safranal administration (0.1, 0.25, and 0.5 ml/kg b.w.) showed anti-ischemia activity due to its antioxidant properties by decreasing MDA levels and increasing antioxidant capacity and total thiol content in rat skeletal muscle tissue.

Besides, Tamaddonfard et al. (2014) compared the antioxidant activity of safranal with that of the vitamin E (α-tocopherol), which is a potent antioxidant compound. The effects of safranal on sciatic nerve function in adult male rats were studied after induction of crush injury, being the oxidative stress one of the main causes of nerve damage. Rats were treated with safranal (0.05, 0.2 and 0.8 mg/kg b.w. per day, intraperitoneally) during 10 consecutive days or with subcutaneous injection of vitamin E (100 mg/kg b.w. per day). Safranal showed a positive effect decreasing blood levels of MDA at doses of 0.2 and 0.8 mg/kg and the same effect was observed in vitamin E in treated groups. The effects of safranal were comparable with those of vitamin E, and both ameliorated sciatic nerve function induced by the modulation of the oxidative stress pathway.

In addition of the rats and mice models, Boskabady, Byrami, and Feizpour (2014) evaluated the effect of safranal on ovalbumin sensitized guinea pigs, who drank water

containing three concentrations of the bioactive compound (4, 8 and 16 µg/ml). The results showed that total NO and nitrite serum levels were significantly reduced in treated groups, reporting that safranal might prevent tracheal responses due to its antioxidant effects.

Therefore, these studies conducted to date showed the antioxidant capacity of safranal against oxidative stress, but further *in vivo* research including human clinical studies is needed to deepen and have a broader knowledge of its mechanisms of action. A summary of the studies conducted to date of the antioxidant effects of safranal is presented in Table 4 and the possible mechanisms of action involved are showed in Figure 3

The effect of saffron supplementation to improve the health

This point explores the potential mechanisms of action of saffron supplementation to prevent several diseases by *in vitro* and *in vivo* studies.

Effects of saffron on cancer

Cancer is the result of uncontrolled cell division due to the effects of genetic and environmental factors. Many strategies have been developed against cancer development, by saffron administration to prevent the growth of cancer cells.

Recently, Akbarpoord et al. (2020) have carried out *in vitro* investigations to evaluate the effects of different saffron distillates concentrations (20, 40 and 100 µg/ml) on the expression of some self-renewal genes in gastric adenocarcinoma tumor cell line. The results showed that saffron administration reduced the expression of self-renewal genes, but this reduction was depended of the concentration of saffron extracts and on the duration of the treatment. The best concentrations were 40 and 100 µg/mL and the incubation time of 48 h. Bathaie et al. (2013) explored the therapeutic potential of saffron aqueous extract on gastric cancer. Three groups of albino rats were administered with 100, 150 and 175 mg/kg b.w. per day, respectively of the extract by intraperitoneal injection for 50 days. The results indicated that the higher doses of saffron aqueous extracts inhibited the cancer progression.

Positive results have also been reported in breast cancer using MCF7 cell line human. Saffron aqueous extract in different concentrations (100, 200, 400 and 800 µg/ml) was tested on matrix metalloproteinases (MMP) gene expression. The MMP play functions by degrading and modifying the cell extracellular matrix and cell-cell contacts. In this *in vitro* test, all concentrations showed an inhibitory effect on MMP gene expression respect to the control sample. However, the inhibitory effect was not dose dependent. The results showed that the MCF-7 cells treated with saffron at 200 µg/ml

showed the best reduction (18%) in gene expression (Mousavi, Baharara, and Asadi-Samani, 2014). The same author investigated the synergic effect of saffron and electromagnetic field (EMF) on the Vascular endothelial growth factor receptor 2 (VEGFR-2) gene expression on the same cell line culture and with the same saffron concentrations. The VEGFR-2 activation is considered critical in the proliferation and survival of the endothelial cells, and is the primary receptor involved in angiogenesis. The results showed that both treatments induced a considerable reduction on VEGFR-2 gene expression respect to the control group when administered separately. However, the synergic administration showed higher reduction on the VEGFR-2 gene expression with 29, 35 and 36% reduction using 200, 400 and 800 µg/ml of saffron, respectively. A higher reduction using the synergy method was observed from the lowest concentration of saffron (100 µg/ml) with a 38% of reduction (Mousavi, Baharara, and Shahrokhabadi, 2014). Therefore, these preliminary investigations proved that saffron could be a good chemotherapeutic agent in breast cancer treatment. A study conducted by Makhlouf et al. (2016) showed that the saffron extract administration on Jurkat cells was better to exert antiproliferative activities against human acute lymphoblastic cells than the crocin and safranal mixture administration. However, these *in vitro* studies must be corroborated by further *in vivo* research.

Effects of saffron on the neurological disorders

Neurological disorders exert a great influence on our society worldwide. Although the symptoms derived from these diseases are well known, the causes and mechanisms are complex and depend on multiple factors (Fernández, Valero-Cases, and Rincón-Frutos, 2019).

Currently, there is a controversy in the results regarding the saffron administration and its beneficial effects on depressive disorder. In one double-blind, controlled clinical trial with 30 mg per day of saffron capsule (containing saffron extract) or 40 mg per day of citalopram during 6 weeks as a treatment of major depressive disorder with anxious distress, 66 patients were recruited during visits to the psychiatric clinic to participate in the study. The results did not show any difference between the two experimental groups. Therefore, the saffron administration could be a good alternative to treat major depressive disorder with an anxious distress (Ghajar et al., 2017). In another double-blind, controlled clinical trial, 40 patients with a mild to moderate depression in post percutaneous coronary intervention were randomized to receive 30 mg per day of saffron in capsules or 40 mg per day of fluoexetine during 6 weeks. The results showed no significant differences between the two experimental groups. Therefore, saffron

administration showed the same efficacy in improving depression compared to treatment with fluoexetine (Shahmansouri et al., 2014).

On the other hand, in a double-blind, controlled clinical trial, patients undergoing coronary artery bypass grafting (CABG) with depression and anxiety were randomized to receive saffron capsules (15 mg twice a day) or placebo during 12 weeks. However, the results did not show significative differences between placebo or saffron groups. Therefore, these results did not support the saffron efficacy in improving anxiety and depression related to CABG (Moazen-Zadeh et al., 2018).

These studies showed controversial results, although they administered the same dose of saffron, and showed the same limitations: short observation periods and reduced sample size. Moreover, the studies reported by Ghajar et al. (2017) and Shahmansouri et al. (2014) did not consider a placebo group in experimentation.

Therefore, to summarize all research carried out to evaluate the saffron effect on mental health parameters and C-reactive protein a recent meta-analysis with 21 randomized clinical trials was conducted by Ghaderi et al. (2020). This meta-analysis concluded that the saffron administration did not affect C-reactive protein levels, Hamilton depression rating scale and Hamilton anxiety rating scale levels. However, saffron administration showed a significant reduction in beck anxiety inventory and Pittsburgh sleep quality index and beck anxiety inventory respect to antidepressant common drugs such as: imipramine, fluoxetine and citalopram. However, the results of this meta-analysis should be interpreted with caution due to heterogeneity of factors such as the period of administration, the concentration of saffron used, the differences between the samples of saffron (crocin, saffron liquid extract or powder) that were used. All these variabilities led to different effects on the parameters of mental health investigated.

In a recent systematic review, the effect of saffron supplementation in relation to cognitive impairment such as mild cognitive impairment or Alzheimer´s disease was also investigated. A total of 5 randomized controlled trials were included: one study with cognitively normal individuals and four studies with subjects with mild cognitive impairment or Alzheimer´s disease. Saffron supplementation was compared with Alzheimer´s medication or placebo. Results showed that saffron supplementation had a similar effect in improving cognitive impairment to the reference Alzheimer´s drug. Those results could be in relation with the potential effect of saffron against oxidative stress that takes place in the Alzheimer´s brain. However, the results should be interpreted carefully because the study presents a potential high risk of bias due to known and unknown factors. Therefore, studies with more individuals to reduce the risk of bias are required

to confirm the supplementation of saffron as an alternative treatment for these diseases (Avgerinos et al., 2020).

Effects of saffron on the cardiovascular diseases

Cardiovascular diseases (CVDs) are the mayor cause of mortality and morbidity worldwide. Among the risk factor of CVDs, diabetes, metabolic syndrome, genetics, high blood pressure, obesity and hyperlipidemia are the most common (Armenian et al., 2017; Chen et al., 2020).

A recent systematic review evaluated the effect of saffron supplementation on the metabolic profile in patients with metabolic syndrome or diabetes mellitus. This systematic review included 14 randomized control trials: one studied the impact of saffron in patients with prediabetes, one with coronary artery disease, four with metabolic syndrome and eight patients with diabetes mellitus. The results showed that the saffron supplementation had an impact on fasting blood glucose. However, authors commented some limitations such as the high heterogeneity in the studies, no report of titration in the administered supplement and few different protocols. Nevertheless, being a systematic review neither analysis nor meta-analysis was carried, only the research trails were evaluated qualitatively (Giannoulaki et al., 2020).

However, a recent meta-analysis conducted by Rahmani, Saberzadeh, and Takhshid (2020) reviewed the saffron effect on fasting plasma glucose, waist circumference (WC) and haemoglobin A1c (HbA1c). These parameters are used to detected and monitor the metabolic syndrome and diabetes mellitus. After removing the studies that were not in compliance with the inclusion criteria, 9 randomized clinical trial studies were selected. The results showed that saffron supplementation has not a significant effect on HbA1c. However, the meta-analysis found beneficial effect on fasting plasma glucose and WC after 12 weeks with saffron supplementation.

At the same time, the effect of saffron supplementation on inflammation and glycemia in patients with type 2 diabetes mellitus (DM2) has been investigated in a randomized double-blind, placebo-controlled clinical trial study. Two groups with 30 patients with DM2 were randomized in each one. For 8 weeks, one group was treated with 100 mg per day of powered saffron and another group, was treated with a placebo. The results showed that saffron supplementation reduced the TNF-α and IL-6, which play an important role as inflammatory markers and are present in a DM2 before detection of the disease. This reduction improved the inflammation status. At the same time, the saffron administration also had a significant effect on the reduction of glucose levels respect to the control placebo group (Mobasseri et al., 2020).

A summary of the studies conducted to date of the effects of saffron extracts supplementation to improve the health is presented in Table 5 and the possible mechanisms of action involved are showed in Figure 3.

Conclusions and future perspectives

The results of the *in vivo* and *in vitro* studies conducted to date about the antioxidant effects of the supplementation with saffron or its bioactive compounds crocin, crocetin and safranal show encouraging effects in the prevention of certain diseases related to oxidative stress, such as cardiovascular and respiratory diseases, cancer and neurological disorders, among others. The acute and sub-acute toxicity of crocin, crocetin and safranal have been studied in different *in vivo* research carried out in mice and rats, after oral or intraperitoneal administration, demonstrating that they did not show any toxic effects. Some studies also revealed that they are not genotoxic or teratogenic.

However, although there are many studies carried out with crocin, crocetin and safranal showing the effects of these compounds as oxidative stress inhibitors, their mechanisms of action are not still completely elucidated. Therefore, further *in vivo* research on human clinical studies with a larger number of individuals is needed, expanding the administration time, and with the inclusion of control groups for a deeper understanding of the pathways and possible mechanisms of action involved in these antioxidant effects. Moreover, the great antioxidant effect against oxidative stress of crocin, crocetin and safranal, makes saffron a promising source of bioactive extracts. Therefore, future research in the field of the potential of saffron derived bioactive ingredients for the development of functional foods is needed. This point explores the potential mechanisms of action of saffron supplementation to prevent several diseases by *in vitro* and *in vivo* studies.

Funding: This paper is supported by the PRIMA programme, project SAFFROMFOOD. The PRIMA programme is supported by the European Union. Thanks to the Spanish-Ministry of Education, Culture and Sport for the FPU-PhD fellowship, and thanks to Generalitat Valenciana and the European Social Found for the Vali+d Postdoctoral fellowship.

Conflicts of Interest: The authors declare no conflict of interest.

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Table 1. Summary of toxicological effects of crocin, crocetin and safranal.

Bioactive compound	Toxicity	Administration	Dose	Effects	Reference
Crocin	Acute	Orally and intraperitoneally in mice and rats, 2 days	1–3 g/kg b.w.	No toxic effects	Hosseinzadeh et al. (2010)
	Sub-acute	Intraperitoneally in mice and rats, 21 days	15-180 mg/kg b.w.	No toxic effects	Hosseinzadeh et al. (2010)
Crocetin	Sub-acute	Orally in healthy adult volunteers, 4 weeks	37.5 mg per day	No changes in blood biochemistry and hematology	Yamashita et al. (2018)
Safranal	Acute	Orally and intraperitoneally in mice and rats, 2 days	0.1 – 0.5 ml/kg b.w.	Low toxicity in intraperitoneal administration	Hosseinzadeh et al. (2013)
	Sub-acute	Orally in mice and rats, 21 days	0.1 – 0.5 ml/kg b.w.	Changes in biochemical and hematological parameters	Hosseinzadeh et al. (2010)

Table 2. Summary of studies conducted to date of the antioxidant effects of crocin.

Experimental model	Crocin administration	Effects	Reference
Adult male rats with chronic stress	Intraperitoneally, with saffron and crocin extract at 30 mg/kg b.w., for 21 days	Reverse the changes in stressed rats and had a positive effect to prevent the induced oxidative stress	Bandegi et al. (2014)
Male mice C57BL/6L with depression induced by corticosterone	Orally, 20 and 40 mg/kg b.w., for 2 weeks	40 mg/kg b.w. of crocin acted as a good antidepressant through the reduction of oxidative stress and inflammation	Xiao et al. (2019)
Male mice with intracerebral haemorrhage induced by collagenase	Oral gavage, 40 mg/kg b.w., 6 h after intracerebral haemorrhage and then every 12 h for up to 7 days	Reduction in the neurological deficit, myelin loss, neuronal degeneration and iron deposition after intracerebral haemorrhage	Duan et al. (2019)
Rats with acute pancreatitis induced by cerulein	30 and 100 mg/kg b.w.	Protection against acute pancreatitis. Enormous reduction in pancreatic edema and the levels of inflammatory pancreatic cytokines	Godugu et al. (2020)
Obese and type 2 diabetic and nondiabetic mice	By gavage, 50 mg/kg b.w., for 8 weeks	The anti-inflammatory properties of crocin showed hypolipidemic, hypoglycemic and renal protective activities	Qiu et al. (2020)
Rats with idiopathic pulmonary fibrosis induced by bleomycin	By gavage, 25 mg/kg b.w, for 28 days	Effective therapy against bleomycin-induced idiopathic pulmonary fibrosis	Mehrabani et al. (2020)
Mice with myocardial fibrosis induced by isoproterenol	Intraperitoneally, 100 and 200 mg/kg b.w., for 14 days	Decrease in oxidative stress, apoptosis and inflammation associated with myocardial fibrosis	Jin et al. (2020)
Male rats with cardiotoxicity induced by doxorubicin	Intraperitoneally, 30, 60 and 120 mg/kg b.w, for 6 days	Protection against cardiotoxicity, correcting cardiomyocyte calcium dysomeostasis, oxidative stress and mitochondria damage	Chu et al. (2020)
Rats with cardiotoxicity induced by isoprenol	Gastric tube, 5, 10 and 15 mg/kg b.w., for 21 days	Cardioprotective potential through modulation of oxidative stress	Goyal et al. (2010)
Rats myocardial infarction-induced	Intraperitoneally, 20, 40, and 60 mg/kg b.w., for 7 days	Improve myocardial infarction in rats treated with crocin	Wang et al. (2018)

Table 3. Summary of studies conducted to date of the antioxidant effects of crocetin.

Experimental model	Crocetin administration	Effects	Reference
In vitro retinal pigment epithelium model (aged ARPE19 cells)	0, 100 and 200 μM, for 24 h	Activation of the extracellular signal-regulated kinase 1/2	Karimi et al. (2020)
Bovine spermatozoa	1, 2.5 and 5 μM, for 2 or 4 h	Improvement in bovine sperm quality and its fertilization ability by modulating ROS [1] production and lipid peroxidation	Sapanidou et al. (2016)
Human umbilical vein endothelial cells	0.1, 1 and 10 μmol/l	Inhibition of nuclear factor-kappa B activation by decreasing ROS [1] generation and increasing antioxidant content	Zheng et al. (2015)
Human neuroblastoma cells	1–125 μM, for 18 h	Decrease in ROS [1] production and repression of caspase-3 activation	Papandreou et al. (2011)
Rat primary cardiac myocytes	1–10 μM, for 48 h	Inhibition of ROS [1]-dependent MAPK/ERK1/2 [2] pathway, GATA binding protein 4 activation and nuclear factor-kappa B signaling	Cai et al. (2009)
Rat primary hepatocytes	5, 10 and 20 μM, for 1 h	Decrease in the lipid peroxidation and superoxide anion formation	Tseng et al. (1995)
Stroke-prone spontaneously hypertensive male rats	Orally, 25 or 50 mg/kg b.w. per day, for 3 weeks	Decrease in 8-hydroxy-2-deoxyguanosine and nitric oxide metabolites nitrite/nitrate levels in urine	Higashino et al. (2014)
Rat with cardiac hypertrophy induced by norepinephrine	Intraperitoneally, 25 and 50 mg/kg b.w., for 15 days	Decrease in the lipid peroxidation and an increase in endogenous antioxidant enzymatic activities in cardiac tissue	Shen and Qian (2006)
Male albino rats with cognitive impairment induced by streptozotocin	Orally, 25 μg/kg b.w., combined with roots of *Nardosatchys jatamansi* and sodium selenite, for 15 days	Decrease in the lipid peroxidation and increase in glutahtione levels and the activity of antioxidant enzymes in rat hippocampus and frontal cortex	Khan et al. (2012)
Male mice with retinal ischemic damage	Orally, 20 mg/kg b.w. twice a day, for 4 days	Reduction in 8-hydroxy-2-deoxyguanosine expression and inhibition of the activation of mitogen-activated protein kinases and redox-sensitive transcriptional factors in the retina	Ishizuka et al. (2013)
Stroke-prone spontaneously hypertensive male rats	Orally, 100 mg/kg b.w.	Decrease in oxidative damage, reducing hydroxyl radicals in the brain	Yoshino et al. (2011)
Month old male Keap1 [3] dependent oxidative stress detector transgenic mice; human skin-derived fibroblasts cells	Orally, 100 mg/kg b.w. at 2, 4, and 6 h after the initiation of ultraviolet -A irradiation; *in vitro*: 1 μM, for 1 h	Decrease in the lipid peroxidation in the skin; *in vitro* effects, reduction in ROS [1] generation and the expression levels of cleaved caspase-3	Ohba et al. (2016)

Male albino mice with mitochondrial damage induced by benzo(a)pyrene	Intraperitoneally, 20 mg/kg b.w., for 18 weeks	Increase in ATPase and mitochondrial enzymes activities and glutathione levels; decrease in ROS [1] generation and lipid peroxides in lungs	Venkatraman et al. (2008)
Lung cancer-bearing male albino mice	Intraperitoneally, 20 mg/kg b.w. dissolved in dimethyl sulphoxide, for 4 weeks	Decrease in the lipid peroxidation, and increase in the activities of antioxidant enzymes and glutathione metabolizing enzymes in lung and liver tissues	Magesh et al. (2006)
Male and female mice with acute lung injury induced by lipopolysaccharide	Intragastrically, 50 and 100 mg/kg b.w., for 1, 12, 24, 36 and 48 h	Reduction in lung myeloperoxidase activity and increase in superoxide dismutase activity in lungs	Yang et al. (2012)
Male rats with intestinal injury burn-induced	Intraperitoneally, 100 and 200 mg/kg b.w., immediately after burn injury	Increase in antioxidant enzymes levels, decrease in the lipid peroxidation and the levels of proinflammatory response in intestinal tissue	Zhou et al. (2015)
Female BALB/c mice with ulcerative colitis induced by 2,4,6- trinitrobenzene sulfonic acid	Intragastrically, 50 mg/kg, for 8 days	Reduction in the lipid peroxidation, the lung myeloperoxidase activity and nitric oxide levels in the colon	Kazi and Qian (2009)

[1]ROS: reactive oxygen species; [2]MAPK/ERK1/2: mitogen-activated protein kinases/extracellular signal-regulated kinase 1/2; [3]Keap1: kelch-like ECH-associated protein 1.

Table 4. Summary of studies conducted to date of the antioxidant effects of safranal.

Experimental model	Safranal administration	Effects	Reference
Bovine aortic endothelium cells	2, 10, 20 and 40 μM, for 24 h	Reduction of intracellular ROS [1] levels and the rate of cell apoptosis	Rahiman et al. (2018)
Primary dopaminergic cells from rat embryos	10, 15, 20, and 50 μg/ml, for 4 h	Decrease of intracellular ROS [1] level and inhibition of apoptosis	Pan, Qiao, and Wen (2016)
Human bronchial epithelial cells asthma-induced; BALB/c mice asthma-induced	10 ng/ml, 100 ng/ml and 1μg/ml; orally, 1 and 10 mg/kg b.w. for 7 days	*In vivo* and *in vitro* effects: suppression of nitric oxide and peroxynitrite ion production and nitric oxide synthase levels	Bukhari et al. (2015)
Rat liver microsomes and red blood cells	0.5, 1 and 2 mM	Decrease of the lipid peroxidation in red blood cells and liver microsomal non-enzymatic lipid peroxidation	Hosseinzadeh, Shamsaie, and Mehri (2010)
Albino rats with chronic stress	Intraperitoneally, 0.25, 0.50 and 0.75 mg/kg b.w. per day, for 21 days	Reduction of MDA [2] and glutathione levels, and increase of the antioxidant enzymes content in the brain	Samarghandian et al. (2017)
Male rats with Alzheimer's disease induced by intrahippocampal amyloid beta	Orally, 0.025, 0.1, and 0.2 ml/kg b.w. per day, for 1 week	Decrease of MDA [2] and ROS [1] hippocampal levels, the protein carbonyl content, the index of protein oxidation, and improvement of superoxide dismutase activity	Baluchnejadmojarad, Mohamadi-Zarch, and Roghani (2019)
Male rats with transient focal cerebral ischemia	Intraperitoneally, 72.5 and 145 mg/kg b.w. at 0, 3, and 6 h after the induction	Reduction of the lipid peroxidation, an increment in the antioxidant capacity and in the total sulfhydryl content	Sadeghnia et al. (2017)
Male rats with oxidative damage induced by quinolic acid in the hippocampus	Intraperitoneally, 145.5 and 291 mg/kg b.w. after the induction	Decrease of the lipid peroxidation and the oxidative DNA damage, and restoration of thiol redox status and the antioxidant power in the hippocampus	Sadeghnia et al. (2013)
Male rats with oxidative damage induced by ischemia-reperfusion injury in the hippocampus	Intraperitoneally, 727.5 mg/kg, 363.75 mg/kg, 145.5 mg/kg, and 72.75 mg/kg, for 72 h after the induction	Increase of the antioxidant capacity and total sulfhydryl content and a decrease of MDA [2] levels in hippocampal tissue	Hosseinzadeh and Sadeghnia (2005)

Male albino rats with diabetic nephropathy induced by high-fat diet and streptozotocin	Intraperitoneally, 30 mg/kg b.w., for 4 weeks	Decrease of the total oxidant capacity, nitric oxide levels and the oxidative stress index values; increase of total antioxidant capacity levels in kidney	Hazman and Bozkurt (2015)
Diabetic and nondiabetic male rats with gastric ulcers induced by indomethacin	Orally, 0.25, 2, 5 ml/kg b.w.	Decrease of the gastric ulcer index and the lipid peroxidation, and increase of glutathione levels in the gastric mucosa	Kianbakht and Mozaffari (2009)
Aged male rats	Intraperitoneally, 0.5 mg/kg b.w. per day, for one month	Increase of the hepatic antioxidant enzymes, and decrease of MDA [2] level in liver homogenate and nitric oxide serum content	Farahmand et al. (2013)
Male albino rats with oxidative damage in heart tissue induced by isoproterenol	Intraperitoneally, 0.025, 0.050, 0.075 ml/kg b.w., for 8 days	Reduction of MDA [2] level in heart tissue	Mehdizadeh et al. (2013)
Male rats with oxidative damage induced by ischemia-reperfusion injury in skeletal muscles	Intraperitoneally, 0.1, 0.25, and 0.5 ml/kg b.w.	Decrease of MDA [2] levels and increase of antioxidant capacity and total thiol content in skeletal muscle tissue	Hosseinzadeh, Modaghegh, and Saffari (2009)
Male rats crush injury induction of sciatic nerve	Intraperitoneally, 0.05, 0.2 and 0.8 mg/kg b.w. per day, for 10 cdays	Decrease of MDA [2] blood levels	Tamaddonfard et al. (2014)
Ovalbumin sensitized guinea pigs	Orally, 4, 8 and 16 µg/ml	Reduction of total nitric oxide and nitrite serum levels	Boskabady, Byrami, and Feizpour (2014)

[1]ROS: reactive oxygen species; [2]MDA: malondialdehyde.

Table 5. Summary of studies conducted to date of the effects of saffron supplementation to improve the health.

Experimental model	Saffron administration	Effects	Reference
Gastric adenocarcinoma tumor cell line	Saffron distillates concentrations, 20, 40 and 100 µg/ml	Reduction in the expression of self-renewal. The best concentrations were 40 and 100 µg/mL and the incubation time of 48 h	Akbarpoord et al. (2020)
Albino rats with gastric cancer	Saffron aqueous extract, intraperitoneally, 100, 150 and 175 mg/kg b.w. per day, for 50 days	The higher doses of saffron aqueous extracts inhibited the cancer progression	Bathaie et al. (2013)
Breast cancer using MCF7 cell line human	Saffron aqueous extract, 100, 200, 400 and 800 µg/ml	MCF-7 cells treated with saffron at 200 µg/ml showed the best reduction (18%) in gene expression	Mousavi, Baharara, and Asadi-Samani (2014)
MCF7 cell line human	Synergic effect of saffron aqueous extract at 100, 200, 400 and 800 µg/ml and electromagnetic field	A higher reduction (38%) on the VEGFR-2 gene expression using the synergy method with the lowest concentration of saffron	Mousavi, Baharara, and Shahrokhabadi (2014)
Jurkat cells	Crocin and safranal mixture administration	Antiproliferative activities against human acute lymphoblastic cells	Makhlouf et al. (2016)
Double-blind, controlled clinical trial	Saffron capsules, 30 mg per day, for 6 weeks	The same efficacy to treat major depressive disorder with an anxious distress compared to reference treatment	Ghajar et al. (2017)
Double-blind, controlled clinical trial	Saffron capsules, 30 mg per day, or fluoexetin, 40 mg per day, for 6 weeks	The same efficacy in improving depression compared to reference treatment	Shahmansouri et al. (2014)
Double-blind, controlled clinical trial	Saffron capsules, 15 mg twice a day, for 12 weeks	The results did not show significant differences between placebo or saffron groups	Moazen-Zadeh et al. (2018)
Meta-analysis	Saffron extract administration	Significant reduction in beck anxiety inventory and Pittsburgh sleep quality index and beck anxiety inventory respect to antidepressant common drugs	Ghaderi et al. (2020)
Systematic review	Saffron extract supplementation	Similar effect in improving cognitive impairment to the reference Alzheimer´s drug	Avgerinos et al. (2020)

Systematic review	Saffron extract administration	Positive impact on fasting blood glucose	Giannoulaki et al. (2020)
Meta-analysis	Saffron extract supplementation	Beneficial effect on fasting plasma glucose and WC after 12 weeks with saffron supplementation	Rahmani, Saberzadeh, and Takhshid (2020)
Randomized double-blind	Powered saffron, 100 mg per day, for 8 weeks	Improve the inflammation status and significant effect on the reduction of glucose levels in patients with type 2 diabetes mellitus	Mobasseri et al. (2020)

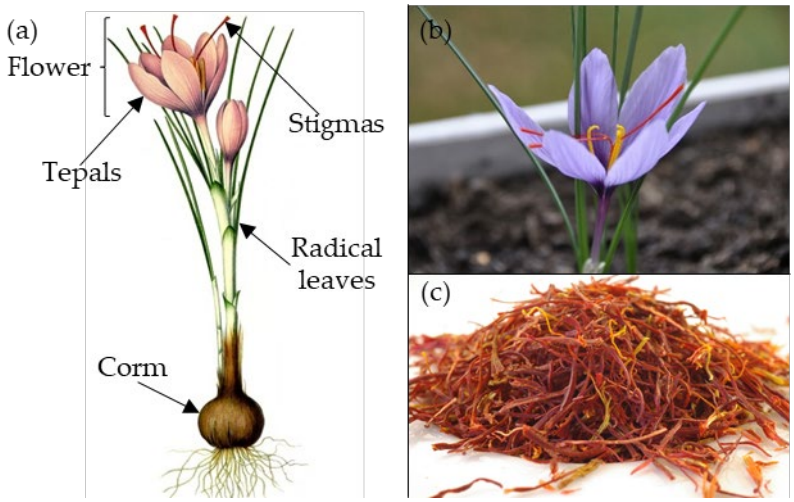


Figure 1. (a) Diagram of the different parts of saffron plant (Brandt et al., 1898); (b) The saffron flower; (c) Dry stigmas used as saffron spice.

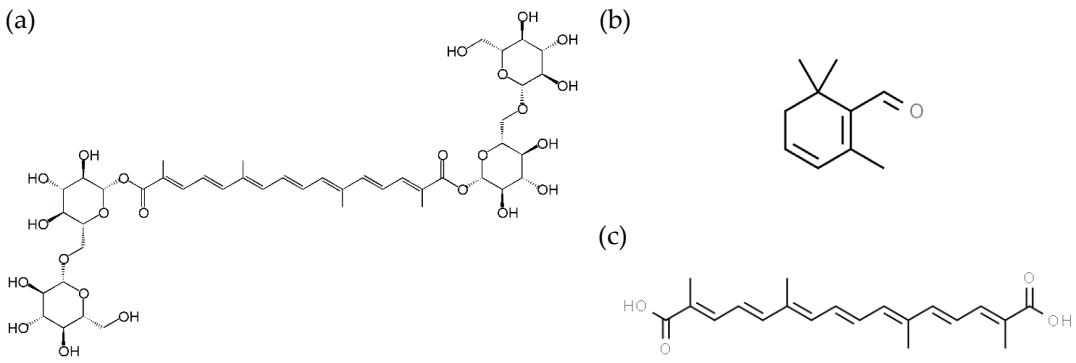
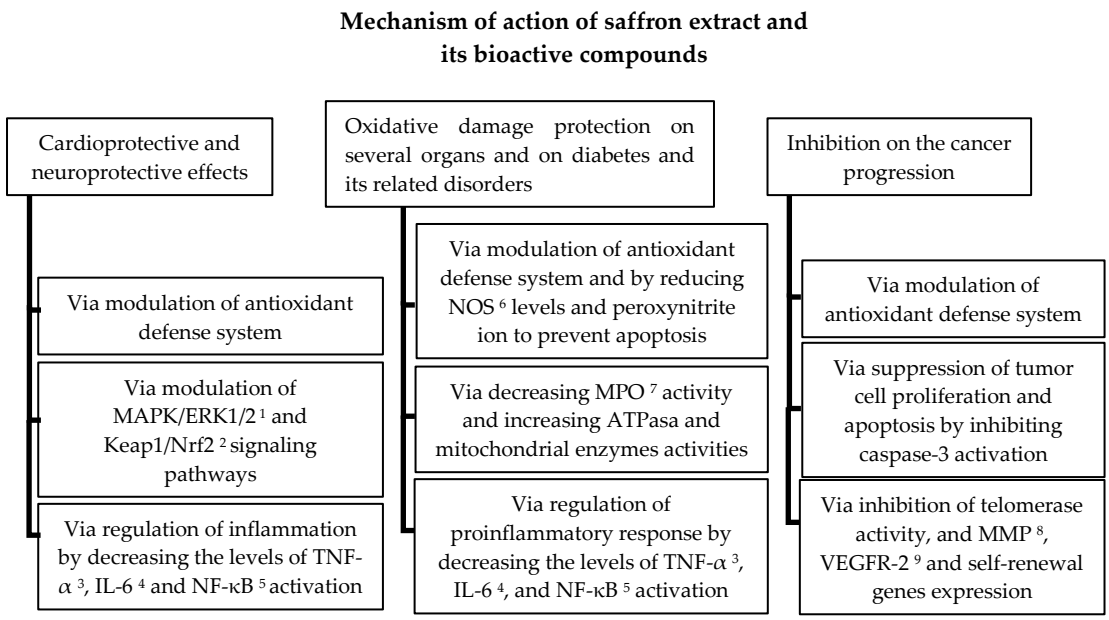


Figure 2. Structural formula of the saffron bioactive compounds: (a) crocin; (b) safranal; (c) crocetin.



[1]MAPK/ERK1/2: mitogen-activated protein kinases/extracellular signal-regulated kinase 1/2; [2]Keap1/Nrf2: kelch-like ECH-associated protein 1/ nuclear factor erythroid 2-related factor 2; [3]TNF-α: tumor necrosis factor-α;[4] IL-6: interleukin-6; [5]NF-κB: nuclear factor-kappa B; [6]NOS: nitric oxide synthases; [7]MPO: myeloperoxidase; [8]MMP: matrix metalloproteinases; [9]VEGFR-2: vascular endothelial growth factor receptor 2.

Figure 3. Possible mechanisms of action involved in the effects of saffron and its bioactive compounds in different diseases.

Publicación 2

Underutilized *Crocus sativus* L. flowers: A hidden source of sustainable high value-added ingredients

Autores	Débora Cerdá-Bernad, Estefanía Valero Cases, Francisca Pérez Llamas, Joaquín Julián Pastor & María José Frutos
Revista	Plant Foods for Human Nutrition
DOI	10.1007/s11130-023-01065-7
Editorial	Springer
Fecha de publicación	30 de Junio de 2023
Categoría JCR®	Plant Science
Cuartil	Q1
Rango	51/239
Factor de impacto	4,000

Plant Foods for Human Nutrition
https://doi.org/10.1007/s11130-023-01065-7

RESEARCH

Underutilized *Crocus Sativus* L. Flowers: A Hidden Source of Sustainable High Value-Added Ingredients

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Accepted: 10 April 2023

Abstract
Crocus sativus L. is used as a spice due to its organoleptic characteristics. Only flower stigmas are used for its production, as the rest of the flower is discarded as waste. This fact represents a lack of sustainability, since around 230,000 flowers are necessary to produce 1 kg of saffron. The main aim of this study was to contribute to the valorization of *Crocus sativus* L. spice and its floral by-products, through the study of their nutritional value and composition, in terms of hydrophilic and lipophilic compounds, as well as their functional properties. The results showed that saffron stigmas and floral bio-residues presented high contents of fiber, and the most abundant macronutrient were the carbohydrates, followed by proteins, and a low content in fats. All samples had high concentrations of glucose, fructose, lactic and malic acids, and minerals, mainly K, Ca and Mg. Furthermore, the polyunsaturated fatty acids were predominant, being linoleic acid (C18:2n6) the most abundant. Therefore, this research provides more in-depth information about the composition of saffron stigmas and floral by-products, to be considered as promising sources for the development of functional ingredients with new applications in the food industry.

Keywords Saffron flower · Bio-residues · Food composition · Sustainability · Functional ingredients

Abbreviations

DF	dietary fiber
dw	dry weight
FA	fatty acid
fw	fresh weight
MUFA	monounsaturated fatty acids
SFA	saturated fatty acids
SFL	saffron floral by-products
PUFA	polyunsaturated fatty acids

Introduction

Saffron (*Crocus sativus* L.), a monocotyledonous herbaceous plant from the Iridaceae family, produces the most expensive spice in the world. The high costs are because producing 1 kg of saffron is necessary around 230,000 flowers (~60–80 kg, being ~78% tepals), as only the dried-up stigmas are used for the spice [1]. The rest of the flower is discarded. Therefore, considering that hundreds of kilograms (~205,000 kg) of saffron arrive to the market each year and the production yields range between 0.02 and 0.03 kg of dry stigmas *per* hectare, the current production system is generating several hundreds of tons of tepal wastes (~9,500 – 12,700 tons/year) [2].

The chemical characteristics of saffron depend on the different geoclimatic conditions and processing techniques used by the growers. However, this plant, originated from southern Europe and south-west Asia, is widely distributed in different areas, due to its high adaptability for cultivation [3]. The chemical composition of saffron is complex, having primary macromolecules, mostly carbohydrates, amino acids and proteins, and lower contents of minerals, fats and

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Published online: 30 June 2023

vitamins; and secondary metabolites such as carotenoids, monoterpenes, and flavonoids [4, 5]. These bioactive compounds are natural antioxidants that could prevent certain diseases related to the oxidative stress, such as cancer, metabolic syndrome, neurological disorders, among others [6]. However, the demonstration of tepals specific activity would convert this waste product into a high value ingredient, increasing the resource efficiency and the competitiveness of this traditional sector. Furthermore, their possible nutritional value may contribute to reducing malnutrition and hunger in the world, using them as a new source of food. Currently, hunger and undernourishment are on the rise, affecting many millions of people around the world, especially in developing countries, so there is a need to explore novel sustainable sources that will decrease this prevalence and have a positive social impact [7]. As a consequence, the main aim of this research was to contribute to the valorization of *Crocus sativus* L. and its floral by-products through their nutritional characterization to provide more in-depth information to develop innovative food ingredients with novel applications that could potentially increase saffron demand on the market and have economic, social and environmental benefits. The proximal and dietary fiber composition, minerals, organic acids, sugar contents, and the lipidic profile were determined in saffron stigmas and saffron floral by-products. Besides, the functional properties of saffron floral by-products were also evaluated. As far as we know, no research has been published to date that delves into the content of lipophilic compounds like the fatty acid profile and the functional properties of saffron floral biowaste.

Materials and Methods

The section of material and methods is provided as Supplementary Materials.

Results and Discussion

Proximate Composition and Dietary Fiber Content of Saffron and its Floral By-Products

Data on the nutritional composition, energetic value and fiber composition of saffron floral by-products and saffron stigmas of different origins are presented in Table 1. Regarding the moisture, an important indicator of shelf life for food, the lyophilized flowers presented lower moisture than saffron stigmas, since the initial moisture values of SFL1 and SFL2 samples before drying were higher than 80 g *per* 100 g of fw (85.45 ± 0.51 for SFL1 and 86.81 ± 0.30 for SFL2). Saffron stigmas had higher moisture contents because they were dehydrated by air under low temperatures (< 60 °C). Spanish, Iranian and Greek saffron showed values lower than 12 g/100 g on dry weight, according to the maximum moisture allowed for saffron threads [8]. Thus, the moisture of all vegetal samples could be adequate for the storage to avoid deterioration because of microbiological, or chemical spoilage.

With respect to ash contents, which provide a measure of the total amount of minerals within a food, SFL2 and saffron stigmas samples presented ash values lower than 8 g/100 g on dry weight, according to the maximum total ash allowed for saffron spice, except for SFL1 (8.39 ± 0.00 g/100 g dw) showing statistically significant differences with the rest the samples (values around 5 g/100 g dw) [8].

Table 1 Proximal composition and dietary fiber in saffron and its floral by-products

	Samples				
	SFL1	SFL2	Spanish Saffron	Iranian Saffron	Greek Saffron
Nutritional value (g/100 g dw)					
Moisture	6.42 ± 0.68bc	5.52 ± 1.63c	8.90 ? 0.14ab	9.01 ? 0.05ab	10.74 ± 0.55a
Ash	8.39 ± 0.00a	4.89 ± 0.31b	5.59 ± 0.09b	5.57 ± 0.26b	4.81 ± 0.14b
Proteins	8.58 ± 0.08d	8.68 ± 0.02d	13.15 ± 0.13b	13.61 ± 0.13a	12.29 ± 0.02c
Lipids	5.81 ± 0.01ab	4.56 ± 0.77b	7.01 ± 0.29a	6.08 ± 0.17ab	6.22 ± 0.20a
Available carbohydrates	70.80 ± 0.75b	76.35 ± 2.07a	65.26 ± 0.25c	65.73 ± 0.16c	65.94 ± 0.23c
Energy (kcal/100 g dw)	369 ± 3b	381 ± 1a	377 ± 2ab	372 ± 2ab	369 ± 3b
Fiber composition (g/100 g dw)					
Total DF	26.59 ± 1.30a	22.56 ± 0.27ab	17.86 ± 2.64b	21.17 ± 0.02ab	17.64 ± 2.51b
Insoluble DF	19.07 ± 0.59a	20.24 ± 1.11a	14.61 ± 0.88b	14.55 ± 0.04b	15.15 ± 0.21b
Soluble DF	7.51 ± 0.71a	2.32 ± 1.38c	3.25 ± 1.76bc	6.61 ± 0.06ab	4.26 ± 0.21abc
Inulin content (g/100 g dw)	1.46 ± 0.47a	1.60 ± 0.51a	1.60 ± 0.65a	1.59 ± 0.15a	1.81 ± 0.67a

Means ± standard deviation in the same line followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each sample ($n = 3$); SFL1, SFL2: Saffron floral by-products from two different producers

Available carbohydrates were the most abundant macronutrients in all the saffron stigmas and flower samples, followed by proteins and the lowest content was for lipids. Flowers, specially SFL2, had amounts of available carbohydrates significantly higher than saffron stigmas (76 g/100 g dw and 65 g/100 g dw, respectively) (Table 1). However, saffron stigmas showed significantly higher protein content (around 13 g/100 g dw), especially Iranian saffron, than flowers (around 8 g/100 g dw). Thus, saffron presented high amounts of protein which is a good source of amino acids for vital physiological functions. Regarding the energy values and lipids, any statistically significant differences were observed in the flowers (5 g/100 g dw of lipids) when compared to stigmas (around 6 g/100 g dw of lipids). These results were in accordance with the ones described by Serrano-Díaz et al. [9] who reported similar values of the proximal composition in saffron bio-residues and saffron stigmas from Castilla-La Mancha (Spain). Besides, these values were comparable with those obtained in the study of Muzaffar et al. [10] regarding Indian saffron stigmas. Furthermore, saffron flowers presented similar proximal composition compared to other edible flowers such as petals of different species (dahlia, rose, calendula and centaurea) that contained carbohydrates as the most abundant macronutrient (81–88%) followed by proteins (6–7%) and the lowest content in fat (0.1-5%) [11].

In addition, the dietary fiber was studied. The consumption of DF has positive effects on human digestive health. Total DF was higher in flowers (22–26 g/100 g dw) than in saffron stigmas (17–21 g/100 g dw). The insoluble DF was the major fraction of the total DF in all samples studied, being significantly higher in the floral by-products (20 g/100 g dw) than in saffron stigmas (15 g/100 g dw). These results were in accordance to those reported by Serrano-Díaz et al. [9] in which saffron and its bio-residues from Spain presented higher content of insoluble DF than soluble DF, being lower in stigmas. Regarding the values of soluble DF, SFL1 had a significant higher content (7 g/100 g dw) compared to SFL2 (2 g/100 g dw) and saffron stigmas from Iran showed a higher soluble DF content (6 g/100 g dw) respect to Spanish (3 g/100 g dw) and Greek saffron (4 g/100 g dw). The differences shown regarding the proximal composition and DF between SFL1 and SFL2 from Spain and between stigmas from Spain, Iran and Greece may be due to the growing conditions, such as temperature, humidity, soil properties, which could noticeably influence the chemical constituents of the plant [12]. Moreover, the content of inulin (as part of the soluble dietary fiber) was studied. Inulin, found naturally in many food plants, is usually used as dietary fiber and as a prebiotic in functional food, since it is not hydrolysed by human intestinal enzymes. Saffron and its floral by-products presented inulin content in similar amounts (1.50 g/100 g dw), showing statistically no-significant differences, suggesting that they could be used as a good source of prebiotic ingredients, stimulating the proliferation of the intestinal microbiota. These values were similar than that found in rye (0.5-1%), barley (0.5–1.5%) and wheat (1–4%) cereals [13].

Mineral Composition of Saffron and its Floral By-Products

The mineral content of saffron and its floral by-products is shown in Table 2. Macrominerals, such as Ca, K, Na and Mg, are relevant for several physiological functions, both in plants and animals. The most abundant mineral in all saffron and its floral by-products samples was K, which may protect against bone loss and reduce the risk of cardiovascular diseases. The K content was significantly higher in saffron floral by-products (around 1500 mg/100 g dw) when compared to the saffron stigmas (around 1000 mg/100 g dw). These values represent 31.91% and 21.28% approximately of the daily intake of potassium in adults (4700 mg *per* day) with a consumption of 100 g of flowers or stigmas, respectively [14]. Besides, SFL1 presented higher

Table 2 Mineral composition in saffron and its floral by-products

	Samples				
	SFL1	SFL2	Spanish Saffron	Iranian Saffron	Greek Saffron
Macrominerals (mg/100 g dw)					
Ca	415.20 ± 25.46a	112.60 ± 1.98b	118.30 ± 25.10b	86.25 ± 6.01b	123.30 ± 63.98b
K	1530 ± 16a	1450 ± 51a	1114 ± 63bc	1136 ± 18b	971.30 ± 31.82c
Mg	120.30 ± 4.95a	103.30 ± 2.68b	100.20 ± 1.77bc	102.50 ± 1.41b	88.70 ± 4.60c
Na	9.00 ± 3.53a	9.20 ± 1.55a	19.00 ± 1.25a	33.75 ± 2.89a	33.25 ± 3.45a
Microminerals (mg/100 g dw)					
Cu	0.57 ± 0.01b	0.55 ± 0.04b	0.35 ± 0.07bc	0.25 ± 0.00c	1.63 ± 0.11a
Fe	46.26 ± 1.44a	6.38 ± 0.03bc	5.45 ± 0.57c	12.28 ± 3.29b	8.13 ± 1.03bc
Mn	2.51 ± 0.21a	0.95 ± 0.01b	1.15 ± 0.01b	1.30 ± 0.00b	1.25 ± 0.07b
Zn	3.89 ± 0.27a	2.10 ± 0.08b	2.65 ± 0.35ab	2.13 ± 0.53b	1.98 ± 0.46b

Means ± standard deviation in the same line followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each sample (n = 3); SFL1, SFL2: Saffron floral by-products from two different producers

amount of Ca (415.20 ± 25.46 mg/100 g dw) respect to SFL2 (112.60 ± 1.98 mg/100 g dw), showing statistically significant differences. Saffron stigmas had a low amount of Ca (around 86–120 mg/100 g dw). These values represent 31.94 and 9.23% approximately of the daily intake of calcium in adults (1300 mg per day) with a consumption of 100 g of flowers or stigmas, respectively [14]. Calcium also plays an important role in the human health especially in the prevention of bone loss and osteoporosis. Regarding Mg, SFL1 presented a significantly higher content (120.30 ± 4.95 mg/100 g dw) respect to SFL2 (103.30 ± 2.68 mg/100 g dw). Spanish and Iranian saffron stigmas showed similar values that SFL2. However, Greek saffron presented the lowest amount (88.70 ± 4.60 mg/100 g dw). These values represent around 28.64 and 24.40% of the daily intake of magnesium in adults (420 mg *per* day) with a consumption of 100 g of flowers or stigmas, respectively [14]. The values of Na were low in all samples, showing statistically no-significant differences between them, being around 9 mg/100 g dw in saffron flowers and 33 mg/100 g dw in stigmas from Iran and Greece, and 19 mg/100 g dw in stigmas from Spain. Fahim et al. [15] also reported high content of K (542.13 ± 0.01 mg/100 g) and Ca (486.25 ± 0.12 mg/100 g) in Iranian saffron petals and low amounts of Na (25.75 ± 0.01 mg/100 g). Regarding the microminerals, the concentration in all samples of Fe, an essential element for human physiology involved in several metabolic processes, was the highest, followed by Zn. SFL1 showed significantly higher levels of Fe (46.26 ± 1.44 mg/100 g dw) with respect to SFL2 (6.38 ± 0.03 mg/100 g dw). Iranian and Greek saffron stigmas presented similar values to SFL2, showing SFL2 statistically no-significant differences with stigmas. However, Spanish saffron presented the lowest concentration (5.45 ± 0.57 mg/100 g dw). These differences could be due to the different cultivation conditions, such as the mineral content in the soil, the use of some fertilizers or the relative bioaccumulation of this mineral in the plant. These values represent around 35.44 and 45.17% of the daily intake of iron in adults (18 mg per day) with a consumption of 100 g of flowers (SFL2) or stigmas, respectively [14]. With respect to Zn, SFL1 presented a significantly higher content (3.89 ± 0.27 mg/100 g dw) than SFL2 (2.10 ± 0.08 mg/100 g dw), which showed similar amounts to saffron stigmas. These values represent 35.36 and 24% approximately of the daily intake of zinc in adults (11 mg per day) with a consumption of 100 g of flowers or stigmas, respectively [14].

Serrano-Díaz et al. [9] reported similar values of Fe in saffron stigmas from Spain (0.011 ± 0.001 g/100 g dw). The Mn and Cu contents were low in all samples, except for SFL1 that showed significantly higher concentrations of Mn (2.51 ± 0.21 mg/100 g dw) compared to SFL2 (0.95 ± 0.01 mg/100 g dw) and stigmas (1.15–1.30 mg/100 g dw). These values represent around 41.30 and 56.52% of the daily intake of manganese in adults (2.3 mg) with a consumption of 100 g of flowers (SFL2) or stigmas, respectively [14]. Regarding Cu, SFL1 had similar concentrations (0.57 ± 0.01 mg/100 g dw) compared to SFL2 (0.55 ± 0.04 mg/100 g dw), showing statistically no-significant differences between them. Except for Greek saffron (1.63 ± 0.11 mg/100 g dw), stigmas showed low amounts of Cu (0.25–0.35 mg/100 g dw). These differences could be also due to the cultivation conditions, but further research would be necessary. Therefore, saffron floral by-products and saffron stigmas showed high concentrations of macrominerals, representing an interesting nutritional quality parameter for the development of new food ingredients.

Hydrophilic Compounds of Saffron and its Floral By-Products

Data on the organic acid composition of the saffron floral by-products and saffron stigmas of different origin are shown in Table 3. Organic acids play an important role in food since they are responsible for essential sensory properties but also have pharmacological actions [16]. Saffron stigmas presented the highest concentrations of lactic acid (5.27–6.02 g/100 g dw) with respect to the flowers, where it was significantly lower (3.59-4.0 g/100 g dw). However, saffron floral by-products had higher content of malic acid, especially SFL2 (5.89 ± 0.72 g/100 g dw), showing statistically significant differences with SFL1 and saffron stigmas, but saffron stigmas also presented elevated concentration of malic acid (2.03–2.72 g/100 g dw). Besides, saffron floral by-products contained propionic acid (2.13–2.34 g/100 g dw) and a low content of fumaric acid (0.17 g/100 g dw). Malic and fumaric acid were not detected in saffron stigmas. However, saffron stigmas showed the presence of formic acid, having the Greek saffron sample significantly higher amounts (7.18 ± 0.39 g/100 g dw) than Spanish and Iranian stigmas. Saffron samples had also oxalic acid, but in the lowest concentrations (around 0.30 g/100 g dw). Formic and oxalic acids were not detected in saffron floral by-products. Therefore, both saffron floral by-products and stigmas showed high amounts of lactic and malic acids, which could exert positive effects on human health and have other technological functionalities: lactic acid could act as preservative towards several spoilage and pathogenic microorganisms, and malic acid as acidulant [17]. Moreover, it should be noted that saffron flowers had propionic acid which has antimicrobial properties, being used as preservative in food [17]. These results are comparable to those obtained by Serrano-Díaz et al. [9] that showed the presence of lactic and malic acids in Spanish saffron bio-residues

Table 3 Organic acid and sugar composition in saffron and its floral by-products

	Samples				
	SFL1	SFL2	Spanish Saffron	Iranian Saffron	Greek Saffron
Organic acids (g/100 g dw)					
Lactic acid	3.59 ± 0.85b	4.00 ± 0.62bc	6.02 ± 0.16a	5.27 ± 0.43ac	5.55 ± 0.77a
Malic acid	3.86 ± 0.88b	5.89 ± 0.72a	2.72 ± 0.31b	2.57 ± 0.65b	2.03 ± 0.62bc
Fumaric acid	0.17 ± 0.03a	0.17 ± 0.01a	nd	nd	nd
Propionic acid	2.13 ± 0.85a	2.34 ± 0.60a	nd	nd	nd
Oxalic acid	nd	nd	0.32 ± 0.02a	0.28 ± 0.02a	0.29 ± 0.04a
Formic acid	nd	nd	4.42 ± 0.45b	4.65 ± 0.27b	7.18 ± 0.39a
Soluble sugars (g/100 g dw)					
Glucose	12.81 ± 1.23a	13.55 ± 1.49a	6.50 ± 0.06b	6.32 ± 0.14b	6.63 ± 0.28b
Fructose	28.67 ± 3.19b	35.33 ± 4.29a	2.82 ± 0.25c	1.58 ± 0.31c	0.63 ± 0.14c

Means ± standard deviation in the same line followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each sample (n = 3); nd: not detected; SFL1, SFL2: Saffron floral by-products from two different producers

and the content of lactic and oxalic acid in saffron stigmas from Spain, and with those obtained by Pires et al. [11] that showed the content of malic acid in high amounts in edible petals from different species such as rose and calendula (1.23 ± 0.02 g/100 g dw and 1.14 ± 0.02 g/100 g dw, respectively). Besides, Jarukas et al. [18] observed the content of lactic, and malic acids in saffron spice from Iran.

Regarding the composition of soluble sugars, all samples presented reducing sugars such as glucose and fructose monosaccharides (Table 3), which play an important role in food, not only in the sensory properties, but also in the food preservation. Besides, they represent a source of energy [19]. Saffron floral by-products had elevated glucose content (12.81–13.55 g/100 g dw) and high concentrations of fructose, especially SFL2 (35.33 ± 4.29 g/100 g dw), showing statistically significant differences with SFL1 and saffron stigmas. In saffron stigmas samples, the concentration of glucose was higher than fructose (6.32–6.63 and 0.63–2.82 g/100 g dw, respectively). These results are comparable to those of other edible plants, such as Dahlia and Rose petals, in which fructose was the soluble sugar found in the highest amount (~4–5 g/100 g dw), followed by glucose (3.23 g/100 g dw) [11].

Lipophilic Compounds of Saffron and its Floral by-products

The fatty acid profile of saffron and its floral by-products is shown in Table 4. The study of fatty acid composition in food is very important since essential fatty acids, which must come from the diet, have an important functional role in human health [20].

Regarding saturated fatty acids, palmitic acid (C16:0) was found in the highest concentration in both saffron flower samples (20.19 g/100 g) and also in all saffron stigmas, with the highest proportion in Greek stigmas (15.18 ± 0.21 g/100 g). With respect to monounsaturated fatty acids, the quantitatively most important FAs were eicosanoic acid (C20:1n-9) and oleic acid (C18:1n-9) in saffron stigmas, as well as in the floral by-products. SFL1 presented higher proportion of eicosanoic acid (11.04 ± 0.38 g/100 g) and of oleic acid (2.57 ± 0.05 g/100 g), compared to SFL2 (5.56 ± 0.17 and 2.13 ± 0.25 g/100 g, respectively). Greek saffron stigmas showed the most elevated concentrations of eicosanoic acid (7.92 ± 0.26 g/100 g) and oleic acid (7.66 ± 0.17 g/100 g) respect to Spanish and Iranian samples. According to the recommendations of the European Food Safety Authority, the daily intake of SFA should be the lowest possible [21]. The proportion of polyunsaturated fatty acids were predominant in all samples, being linoleic acid (C18:2n6) the major FA found. Linoleic acid, an essential fatty acid produced in plants, is the most prevalent n-6 PUFA in the human diet. SFL2 presented an amount of linoleic acid (39.62 ± 2.83 g/100 g) higher than SFL1 (35.12 ± 0.26 g/100 g). Besides, Greek saffron stigmas (45.43 ± 1.08 g/100 g) showed an elevated concentration of linoleic acid, that was higher in Spanish (35.76 ± 0.72 g/100 g) and Iranian stigmas (40.24 ± 7.20 g/100 g). Traditional sources of linoleic acid are vegetable oils, such as sunflower oil containing around 65% of this FA whose consumption may reduce the risk of cardiovascular diseases. According to the recommendations of the European Food Safety Authority, the adequate intake per day of linoleic acid should be 4% of total energy intake [22].

Regarding n-3 PUFA, the essential α-linolenic acid (C18:3n-3), mostly found in plant foods such as flaxseed, walnuts and vegetable oils, was predominant in saffron (5.77–8.50 g/100 g) and its floral-by-products (6.87–9.28 g/100 g). Thus, the diet richness in PUFA, replacing dietary SFA, contribute to lower LDL cholesterol and it is associated with a lower cardiovascular risk. These results were in accordance with other studies, showing the same

Table 4 Fatty acid profile (main groups and ratios) for saffron and its floral by-products

	Samples				
Fatty acids (g/100 g total FAs)	SFL1	SFL2	Spanish Saffron	Iranian Saffron	Greek Saffron
Myristic acid (C14:0)	1.41 ± 0.03	3.63 ± 2.19	1.51 ± 0.03	2.45 ± 1.38	2.82 ± 1.13
Pentadecanoic acid (C15:0)	0.18 ± 0.00	0.37 ± 0.24	0.12 ± 0.00	0.22 ± 0.11	0.21 ± 0.02
Palmitic acid (C16:0)	20.19 ± 0.08	20.19 ± 2.09	12.40 ± 0.25	14.19 ± 2.62	15.18 ± 0.21
Stearic acid (C18:0)	2.56 ± 0.04	3.15 ± 0.85	1.57 ± 0.03	0.85 ± 0.17	0.89 ± 0.02
Behenic acid (C22:0)	1.64 ± 0.06	1.12 ± 0.03	0.49 ± 0.01	0.73 ± 0.13	0.93 ± 0.01
Lignoceric acid (C24:0)	2.26 ± 0.06	1.01 ± 0.02	0.18 ± 0.00	0.54 ± 0.12	0.56 ± 0.00
∑ SFA	**28.55 ± 0.19ab**	**29.64 ± 5.15a**	**16.27 ± 0.33c**	**18.98 ± 4.53bc**	**20.70 ± 1.03ac**
Hypogeic acid (C16:1n-9)	0.61 ± 0.00	1.27 ± 0.87	0.35 ± 0.01	0.85 ± 0.24	0.87 ± 0.16
Palmitoleic acid (C16:1n-7)	1.69 ± 0.01	2.02 ± 0.55	0.30 ± 0.01	0.40 ± 0.06	0.40 ± 0.05
Oleic acid (C18:1n-9)	2.57 ± 0.05	2.13 ± 0.25	4.39 ± 0.09	5.51 ± 0.94	7.66 ± 0.17
Vaccenic acid (C18:1n-7)	1.52 ± 0.02	1.35 ± 0.13	2.88 ± 0.06	2.93 ± 0.52	4.00 ± 0.08
Eicosanoic acid (C20:1n-9)	11.04 ± 0.38	5.56 ± 0.17	5.23 ± 0.10	6.37 ± 1.31	7.92 ± 0.26
Cis-14-tricosenoic acid (C23:1n-9)	5.59 ± 0.03	2.24 ± 0.45	0.38 ± 0.01	0.91 ± 0.18	0.96 ± 0.01
Erucic acid (C22:1n-9)	1.26 ± 0.02	0.55 ± 0.20	0.28 ± 0.00	0.35 ± 0.08	0.29 ± 0.00
Nervonic acid (C24:1n-9)	0.84 ± 0.02	0.63 ± 0.01	0.47 ± 0.01	0.77 ± 0.16	0.59 ± 0.01
∑ MUFA	**25.13 ± 0.37a**	**15.76 ± 2.22c**	**14.28 ± 0.29c**	**18.16 ± 3.39bc**	**22.79 ± 0.18ab**
Linoleic acid (C18:2n-6)	35.12 ± 0.26	39.62 ± 2.83	35.76 ± 0.72	40.24 ± 7.20	45.43 ± 1.08
γ-Linolenic acid (C18:3n-6)	nd	7.16 ± 0.82	25.95 ± 0.52	12.94 ± 1.30	nd
Eicosadienoic acid (C20:2n-6)	0.25 ± 0.03	0.10 ± 0.03	0.36 ± 0.01	0.19 ± 0.07	0.43 ± 0.00
Arachidonic acid (C20:4n-6)	nd	nd	0.26 ± 0.00	nd	0.35 ± 0.06
Docosadienoic acid (C22:2n-6)	0.15 ± 0.00	nd	0.93 ± 0.02	0.59 ± 0.03	1.12 ± 0.04
∑ n-6 PUFA	**35.94 ± 0.23b**	**47.12 ± 7.77ab**	**63.26 ± 1.27a**	**54.05 ± 10.13ab**	**47.33 ± 1.19ab**
α-Linolenic acid (C18:3n-3)	9.28 ± 0.40	6.87 ± 0.68	5.77 ± 0.12	7.76 ± 1.38	8.50 ± 0.10
Eicosatrienoic acid (C20:3n-3)	0.28 ± 0.04	0.33 ± 0.11	0.23 ± 0.01	0.50 ± 0.08	0.39 ± 0.03
∑ n-3 PUFA	**10.38 ± 0.41a**	**7.48 ± 0.39ab**	**6.19 ± 0.12b**	**8.80 ± 2.21ab**	**9.18 ± 0.35ab**
∑ PUFA	**46.31 ± 0.18b**	**54.60 ± 7.37ab**	**69.45 ± 1.39a**	**62.85 ± 7.92ab**	**56.52 ± 0.85ab**

Means ± standard deviation in the same line followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each sample (n = 3); nd: not detected; SFL1, SFL2: Saffron floral by-products from two different producers

tendency in fatty acids content of some edible plants, being polyunsaturated fatty acids group the predominant (46–57%) in petals from different species (dahlia, rose, calendula and centaurea) and were similar to the content of vegetable oils like soybean oil (6%) [11]. Therefore, due to the high content of n-6 and n-3 fatty acids and the lower content of SFA, the intake of saffron and its floral by-products may provide additional benefits for cardiovascular health.

Expected Impacts of Saffron Waste Valorization

Currently, the management of food by-products through sustainable solutions is one of the main challenges of agro-food industries which are continuously searching for innovative solutions to obtain zero waste. As shown in Fig. 1, only the stigmas of *Crocus sativus* L. are used to obtain the commercial saffron spice. However, several tons of floral bio-residues are generated for each kg of spice, but these biomaterials are a source of valuable compounds such as proteins, PUFA, organic acids, and dietary fibers. Therefore, the valorization of these saffron floral by-products must take full advantage of their nutritional and functional potential in order to achieve economic, social and environmental positive impacts: (1) social benefits, since the use of this new food source as healthy and sustainable natural ingredients for human food may contribute to reduce hunger and undernourishment in developing countries, as well as contributing to the demand for new foodstuffs due to the growing world population; (2) financial benefits, since the added value generated in saffron floral by-products could create new companies, generating job opportunities and could be a new income for saffron farmers and for the saffron industry, which might lead to an increase in wages and the employment rate; (3) environmental impact minimization, since their valorization contributes to the reduction in waste accumulation, taking advantage of a biomass that is unexploited [7]. At the same time, saffron production will become more sustainable and profitable.

Therefore, saffron floral waste could be used as high-added value ingredients adding them into the food matrices previously freeze-dried to improve the safety of food by-products, separated individually from the biological matrix

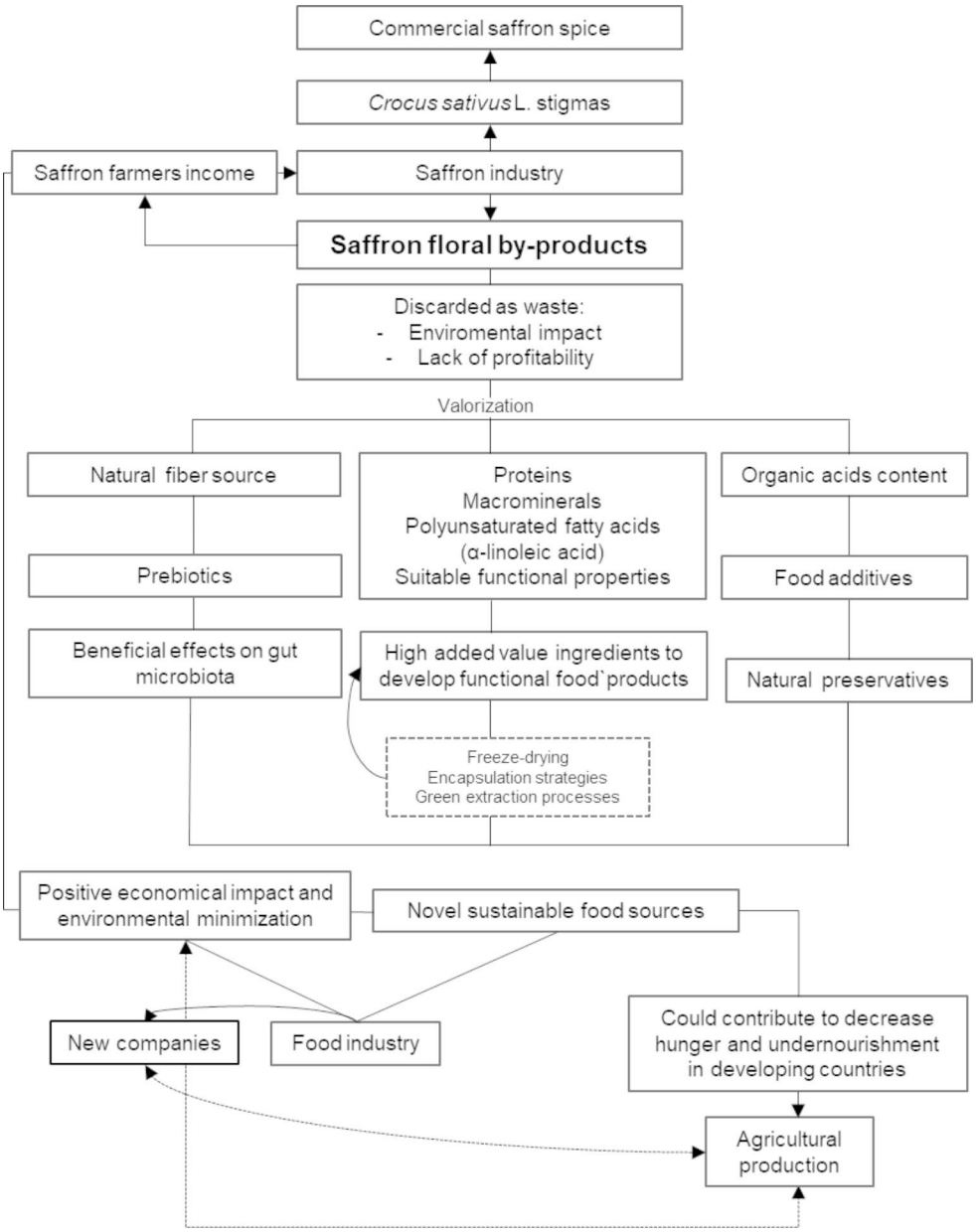

Fig. 1 Diagram about the economic, social and environmental expected impacts from the valorization of saffron floral by-products

by green and sustainable strategies or by microencapsulation strategies to decrease any oxidation of bioactive, nutritional and functional components contained in them when in contact environmental factors [23]. In order to contribute to this approach, this study provides new information emphasizing the nutritional characterization of saffron floral by-products to unveil a deeper knowledge of their application as high-added value ingredients, which could generate economic gains for the industry, and contribute to reducing nutritional and environmental problems.

Conclusion

This research provides new information on the nutritional value and composition of saffron spice and its floral by-products, in terms of hydrophilic and lipophilic compounds, revealing their potential as promising sources that could be further processed to be incorporated into different food matrices, due to their good composition of natural fiber, organic acids and soluble sugars, macrominerals and n-6 and n-3 fatty acids. In addition to the nutritional potential of saffron flowers, their suitable functional properties could increase the future perspectives to use them as sustainable ingredients. Nevertheless, further research would be necessary focused on the beneficial effects of saffron floral

by-products on human health, such as intestinal health due to their fiber content or on cardiovascular health because of their content of n-6 and n-3 fatty acids. Furthermore, through the valorization of saffron floral by-products, this research could also contribute to the improvement of the sustainability of the saffron spice production and to the profitability of this agro-industrial sector taking advantage of a high-value biomass that is currently unexploited and discarded representing an environmental problem.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11130-023-01065-7.

Acknowledgements Thanks to the Spanish-Ministry of Education, Culture and Sport for the FPU-PhD fellowship (FPU18/02225) and thanks to the Spanish company Verdú Cantó Saffron Spain, S.L. for the supply of samples.

Author Contributions Conceptualization: D.C.B.; methodology: D.C.B.; writing-original draft preparation: D.C.B. and M.J.F.; writing—review and editing: D.C.B., and M.J.F.; supervision: M.J. F, F.P.L., J.J. P. and E.V.C.; project administration: M.J.F. All authors have read and agreed to the published version of the manuscript.

Funding Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature.

Data Availability Data available upon request.

Declarations

Conflict of Interest The authors declare no conflicts of interest.

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication All authors consent for publication.

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SUPPLEMENTARY MATERIAL

Material and Methods

Plant material

Saffron floral by-products were obtained from two different producers (SFL1, SFL2) of a village belonging to the Toledo province (Castilla-La Mancha region, Spain), during the 2020 harvest season and cultivated following the requirements established by the Protected Designation of Origin “La Mancha Saffron” according to DOCM [1]. Saffron floral by-products were composed of all the parts of saffron flowers (tepals, stamens and styles), except for the stigmas that were detached manually from the rest of the flower by hand, following traditional procedures according to DOCM [1]. All fresh flowers were frozen in liquid nitrogen and kept at -80 °C until freeze-dried in a freeze-dryer Christ Alpha 2-4 (B. Braun Biotech International, Melsungen, Germany) for 48 h to constant weight. The conditions of the freeze dryer were: initial temperature -25 ± 2 °C and pressure 0.220 mbar. Then, they were crushed and sieved through a 500 μm mesh size, and stored in polyethylene bags at -20 °C until further analysis. Stigmas were obtained from flowers of *Crocus sativus* L. from Spanish (Castilla-La Mancha region), Greek (Kozani area) and Iranian cultivations (Torbat zone), which were supplied by the Spanish company Verdú Cantó Saffron Spain, S.L.

Proximal composition and dietary fiber

The moisture and ash content of freeze-dried flower samples and saffron stigmas were determined according to ISO 3632 [2]. Protein content (Kjeldahl method using a conversion factor of 6.25), fat content and total dietary fiber, insoluble dietary fiber and soluble dietary fiber were estimated according to AOAC [3]. Available carbohydrates were calculated by the difference (meaning 100-the sum of moisture, ash, protein and fat), and the energy, total caloric values (kcal), were determined on the basis of a 100 g portion using values for protein (×4 kcal/g), carbohydrate (×4 kcal/g) and fat (×9 kcal/g) [4].

Extraction organic acids and sugars

The extractions were prepared using ultrapure water, and a sample/water ratio 1:20 (*w/v*) for the freeze-dried flowers and a ratio 1:50 (*w/v*) for saffron stigmas. The extracts were

shaken for 1 h in the dark at 400 rpm on a magnetic stirrer at room temperature (Ovan, mod. MultiMix Heat D-MMH30E, Barcelona, Spain) and then sonicated for 15 min and centrifuged at 11200 x g for 10 min at 4 °C. Then, the supernatants were filtered (0.45 µm PTFE filter, Millipore, Spain) and stored at -20 °C. All extractions were done in triplicate.

Analysis of organic acids and sugars

The identification and quantification of sugars and organic acids were carried out by high performance liquid chromatography using Hewlett-Packard HPLC series 1100 equipment (Woldbronn, Germany) equipped with a Supelcogel C-610H column (30 cm x 7.8 mm) and a Supelcoguard C-610H pre-column (5 cm x 4.6 mm) (Supelco, Sigma Aldrich, St. Louis, MO, USA). The organic acids were measured at 210 nm in UV-VIS with diode array detector (DAD G1315A). For sugars, a refractive index detector (G1362A RID) was used. As a mobile phase, 0.1% orthophosphoric acid was used with an injection volume of 20 µl and the flow rate of 0.5 mL/min under isocratic conditions according to the method described by Cerdá-Bernad et al. [5]. The concentrations were calculated through calibration curves with the standards for sugars and organic acids (Sigma Aldrich, St. Louis, MO, USA). The results were expressed as mg/g of dry weight of sample.

Minerals

The minerals composition were determined according to Serrano-Díaz et al. [4], with slight modifications. Freeze-dried flower samples weighing 0.5 g were digested with 10 ml of 65 % HNO_3 (*v/v*), and 0.1 g of saffron were digested with 5 ml of 65 % HNO_3 (*v/v*) using a microwave reactor digestor (CEM Mars one, NC, USA) for 30 min with a temperature ramp whose final temperature was 200 °C. All samples were filtered (Whatman qualitative filter paper 90 mm) and diluted with ultrapure deionized water 1:50 (*v/v*) and stored at 4 °C. Total concentrations of macronutrients (Ca, Mg, Na and K) and micronutrients (Zn, Cu, Mn and Fe) in the previously mineralized samples were quantified with an Inductively Coupled Plasma Mass Spectrometer (ICPMS-2030, Shimadzu, Kyoto, Japan).

Fatty acid profile

Fatty acids were extracted from 0.5 g of freeze-dried flowers or saffron stigmas, by homogenizing them in a vortex with 20 ml of chloroform/methanol (2:1 *v/v*). Total lipids were extracted according to Folch et al. [6] and non-lipid impurities were removed by washing with 0.88% KCl (*w/v*). Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification of total lipids according to the method described by Christie [7]. FAME were separated and quantified by gas–liquid chromatography using an SP™ 2560 flexible fused silica capillary column (100 m long, internal diameter of 0.25 mm and film thickness of 0.20 mm) (Supelco 2560 SPTM, Bellefone, PA, USA) in a Hewlett Packard 5890 gas chromatograph (Bellefone, PA, USA).

Functional properties

Water-holding capacity and water solubility

The water-holding capacity (WHC) was determined mixing 0.25 g of freeze-dried saffron floral by-products in 10 ml of distilled water during 1 min in a vortex mixer and keeping samples to hydrate at room temperature for 30 min, prior to centrifugation at 2000 x g for 30 min. Excess supernatant was decanted and WHC was expressed as g water/g dry sample. Solubility was measured following the methodology reported by Garau et al. [8], as % loss in the initial freeze-dried sample weight, used previously to determine WHC, after the recovery of insoluble material (precipitate) after the centrifugation process.

Oil-holding capacity

The oil-holding capacity (OHC) was conducted according to Mallek-Ayadi et al. [9], mixing 0.25 g of freeze-dried saffron floral by-products in 10 ml of sunflower oil during 1 min in a vortex mixer, prior to centrifugation at 2000 x g for 30 min. Excess supernatant was decanted and OHC was expressed as g oil/g dry sample.

Swelling capacity

To study the swelling capacity (SC), 0.1 g of freeze-dried saffron floral by-products were added to 10 ml of distilled water and heated in a water bath at 60 °C for 30 min with stirring, following the methodology reported by Kusumayanti et al. [10]. Then, samples were

centrifuged at 1000 x g for 15 min and the precipitate was weighed, calculating SC using the Eq. 1:

Swelling capacity (g/g) = precipitate weight/initial sample weight (1)

Statistical analysis

All determinations were done in triplicate. Results were expressed as the mean ± standard deviation. The mean comparisons were carried out using an analysis of variance (ANOVA) and by the Tukey multiple range test, using SPSS version 21.0 software package (SPSS Inc., Chicago, IL). The significant differences were established as ($p \leq 0.05$).

Results and Discussion

Functional properties of saffron floral by-products

In order to use saffron floral by-products as new ingredients to develop enriched products, the study of their functional properties is very relevant to provide information regarding the interactions between components, structure and physicochemical properties of food components with the nature of the environment or food matrices [9]. The functional properties of freeze-dried saffron floral by-products are presented in Fig. S1.

WHC was evaluated in order to study the optimum amount of water to obtain a quality food product regarding its consistency. Samples showed statistically significant differences in WHC values, being around 15 g water/g dw for SFL1 and 10 g water/g dw for SFL2. These differences could be attributed to different concentration of hydrophilic compounds between SFL1 and SFL2. However, OHC values were similar in SFL1 and SFL2 (7 g oil/g dw), and statistically no-significant differences were found. It is essential to evaluate oil-holding capacity to use these ingredients in the formulation of foods with high content of fat and emulsion to improve their palatability and flavor retention (meat or bakery products) [11].

To develop bakery products, swelling capacity is a relevant quality parameter which could be influence by the particle size and the starch content of samples. Due to their high concentration in available carbohydrates, saffron floral by-products showed values around 12 g/g dw of SC, without finding statistically significant differences. These results were higher than

that reported in other studies for different starch sources like potato flours, in which values ranged from 3.40 to 3.67 g/g [10].

In addition, water solubility is related to the digestibility of food in order to develop infant formula and food [11]. The results of solubility of saffron floral by-products indicated a high solubility which could be present a high digestibility, being excellent ingredients to develop new food products. Values ranged from 34 to 37 % for SFL2 and SFL1, respectively, showing statistically significant differences, which may be also due to the content of hydrophilic compounds.

Therefore, saffron floral by-products presented adequate and suitable functional properties, so they could be exploited as food ingredients to add in several food matrices developing novel functional food products with enhanced nutritional values and functional characteristics.

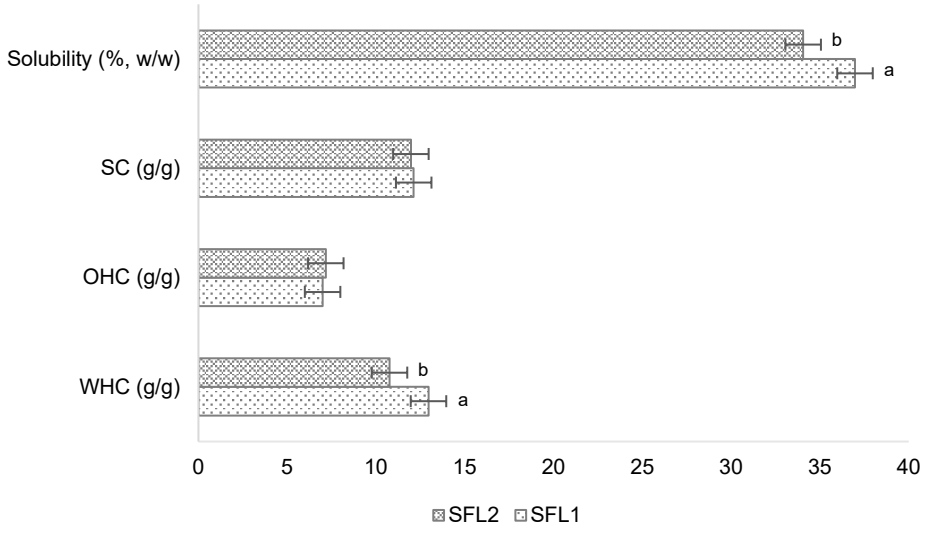

Fig. S1 Functional properties of saffron floral by-products. Error bars represent standard deviation and different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each sample (n = 3); SFL1, SFL2: Saffron floral by-products from two different producers; SC: Swelling capacity; OHC: Oil-holding capacity; WHC: Water-holding capacity.

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Publicación 3

Novel insight into the volatile profile and antioxidant properties of *Crocus sativus* L. flowers

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Revista	Antioxidants
DOI	10.3390/antiox11091650
Editorial	MDPI
Fecha de publicación	25 de Agosto de 2022
Categoría JCR®	Food Science and Technology
Cuartil	Q1
Rango	13/142
Factor de impacto	7,000

antioxidants

MDPI

Article

Novel Insight into the Volatile Profile and Antioxidant Properties of *Crocus sativus* L. Flowers

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Abstract: The current production system of saffron spice generates hundreds of tons of waste. Thus, the aim of this study was to value both saffron and its floral by-products as a source of natural bioactive extracts, studying the in vitro antioxidant capacity, the composition of the volatile fraction by GC-MS/MS, and the determination of crocetins esters by HPLC-PDA. Saffron stigmas and floral by-products showed a high content of polyphenols and different antioxidant properties. Floral bio-residues (tepals, stamens, and styles) presented a high concentration of anthocyanins, and stigmas had high levels of flavonoids, β-carotene, and total crocins. In stigmas, 25 different volatile components were found, with safranal the most relevant. Floral by-products volatile composition consisted of 55 compounds with varying amounts depending on the drying treatment; all the samples presented acetic acid, 2(5H)-furanone, and phenylethyl alcohol. Therefore, saffron stigmas and flower by-products represent a sustainable source of bioactive ingredients for innovative healthy food formulations.

Keywords: saffron; floral bio-residues; volatile compounds; polyphenols; flavonoids; anthocyanins; apocarotenoids; sustainability; functional food; high added-value ingredients

Citation: Cerdá-Bernad, D.; Clemente-Villalba, J.; Valero-Cases, E.; Pastor, J.-J.; Frutos, M.-J. Novel Insight into the Volatile Profile and Antioxidant Properties of *Crocus sativus* L. Flowers. *Antioxidants* **2022**, *11*, 1650. https://doi.org/10.3390/antiox11091650

Academic Editor: Renan Campos Chisté

Received: 2 August 2022
Accepted: 22 August 2022
Published: 25 August 2022

1. Introduction

Saffron (*Crocus sativus* L.), a traditional Mediterranean plant, is a perennial herb that belongs to the Iridaceae family. It is employed as a spice, natural colorant in food, and a flavoring agent. This spice is obtained from the flower of *C. sativus*, which is composed of three golden yellow stamens, six purple tepals, and one red pistil that culminates with three red branched stigmas, whose length exceeds that of the tepals, which, when dried up, constitute the saffron spice [1]. Only flower stigmas are used for saffron production, while tepals and stamens are simply discarded. For the production of 1 kg of saffron, around 350 kg of tepals are generated as a by-product. Thus, the current production system is not sustainable since it generates hundreds of tons of waste, with a high environmental impact [2].

In addition to its organoleptic properties, saffron is occasionally used as a textile dye, in perfumes, and in medicine due to its therapeutic properties since it has been considered for centuries as a medicinal plant in many cultures [3]. The therapeutic activity of saffron is mainly due to its valuable bioactive compounds (carotenoids, terpenes, and flavonoids). The main chemical components are crocin, crocetin and safranal. Crocetin, the principal apocarotenoid in saffron, is the precursor of crocin, and the most abundant compound is *trans*-crocetin di(β-D-gentiobiosyl) ester. The volatile compounds, including terpenoids, along with phenolic compounds that are widely distributed in plants, are responsible for

their sensory properties. In saffron, 3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl-, known as safranal, is the major volatile compound contributing to saffron aroma [4].

One of the greatest interests of these bioactive compounds is due to their high antioxidant capacity and free radical scavenging activity. The generation of reactive oxygen species is a normal process in cells, but the uncontrolled generation and concomitant increase in reactive oxygen species (ROS) level in the body results in "oxidative stress", which is considered to be the main cause of various diseases [5]. The antioxidant substances play a role in protecting biological systems against the effects of oxidative processes on macromolecules. Many of those substances are plant-derived natural molecules, such as carotenoids or polyphenolic compounds, that could contribute to preventing and fighting against several diseases in which ROS are involved [6].

Currently, some studies have reported that tepals, considered waste, contain valuable bioactive compounds such as flavonoids and polyphenols with potential antioxidant activity [7]. Therefore, saffron floral by-products could be natural antioxidant sources to be used in food, increasing the resource efficiency and the competitiveness of this sector. This waste raw material could be valorized through the development of innovative high added-value food ingredients, and this could potentially increase saffron demand on the market.

The main objective of this study was to value both saffron and its floral by-products through their use as a source of bioactive extracts, studying the in vitro antioxidant capacity, the metal chelating activity, the composition of volatile compounds by GC-MS/MS, and the determination of crocetins esters and total crocins by HPLC in saffron and its floral by-products. As far as we know, no research has been published to date that delves into the composition of the volatile fraction in saffron floral by-products and into the changes in the volatile profile depending on the drying treatment used. Moreover, the results of this research would support the production of saffron as a more sustainable and profitable agronomic resource.

2. Materials and Methods

2.1. Chemicals

Methanol, ethanol, n-hexane, acetone, and acetonitrile solutions were HPLC grade (J.T. Baker, Madrid, Spain), and hydrochloric acid 37% was obtained from Panreac (Barcelona, Spain).

Trolox, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate ($K_2S_2O_8$), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), iron(III) chloride solution ($FeCl_3$), iron(II) chloride solution ($FeCl_2$), sodium acetate trihydrate ($CH_3COONa{\cdot}3H_2O$), gallic acid analytical standard (≥98.0%), catechin analytical standard (≥99.0%), sodium nitrate ($NaNO_2$), aluminum chloride ($AlCl_3$), sodium hydroxide (NaOH), delphinidin chloride analytical standard (≥95.0%), sodium bisulfite ($NaHSO_3$), ferrozine iron reagent, isoamyl acetate (≥95%), *trans*-crocetin di(β-D-gentiobiosyl) ester and *trans*-crocetin (β-Dglucosyl)-(β-D-gentiobiosyl) ester standards (≥95%, HPLC grade) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Folin–Ciocalteu reagent, sodium bicarbonate (Na_2CO_3), potassium dihydrogen phosphate (KH_2PO_4), and dipotassium hydrogen phosphate (K_2HPO_4) were purchased from Merck Millipore (Darmstadt, Germany). A commercial alkane standard mixture (C_6-C_{20}) for Gas Chromatography-Mass Spectrometry compounds identification was also purchased from Merck Millipore (Darmstadt, Germany).

For all the experiments, ultrapure Milli-Q water (Millipore Corp., Bedford, MA, USA) was used.

2.2. Plant Material

Saffron floral by-products (SFL1, SFL2) were obtained from different producers in Castilla-La Mancha region (Spain) during the 2020 harvest season and cultivated following the requirements established by the Protected Designation of Origin "La Mancha Saffron" according to DOCM [8]. The flowers were composed of all the parts of saffron flowers

(tepals, stamens, and styles), except for the stigmas that were detached manually from the rest of the flower after being harvested, following the traditional procedure.

All fresh flowers were frozen in liquid nitrogen and stored at −80 °C until freeze-dried in a freeze-dryer Christ Alpha 2–4 (B. Braun Biotech International, Melsungen; Germany) for 48 h to constant weight (initial temperature −25 ± 2 °C and pressure 0.220 mbar). Then, they were crushed and sieved through a 500 µm mesh size and stored at −20 °C until further analysis. For the volatile composition analysis, in addition to fresh and freeze-dried samples, oven-dried samples were studied, which were dried for 24 h at 60 °C in an air oven.

Saffron stigmas were supplied dehydrated by the Spanish company Verdú Cantó Saffron Spain, S.L and were from Spanish (Castilla-La Mancha region), Greek (Kozani area), and Iranian cultivations (Torbat zone). The moisture of all samples was lower than 11%, and saffron threads were crushed and sieved through a 500 µm mesh size and stored at 4 °C until further analysis.

2.3. Extraction of Bioactive Compounds

2.3.1. Polyphenolic Compounds

The extractions were prepared using a methanol solution and a sample/methanol ratio 1:20 (*w*/*v*) for the freeze-dried flowers and a ratio 1:50 (*w*/*v*) for saffron stigmas. The extracts were shaken for 1 h in the dark at 400 rpm with a magnetic stirrer (Ovan, mod. MultiMix Heat D-MMH30E) and then sonicated for 15 min and centrifuged at 10,000× *g* for 10 min at 4 °C. Then, the supernatants were filtered (0.45 µm PTFE filter, Millipore, Madrid, Spain) and stored at −20 °C. All extractions were performed in triplicate.

2.3.2. Beta-Carotene

The extractions were prepared using n-hexane:acetone:ethanol (50:25:25, *v*/*v*/*v*) solution and a sample/solvent ratio 1:20 (*w*/*v*) for the freeze-dried flowers and a ratio 1:50 (*w*/*v*) for saffron stigmas. The extracts were shaken for 10 min at 400 rpm with a magnetic stirrer (Ovan, mod. MultiMix Heat D-MMH30E), keeping them in ice, and then centrifugated at 14,460× *g* for 20 min at 4 °C. Then, the supernatants were filtered (0.45 µm PTFE filter, Millipore, Madrid, Spain) and stored at −20 °C. All extractions were performed in triplicate.

2.3.3. Anthocyanins

The extractions were prepared using 50% ethanol (0.1% HCl) solution and a sample/solvent ratio 1:20 (*w*/*v*) for the freeze-dried flowers. The extracts were sonicated for 30 min and centrifugated at 10,000× *g* for 15 min. Supernatants were filtered (0.45 µm PTFE filter, Millipore, Madrid, Spain). All extractions were performed in triplicate.

2.4. In Vitro Antioxidant Properties

For the study of the antioxidant properties, the extracts obtained from polyphenolic compounds extraction (Section 2.3.1.) were used.

2.4.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Method

The free radical scavenging activity was determined using DPPH method, following the methodology from Brand-Williams et al. [9], with some modifications. The DPPH radical was prepared dissolving 0.0035 g with 10 mL of methanol. The mixture was shaken and kept in dark for 30 min. The absorbance decrease was measured at 515 nm (UV/Vis Spectrophotometer T80; PG Instruments Limited, UK). Trolox (10 mM) was used as a reference standard at different concentrations (0.50–4.00 mmol/L). The results were expressed as mmol of Trolox Equivalents (TE) per 100 g dw (dry weight) of sample.

2.4.2. 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Free Radical Scavenging Method

The ABTS cation radical method for measuring the antioxidant capacity was performed by adapting the methodology from Re et al. [10]. ABTS radical was prepared mixing ABTS (7 mM) with $K_2S_2O_8$ (2.45 mM), and they reacted for 16 h in dark at room temperature. Subsequently, the solution was diluted with ultrapure water until its absorbance was adjusted to 0.70 ± 0.02 at 734 nm. Trolox (10 mM) was used as a reference standard in different concentrations (0.20–3.00 mmol/L). The results were expressed as mmol of TE per 100 g dw of sample.

2.4.3. Ferric Reducing Antioxidant Power (FRAP) Method

The FRAP method adopted from Benzie and Strain [11] was used. Briefly, the FRAP reagent was prepared fresh daily by mixing 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L TPTZ solution in 40 mmol/L HCl, and 20 mmol/L $FeCl_3 \cdot 6H_2O$ solution in a volume ratio of 10:1:1, respectively. The absorbance was measured at 593 nm. Trolox (10 mM) was used as standard solution in the range of 0.01−5.00 mmol/L. The results were expressed as mmol of TE per 100 g dw of sample.

2.5. Bioactive Compounds Content

2.5.1. Total Polyphenols Content (TPC)

The total polyphenols were determined using the Folin–Ciocalteu methodology following the method described by Singleton et al. [12]. The Folin–Ciocalteu reagent was mixed with ultrapure water 1:10 (*v*/*v*). Gallic acid (1 mM) was used as a reference standard in the range of 0.00–4.72 mg/L. For the assays, 100 µL of the different extracts were mixed with 400 µL of phosphate buffer (50 mM) at pH 7.8, and 2.5 mL of Folin–Ciocalteu reagent was added. After 2 min, 2 mL of Na_2CO_3 (75 g/L) were added and kept at 50 °C for 10 min. The absorbance was measured at 760 nm in a spectrophotometer (UV/Vis), and the results were expressed as mg Gallic Acid Equivalents (GAE) per g dw of sample.

2.5.2. Total Flavonoids Content (TFC)

The total flavonoid content was determined as described by Çam and Hışıl [13]. Catechin was used as standard; in order to calculate the flavonoid content, a calibration curve ranging from 20–100 mg/L was prepared. The absorbance was measured at 510 nm, and the results were expressed in mg of Catechin Equivalents (CE) per g dw of sample.

2.5.3. Total Anthocyanins Content (TAC)

The total anthocyanin content was determined by the method from Figueira et al. [14] with some modifications. Delphinidin was used as an external standard, and the total anthocyanin content was calculated using a calibration curve (10–150 mg/L). The final absorbance was determined by the difference between the measured reference absorbance and the measured sample absorbance. The absorbance was measured at 520 nm, and the results were expressed in mg of Delphinidin Equivalents (mg DE) per g dw of sample.

2.5.4. Total Beta-Carotene Content

The change in absorbance was measured using a UV-Vis spectrophotometer at 450 nm. Carotene content was determined using the Beer-Lambert law. The carotene concentration was calculated using the extinction coefficient of beta-carotene in hexane (2.505 $M^{-1}cm^{-1}$). The results were expressed as mg of beta-carotene per 100 g dw of sample (%).

2.6. Iron (II) Chelating Activity

For the metal chelating activity, the extractions were performed in the same procedure previously explained for the extraction of polyphenolic compounds but using ultrapure water as extracting agent.

The chelation of iron (II) ions was performed as described by Carter [15]. Briefly, a final concentration of 0.02 g/mL of extract were used and mixed with 100 µL of 2.0 mM aqueous $FeCl_2$ and 900 µL methanol. After an incubation of 5 min, the reaction was initiated adding 400 µL of 5.0 mM ferrozine. Then, after a 10 min equilibrium period, the absorbance was measured at 562 nm. The iron chelation activities were calculated from the absorbance of the control (*Ac*) and of the sample (*As*) using the following equation and expressed as % inhibition:

$$\%\ Inhibition = \frac{A_c - A_s}{A_c} \cdot 100 \quad (1)$$

2.7. Color

The color was measured with a Minolta CR-300 Chroma Meter (Japan) colorimeter, using L*, a*, b* scale (CIELAB system). The results were expressed as luminosity L*, a* (greenness/redness), b* (blueness/yellowness), Hue angle (*h*) and Chroma (C*), which were calculated according to the following equations, respectively:

$$h = \left(\arctan\left(\frac{b^*}{a^*}\right)\right) \times \frac{180}{\pi} \quad (2)$$

$$C^* = \left(a^{*2} + b^{*2}\right)^{1/2} \quad (3)$$

2.8. Determination of Crocetin Esters by HPLC-PDA

2.8.1. Extract Preparation

The aqueous extracts of saffron stigmas and flowers were prepared according to ISO 3632 [16]. Briefly, 0.5 g of sample was mixed with 900 mL of ultrapure water in a 1 L volumetric flask. The solutions were shaken for 1 h in the dark at 1000 rpm on a magnetic stirrer. The flask was filled to 1 L and homogenized through agitation. Then, 20 mL of solution were transferred to a 200 mL volumetric flask which was filled with ultrapure water. The extractions were filtered (0.45 µm PTFE filter, Millipore, Madrid, Spain) and transferred into a vial for HPLC analysis. All extractions were performed in triplicate.

2.8.2. HPLC-PDA

The identification and quantification of crocetin esters were carried out by high-performance liquid chromatography (HPLC) using HPLC Altus^{TM} 10 PerkinElmer (Waltham, MA, USA) equipped with a C18 KromaPhase column (150 × 4.6 mm inner diameter, 3.5 µm) (Scharlab, Barcelona, Spain) that was equilibrated at 30 °C. The analysis were carried out as described by Valle García-Rodríguez et al. [17] with some modifications. The eluents used were acetonitrile (A) and water (B), with a proportion of 20% A and 80% B. The flow rate was 0.8 mL/min, and the injection volume of saffron extracts was 25 µL. Crocetin esters were measured at 440 nm in UV-VIS (UV-6300PC double beam spectrophotometer) with the Photodiode Array (PDA) Detector (PerkinElmer, Waltham, MA, USA).

The identifications of crocetin esters, such as *trans*-crocetin di(β-D-gentiobiosyl) ester (*trans*-4-GG), and *trans*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester (*trans*-3-Gg), were determined using the UV−VIS spectrum, and the retention time was carried out by the HPLC−PDA method at 440 nm. Their quantification was performed through calibration curves with the standards, y = 0.0075x − 0.0080 (R value = 0.99) for *trans*-4-GG in the range from 0.8 to 50 mg/L, and y = 0.0071x− 0.0047 (R value = 0.99) for *trans*-3-Gg concentration in the range from 0.8 to 25 mg/L.

2.9. Volatile Composition

The volatile composition was determined using headspace solid phase micro-extraction (HS-SPME). After several preliminary tests to optimize the extraction system, accurately amounts between 20 and 40 mg of saffron stigmas and between 120 and 140 mg of saffron flowers were weighted and added into a 40 mL vial with polypropylene caps and PTFE/silicone septa, and isoamyl acetate (1000 mg/L, internal standard for semi-

quantification of compounds). Fresh, freeze-dried, and air oven-dried samples of saffron flowers were used in the analyses. Then, the vial was placed in an AOC-6000 Plus autosampler (Shimadzu Corporation, Kyoto, Japan), and after 5 min of equilibration time, a 50/30 µm DVB/CAR/PDMS fiber (1 cm) was exposed to the sample headspace for 45 min at 40 °C (with agitation, 250 rpm).

The separation and identification of compounds were performed by GC2030 (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) in a Sapiens X5MS column (Teknokroma, Barcelona, Spain), 30 m × 0.25 mm i.d., 0.25 µm f.t., and coupled with a mass spectrometer detector (TQ8040 NX triple quadrupole mass spectrometer; Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). Only the single quadrupole acquisition mode was used on the TQ8040 NX (Q3 Scan; scan speed 5000 amu/s; mass range 40–400 m/z; event time 0.100 s). The oven temperature program was as follows: (i) initial temperature of 35 °C and hold 5 min; (ii), increment of 5 °C/min up to 150 °C/min, and hold 1 min; (iii) increment of 10 °C/min up to 280 °C and hold for 5 min. Helium column head pressure was 47.6 kPa (constant linear velocity mode of 36 cm/s). Injector, ion source, and interface were at 250, 230, and 280 °C, respectively. Helium was used as gas carrier, column flow 1 mL/min, with split ratio 1:50 and purge flow of 6 mL/min.

Retention indexes of a commercial alkane standard mixture were used to identify the compounds, as well as the National Institute of Standards and Technology (NIST) 17 Mass Spectral and Retention Index Libraries. The identification was considered tentative when it was based only on mass spectral data, and only compounds with spectra similarity > 90% were considered correct hits. Linear retention similarity filter was set at ±10 units. This volatile compound extraction method has been previously used for the analysis of different food matrices, according to Clemente-Villalba et al. [18].

2.10. Statistical Analysis

All determinations were performed in triplicate. Results were expressed as the mean ± standard deviation. The mean comparisons were carried out using an analysis of variance (ANOVA) and by the Tukey multiple range test using SPSS version 21.0 software package (SPSS Inc., Chicago, IL, USA). The significant differences were established as $p < 0.05$.

3. Results and Discussion

3.1. Antioxidant Properties, Bioactive Content and Iron (II) Chelating Activity

Polyphenols, which are plant-derived natural molecules found as secondary metabolites, present important biological activities, with their antioxidant capacity one of the most important properties for physiological function. The total polyphenols and flavonoid content, as well as the antioxidant effects, were studied in saffron and its floral by-products extracts.

The results of the antioxidant activity and bioactive content are shown in Table 1. Saffron floral by-products and saffron stigmas presented a high concentration of total polyphenols, between 32–36 mg GAE/g dw, except for Spanish saffron, which had a significant highest amount (44.80 ± 2.30 mg GAE/g dw). Regarding the total flavonoid content, saffron stigmas from Spain, Iran, and Greece showed high levels of flavonoids in the range of 15–18 mg CE/g dw. However, saffron flowers had a lower concentration of total flavonoids (4–5 mg CE/g dw) than saffron stigmas.

These results obtained for saffron flowers were in accordance with the ones described by Sun et al. [19], reporting values about 30 mg/g for TPC and values lower than 10 mg/g for TFC in saffron tepal methanol extracts from China. Moreover, the values obtained for saffron stigmas were higher than those reported in the study of Karimi et al. [6], in which saffron stigmas methanol extracts from Iran presented 6.5 ± 0.02 mg GAE/g dw for TPC and 5.8 ± 0.12 mg rutin equivalents/g dw for TFC.

Table 1. Antioxidant capacity and bioactive compounds content in saffron floral by-products and stigmas [1].

	SFL1	SFL2	Spanish Stigmas	Iranian Stigmas	Greek Stigmas
DPPH [2]	98.82 ± 4.79d	107.40 ± 1.95d	201.27 ± 7.71b	278.92 ± 8.37a	145.58 ± 21.98c
ABTS [2]	50.99 ± 2.51c	48.41 ± 4.67c	110.88 ± 9.99b	142.22 ± 5.23a	124.95 ± 27.44ab
FRAP [2]	1250 ± 88b	1181 ± 26b	3667 ± 319a	3471 ± 123a	3445 ± 274a
TPC [3]	32.42 ± 6.90b	32.82 ± 2.23b	44.80 ± 2.30a	36.35 ± 3.47b	34.00 ± 3.22b
TFC [4]	3.99 ± 0.79b	5.37 ± 0.19b	18.74 ± 1.22a	15.32 ± 1.73a	17.39 ± 4.14a
TAC [5]	39.17 ± 2.98b	69.02 ± 4.34a	n.d.	n.d.	n.d.
TBC [6]	28.39 ± 2.17c	39.59 ± 1.47c	77.51 ± 14.93ab	71.06 ± 6.47b	90.82 ± 8.37a

[1] Means ± standard deviation in the same line followed by different lowercase letters indicate statistically significant differences at $p \leq 0.05$ for each sample ($n = 3$). [2] The antioxidant capacity is expressed as mmol Trolox Equivalents (TE) per 100 g dw of sample. [3] The Total Polyphenols Content (TPC) are expressed as mg Gallic Acid Equivalents (GAE) per g dw of sample. [4] The Total Flavonoids Content (TFC) are expressed as mg of Catechin Equivalents (CE) per g dw of sample. [5] The Total Anthocyanin Content (TAC) were expressed in mg of Delphinidin Equivalents (mg DE) per g dw of sample. [6] The total bta-carotene is expressed as mg of beta-carotene per 100 g dw of sample (%). SFL1, SFL2: Saffron floral by-products from two different producers; n.d.: not determined.

Within the group of flavonoids, anthocyanins are one class of pigments that also present antioxidant properties [20]. Saffron flowers without stigmas, SFL1 and SFL2, contained high levels of anthocyanins, showing SFL2 a significantly higher concentration than SFL1. Serrano-Díaz et al. [21] also found the highest anthocyanin content in saffron tepals from Spain and were also detected in whole flowers and floral bio-residues.

Regarding carotenoids, they are organic pigments from the group of isoprenoids that are found naturally in plants and exert several beneficial functions in the body due to their antioxidant properties, among others. Based on their structures, carotenes are one of the main subclasses of carotenoids, with β-carotene and α-carotene the two major types. Beta-carotene was found to be the precursor of crocins. Crocins, which are glycosyl esters of crocetin, are the main chemical components of saffron, responsible for many of its pharmacological and biomedical properties. The synthesis of these apocarotenoids involves several reactions, including the cleavage of beta-carotene and zeaxanthin, oxidation and glycosylation steps [22].

Saffron stigmas showed a high proportion of total beta-carotene being higher than 70%. Greek saffron presented the highest concentration (90.82 ± 8.37%) and Iranian saffron the lowest, having 20% less total beta-carotene than Greek saffron (71.06 ± 6.47%) (Table 1). With respect to the crocetin esters content, the *trans* isomers, which are the majority, were studied. *Trans*-4-GG crocin was found in higher concentrations than *trans*-3-Gg-crocin in all saffron samples. Spanish stigmas presented the significant highest proportion of total crocins (17.04 ± 0.18%) and of *trans*-4-GG crocin (11.91 ± 0.13%) (Table 2). The same tendency for beta-carotene content was shown by Iranian stigmas, having the lowest proportion of total crocins (13.19 ± 0.78%) and of *trans*-4-GG crocin (9.22 ± 0.30%) (Table 2).

Table 2. Crocins in saffron floral by-products and saffron stigmas [1].

	trans-4-GG (% *w*/*w*)	*trans*-3-Gg (% *w*/*w*)	Total Crocins (% *w*/*w*)
SFL1	0.009 ± 0.002d	0.034 ± 0.005b	0.042 ± 0.007c
SFL2	n.d.	n.d.	-
Spanish Stigmas	11.91 ± 0.13a	5.13 ± 0.05a	17.04 ± 0.18a
Iranian Stigmas	9.22 ± 0.30c	3.98 ± 0.48a	13.19 ± 0.78b
Greek Stigmas	10.74 ± 0.24b	3.93 ± 0.53a	14.67 ± 0.77b

[1] Means ± standard deviation in the same column followed by different lowercase letters indicate statistically significant differences at $p \leq 0.05$ for each sample ($n = 3$). SFL1, SFL2: Saffron floral by-products from two different producers; n.d.: not detected.

These results were in accordance with those obtained by Valle García-Rodríguez et al. [17], that have reported the quantification of these two crocins (*trans*-4-GG and *trans*-3-Gg) in saffron from Italy, Iran, Greece, and Spain by HPLC. Furthermore, the values of Spanish saffron were consistent with the content of crocetin esters reported by Moratalla-López et al. [23], in which these compounds represented 16–28% of the saffron composition. Thus, the level of apocarotenoids may vary because of different geographical origins, processing, and storage conditions.

Saffron flowers presented low amounts of total beta-carotene (28–39%) that are mainly located in the yellow stamens. Nevertheless, beta-carotene was reported in higher quantity in other Crocus species such as *C. ancyrensis* also due to the yellow color of the tepals [22] (Table 1). Moreover, in saffron floral by-products, crocins were only detected in SFL1 but in very low amounts (0.042%) due to the presence of small fragments of stigmas remaining after their detachment (Table 2). However, Rubio Moraga et al. [22] reported that crocins in *C. sativus* saffron floral by-products from Spain were not detected.

DPPH, ABTS, and FRAP assays results revealed the different antioxidant activities of saffron and its floral by-products (Table 1). DPPH and ABTS tests evaluate the in vitro antiradical activity, and FRAP assay the reducing potential of the extracts. Saffron stigmas presented stronger antioxidant capacity than the floral by-products, which could be related to the higher amounts of total flavonoids and crocins content since previous studies have demonstrated the significant antioxidant activity of flavonoids and crocins [24]. Nevertheless, saffron flowers, SFL1 and SFL2, showed a good antioxidant ability (by DPPH, ABTS, and FRAP) that could be related to their high polyphenols content. These results are similar to other studies that reported the antioxidant capacity of Indian and Iranian saffron stigmas [6,25], the potential antioxidant activity of commercial saffron powder from the large-scale Italian market [26], and also to that of Sun et al. [19] in which it is revealed the strong antioxidant ability of saffron tepals from China.

Regarding the ability of the saffron extracts to chelate iron (II), the results are shown in Figure 1. Bivalent transition metal ions, such as iron, play an important role as catalysts of oxidative processes, participating in hydroxyl radical generation via the Fenton reaction [27]. Thus, excess metal ions could lead to the formation of free radicals generating high levels of oxidative stress, but these processes can be delayed by iron chelation. Apart from the ferric reducing power activity (FRAP) assay determination that indicated the reduction potential of Fe^{3+} to Fe^{2+}, the iron (II) chelating activity of saffron stigmas and flowers was also studied.

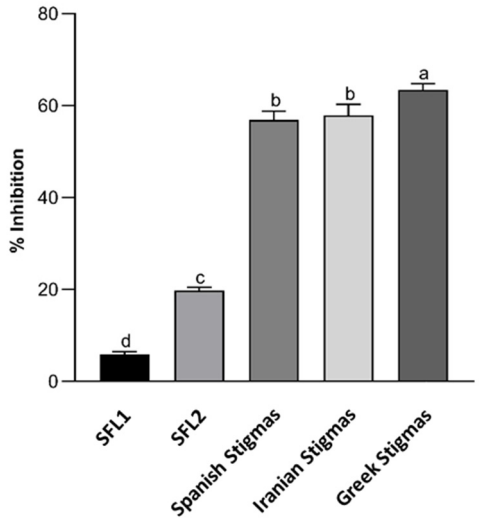

Figure 1. Iron (II) chelating activity of saffron floral-by products and saffron stigmas. The error bars represent the standard deviation and the different lowercase letters indicate statistically significant differences at $p \leq 0.05$ for each sample (n = 3). SFL1, SFL2: Saffron floral by-products from two different producers.

Saffron stigmas presented a good chelating activity, around 60% of inhibition, with the highest for the Greek saffron (Figure 1). This ability could be due to their flavonoid content since previous research demonstrated that these bioactive compounds have the capacity to neutralize reactive radicals and sequestrate metal ions, suppressing Fenton reactions [28]. Saffron flowers showed a low iron chelating activity (less than 20% of inhibition), with the capacity of SFL2 significantly higher than SFL1 (Figure 1). This fact might be related to the anthocyanins content of SFL2 (Table 1). These results were similar to those reported by Sánchez-Vioque et al. [29], indicating that saffron tepals from Spain had very low chelating activity.

Therefore, the chelating activity of Fe^{2+} and antioxidant capacity that presented saffron and its floral by-products could prevent oxidative damage, protecting against oxidative stress. These abilities may be linked to their bioactive compounds content that were located differently on the flower; stigmas had carotenoids and flavonoids, and the rest of the flower presented anthocyanins and other polyphenolic compounds.

3.2. Color

The color parameters of saffron and its floral by-products are shown in Table 3. The L* values were around 30 in all samples indicating a low luminosity and lightness. Regarding powdered saffron floral by-products, SFL1 and SFL2, positive a* values and negative b* values represented a color in the ranges of red and blue, respectively, which were characteristic of the violet color of tepals. It should be noted that the b* value of SFL2 (-4.77 ± 0.14) indicated a higher blue color intensity with respect to SFL1 (-1.63 ± 0.25). This fact may be mainly related to the high concentration of anthocyanins pigments found in SFL2, which render a blue, red, or purple color [30]. Moreover, *h* values (°) of SFL1 (334.50 ± 2.46) and SFL2 (313.00 ± 0.19) represented a tone in the blue/purple range, and the color was more saturated and intense in SFL2 than SFL1, represented by the chroma (C*) values (6.53 ± 0.18 and 3.78 ± 0.22, respectively).

Table 3. Color parameters of the powdered saffron floral by-products (**A**) and saffron stigmas (**B**) [1].

(A)	SFL1	SFL2	(B)	Spanish Stigmas	Iranian Stigmas	Greek Stigmas
L*	32.29 ± 0.16a	30.94 ± 0.24b	L*	29.64 ± 0.12a	28.86 ± 0.18b	29.54 ± 0.15a
a*	3.41 ± 0.13b	4.45 ± 0.11a	a*	10.69 ± 0.01a	9.26 ± 0.17b	10.60 ± 0.10a
b*	−1.63 ± 0.25a	−4.77 ± 0.14b	b*	5.80 ± 0.13a	4.56 ± 0.09b	5.72 ± 0.16a
h (°)	334.50 ± 2.46a	313.00 ± 0.19b	*h* (°)	28.47 ± 0.34a	26.23 ± 0.25b	28.38 ± 0.41a
C*	3.78 ± 0.22b	6.53 ± 0.18a	C*	12.16 ± 0.15a	10.32 ± 0.19b	12.05 ± 0.25a

[1] Means ± standard deviation in the same line followed by different lowercase letters indicate statistically significant differences at $p \leq 0.05$ for each sample ($n = 3$). SFL1, SFL2: Saffron floral by-products from two different producers.

Saffron stigmas from Spain, Iran, and Greece showed positive a* and b* values, representing colors ranging from red to yellow, respectively, which are characteristic of saffron spice. The *h* values were around 26–28° which indicated a red-orange tone that was saturated and intense in all saffron samples (C* values around 10–12). This yellow to red color observed in the stigmas of *Crocus sativus* L. was due to the presence of a high amount of carotenoids, such as crocetin and crocins, which are responsible for the coloring power of the saffron spice exhibiting red, orange, and yellow colors [22].

The results showed that the color parameters variability within the flowers and stigmas of *Crocus sativus* L. is related to the number of bioactive compounds with coloring properties in the different parts of the plant.

3.3. Volatile Composition

The evaluation of volatiles is an important aspect that contributes to the aroma of foods. Furthermore, volatile secondary metabolites also present important bioactive properties, such as antioxidant, antimicrobial, and anticancer activities [31].

The results of the volatile composition of saffron stigmas from Spain, Iran, and Greece are shown in Table 4.

Table 4. Identification, concentration (μg/g) and descriptors of volatile compounds found in saffron stigmas [1].

Code	Compound	CF	RT	KI (EXP)	KI (LIT)	Spanish Stigmas (μg/g)	Iranian Stigmas (μg/g)	Greek Stigmas (μg/g)	Odor Descriptors [2]
V1	Acetic acid	Organic acid	2.422	645	646	18.30c	40.40a	35.80b	Pungent, sour, vinegar
V2	1-Butanol, 3-methyl- (isoamyl alcohol)	Alcohol	5.445	744	750	311c	770b	1018a	Alcoholic, whiskey, fruity, banana
V3	Cyclopetanone	Ketone	6.750	774	767	2.01a	0.63c	1.65b	Minty
V4	2(5H)-Furanone	Ester	12,.27	903	913	173a	123b	63.50c	Buttery
V5	Hexyl acetate	Ester	16.070	1012	1012	3.62c	10.10a	9.36b	Sweet, green, fruity, banana
V6	3-Cyclohexen-1-one, 3,5,5-trimethyl- (beta isophorone)	Ketone	17.041	1040	1044	5.40c	7.60b	8.92c	Woody, sweet, camphoreous, musty
V7	Linalool	Terpene	19.026	1098	1098	5.50a	3.97b	5.26a	Floral, citrus, rose
V8	Nonanal	Aldehyde	19.159	1101	1101	7.40a	1.71c	6.08b	Waxy, aldehydic, citrus, fresh
V9	Cyclohexene, 1-methyl-4-(1-methylethylidene)- (Terpinolene)	Terpene	19.256	1105	1098	49.00b	49.20b	59.50a	Herbal, fresh, sweet, pine
V10	Phenylethyl Alcohol	Alcohol	19.389	1109	1110	22.80c	39.80b	110a	Floral, rose
V11	Isophorone	Ketone	19.687	1119	1118	1477b	1531b	1936a	Woody, sweet, camphoreous, musty
V12	2,6,6-Trimethyl-2-cyclohexene-1,4-dione (4-ketoisophorone)	Ketone	20.409	1142	1139	273c	690b	789a	Musty, woody, sweet
V13	2-Hydroxy-3,5,5-trimethyl-cyclohex-2-enone	Ketone	20.504	1145	1149	20.10b	39.70a	14.80c	Woody, dry nutty, tobacco
V14	1,4-Cyclohexanedione, 2,2,6-trimethyl- (Dihydrooxophorone)	Ketone	21.181	1166	1168	748c	848a	792c	Woody, musty, sweet
V15	Benzaldehyde, 2,4-dimethyl-	Aldehyde	21.693	1183	1180	17.30a	13.00b	12.90b	Naphthyl, cherry, almond, spice, vanilla
V16	1,3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl- (Safranal)	Terpene	22.191	1198	1197	7443a	7656a	7429a	Fresh, herbal, saffron, spicy
V17	2,4-Cycloheptadien-1-one, 2,6,6-trimethyl- (Eucarvone)	Terpene	22.817	1220	1222	54.00c	120b	127a	Minty
V18	Acetic acid, 2-phenylethyl ester	Ester	23.757	1252	1250	8.30c	31.60b	72.90a	Floral, honey, fruity, tropical
V19	4-Hydroxy-3,5,5-trimethylcyclohex-2-enone	Ketone	25.339	1307	1317	16.60c	54.10a	35.40b	Camphor
V20	Benzaldehyde, 2,4,6-trimethyl-	Aldehyde	25.563	1315	1323	4.40c	6.89a	5.01b	Naphthyl, cherry, almond, vanilla
V21	α-Cubebene	Terpene	27.321	1379	1372	4.00a	1.36c	3.20b	Herbal, waxy
V22	4-hydroxy-2,6,6-trimethyl-3-oxocyclohexa-1-ene-1-carbaldehyde	Ketone	27.429	1383	1396	289b	286b	311a	Citrus, vegetable
V23	4-Hydroxy-2,6,6-trimethylcyclohex-1-enecarbaldehyde (HTTC)	Terpene	28.380	1418	1431	140c	172b	211a	Tropical, saffron, herbal
V24	2-Butanone, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	Terpene	28.802	1434	1433	7.90a	3.80c	5.68b	Earthy, woody
V25	3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	Terpene	30.048	1480	1486	3.30c	4.00b	4.43c	Woody, sweet, fruity, berry
	Total					11,104.03	12,503.86	13,067.39	

[1] Means in the same line followed by different lowercase letters indicate statistically significant differences at $p \leq 0.05$ for each sample ($n = 3$); CF: Chemical Family; RT: Retention Time; KI: Kovats Index; EXP: Experimental; LIT: Literature; [2] Commercial flavor descriptors. or online according to: Flavornet (http://www.flavornet.org/flavornet.html) (accessed on 2 July 2022); Bedoukian Research (http://www.bedoukian.com/) (accessed on 2 July 2022); Sigma Aldrich SAFC. Flavors and Fragrances (http://www.safcglobal.com/safc-supply-solutions/en-us/home/flavors-and-fragrances.html) (accessed on 2 July 2022) and The Good Scents Company (http://www.thegoodscentscompany.com/) (accessed on 2 July 2022); FAO/WHO Expert Committee on Food Additives (JECFA) (https://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/en/) (accessed on 2 July 2022).

In all saffron samples, a total of 25 different volatile compounds were identified and quantified, with the more representative chemical families, ketones (n = 8), terpenes (n = 8), aldehydes (n = 3), esters (n = 3), alcohols (n = 2) and acids (n = 1). Regarding the concentration of the different volatile compounds, there were significant differences between the samples, except for safranal (1,3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl-, V16) which was the compound found in a higher amount in all the samples regardless of the saffron origin (7443, 7656 and 7429 μg/g for Spanish, Iranian and Greek saffron, respectively). The concentrations of safranal were higher than those reported by other studies. Kanakis et al. [32] indicated maximum values of safranal in Greek saffron around 6879 μg/g, Culleré et al. [33] found 1365 ± 81.9 μg/g in Spanish saffron, and Jalali-Heravi et al. [34] showed values of 4356 μg/g for Iranian samples. These differences could be due to the post-harvest processing, such as the dehydration procedures since safranal is formed at elevated temperatures from picrocrocin (50–55 °C), but after this process, safranal could still be generated via *trans*-crocetin esters from other volatile compounds [4].

Other important volatile compounds presented in saffron stigmas belong to the family group of ketones (3) and alcohols (1), such as isophorone (V11); 1,4-cyclohexanedione, 2,2,6-trimethyl-(dihydrooxophorone, V14); 1-butanol, 3-methyl-(isoamyl alcohol, V2) and 2,6,6-trimethyl-2-cyclohexene-1,4-dione (4-keitoisophorone, V12). Regarding isophorone, isoamyl alcohol, and 4-keitoisophorone content, Greek saffron had a significant highest concentration of those compounds (1936 μg/g, 1018 μg/g, and 789 μg/g, respectively), with these values consistent with the results reported by Jalali-Heravi et al. [34] in Greek saffron. Other studies also reported that safranal, isophorone, 4-ketoisophorone, and dihydrooxophorone were the main volatiles found in saffron stigmas from central Italy [35]. Moreover, isophorone also has antimicrobial and antioxidant properties [36]. With respect to dihydrooxophorone, the significant highest content was found in Iranian saffron with a concentration of 848 μg/g; this value was higher than those obtained in other studies for Iranian saffron (591 μg/g) [37]. The formation of oxidized and reduced isophorone-related compounds might be produced through an enzymatic process or may occur via oxidation and decarboxylation of safranal and further chemical reactions of other compounds [4].

The volatile compounds identified in the saffron samples were comparable to those reported in previous research. Anastasaki et al. [38] reported that some of the major compounds in Spanish, Greek, Italian and Iranian samples were safranal, isophorone, dihydrooxophorone, but also 4-hydroxy-2,6,6-trimethyl-3-oxocyclohexa-1,4-diene-1-carboxaldehyde and 4-hydroxy-2,6,6-trimethylcyclohex-1-enecarbaldehyde (HTTC). Nevertheless, in the saffron samples studied, 4-hydroxy-2,6,6-trimethyl-3-oxocyclohexa-1,4-diene-1-carboxaldehyde (V22) and HTCC (V23) appeared in lower concentrations. HTCC is derived from picrocrocin, which is also the precursor of safranal and is formed under heat treatment by deglycosylation of picrocrocin or by hydrolysis [39]. Therefore, during the drying process, picrocrocin may produce safranal in high levels, thus producing lower concentrations of HTCC in saffron samples.

In summary, saffron from Greece had the highest concentration of total volatile compounds (around 13,067 μg/g) with respect to the Iranian and Spanish saffron samples (around 12,503 μg/g and 11,104 μg/g, respectively). These differences could be related to the geographical origin, which is considered an essential factor in the concentration of volatiles, among others, such as the harvest season, dehydration temperature and duration, and the storage time and conditions [39].

The aroma of saffron is mainly due to the contribution of aldehydes of saffron, such as safranal and its derivative 4-hydroxy-2,6,6-trimethyl-1-cyclohexene 1-carboxaldehyde, but other compounds could contribute as well, with the characteristic saffron aroma developed during the post-harvest treatment. With respect to the odor descriptors, the saffron aroma is especially herbal, spicy, sweet, fresh, woody, floral, and musty, among others (Table 4).

Regarding the flower by-products samples from Spain fresh and dried by two different methods (freeze-dried and air oven-dried), 55 volatile compounds were isolated, identified, and quantified (Table 5). The identification parameter of these compounds,

namely Kovats index, retention time, chemical family, and odor descriptors, were indicated in Supplementary Table S1.

The chemical families more representative found in all samples were: esters (n = 13), aldehydes (n = 12), and acids and alcohols (n = 5). All floral by-product samples presented acetic acid (V1), 2(5H)-furanone (V14), and phenylethyl alcohol (V31). SFL2 (air-oven dried) had the highest concentration of acetic acid (161.55 μg/g), followed by fresh SFL1 (145.21 μg/g). The production of this organic acid could occur by the conversion of some carbohydrates in the presence of oxygen.

SFL2 (air-oven dried) presented a significant highest level of 2(5H)-furanone (V14) (452 μg/g), while SFL1 (air-oven dried) showed the lowest concentration (4.11 μg/g). However, the generation of furanone, such as 2(5H)-furanone, may be due to several processes: via oxidation reactions, by microorganisms, or spontaneous formation via the Maillard reaction between sugars and amino acids during heating [40]. Thus, the content of this volatile compound could be related mainly to the sugar and amino acid composition of the fresh flowers SFL1 and SFL2, leading to different concentrations of 2(5H)-furanone in the air-oven dried flowers. Moreover, 2(5H)-furanone is authorized as a flavoring substance by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and is defined in its specification as a "rich winey meat-like aroma". This fact could be interesting for the use of dried saffron flowers as food ingredients. This compound has also been studied as bactericidal [41].

Phenylethyl alcohol was present in the SFL2 (air-oven dried) sample in high concentrations (35.63 μg/g). However, minimal concentrations were found in the SFL1 (air-oven dried) sample (0.49 μg/g). This volatile was also found in other flower extracts (rose, hyacinth, geranium), presenting a rose-like odor and antimicrobial and antifungal properties, and it is widely used in foods and cosmetics [42].

It should be noted that safranal (V39) was only found in SFL2 (fresh and air-oven dried) but in very low amounts (0.11 and 7.53 μg/g, respectively). This could be due to the presence of traces of saffron stigmas in the floral by-products, with the amount of safranal higher in SFL2 (air-oven dried) since it is generated at the temperatures used (50–55 °C) for drying. Moreover, isophorone (V35) was also present in SFL2 (22.28 μg/g) which may be produced from safranal and/or from other precursors formed in the oven during the heating process.

Other compounds that should be highlighted due to their high concentration were 3-hydroxy 2-butanone (acetoin, V4) and butanoic acid, 3-methyl (isovaleric acid, V9). These volatiles were found in fresh SFL1 at 299.42 μg/g and 177.52 μg/g, respectively. Acetoins, which exist widely in nature, are mainly found in higher plants that have the ability to synthesize them using different enzymes, but their biological mechanisms remain unclear; however previous studies have shown their antimicrobial actions [43]. Isovaleric acid is an isomer of valeric acid, which is a compound naturally present in plants as a metabolite that could be formed by the secondary metabolism of plants [44]. Moreover, these volatiles were only found in fresh samples, meaning that they may be degraded by heat.

In summary, some volatiles present in fresh flowers increased by air drying in the oven due to the thermal treatment, and new volatiles also appears in the air-dried flowers. Some volatile compounds (V3, V17, V26, V32, V44) were only found in SFL2 (air-oven dried) samples, which showed the highest concentration of total volatile compounds (833.49 μg/g). These results are justified by the generation of volatiles from other non-volatile precursors by heat processing. Thus, the post-harvest procedures, such as drying, are very important when considering the volatile quality in terms of the contribution of the volatile compounds that are generated during this processing step.

Table 5. Identification and concentration (μg/g) of volatile compounds found in saffron floral by-products [1].

Code	Compound	RT	SFL1 Fresh (μg/g)	SFL1 Freeze-Dried (μg/g)	SFL1 Air-Oven Dried (μg/g)	SFL2 Fresh (μg/g)	SFL2 Freeze-Dried (μg/g)	SFL2 Air-Oven Dried (μg/g)
V1	Acetic acid	2.441	145.21b	5.09cd	0.26d	8.20c	0.83d	161.55a
V2	Butanal, 3-methyl-(Isovaleraldehyde)	3.370	13.66b	n.d.	n.d.	n.d.	n.d.	28.30a
V3	Butanal, 2-methyl-	3.531	n.d.	n.d.	n.d.	n.d.	n.d.	50.93
V4	3-hydroxy-2-butanone (Acetoin)	4.531	299.42	n.d.	n.d.	n.d.	n.d.	n.d.
V5	1-Butanol, 2-methyl-	5.617	5.84	n.d.	n.d.	n.d.	n.d.	n.d.
V6	Propanoic acid, 2-methyl-	6.163	3.13	n.d.	n.d.	n.d.	n.d.	n.d.
V7	2,3-Butanediol	7.405	2.61	n.d.	n.d.	n.d.	n.d.	n.d.
V8	Hexanal	7.888	n.d.	1.52	n.d.	n.d.	n.d.	n.d.
V9	Butanoic acid, 3-methyl- (Isovaleric acid)	10.222	177.52	n.d.	n.d.	n.d.	n.d.	n.d.
V10	Butanoic acid, 2-methyl-	10.364	8.26	n.d.	n.d.	n.d.	n.d.	n.d.
V11	1,2-Propanediol, 2-acetate	11.489	2.51	n.d.	n.d.	n.d.	n.d.	n.d.
V12	Heptanal	11.941	n.d.	3.96	n.d.	n.d.	n.d.	n.d.
V13	4-Penten-1-yl acetate	11.962	n.d.	n.d.	3.67	n.d.	n.d.	n.d.
V14	2(5H)-Furanone	12.104	4.42b	38.01b	4.11b	14.64b	4.95b	452a
V15	Butyrolactone	12.127	n.d.	24.47	n.d.	n.d.	n.d.	n.d.
V16	Acetic acid, pentyl ester	12.449	3.40e	3.95d	11.12a	8.85b	5.81c	n.d.
V17	2-Furancarboxaldehyde, 5-methyl-	14.186	n.d.	n.d.	n.d.	n.d.	n.d.	6.92
V18	1-Butanol, 3-methyl-, propanoate	14.551	n.d.	0.56d	3.16a	2.20b	0.84c	n.d.
V19	Carbolic acid	14.816	7.60	n.d.	n.d.	n.d.	n.d.	n.d.
V20	Diisoamyl ether	15.746	n.d.	n.d.	1.13	n.d.	n.d.	n.d.
V21	Acetic acid, hexyl ester	16.080	n.d.	1.13c	4.22a	2.86b	0.90d	n.d.
V22	1-Hexanol, 2-ethyl-	16.653	n.d.	n.d.	n.d.	n.d.	13.38	n.d.
V23	D-Limonene	16.657	n.d.	n.d.	n.d.	0.59	n.d.	n.d.
V24	Benzeneacetaldehyde	17.099	n.d.	3.86c	n.d.	4.27b	1.11d	13.39a
V25	Butanoic acid, pentyl ester	17.596	n.d.	n.d.	0.65a	0.58b	n.d.	n.d.
V26	Ethanone, 1-(1H-pyrrol-2-yl)-	17.717	n.d.	n.d.	n.d.	n.d.	n.d.	25.28
V27	Benzaldehyde, 4-methyl-	18.448	n.d.	0.53	n.d.	n.d.	n.d.	n.d.
V28	2-Nonanone	18.734	3.44	n.d.	n.d.	n.d.	n.d.	n.d.
V29	Linalool	19.031	2.46a	n.d.	n.d.	n.d.	0.77b	n.d.
V30	Nonanal	19.193	n.d.	12.40b	0.62c	n.d.	0.94c	14.98a
V31	Phenylethyl Alcohol	19.404	7.37d	10.03c	0.49f	16.64b	3.89e	35.63a
V32	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-(DDMP)	20.326	n.d.	n.d.	n.d.	n.d.	n.d.	62.83
V33	Acetic acid, 2-ethylhexyl ester	20.559	n.d.	n.d.	n.d.	0.52	n.d.	n.d.
V34	2(3H)-Furanone, dihydro-4-hydroxy-	20.609	n.d.	109.82a	n.d.	n.d.	9.01b	n.d.
V35	Isophorone	20.675	n.d.	n.d.	n.d.	n.d.	n.d.	22.28
V36	Cyclohexanone, 5-methyl-2-(1-methylethyl)-, trans- (trans-Menthone)	20.859	n.d.	n.d.	n.d.	n.d.	0.60	n.d.
V37	Acetic acid, phenylmethyl ester	21.000	n.d.	n.d.	n.d.	0.30b	0.84a	n.d.
V38	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1α,2β,5α)-(Menthol)	21.529	n.d.	n.d.	n.d.	n.d.	0.98	n.d.

Table 5. *Cont.*

Code	Compound	RT	SFL1 Fresh (μg/g)	SFL1 Freeze-Dried (μg/g)	SFL1 Air-Oven Dried (μg/g)	SFL2 Fresh (μg/g)	SFL2 Freeze-Dried (μg/g)	SFL2 Air-Oven Dried (μg/g)
V39	1,3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl-(Safranal)	22.180	n.d.	n.d.	n.d.	0.11b	n.d.	7.53a
V40	Benzofuran, 2,3-dihydro- (Coumaran)	22.630	2.59	n.d.	n.d.	n.d.	n.d.	n.d.
V41	2-Dodecene, (Z)-	22.375	n.d.	n.d.	n.d.	n.d.	1.20	n.d.
V42	3-Dodecene, (Z)-	22.634	n.d.	n.d.	n.d.	n.d.	1.14	n.d.
V43	Benzaldehyde, 2,4-dimethyl-	22.659	n.d.	n.d.	0.32	n.d.	n.d.	n.d.
V44	2-Cyclohexen-1-one, 2-methyl-5-(1-methylethyl)-, (S)-	23.656	n.d.	n.d.	n.d.	n.d.	n.d.	14.98
V45	Benzene, 1,3-bis(1,1-dimethylethyl)-	23.668	n.d.	3.75a	0.47b	0.55b	n.d.	n.d.
V46	Acetic acid, 2-phenylethyl ester	23.773	9.96b	2.34d	n.d.	32.42a	3.72c	n.d.
V47	Benzeneacetaldehyde, α-ethylidene-	24.200	n.d.	n.d.	n.d.	0.97	n.d.	n.d.
V48	2-Propenal, 3-phenyl-	24.337	n.d.	n.d.	n.d.	0.50	n.d.	n.d.
V49	Dodecane, 4,6-dimethyl-	24.450	n.d.	n.d.	0.34c	0.67bc	0.92b	16.12a
V50	2-Undecanone	24.903	0.73	n.d.	n.d.	n.d.	n.d.	n.d.
V51	α-Cubebene	27.334	n.d.	n.d.	n.d.	1.00a	0.53b	n.d.
V52	Dodecanal	28.113	n.d.	n.d.	n.d.	n.d.	1.79	n.d.
V53	1-Dodecanol	29.900	n.d.	n.d.	n.d.	n.d.	4.63	n.d.
V54	Lauryl acetate	32.679	n.d.	n.d.	0.48	n.d.	n.d.	n.d.
V55	Hexadecanoic acid, methyl ester	37.034	0.93	n.d.	n.d.	n.d.	n.d.	n.d.
	Total		700.13	217.46	27.37	93.90	58.78	833.49

[1] Means in the same line followed by different lowercase letters indicate statistically significant differences at $p \leq 0.05$ for each sample ($n = 3$); RT: Retention Time; SFL1, SFL2: Saffron floral by-products from two different producers; n.d.: not detected.

4. Conclusions

The studied Spanish, Iranian, and Greek saffron stigmas and Spanish saffron floral by-products could have a great potential to develop new high-added-value ingredients due to their antioxidant properties and bioactive content. Floral by-products showed a good concentration of total anthocyanins, and saffron stigmas had a high concentration of total flavonoids, β-carotene, and crocetin esters. Furthermore, among the volatile composition, the saffron studied presented high levels of safranal, which contributes to the bioactivity and aroma of this spice. The floral by-products volatile composition from fresh to dried samples was highly influenced by the drying method, of which some may present biological activities, especially antioxidant and antimicrobial properties. Therefore, these results suggest that saffron and its floral by-products are natural sources of antioxidant compounds that could be used as sustainable, innovative ingredients to apply in food for the development of novel functional food products or for other human health applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox11091650/s1, Table S1: Identification, quantification and descriptors of volatile compounds found in saffron floral by-products.

Author Contributions: Conceptualization: D.C.-B., E.V.-C., J.-J.P. and M.-J.F.; methodology: D.C.-B. and J.C.-V.; writing-original draft preparation: D.C.-B. and J.C.-V.; writing—review and editing: D.C.-B. and M.-J.F.; supervision: E.V.-C., J.-J.P. and M.-J.F.; project administration: M.-J.F. All authors have read and agreed to the published version of the manuscript.

Funding: This article is supported by the Partnership for Research and Innovation in the Mediterranean Area (PRIMA) program, project 174-SAFFROMFOOD. The PRIMA program is supported by the European Union. The research was also supported by the regional agency "Diputación de Alicante", Alicante, Spain. Grant EQC2018-004170-P funded by MCIN/AEI/10.13039/501100011033 and by ERDF A way of making Europe for GC-MS analysis.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: Thanks to the Spanish-Ministry of Education, Culture and Sport for the FPU-PhD fellowship (FPU18/02225) and thanks to the Spanish company Verdú Cantó Saffron Spain, S.L. for the collaboration.

Conflicts of Interest: The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Identification, quantification and descriptors of volatile compounds found in saffron floral by-products.

Code	Compound	CF	RT	KI LIT	KI EXP	Odor descriptors [1]
V1	Acetic acid	Acid	2.441	645	646	Pungent, sour, vinegar
V2	Butanal, 3-methyl- (Isovaleraldehyde)	Aldehyde	3.370	690	686	Chocolate, peach, fatty
V3	Butanal, 2-methyl-	Aldehyde	3.531	699	688	Cocoa, musty, coffee
V4	3-hydroxy-2-butanone (Acetoin)	Ketone	4.531	722	721	Buttery, dairy, fatty
V5	1-Butanol, 2-methyl-	Alcohol	5,617	748	748	Fusel, alcoholic
V6	Propanoic acid, 2-methyl-	Acid	6.163	760	757	Sour, cheesy, rancid
V7	2,3-Butanediol	Alcohol	7.405	789	786	Creamy, fruity, buttery
V8	Hexanal	Aldehyde	7.888	800	800	Green, fresh, fatty
V9	Butanoic acid, 3-methyl- (Isovaleric acid)	Acid	10.222	858	858	Cheesy, sour
V10	Butanoic acid, 2-methyl-	Acid	10.364	862	861	Cheesy, acidic
V11	1,2-Propanediol, 2-acetate	Ester	11.489	889	891	Fruity
V12	Heptanal	Aldehyde	11.941	894	893	Green, fresh, fatty
V13	4-Penten-1-yl acetate	Ester	11.962	895	890	Green, plastic, vegetable
V14	2(5H)-Furanone	Ester	12.104	906	913	Buttery
V15	Butyrolactone	Ester	12.127	906	908	Creamy, oily, fatty
V16	Acetic acid, pentyl ester	Ester	12.449	914	912	Fruity, banana, pear
V17	2-Furancarboxaldehyde, 5-methyl-	Aldehyde	14.186	960	961	Caramellic, sweet, coffee
V18	1-Butanol, 3-methyl-, propanoate	Acid	14.551	970	969	Fruity, tropical, banana
V19	Carbolic acid	Acid	14.816	977	976	Plastic
V20	Diisoamyl ether	Ether	15.746	1002	1002	Floral
V21	Acetic acid, hexyl ester	Ester	16.080	1012	1011	Fruity, green
V22	1-Hexanol, 2-ethyl-	Alcohol	16.653	1029	1029	Citrus, fresh, floral
V23	D-Limonene	Terpene	16.657	1029	1029	Citrus, fresh
V24	Benzeneacetaldehyde	Aldehyde	17.099	1042	1043	Green, sweet, floral
V25	Butanoic acid, pentyl ester	Ester	17.596	1056	1059	Fruity, banana, tropical
V26	Ethanone, 1-(1H-pyrrol-2-yl)-	Ketone	17.717	1060	1060	Musty, nut
V27	Benzaldehyde, 4-methyl-	Aldehyde	18.448	1081	1080	Fruity, cherry
V28	2-Nonanone	Ketone	18.734	1089	1089	Fresh, sweet, fruity
V29	Linalool	Terpene	19.031	1098	1098	Floral, citrus, rose
V30	Nonanal	Aldehyde	19.193	1103	1102	Waxy, aldehydic, citrus, fresh
V31	Phenylethyl Alcohol	Alcohol	19.404	1110	1110	Floral, rose
V32	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (DDMP)	Other	20.326	1139	1140	-
V33	Acetic acid, 2-ethylhexyl ester	Ester	20.559	1147	1157	Earthy, herbal
V34	2(3H)-Furanone, dihydro-4-hydroxy-	Ester	20.609	1148	1153	-
V35	Isophorone	Ketone	20.675	1150	1146	Woody, sweet, camphoreous, musty
V36	Cyclohexanone, 5-methyl-2-(1-methylethyl)-, trans- (trans-Menthone)	Cetone	20.859	1156	1152	Minty
V37	Acetic acid, phenylmethyl ester	Ester	21.000	1161	1161	Floral, jasmine
V38	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1α,2β,5α)- (Menthol)	Cetone	21.529	1177	1171	Minty
V39	1,3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl- (Safranal)	Terpene	22.180	1198	1201	Fresh, herbal, saffron, spicy

V40	Benzofuran, 2,3-dihydro- (Coumaran)	Other	22.630	1213	1219	Sweet
V41	2-Dodecene, (Z)-	Alkene	22.375	1205	1213	-
V42	3-Dodecene, (Z)-	Alkene	22.634	1214	1222	-
V43	Benzaldehyde, 2,4-dimethyl-	Aldehyde	22.659	1214	1208	Naphthyl, sweet
V44	2-Cyclohexen-1-one, 2-methyl-5-(1-methylethyl)-, (S)-	Terpene	23.656	1249	1250	-
V45	Benzene, 1,3-bis(1,1-dimethylethyl)-	Other	23.668	1249	1249	-
V46	Acetic acid, 2-phenylethyl ester	Ester	23.773	1252	1254	Floral, honey, fruity, tropical
V47	Benzeneacetaldehyde, α-ethylidene-	Aldehyde	24.200	1267	1273	Green, floral, woody
V48	2-Propenal, 3-phenyl-	Aldehyde	24.337	1272	1278	Spicy, sweet
V49	Dodecane, 4,6-dimethyl-	Alkane	24.450	1276	1285	-
V50	2-Undecanone	Cetone	24.903	1291	1291	Fruity, waxy,fatty
V51	α-Cubebene	Terpene	27.334	1379	1380	Herbal, waxy
V52	Dodecanal	Aldehyde	28.113	1408	1409	Soapy, waxy
V53	1-Dodecanol	Alcohol	29.900	1475	1474	Waxy, earthy, soapy
V54	Lauryl acetate	Ester	32.679	1606	1605	Waxy, sweet
V55	Hexadecanoic acid, methyl ester	Ester	37.034	1921	1921	Waxy, oily, fatty

[1] Commercial flavor descriptors. or online according to: Flavornet (http://www.flavornet.org/flavornet.html); Bedoukian Research (http://www.bedoukian.com/); Sigma Aldrich SAFC. Flavors and Fragrances (http://www.safcglobal.com/safc-supply-solutions/en-us/home/flavors-and-fragrances.html) and The Good Scents Company (http://www.thegoodscentscompany.com/); FAO/WHO Expert Committee on Food Additives (JECFA) (https://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/en/) CF: Chemical Family; RT: Retention Time; KI: Kovats Index; EXP: Experimental; LIT: Literature.

Publicación 4

Evaluation of microwave-assisted extraction as a potential green technology for the isolation of bioactive compounds from saffron (*Crocus sativus* L.) floral by-products

Autores	Débora Cerdá-Bernad, João Paulo Baixinho, Naiara Fernández & María José Frutos
Revista	Foods
DOI	10.3390/foods11152335
Editorial	MDPI
Fecha de publicación	5 de Agosto de 2022
Categoría JCR®	Food Science and Technology
Cuartil	Q1
Rango	34/142
Factor de impacto	5,200

foods

MDPI

Article

Evaluation of Microwave-Assisted Extraction as a Potential Green Technology for the Isolation of Bioactive Compounds from Saffron (*Crocus sativus* L.) Floral By-Products

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Abstract: The saffron flower stigmas are used for the saffron spice production while the remaining saffron floral by-products, that are a valuable source of natural bioactive compounds, remain underutilized. The aim of this study was to evaluate the microwave-assisted extraction (MAE) through response surface methodology to obtain high value-added compounds from saffron tepals as ingredients with potential application in the food, pharmaceutical and/or cosmetic industries. A central composite design was applied to optimize process variables: temperature, time and ethanol solvent concentration. Extracts were characterized in terms of total phenolic and total flavonoid content, and antioxidant capacity (ORAC and HOSC assays), being the maximum values obtained: 126.20 ± 2.99 mg GAE/g dry matter; 8.05 ± 0.11 mg CE/g dry matter; 6219 ± 246 µmol TEAC/dry matter; 3131 ± 205 µmol TEAC/dry matter, respectively. Results indicated that the optimal extraction conditions were the combination of low temperature (25 °C)—high extraction time (5 min) using ethanol as solvent (100%). MAE revealed to be an efficient technique to isolate bioactive compounds from saffron floral by-products with a low energy footprint.

Keywords: green chemistry; sustainability; antioxidant activity; high value-added ingredients; bio-residues; *Crocus sativus* L.; microwave-assisted extraction; valorization; food by-products; polyphenols

Citation: Cerdá-Bernad, D.; Baixinho, J.P.; Fernández, N.; Frutos, M.J. Evaluation of Microwave-Assisted Extraction as a Potential Green Technology for the Isolation of Bioactive Compounds from Saffron (*Crocus sativus* L.) Floral By-Products. *Foods* **2022**, *11*, 2335. https://doi.org/10.3390/foods11152335

Academic Editors: Jelena Vladić, Stela Jokić and Igor Jerković

Received: 20 July 2022
Accepted: 29 July 2022
Published: 5 August 2022

1. Introduction

Saffron (*Crocus sativus* L.) is used as a spice due to its coloring and flavoring properties, being a traditional Mediterranean plant widely cultivated in different areas, such as Iran, Spain, Greece, Italy, India, Morocco, Algeria and Turkey, among others. The flower of *Crocus sativus* L. is composed of six violet tepals, three golden-yellow stamens, and a white filiform style which culminates in a red stigma divided into three filaments [1].

Saffron is one of the most expensive spices in the world due to its high production costs as the saffron spice stigmas have to be detached manually from the rest of the flower. Around 165.000–230.000 flowers are necessary to produce 1 kg of saffron [2].

In addition to its applications in food, saffron has been traditionally used for health care due to its therapeutic and pharmacological properties [3]. Bioactive compounds such as carotenoids, terpenes, and flavonoids are the major compounds reported in saffron varieties [4]. Despite the well-known saffron characterization, recent studies showed that saffron floral by-products, such as tepals, are rich in valuable bioactive molecules such as flavonols, flavonoid glycosides and anthocyanins, which have positive biological effects such as their antioxidant and antimicrobial activities [5,6]. The valorization of this by-product through the development of innovative high added-value functional ingredients with new applications in food, pharmaceutical and or cosmetic industries could lead to

the minimization of the environmental impact and to the potentially increase of saffron demand on the market [7,8].

The extraction of bioactive compounds from saffron floral by-products has been carried out using different conventional extraction methods such as maceration, distillation, and Soxhlet extraction [9]. The need to avoid toxic solvents using environmentally friendly technologies has motivated the development of new processing methods that can be transferred into industrial scale.

In this field, new green energy-saving technologies such as the microwave-assisted extraction (MAE), present several advantages compared to conventional technologies: increase of the extraction kinetics, a shorter extraction time and rapid temperature increase, a higher efficiency and extraction yield as well as a lower energy consumption and cost [10–12].

MAE consist on the combination of microwave heating with traditional solid-liquid extraction. This technique is based on the mechanisms of energy transfer as dipole rotation and ionic conduction. The radiation causes the rupture of cells which allow the solvent penetration through the plant matrix. On the other hand, plant material flow outside the cells into the solution [9]. Some of the major factors that could affect the MAE process are the solvent type, temperature, extraction time, and power. Thus, MAE is considered a green technology to save costs, time and energy.

The main focus of this research was to evaluate MAE process efficiency to obtain high added-value antioxidant compounds from saffron floral by-products at laboratory scale. A Central Composite Orthogonal design was used to optimize MAE process conditions (extraction time, temperature, and solvent ratio) for the isolation of bioactive compounds and to study the antioxidant potential of saffron floral by-products. Extracts were characterized in terms of total phenolic (TPC) and flavonoids content (TFC) and antioxidant capacity, evaluated by oxygen radical absorbance capacity and hydroxyl radical scavenging capacity. Therefore, the valorization of this biomass that is currently unexploited could lead to its use as a source of added-value extracts for the potential development of novel bioactive ingredients.

2. Materials and Methods

2.1. Plant Material

Saffron flowers were obtained during the 2020 harvest season from Castilla La-Mancha region (Spain), cultivated following the requirements established by the Protected Designation of Origin "La Mancha Saffron" and supplied by the Spanish company Verdú Cantó Saffron Spain, S.L. [13]. The flowers were composed of tepals, stamens and styles. Stigmas were detached manually by hand following traditional procedures [13] and the remaining fresh floral by-products were frozen in liquid nitrogen and kept at −80 °C until freeze-dried.

Freeze-drying step took 48 h to constant weight (initial temperature −25 ± 2 °C and pressure 0.220 mbar) in a Christ Alpha 2–4 (B. Braun Biotech International, Melsungen; Germany). The freeze-dried flowers were crushed and sieved through a 500 µm mesh size and kept at −20 °C in polyethylene bags until further analysis.

2.2. Experimental Design

Response Surface Methodology (RSM), a collection of statistical and mathematical techniques for improvement and optimize processes, was used to optimize the extraction process and predict the responses which are affected by experimental variables. Using MODDE® software version 12.1 (Sartorius Stedim Biotech, Göttingen, Germany), a Central Composite Orthogonal design (CCO) with three independent variables was applied to evaluate the effect of time (X_1), temperature (X_2) and ethanol concentration (X_3) on the extraction yield, bioactive content and antioxidant activity of saffron flowers. The coded variable levels are summarized in Table 1.

Table 1. The CCO matrix of MAE of the experimental design.

	Independent Variables: MAE Conditions		
Experiments	**Time (min) (X_1)**	**Temperature (°C) (X_2)**	**Ethanol Concentration (%) (X_3)**
1	0.5 (−1)	25 (−1)	0 (−1)
2	5 (+1)	25 (−1)	0 (−1)
3	0.5 (−1)	100 (+1)	0 (−1)
4	5 (+1)	100 (+1)	0 (−1)
5	0.5 (−1)	25 (−1)	100 (+1)
6	5 (+1)	25 (−1)	100 (+1)
7	0.5 (−1)	100 (+1)	100 (+1)
8	5 (+1)	100 (+1)	100 (+1)
9	6.2 (+1.35)	62.5 (0)	50 (0)
10	3.15 (0)	11.75 (−1.35)	50 (0)
11	3.15 (0)	113.24 (+1.35)	50 (0)
12	3.15 (0)	62.5 (0)	50 (0)
13	3.15 (0)	62.5 (0)	50 (0)
14	3.15 (0)	62.5 (0)	50 (0)

2.3. Extraction Procedure

MAE was conducted using Discover SP-CEM MW system (CEM Co., Charlotte, NC, USA), operating at 500 PSI with a maximum output power of 300 W. The variable parameters included radiation time (0.5−5 min), temperature (25−100 °C) and ethanol solvent concentration (0–100%). The mass: solvent ratio (1:10, *w*/*v*) was fixed and the assay was performed with 2 g of sample dissolved in 20 mL of solvent. In this case, 14 extractions were carried out, following the designed conditions indicated in Table 1. Once the extraction was completed, the extracts were dried by a CentriVap® Concetrator (Labconco, Kansas City, MO, USA) at 37 °C during 24 h.

The extraction yield was determined using Equation (1). The characterization experiments (bioactive composition and antioxidant capacity) were conducted in triplicate. For these assays dry extracts were reconstituted in 50% ethanol (*w*/*v*).

$$\text{Yield\%} = ((\text{weight of extract dried})/(\text{initial weight of extract})) \times 100 \quad (1)$$

2.4. Extract Characterization

2.4.1. Total Phenolic Content

TPC was determined using the Folin Ciocalteau colorimetric method [14]. Briefly, 10 µL of the saffron floral extracts were mixed with 230 µL of milli-Q water, 15 µL of Folin-Ciocalteu's reagent and 45 µL of sodium carbonate solution (35%). The samples were stirred and incubated at room temperature during 1 h under dark conditions. Gallic acid (1000 mg/L) was used as a reference standard (7.5–240 mg/L) and absorbance was measured at 765 nm in a microplate reader (SynergyHT, Biotek, Winooski, VT, USA). The results were expressed as mg gallic acid equivalent (mg GAE) per gram of dry matter.

2.4.2. Total Flavonoid Content

TFC was determined as described by Çam and Hışıl [15]. Briefly, 1 mL of sample were mixed with 4 mL of water and 300 µL of sodium nitrite (5%) solution. After 5 min, 300 µL of aluminum trichloride (10%) solution were added. After more 6 min, 2 mL of sodium hydroxide (1M) solution were added, and the volume was adjusted to 10 mL with water. Catechin (1000 mg/L) was used for quantification (20–100 mg/L) and absorbance was measured at 510 nm in a spectrophotometer (UV/Vis Spectrophotometer T80; PG Instruments Limited, Lutterworth, UK). The results were expressed as mg of catechin equivalents (mg CE) per gram of dry matter.

2.4.3. Oxygen Radical Absorbance Capacity Assay

The ORAC assay method measures the capacity of the antioxidant molecules, present in the extracts, to protect the disodium fluorescein (FL) from oxidation by peroxyl radicals. The assay was carried out following the method described by Serra et al. [16]. This assay measured the ability to inhibit the oxidation of fluorescein (3×10^{-4} mM) catalyzed by peroxyl radicals generated from AAPH (2,2-Azobis (2-methylpropionamidine) dihydrochloride) using a microplate fluorescent reader (FL800 Bio-Tek Instruments, Winooski, VT, USA). Trolox (1000 µM) was used as a reference standard (5–30 µM) and the results were expressed as micromoles of trolox equivalents antioxidant capacity (µmol TEAC) per gram of dry matter.

2.4.4. Hydroxyl Radical Scavenging Capacity Assay

HOSC method was used to evaluate the hydroxyl radical scavenging capacity of samples. The assay was performed using the method described in Moore et al. [17]. Briefly, in a microplate fluorescent reader, fluorescein (9.96×10^{-8} M) was used as a probe and the reaction of ferric chloride (3.42 mM) and hydrogen peroxide (0.20 M) as a source of hydroxyl radicals. Trolox (1000 µM) was used as a reference standard (5–30 µM) and the results were expressed as micromoles of trolox equivalents antioxidant capacity (µmol TEAC) per gram of dry matter.

2.5. Statistical Analysis

Results were expressed as the mean ± standard deviation. The mean comparisons were carried out using an analysis of variance (ANOVA) and the Tukey multiple range test, using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, San Diego, CA, USA). The significant differences were stablished as ($p < 0.05$). All determinations were carried out in triplicate.

3. Results and Discussion

3.1. Bioactive Content

A design of experiments (DOE) was planned to obtain high added value compounds from saffron floral by-products. To optimize the extraction procedure and evaluate MAE as a green extraction method, the effect of process variables on the mass yield, TPC and TFC, and the antioxidant capacity (ORAC and HOSC) were evaluated on a CCO design with three independent variables, time (X_1), temperature (X_2) and ethanol concentration (X_3).

The results of the interaction effects of the three independent variables studied in MAE on phenolics compounds extraction are shown in Figure 1. As it can be seen in the response surface plot, the interaction between temperature and time, keeping constant the solvent ratio (ethanol concentration), can be exploited to increase the affinity and specificity of the extraction. By changing the conditions, we can adjust the solvent polarity for the desired extraction. Nevertheless, the values were lower using a high temperature and time (blue surfaces in the Figure 1A). This fact might be due either to an initial thermal degradation of free phenolic compounds in the floral extracts at 5 min, since other studies have showed a significant decrease in TPC values during the thermal treatment on grape marc at 80 °C [18], and to the decrease in the dielectric constant and polarity of water and ethanol solvents at high temperatures with a reduction in their capability to dissolve polar compounds [19]. TPC indicated that increasing the extraction time while decreasing the temperature and keeping constant the ethanol ratio at 50 or 100% (Figure 1B,C, respectively), lead to an increase in the extraction yield of these bioactive compounds (red surfaces).

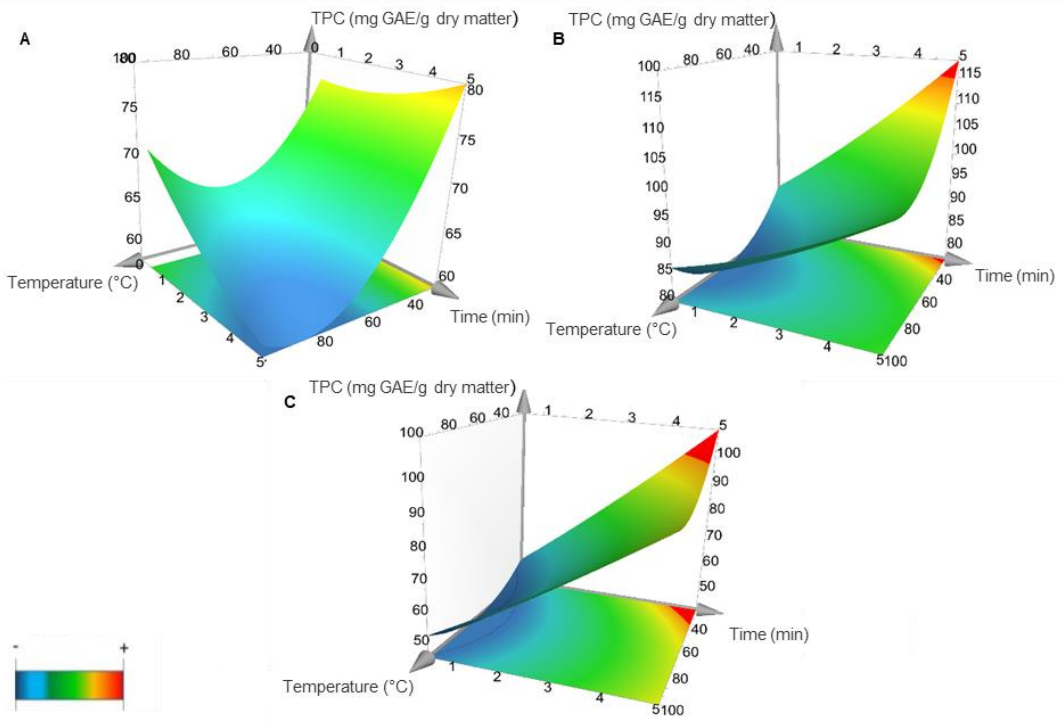

Figure 1. Response surface plot representing the effects of time, temperature and solvent ratio on Total Phenolic Content (TPC) from saffron floral by-products. (**A**) Ethanol concentration was kept constant at 0%. (**B**) Ethanol concentration was kept constant at 50%. (**C**) Ethanol concentration was kept constant at 100%. Lower values are represented in blue and higher values in red.

The empirical values of the TPC obtained for the MAE of bioactive compounds are showed in Table 2. The values of TPC obtained by MAE from Spanish saffron flowers (maximum value: 126.20 ± 2.99 mg GAE/g dry matter) were higher than those reported in previous research using saffron floral bio-residues from Italy, where values of 4000 mg GAE/100 g of dry matter were reported [20] when MAE was used as extraction method, and a TPC richness of 40–50 mg GAE/g of dry extract, using MAE as a pretreatment to a conventional solid–liquid extraction reported by Álvarez et al. [12].

Table 2. Empirical results of total phenolic content (TPC), total flavonoid content (TFC), yield, and ORAC and HOSC assays for the microwave-assisted extraction of bioactive compounds from saffron floral-by products [1].

Extraction	Time (min)	Temperature (°C)	Ethanol (%)	TPC (mg GAE/g Dry Matter)	TFC (mg CE/g Dry Matter)	Yield (%)	ORAC (μmol TEAC/g Dry Matter)	HOSC (μmol TEAC/g Dry Matter)
1	0.50	25.00	0	93.87 ± 3.33 cd	3.56 ± 0.23 efg	20.65	4777 ± 352 bcd	2286 ± 583 abc
2	5.00	25.00	0	80.54 ± 1.56 ef	3.33 ± 0.039 fg	25.15	2679 ± 504 fg	2034 ± 253 cd
3	0.50	100.00	0	54.82 ± 4.33 gh	3.26 ± 0.25 fg	36.40	2170 ± 304 g	1281 ± 230 d
4	5.00	100.00	0	58.62 ± 4.44 g	3.13 ± 0.03 g	32.35	2175 ± 268 g	2117 ± 127 bcd
5	0.50	25.00	100	52.12 ± 1.75 gh	5.62 ± 0.02 c	23.65	2019 ± 439 g	1716 ± 44 cd
6	5.00	25.00	100	126.20 ± 2.99 a	6.80 ± 0.33 b	17.10	5128 ± 303 bc	3131 ± 205 a
7	0.50	100.00	100	49.19 ± 1.67 h	7.41 ± 0.35 ab	29.70	3451 ± 443 ef	1623 ± 277 cd
8	5.00	100.00	100	75.47 ± 1.02 f	8.05 ± 0.11 a	27.00	4026 ± 84 de	2124 ± 383 bcd
9	6.20	62.50	50	105.50 ± 3.05 b	4.23 ± 0.14 de	36.75	5027 ± 351 bc	2444 ± 112 abc
10	3.15	11.75	50	85.13 ± 2.13 e	4.15 ± 0.42 de	31.80	5641 ± 384 ab	2407 ± 315 abc
11	3.15	113.24	50	120.70 ± 3.69 a	3.67 ± 0.15 defg	27.75	4556 ± 390 cd	1779 ± 272 cd
12	3.15	62.50	50	86.97 ± 1.38 de	4.34 ± 0.38 d	23.60	4637 ± 37 cd	1995 ± 131 cd
13	3.15	62.50	50	96.77 ± 3.46 bc	3.99 ± 0.22 def	34.15	3278 ± 121 ef	2240 ± 265 bc
14	3.15	62.50	50	79.28 ± 3.14 ef	4.14 ± 0.31 de	25.70	6219 ± 246 a	2944 ± 407 ab

[1] Means ± standard deviation in the same column followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each extraction ($n = 3$).

With respect to TFC, the results of the interaction effects of the three independent variables studied in MAE from saffron floral by-products are shown in Figure 2. It illustrates the interaction between temperature and time, keeping constant solvent ratio during the extraction process. The regression analysis of data showed that, for a 95% confidence level ($p < 0.05$), flavonoids concentration in the extract was positively affected by temperature and solvent ratio. As it can be seen in the response surface plot, the shape of the Figure 2C indicated that, keeping constant ethanol ratio at a level of 100%, flavonoids extraction was enhanced (red surface) with an increase in the extraction temperature.

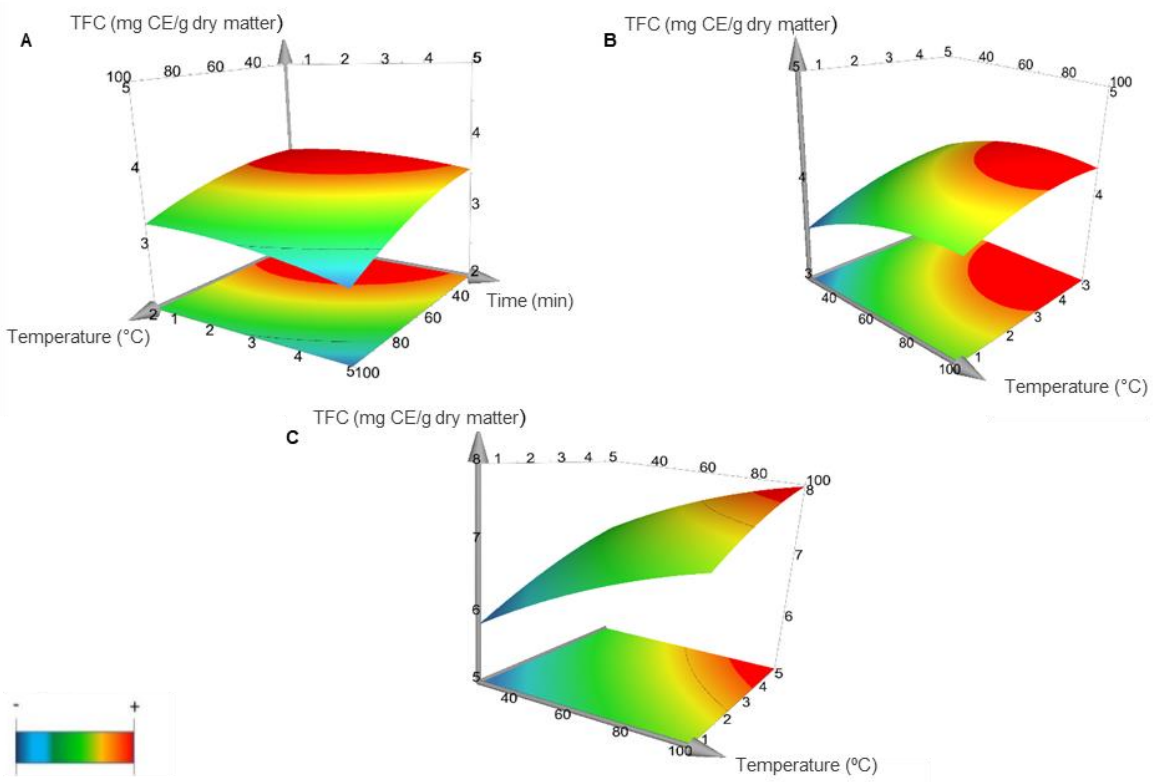

Figure 2. Response surface plot representing the effects of time, temperature and solvent ratio on Total Flavonoids Content (TFC) from saffron floral by-products. (**A**) Ethanol concentration was kept constant at 0%. (**B**) Ethanol concentration was kept constant at 50%. (**C**) Ethanol concentration was kept constant at 100%. Lower values are represented in blue and higher values in red.

The empirical values of TFC obtained for the MAE of bioactive compounds are shown in Table 2. The results of TFC were more similar between the different extraction experiments than those of TPC. However, the highest concentrations of flavonoids were found in the extractions 5, 6, 7 and 8, being the values between 5.62–8.05 mg CE/g dry matter where the concentration of ethanol used was the 100%. These results are in accordance with previous research in the literature by other authors that had also studied the variables of temperature, time and solvent ratio for MAE to extract anthocyanins from Iranian saffron tepals. The results indicated that the solvent ratio was the factor which had the most significant effect in the extraction of bioactives [21]. Furthermore, all experiments were conducted at a maximum irradiation power (300 W), however, its effects on flavonoids extraction should also be studied. Previous research evaluated the effect of the power in MAE and has suggested that the microwave power has a significant effect on extracting bioactive compounds from saffron petals because of the fact that the irradiation of microwave may accelerate the rupture of plant cells due to a rapid increase of pressure and temperature [22].

The values of TFC obtained by MAE were in accordance with the ones described by Sun et al. [23], reporting values lower than 10 mg/g for TFC in saffron tepals methanol

extracts obtained with ultrasound agitation (150 W, 40 °C, 40 min). Thus, MAE was an efficient technique to extract saffron bioactives in shorter times compared to other processes.

Additionally, according to Table 2 and to the RSM results, the extraction yield increased by the increment of the extraction temperature (62.5 °C and 100 °C), being the experiment 9 (62.5 °C and 50% ethanol), the one with the optimum conditions leading to a 36.75% of yield. The regression analysis of data showed that, for a 90% confidence level ($p < 0.10$), extraction yield was only significantly affected by extraction temperature. The extraction yields obtained were higher than those reported by Hashemi Gahruie et al. [22] in which the yield values using MAE to extract bioactive compounds from Iranian saffron tepals were between 8.07 and 19.42%.

Therefore, according to the results shown in Table 2, the parameters used in the experiment 6 in MAE (25 °C, 5 min, 100% ethanol) were the optimum for increasing the extraction yield of both TPC and TFC. The maximum concentrations obtained for TPC and TFC was 126.20 ± 2.99 mg GAE/g dry matter and 6.80 ± 0.33 mg CE/g dry matter, respectively. The regression analysis of data showed that, for a 90% confidence level ($p < 0.10$), phenolic concentration in the saffron floral by-product extract was positively influenced by extraction time and ethanol concentration. However, the effect of temperature on the phenolic concentration was not significant while it had a significantly positive effect on mass yield.

The analysis of the model indicated that the optimal extraction conditions, for these bioactive compounds, were the combination of low temperature (25 °C) using only ethanol as solvent (100%) with longer extraction times (5 min). However, looking in more detail into the TPC extraction, it can be observed that to obtain a high concentration of phenolic compounds, the conditions varied according to the extraction temperature chosen, so in order to reach values higher than 80 mg GAE/g dry matter from saffron flowers, combinations with higher temperatures would be suitable, given to the fact that high temperatures will increase the extraction yield. TPC was enhanced with half concentration of ethanol at high temperatures, that could be explained by the increase of the pressure of the solvent system, which led to the breakdown of the cell walls of the plant material, improving the penetration of solvent across the sample matrix and increasing the release of the bioactive compounds. Since the boiling point of ethanol is lower than water (78 °C), this increase in pressure only occur when using ethanol as primary solvent (50 or 100%) [24].

Saffron floral by-products revealed to be a high-level source of phenolics and flavonoids compounds, efficiently isolated by MAE, a greener extraction method than the commonly used organic solvents. These saffron floral bio-residues could be used as natural sources of bioactive compounds with different biological activities, that can be used in the development of innovative functional foods, nutraceuticals or in cosmetic applications.

3.2. Antioxidant Activity

A number of methods have been developed to measure the efficiency of dietary antioxidants either as pure compounds or in food extracts. These methods focus on different mechanisms of the antioxidant defense system, i.e., scavenging of oxygen and hydroxyl radicals, reduction of lipid peroxyl radicals, inhibition of lipid peroxidation, or chelation of metal ions. One of the in vitro methods commonly used to measure the antioxidant capacity of food constituents is ORAC assay, since it determines the potential to scavenge harmful oxygen reactive species that are biologically relevant radicals, such as peroxyl radicals, that are involved in the lipid oxidation in food systems [25]. Other in vitro method to determine the antioxidant capacity is HOSC assay that measures the ability of the extracts to scavenge hydroxyl radicals generated by hydrogen peroxide. In this work, the two methods were used to evaluate the antioxidant capacity of the saffron floral extracts obtained by MAE.

The empirical results for ORAC and HOSC assays are shown in Table 2. The regression analysis of data showed that, for a 95% confidence level ($p < 0.05$), the antioxidant activity evaluated by ORAC was significantly affected by solvent ratio, namely ethanol

concentration in the extraction process. Moreover, antioxidant activity evaluated by HOSC was significantly affected by extraction time and temperature. The use of two methods for screening the antioxidant activity of the extracts can reveal that various distinct compounds are extracted under different conditions. According to the results, the parameters used in the experiment 6 for MAE were the optimum regarding both, ORAC and HOSC assays, reaching values of 5128 ± 303 μmol TEAC/g dry matter and 3131 ± 205 μmol TEAC/g dry matter, respectively. In all these experiments, the temperature was below 100 °C, and the ethanol solvent ratio was always above 50%.

The reactions involved in those analytical methods are associated to enzymatic and non-enzymatic oxidation reactions, these results can be explained by the oxidation of phenolic compounds at high temperatures that lead to a loss in their antioxidant activity [26]. This information was in accordance with previous studies of natural compounds that have investigated the effect of drying temperatures in the antioxidant activity of grape pomace peels extracts. The authors have found that the antioxidant activity at 120 °C was lower (1.7 times) than that obtained at 20 °C [27,28].

ORAC results for the floral extracts were comparable with those reported by Sun et al. [23] in which saffron tepals showed a strong oxygen radical scavenging ability, even higher than that of the stamen extracts, but for HOSC assay, any study has been published to date for saffron floral samples.

Many natural substances such as carotenoids, tocopherols, and polyphenols can act as antioxidants and are widely spread within food and plants. Flavonoids and other polyphenols can scavenge free radicals thus delaying lipid autoxidation. Several studies have been carried out to correlate polyphenolic composition with its antioxidant properties of natural extracts.

In this work, the antioxidant activity showed correlation with TPC values, since in the experiments 6 and 9, saffron flowers extracts presented a high concentration of phenolic compounds and a high antioxidant ability. Regarding TFC values, the results showed that the optimal experiment was also number 6, using 100% of ethanol as extraction solvent during 5 min at low temperature (25 °C).

The best extraction results and the interaction effects of the variables studied for TPC, TFC and antioxidant activity are illustrated in Figure 3. As it can be seen in the response contour plot, at low temperatures (25 or 62.50 °C), TPC and antioxidant capacities by ORAC and HOSC assays were higher (red surface) than those observed at 100 °C.

These findings were comparable to previous research by Sólyom et al. [18] that reported that the antioxidant activity and the total polyphenol content on grape marc decreased after a treatment at high temperature, that may be due to a degradation of the bioactive compounds. Gallo et al. [29] also applied MAE for the recovery of saffron phenolic compounds. Using ethanol (50%) at 200 W and low temperatures (50 °C) during 18 min obtaining extracts rich in total phenolic content with high antioxidant activity. Their results also suggested that extracts obtained by MAE showed a higher antioxidant ability (45 times) than the one obtained by ultrasound assisted extraction. MAE was a better technique to isolate interesting bioactive compounds from *Crocus sativus* L., most likely because of the good interaction between the solvent and the plant matrix during MAE.

The ORAC and HOSC results revealed the antioxidant activities of saffron floral by-products. This antioxidant power would be due to their bioactive content, such as polyphenols, since several research have reported that spices and herbs, with high phenolic content, are excellent sources of natural antioxidant compounds [30]. Nevertheless, the relationship between the structure of polyphenols and their antioxidant power is not yet well known [28]. Therefore, saffron floral by-products could be a source of natural antioxidants resulting from the saffron spice handling and processing and can be used to develop nutraceutical or functional food products with positive effects in human health such as the protection against oxidative stress [31].

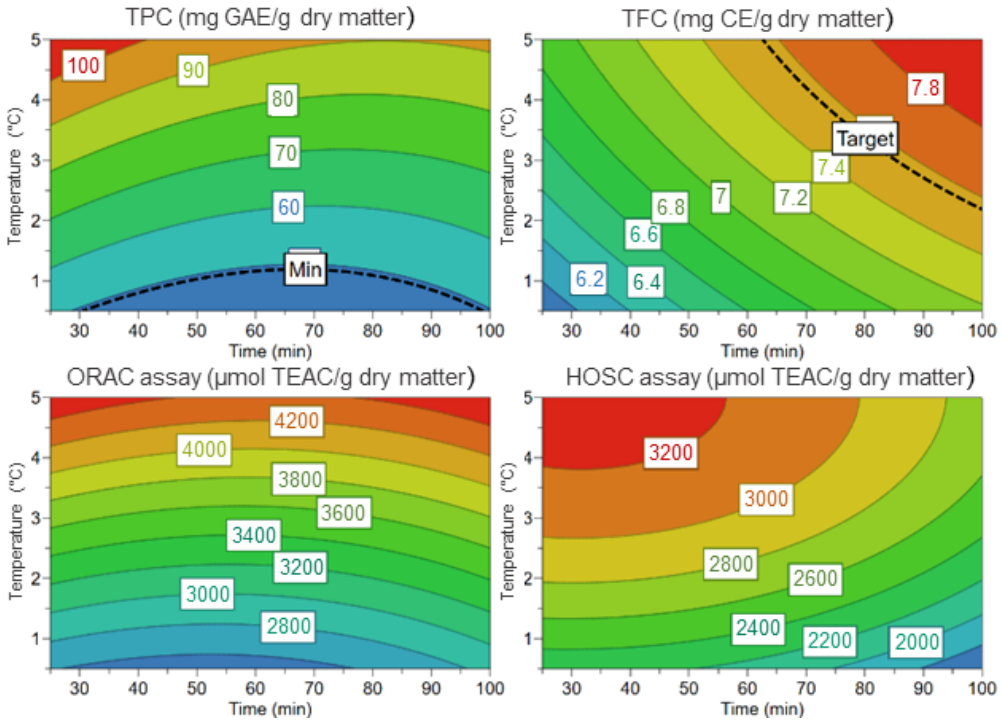

Figure 3. Response contour plots representing the effects of time and temperature at a constant ethanol concentration (100%), on all extraction responses. Lower values are represented in blue and higher values in red.

4. Conclusions

This study explored the effects of different processing parameters such as time, temperature and ethanol concentration in MAE on saffron floral by-products, in terms TPC, TFC, and antioxidant activity (ORAC and HOSC assays). At laboratory scale, the results showed that the optimal MAE conditions for the extraction bioactive compounds were using ethanol as primary solvent (50 or 100%) and low temperatures. Therefore, this information could help to choose the most appropriate extraction method and parameters to obtain compounds of interest from natural plant sources, including scale-up to industrial level.

From these findings, it can be concluded that MAE was an efficient technique that allows to obtain high added value compounds from saffron floral by-products with low energy footprint. Additionally, this research provides new information about the functional compounds present in saffron floral by-products extracts, representing an important source of natural antioxidant compounds and that could be considered as a source of promising bioactive ingredients for the development of functional foods and for other human health applications.

Author Contributions: Conceptualization: D.C.-B. and N.F; methodology: D.C.-B.; writing-original draft preparation D.C.-B. and J.P.B.; writing—review and editing: N.F. and M.J.F.; supervision: N.F. and M.J.F.; project administration: M.J.F. All authors have read and agreed to the published version of the manuscript.

Funding: This article is supported by the Partnership for Research and Innovation in the Mediterranean Area (PRIMA) program, project 174-SAFFROMFOOD. The PRIMA program is supported by the European Union. The research was also supported by the regional agency "Diputación de Alicante", Alicante, Spain.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: Thanks to Green Chemical Engineering Network towards upscaling sustainable processes (CA18224)—GREENERING funded by COST (European Cooperation in Science and Technology) for the STSM grant and thanks to the Spanish-Ministry of Education, Culture and Sport for the FPU-PhD fellowship (FPU18/02225). The authors acknowledge the financial support received funded by Fundação para a Ciência e Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior (FCT/MCTES, Portugal) through national funds to iNOVA4Health (UIDB/04462/2020 and UIDP/04462/2020), the Associate Laboratory LS4FUTURE (LA/P/0087/2020) and INTERFACE Programme, through the Innovation, Technology and Circular Economy Fund (FITEC).

Conflicts of Interest: The authors declare no conflict of interest.

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Publicación 5

Novel chitosan/alginate hydrogels as carriers of phenolic-enriched extracts from saffron floral by-products using natural deep eutectic solvents as green extraction media

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Revista	Current Research in Food Science
DOI	10.1016/j.crfs.2023.100469
Editorial	Elsevier
Fecha de publicación	26 de Febrero de 2023
Categoría JCR®	Food Science and Technology
Cuartil	Q1
Rango	21/142
Factor de impacto	6,300

Current Research in Food Science 6 (2023) 100469

Contents lists available at ScienceDirect

Current Research in Food Science

journal homepage: www.sciencedirect.com/journal/current-research-in-food-science

"Novel chitosan/alginate hydrogels as carriers of phenolic-enriched extracts from saffron floral by-products using natural deep eutectic solvents as green extraction media"

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ARTICLE INFO

Keywords:
NaDES
Sustainability
Crocus sativus L.
Antioxidant capacity
Bioactive compounds
Environmental impact

ABSTRACT

The current saffron production system is generating several hundreds of tons of tepal waste, because only stigmas are used for food. Consequently, the valorization of saffron floral by-products by developing stable functional ingredients could lead to the environmental impact minimization. Thus, the main aim of this study was to develop innovative green extraction processes from saffron floral by-products by using Natural Deep Eutectic Solvents (NaDES) and ultrasound-assisted extraction (UAE) as ecological extraction method. Response surface methodology was used to optimize process parameters. To improve the stability of the optimal extracts, they were incorporated into chitosan/alginate hydrogels, studying their water-uptake and water retention capacity and the total phenolic content (TPC) during the *in vitro* digestion. The results indicated that the optimal extraction, regarding total phenolic and flavonoid content, was achieved in 20 min, using 180 W ultrasound power and 90% of NaDES. The results of the DPPH assay revealed the potent antioxidant activity of saffron floral by-products. The chitosan/alginate hydrogels incorporating the as-obtained NaDES extracts showed favorable properties whereas the TPC remained stable under intestinal conditions. Therefore, NaDES combined with UAE was an efficient technique to isolate high added-value compounds from saffron flowers, succeeding also the valorization of discarded waste by using green and low-cost strategies. Furthermore, these novel hydrogels could be used as promising candidates for food or cosmetic applications.

1. Introduction

Saffron, the most expensive spice in the world, is widely cultivated in Iran, India, Spain, Greece, Italy and Turkey. Apart from its applications in food, it has been used in medicine due to its bioactive compounds content (Melnyk et al., 2010). It is produced from the red dried stigmas of *Crocus sativus* L. flowers, but the rest of the flower, mostly composed of six purple tepals and three yellow stamens, is considered as waste material (Fig. 1) (Mathew, 1977). Nevertheless, recent research showed that saffron processing waste are promising natural sources of bioactive compounds, such as polyphenols including flavonol glycosides, anthocyanins and phenolic acids (Table 1) (Cerdá-Bernad et al., 2022; Da Porto and Natolino, 2018; Lakka et al., 2019). Then, one of the greatest interests of saffron bioactive compounds is because of their benefits on the human health through their high antioxidant capacity with potential applications in food, cosmetic and pharmaceutical industries (Cerdá-Bernad et al., 2022). In the post-pandemic food sector, the valorization of a wide range of antioxidant bioresources (such as spices, medicinal plants, cereal processing by-products, edible and wild flowers, among others) through their use as functional food ingredients is one of the innovations with the greatest potential, since their bioactive composition content may play an important role in human health, highlighting even antiviral activity (Galanakis, 2022; Galanakis et al., 2020, 2021). Moreover, the valorization of saffron bio-residues could improve the sustainability and profitability of saffron industry since to produce 1 kg of saffron are necessary around 350 kg of tepals

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https://doi.org/10.1016/j.crfs.2023.100469
Received 16 December 2022; Received in revised form 13 February 2023; Accepted 22 February 2023
Available online 26 February 2023

Fig. 1. Parts of *Crocus sativus* L. flower.

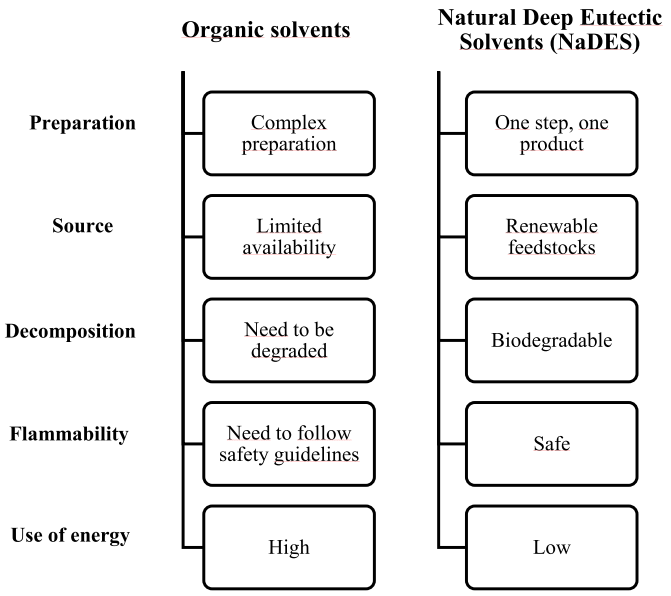

Fig. 2. Comparison of organic solvents vs NaDES.

(Sánchez-Vioque et al., 2012).

The extraction process of bioactive compounds from saffron floral bio-residues has been done through conventional extraction methods (maceration, distillation, Soxhlet extraction) (Ozkan et al., 2021). However, to minimize the environmental impact of common volatile organic solvents and to improve the extraction efficiency there is a need of new green and energy-saving technologies that can be transferred to industrial scale such as ultrasound-assisted extraction, and to use environmentally friendly solvents such as NaDES to obtain phenolic-enriched extracts. Ultrasound-assisted extraction process presents several advantages in contrast to conventional technologies: higher extraction efficiency, lower energy consumption and cost, as well as shorter extraction time and rapid temperature rise leading to a lower thermal degradation of compounds of interest (Nagarajan et al., 2019; Sarfarazi et al., 2020).

NaDES, a novel category of green and sustainable solvents, are mixtures of two or more naturally occurring compounds, a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) (e.g., organic acids, amino acids, sugars), in specific molar ratios (Choi et al., 2011). These mixtures can form intermolecular hydrogen bonds and van der Waals interactions that cause a considerable reduction of the melting point compared to that of each initial compound. NaDES have exceptional properties (Fig. 2): biodegradability, non-volatility, non-flammability, ability to dissolve several compounds, lack of toxicity and low cost, and have the striking ability to act as effective extraction media and protective carriers of the extracted bioactive phytochemicals (Koutsoukos et al., 2019; Skarpalezos and Detsi, 2019; Tzani et al., 2021).

NaDES extractions are usually combined with high-energy non-conventional extraction techniques like ultrasound-assisted extraction to improve the extraction yield and to reduce the extraction time, the degradation of bioactives and any adverse ecological effects. The application of ultrasonic energy causes the formation and collapse of cavitation bubbles, and the generation of high pressure and temperature that accelerates the disruption of cell walls of plants, promoting the release of their bioactive content and improving the mass transfer (Wen et al., 2018).

To improve the stabilization of the bioactive ingredients, gel-like structures are used for their encapsulation. Hydrogels are three-dimensional network structures obtained by polymers that can absorb large amounts of water. Recently hydrogels are being studied as delivery systems of different active ingredients with potential application in the food industry (Ćorković et al., 2021). Chitosan is a cationic biopolymer, considered as GRAS (Generally Recognized as Safe) with good properties such as low toxicity, biocompatibility, biodegradability, and the ability, under acidic conditions, to be manufactured into various forms such as hydrogels, films, emulsions, among others (Detsi et al., 2020). Sodium alginate is an anionic biopolymer, commonly used as encapsulation matrix showing simple gelation with divalent cations such as Ca^{2+}, and it is cheap, available, non-toxic, biocompatible, biodegradable and can be processed easily in hydrophilic solvents like water. Thus, the combination of alginate, using $CaCl_2$ as ionic cross-linker with chitosan developing chitosan/alginate hydrogels could have a potential use as new drug delivery systems.

The main aim of this research was to evaluate innovative eco-friendly extraction processes as UAE process efficiency, at laboratory scale, to obtain high-added value compounds from saffron floral by-products, using a central composite design to optimize UAE process conditions (extraction time, power, and NaDES:water ratio). Extracts were characterized regarding total phenolic and flavonoids content (TFC) and antioxidant activity by DPPH assay. The best NaDES-extract in terms of bioactive content was incorporated into chitosan/alginate hydrogels to obtain new stabilized bioactive ingredients to examine their potential for practical applications. Therefore, this research contributes to the valorization of a currently unexploited biomass which could improve the sustainability of the saffron spice production and

Table 1
Summary of the main bioactive compounds presented in saffron stigmas and saffron floral by-products (tepals and stamens).

Bioactive Compounds		Saffron stigmas	Saffron floral by-products
(Poly) phenols	Flavonoids	Flavonols: kaempferol, quercetin and isorhamnetin glycosides Flavanols: epicatechin	Flavonols: kaempferol, quercetin, myricetin and isorhamnetin glycosides Anthocyanins: delphinidin, petunidin and malvidin glycosides
	Phenolic acids	Gallic acid, hydroxybenzoic acid, coumaric acid, rosmarinic acid, vanillic acid, caffeic acid	Gallic acid, hydroxybenzoic acid, coumaric acid, syringic acid, hydroxycinnamic acids
Carotenoid-related		Crocetin, crocin, β-carotene, zeaxanthin	Lutein diesters with lauric, myristic, palmitic and stearic acids
Terpenoids		Picrocrocin, safranal	–

profitability of this industrial sector, optimizing the environmental impact and developing new high added-value ingredients.

2. Material and methods

2.1. Plant material and reagents

Saffron floral by-products were obtained from Spain (Castilla-La Mancha region, 2020 harvest season), and composed mainly of tepals and stamens. Stigmas were already detached following traditional procedures (DOCM, 1999). All fresh flowers were frozen in liquid nitrogen and kept at −80 °C until freeze-dried during 48 h (Christ Alpha 2–4, B. Braun Biotech International, Melsungen; Germany). Freeze-dried flowers were crushed and sieved (500 μm mesh size), and kept at −20 °C until further analysis.

Saffron stigmas were supplied by the Spanish company Verdú Cantó Saffron Spain, S.L and were from Greek (Kozani area) cultivation, being the moisture lower than 11%. Saffron threads were crushed and sieved (500 μm mesh size), and stored at 4 °C until further analysis.

Reagents: L-proline (Panreac, Barcelona, Spain), D,L-lactic acid (LabKem, Barcelona, Spain, 80% aq. Sol.), glycerol anhydrous (Penta, Katovice, Czech Republic, 99.9%), anhydrous betaine (Alfa Aesar, Ward Hill, MA, USA), anhydrous citric acid (Fluka, Charlotte, NC, USA), anhydrous D(+)-Glucose (Sigma Aldrich, St. Louis, MO, USA), Folin-Ciocalteu reagent (Merck Millipore, Darmstadt, Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Catechin (Sigma Aldrich, St. Louis, MO, USA), alpha-amylase, bile salts and pancreatin (Sigma Aldrich, St. Louis, MO, USA), phosphate-buffered saline (PBS) and pepsin (Thermo Fisher Scientific, Waltham, MA, USA), hydrochloric acid and sodium bicarbonate (Panreac, Barcelona, Spain).

2.2. NaDES

The synthesis of NaDES was done using the heating method as described by Tzani et al. (2021) with slight modifications, mixing appropriate amounts of starting materials under continuous and vigorous stirring for 1–4 h at temperatures between 50 and 80 °C under an inert atmosphere in a round-bottom flask (Table 2). The homogenous mixtures were transferred into glass vials, hermetically closed and stored in dark at room temperature until their further use. The pH of the prepared NaDES was measured by a 744 pH Meter Metrohm at 25 ± 2 °C. The NADESs viscosities were measured in triplicate at 25 °C by the Brookfield DV1 Viscometer using the spindle SC4-18. The temperature of the samples was maintained using a Julabo F12 thermostat bath (± 0.01 °C). Each measurement was carried out at a constant time of about 7 min and the viscosities were measured with an accuracy of ± 2%.

2.3. Box–Behnken experimental Design—Response Surface Methodology

Response Surface Methodology was employed to optimize the extraction process and predict the responses which are affected by experimental variables. Using Design-Expert® software version 12.0 (trial version) (Stat-Ease, Inc., Minneapolis, USA), a Box–Behnken design (BBD) with three independent variables was applied to evaluate the effect of time (A), power (B) and % NaDES (w/w) in the NaDES:H_2O mixture (C) on the bioactive content (total phenolic content and total flavonoid content) and antioxidant activity of saffron flowers. The coded variable levels are summarized in Table 3. The experimental data were fitted to a third-order polynomial, which correlated each response to the factors.

Table 2
Synthesized NaDES.

No.	NaDES	HBA	HBD	Molar ratio	Conditions
1	Bet/LA	Betaine	D,L-Lactic acid[a]	1:2:2.5	50 °C, 1 h
2	Glu/LA	D-(+)-Glucose	D,L-Lactic acid[a]	1:5:6.2	65 °C, 2 h
3	Bet/Gly/Water	Betaine	Glycerol	1:3:1	70 °C, 3 h
4	Pro/CA/Water	L-proline	Citric acid	2:1:3	80 °C, 4 h
5	Pro/Gly	L-proline	Glycerol	1:2	65 °C, 3 h

[a] Lactic acid is 80% aqueous solution, the composition of water is considered as it could participate in the hydrogen bonding of the NaDES.

2.4. NaDES—Ultrasound assisted extraction process

Extractions of freeze-dried saffron flowers were carried out using a Vibra-Cell VCX 400 (400 W) Ultrasonics High Intensity Processor (Sonics and Materials Inc., Newtown, USA), equipped with a piezoelectric converter and a 13 mm diameter titanium alloy (Ti–6Al–4V) probe. The variable parameters included time (5−35 min), power (60−180 W) and NaDES concentration (30–90%). The mass:solvent ratio was set at 1:20 (w/v), and the samples were kept in an ice bath during the UAE to avoid overheating. The pulse sequence was set at 6 s on – 2 s off. Sixteen extractions were carried out, following the designed conditions presented in Table 3. Once the extraction was completed, by centrifugation at 10000 rpm for 10 min and by filtration under vacuum, the supernatant was recovered from the solid material. All the extracts were stored in dark at 4 °C until further analysis.

Once the extracts were characterized in terms of bioactive compounds content, the best parameters UAE conditions (20 min, 180 W, 90% NaDES (Pro/Gly)) were used for Greek saffron stigma extraction, following the same procedure explained. The extractions were performed in triplicate.

Initially, a screening was carried out to select the optimal NaDES using UAE. The five synthesized NaDES (Table 2) were studied, using as UAE variables: 15 min, 160 W and 65% w/w of NaDES in the NaDES-water system. The mass:solvent ratio was set at 1:20 (w/v), following the same procedure as above. The extracts were characterized in terms of TPC and TFC, and the optimal NaDES selected was Pro/Gly (1:2) which was employed for the 16 experiments.

2.5. Extract characterization

2.5.1. Total phenolic content

TPC was determined using the Folin Ciocalteau colorimetric method as described by Tzani et al. (2021), with slight modifications. Briefly, 50 μL of the saffron floral extracts were mixed with 3 mL of milli-Q water and 250 μL of Folin-Ciocalteu's reagent and the solutions were stirred in

Table 3
The BBD matrix of UAE of the experimental design.

Experiments	Independent variables: UAE conditions		
	Time (min) (A)	Power (Watt) (B)	NaDES %(w/w) (C)
1	35 (+1)	180 (+1)	60 (0)
2	20 (0)	60 (−1)	30 (−1)
3	20 (0)	120 (0)	60 (0)
4	20 (0)	120 (0)	60 (0)
5	35 (+1)	60 (−1)	60 (0)
6	20 (0)	180 (+1)	30 (−1)
7	5 (−1)	60 (−1)	60 (0)
8	20 (0)	60 (−1)	90 (+1)
9	20 (0)	180 (+1)	90 (+1)
10	35 (+1)	120 (0)	90 (+1)
11	20 (0)	120 (0)	60 (0)
12	20 (0)	120 (0)	60 (0)
13	35 (+1)	120 (0)	30 (−1)
14	5 (−1)	120 (0)	90 (+1)
15	5 (−1)	180 (+1)	60 (0)
16	5 (−1)	120 (0)	30 (−1)

dark for 2 min. Then, 750 μL of saturated aqueous Na_2CO_3 solution were added and were stirred again and the final volume of the solution was adjusted to 5 mL with milli-Q water. Protected from light, samples were stirred and incubated at room temperature during 1 h. Gallic acid (1 mg/mL) was used as a reference standard (7.5–240 mg/L) and absorbance was measured at 755 nm using a Jasco V-770 UV–Vis/NIR spectrophotometer. The experiments were performed in triplicate and the results were expressed as mg gallic acid equivalent (mg GAE) per gram of dry weight (dw).

2.5.2. Total flavonoid content

TFC was determined as described by Çam and Hışıl (2010) with some modifications. Briefly, 0.2 mL of sample were mixed with 0.8 mL of water and 60 μL of sodium nitrite (5%) solution. After 5 min, 60 μL of aluminum trichloride (10%) solution were added. After 6 min, 0.4 mL of sodium hydroxide (1 M) solution were added and the volume was adjusted to 2 mL with water. Catechin (1 mg/mL) was used for quantification (20–100 mg/L) and absorbance was measured at 510 nm using a Jasco V-770 UV–Vis/NIR spectrophotometer. The experiments were performed in triplicate and the results were expressed as mg of catechin equivalents (mg CE) per gram of dw.

2.5.3. DPPH radical scavenging ability

The antioxidant capacity by the radical scavenging capacity measuring method by the 2-2,diphenyl-1-picryl-hydrazide (DPPH) stable radical, were carried out in the optimal extracts in terms of their bioactive content, following the method described by Boly et al. (2016). Briefly, 5 mg of DPPH were dissolved in ethanol with a final volume of 50 mL. In a 96-well plate, 100 μL of DPPH solution were added to 100 μL of saffron extracts diluted to 2% v/v in ethanol (initial concentration C) and then the same procedure was followed for samples diluted in ethanol, with concentrations 0.8C, 0.6C, 0.4C and 0.2C. The samples were incubated for 30 min in dark at room temperature and then the absorbance was measured at 515 nm using a Molecular Devices SpectraMax 250 Microplate UV/Vis Reader. The percentage of inhibition was calculated using the following Eq. (1):

$$\%\ Inhibition = \frac{A_{blank-A_{sample}}}{A_{blank}} x\ 100 \quad (1)$$

where A_{blank} is the absorbance of the blank samples containing ethanol/DPPH and A_{sample} is the absorbance of each sample containing extract. A curve of % inhibition against samples was plotted and the concentration of the sample required for 50% inhibition of DPPH radicals was determined (IC_{50}), expressed as mg of saffron flower extracts per mL of sample. The experiments were performed in triplicate.

2.6. Chitosan/alginate hydrogels synthesis

A solution of chitosan (0.2% w/v) in aqueous lactic acid (3% v/v) was prepared. To this solution, the NADES extract was added in order to prepare a solution of final concentration 2% v/v (solution A). Next, a 3% w/v aqueous solution of sodium alginate (SA) was prepared by adding 3 g of SA powder to 100 mL of distilled water and stirring at 70 °C until the mixture became homogeneous (solution B). Then, 2 mL of solution A and 5 mL of solution B were mixed and an aqueous solution of calcium chloride (1% w/v), used to crosslink the alginate and chitosan, were added dropwise to the mixture. After 24 h, the excess of $CaCl_2$ was drained out and hydrogels were obtained. These hydrogels were washed 3 times with distilled water and the water remaining on the surface was subsequently removed. The hydrogels were lyophilized using a freeze drier (ModulyoD Thermo Fisher Scientific, Waltham, MA, USA) for 48 h.

2.7. Structure of hydrogels

The shape and surface of the hydrogels was observed with a stereomicroscope Leica MZ95 (Leica, Spain).

2.8. Water-uptake and water-retention capacity

The water-uptake capacity of the hydrogel after drying was measured by a conventional gravimetric method. The weight of the dried hydrogel sample was accurately weighed, and then the dry samples were soaked in deionized water. The water-uptake capacity of the prepared Chit/Alg/extract/NaDES hydrogels was measured in PBS solution (pH = 7.4), at room temperature, and calculated by the following Eq. (2):

$$Water-uptake\ (\%) = \frac{m_1 - m_0}{m_0} x\ 100 \quad (2)$$

where m_0 is the initial weight of dry sample and m_1 is the mass swollen weight of the hydrogel at time *t*. After the predetermined time points the samples were extracted from the solution and weighted quickly.

The Chit/Alg/extract/NaDES hydrogels after reaching its maximum water-uptake capacity (equilibrium) at pH = 7.4, 37 °C it was used in the following water retention experiments. When water-uptake capacity (%) is the maximum, we assume that the water-retention (%) of the hydrogel is 100%. Thus, we calculate the water-retention with the aid of the following Eq. (3):

$$Water-retention\ (\%) = \frac{m_1 - m_0}{m_2 - m_0} x\ 100 \quad (3)$$

where m_0 is the initial weight of dry sample, m_1 is the mass swollen weight of the hydrogel at time *t*, and m*2* is the weight of the hydrogel at the time of maximum water-uptake capacity (equilibrium). All the experiments were performed in triplicate.

2.9. In vitro digestion of hydrogels

The *in vitro* digestion was performed according to Gawlik-Dziki et al. (2009) and Cerdá-Bernad et al. (2021) with slight modifications. Briefly, the hydrogels were homogenized in a stomacher laboratory blender for 30 s to simulate mastication with the presence of 5 mL of PBS (hydrogel: PBS 1:50 w/v). The solution was adjusted to pH 6.75 and alpha-amylase (E.C. 3.2.1.1.) was added to obtain 100 U per mL of enzyme activity. Then, the samples were subjected to simulated gastric digestion for 60 min at 37 °C under stirring, adjusting the solution to pH 3 with 1 M HCl, and pepsin was added to reach a concentration of 3 g/L. For intestinal simulation, the pH was increased to 7 with 1 M $NaHCO_3$ and 4.5 g/L of bile salts and 1 g/L of pancreatin were added. The samples were incubated during 120 min at 37 °C. Samples were taken at the different stages of *in vitro* digestion and filtered for TPC analysis: after oral digestion, after 60 min of gastric digestion, and after 60 min and 120 min of intestinal digestion.

2.10. Statistical analysis

Results were expressed as the mean ± standard deviation. The mean comparisons were carried out using an analysis of variance (ANOVA) and by the Tukey multiple range test, using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, USA). The significant differences were stablished as ($p < 0.05$).

3. Results and discussion

3.1. Preliminary extraction experiments

In this study, the influence of solvent, pH of the extraction solvent systems and viscosity in the recovery of phenolics and flavonoids from saffron floral by-products was examined (Table 4).

Bet/Gly/Water (1:3:1) and Pro/Gly (1:2) were some of the optimal

Table 4
Total phenolic content (TPC) and total flavonoid content (TFC) obtained from preliminary NaDES-UAE (extraction conditions: 15min, 160W, NaDES/water = 65:35 w/w) and pH of the extraction solvent systems at 25 °C[a].

No.	NaDES	pH (NaDES/water = 65:35 w/w)	Viscosity (mPa·s)	TPC (mg GAE/g dw)	TFC (mg CE/g dw)
1	Bet/LA/Water	2.85	139.8	18.90 ± 0.30b	1.30 ± 0.05c
2	Glu/LA/Water	1.32	411.1	21.88 ± 0.58b	1.16 ± 0.08c
3	Bet/Gly/Water	6.36	519.0	31.73 ± 0.08a	5.85 ± 0.17b
4	Pro/CA/Water	n.m[b]	n.m[b]	33.56 ± 0.45a	8.47 ± 0.10a
5	Pro/Gly	7.59	5064.0	35.15 ± 1.99a	8.04 ± 0.49a

[a] Means ± standard deviation in the same column followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each sample (n = 3).
[b] n.m.: not measured due to the very high viscosity.

solvents to isolate phenolic and flavonoids compounds by UAE from saffron flowers. Then, the pH could strongly influence the recovery of these antioxidant compounds, since using acidic solvents, TPC and TFC values were lower than those obtained using solvents with a neutral pH. A plausible explanation is that the low pH of some NaDES may affect the chemical composition of extracts by release of other bioactive compounds or cause structural changes (Inada et al., 2015). However, using the acid-containing NaDES solvent Pro/CA/Water (2:1:3) a high TPC and TFC values were obtained, so other factors such as viscosity may have influenced the extraction process of bioactive compounds.

The NaDES Pro/Gly (1:2) was selected as a promising green solvent to implement the extraction process and its optimization through the experimental design.

3.2. Experimental design

To optimize the extraction of secondary metabolites from saffron flowers through innovative eco-friendly extraction processes and by using ecological extraction media to obtain high-value bioactive compounds, the extraction method should be considered, as well as the conditions of the extraction process, since they have a strong influence on the extraction yield.

A design of experiments (DOE) was carried out to isolate bioactive compounds from *Crocus sativus* L. flowers. To optimize the extraction procedure and to evaluate UAE as a green extraction method and NaDES as green extraction media, the effect of process variables (TPC and TFC, and the antioxidant capacity by DPPH assay) were evaluated on a BBD with three independent variables: time (A), power (B) and NaDES:water solvent ratios (C).

3.2.1. Bioactive content

The results of the effects of the three independent variables studied in UAE on phenolics compounds extraction are shown in Fig. 3. As it can be seen in the 3D surface response plots which depict the correlation between the factors of Time (A) and % NaDES (B) in stable Power (C) conditions each time at 60 W, 120 W and 180 W which are the 3 levels of factor C. Then, the study of the interaction between time and NaDES solvent ratio keeping constant the power allows to adjust the solvent polarity to increase the affinity and specificity of the UAE. The values of TPC were lower using as extraction factors shorter time, 30% of NaDES solvent ratio and 60 or 120 W of power (blue surfaces in Fig. 3A and 3B). Nevertheless, the values were higher using 20 or 35 min, 60 or 90% of NaDES solvent and 180 W of power during the UAE (red surfaces in

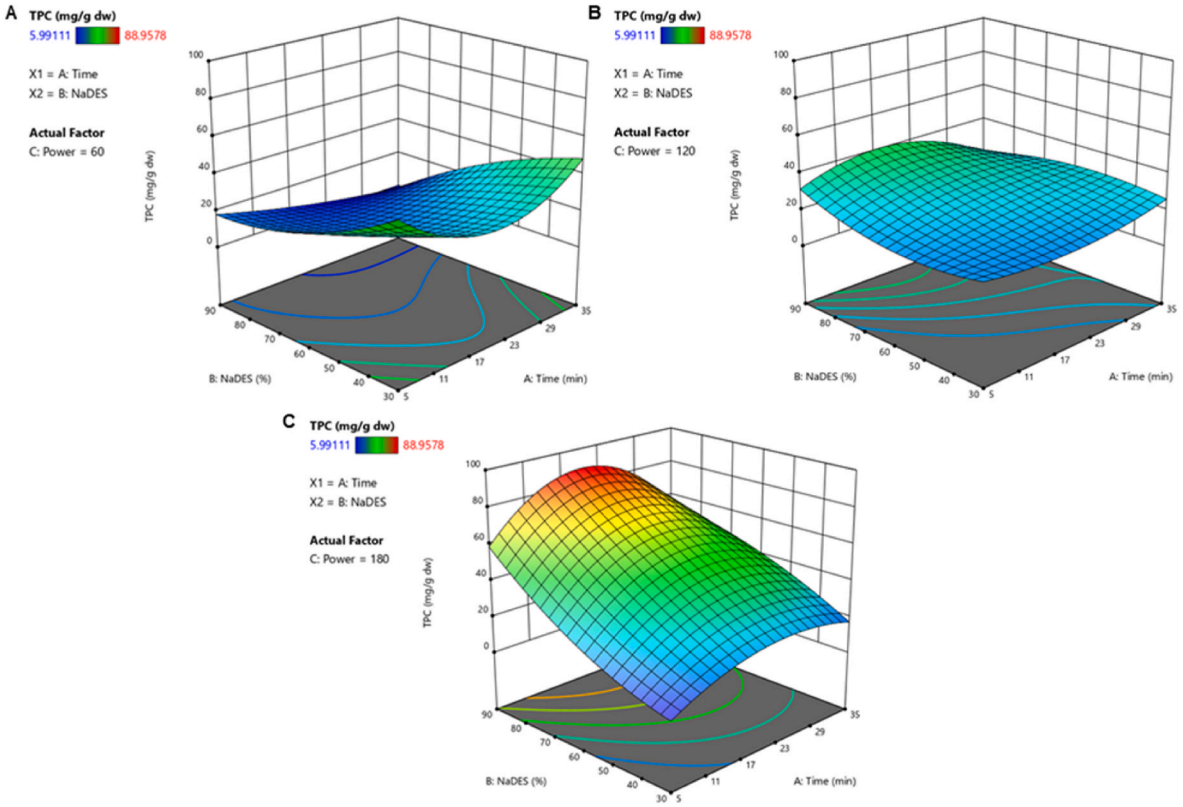

Fig. 3. Response surface plot representing the effects of time, temperature and NaDES solvent ratio on Total Phenolic Content (TPC) from saffron floral by-products. (A) Power was kept constant at 60 W. (B) Power was kept constant at 120 W. (C) Power was kept constant at 180 W. Lower values are represented in blue and higher values in red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3C). The optimal extraction regarding TPC was using 20 min, 180 W and 90% of NaDES (88.96 ± 1.08 mg GAE/g dw), indicating that increasing the extraction time and NaDES solvent ratio while keeping constant the power at 180 W, lead to an increase in the extraction yield of these bioactive compounds. This fact may be due to the significant effect of power on extracting bioactive compounds from saffron flowers by some interconnected mechanisms: accelerating the breakdown of plant cells due to a fast-localized increase in temperature and pressure, increasing the permeability and release of intracellular compounds and improving the mass transfer and diffusion (González-Centeno et al., 2015). Besides, a higher ratio of NaDES solvent than water could improve the solubilization of non-water-soluble metabolites of plant cells.

The experimental values of the TPC obtained for the UAE of bioactive compounds are showed in Table 5. The statistical analysis of the 16 runs revealed that the TPC is best described by the reduced cubic model, being the independent variables: time (A), power (B) and NaDES:water solvent ratios (C):

$$TPC = 28.01 + 5.44\ A + 11.23\ B + 19.31\ C - 4.06\ AB + 4.08\ AC + 22.18\ BC - 4.57\ A^2 + 1.29\ B^2 + 6.95\ C^2 - 10.09\ A^2B - 16.94\ A^2C - 7.07\ AB^2$$

The proposed model was significant with the F-value equal to 417.92 and the *p*-value less than 0.05. The coefficient R^2 was 0.9994, indicating accuracy and a good fit of the experimental data with the calculated ones, and the adjusted coefficient R^2 0.9970 which verifies the adequacy of the model.

According to the results, NaDES:water solvent ratios (C) is the factor that contributes the most to the extraction of phenolic content. Based on the TPC results (Table 5), the values were higher than those reported in previous research using dried saffron tepals from Italy extracted by UAE during 15 min (23 kHz) using water as extracting agent (1150.63 ± 11.23 mg GAE/100 g dw) (Stelluti et al., 2021) or Iranian saffron petals extracted by UAE during 40.61 min (135.5 W), using water as extracting agent (863 mg/100 g) (Hashemi Gahruie et al., 2020). It should be noted that there is also an improvement of TPC extraction respect to conventional extraction methods as sonication, in Spanish saffron floral by-products (32.82 ± 2.23 mg GAE/g dw) (Cerdá-Bernad et al., 2022).

Regarding TFC, the results of the interaction effects of the three independent variables studied in UAE on flavonoids content are shown in Fig. 4. This figure illustrates the interaction between time and NaDES solvent ratio, keeping each time the power during the extraction process constant in the 60 W, 120 W and 180 W which are the 3 levels set in the Power factor. The flavonoids concentration in the extract was positively affected by temperature and solvent ratio. As it can be seen in the response surface plot, the shape of the Fig. 4C indicated that, keeping the power constant at 180 W, flavonoids extraction was enhanced (red surface) with 20 min of extraction time and 60% of NaDES solvent.

The experimental values of the TFC obtained by UAE from saffron flowers are showed in Table 5. The statistical analysis of the 16 runs revealed that the TFC is best described by the reduced cubic model, being the independent variables: time (A), power (B) and NaDES:water solvent ratios (C):

$$TFC = 4.69 - 0.1819\ A - 0.1469\ B + 0.7783\ C - 0.6010\ AB - 0.0956\ AC + 0.7501\ BC - 0.5864\ A^2 - 1.77\ B^2 - 0.4628\ A^2B - 1.35\ A^2C$$

The proposed model was significant with the F-value equal to 4.86 and the *p*-value less than 0.05. The coefficient R^2 was 0.9068, and the adjusted coefficient R^2 0.7203.

According to the TFC results (Table 5), the values were higher than other studies that showed TFC values for 130 mg/100 g of Iranian saffron petals extracted by UAE (Hashemi Gahruie et al., 2020). Furthermore, TFC results were similar to those reported by that study Spanish saffron floral by-products extracts obtained by MAE using ethanol as extracting agent (Cerdá-Bernadet al., 2022).

Regarding Greek saffron stigmas, they were extracted following the UAE conditions of extraction number 9 (Table 5), since it was the optimum extraction considering the simultaneous maximization of the TFC and TPC values for saffron floral by-products (20 min, 90% of NaDES and 180 W). The results showed that high content of total phenols (95.66 ± 9.34 mg GAE/g dw) and flavonoids (9.56 ± 0.60 mg CE/g dw) were obtained from saffron stigmas. The TPC obtained is higher than the ones reported by other studies, in which Greek saffron stigmas extracts had values of 34.00 ± 3.22 mg GAE/g dw, extracted by usual methods as sonication, but TFC values were lower (17.39 ± 4.14 mg CE/g dw) (Cerdá-Bernad et al., 2022). However, TFC values obtained for saffron stigmas are higher than those presented by Karimi et al. (2010) for saffron stigmas methanol extracts from Iran (5.8 ± 0.12 mg rutin equivalents/g dw).

Therefore, NaDES combined with UAE efficiently extracted bioactive compounds from saffron floral by-products, providing new information about the optimal factors of UAE at laboratory scale. Moreover, UAE is a method that could be easier scaled up at an industrial scale comparing to

Table 5
Experimental results of total phenolic content (TPC), total flavonoid content (TFC) and DPPH assay for the ultrasound-assisted extraction of bioactive compounds from Spanish saffron floral-by products, and for Greek saffron stigmas extracted with the optimal UAE conditions[a].

	Extraction	UAE Conditions			Responses		
		Time (min)	Power (W)	NaDES (Pro/Gly 1:2) (%)	TPC (mg GAE/g dw)	TFC (mg CE/g dw)	DPPH Scavenging Ability, IC_{50} (mg of extract/mL)
Saffron floral by-products	1	35	180	60	42.27 ± 2.55b	3.60 ± 0.32cde	6.64 ± 0.24b
	2	20	60	30	27.89 ± 0.61cde	3.10 ± 0.07e	–
	3	20	120	60	27.10 ± 0.83cde	3.67 ± 0.08cde	–
	4	20	120	60	28.21 ± 2.41cd	4.60 ± 0.04abc	–
	5	35	60	60	29.38 ± 2.13c	4.93 ± 0.25 ab	–
	6	20	180	30	22.15 ± 0.02efg	3.15 ± 0.08e	–
	7	5	60	60	26.66 ± 0.45cdef	4.54 ± 0.03abcd	–
	8	20	60	90	5.99 ± 0.15h	1.30 ± 0.06f	–
	9	20	180	90	88.96 ± 1.08a	4.36 ± 0.48bcd	2.06 ± 0.15a
	10	35	120	90	20.17 ± 0.67g	0.60 ± 0.11f	–
	11	20	120	60	29.26 ± 0.58c	4.77 ± 0.14 ab	–
	12	20	120	60	27.49 ± 2.23cde	5.49 ± 0.05[a]	–
	13	35	120	30	26.01 ± 2.57cdefg	3.02 ± 0.13e	–
	14	5	120	90	31.56 ± 0.09c	2.73 ± 0.24e	6.36 ± 0.51b
	15	5	180	60	23.24 ± 1.68defg	3.59 ± 0.42de	–
	16	5	120	30	21.17 ± 0.78 fg	2.75 ± 0.56e	–
Saffron stigmas		20	180	90	95.66 ± 9.34	9.56 ± 0.60	2.74 ± 0.47

[a] Means ± standard deviation in the same column followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each sample (n = 3).

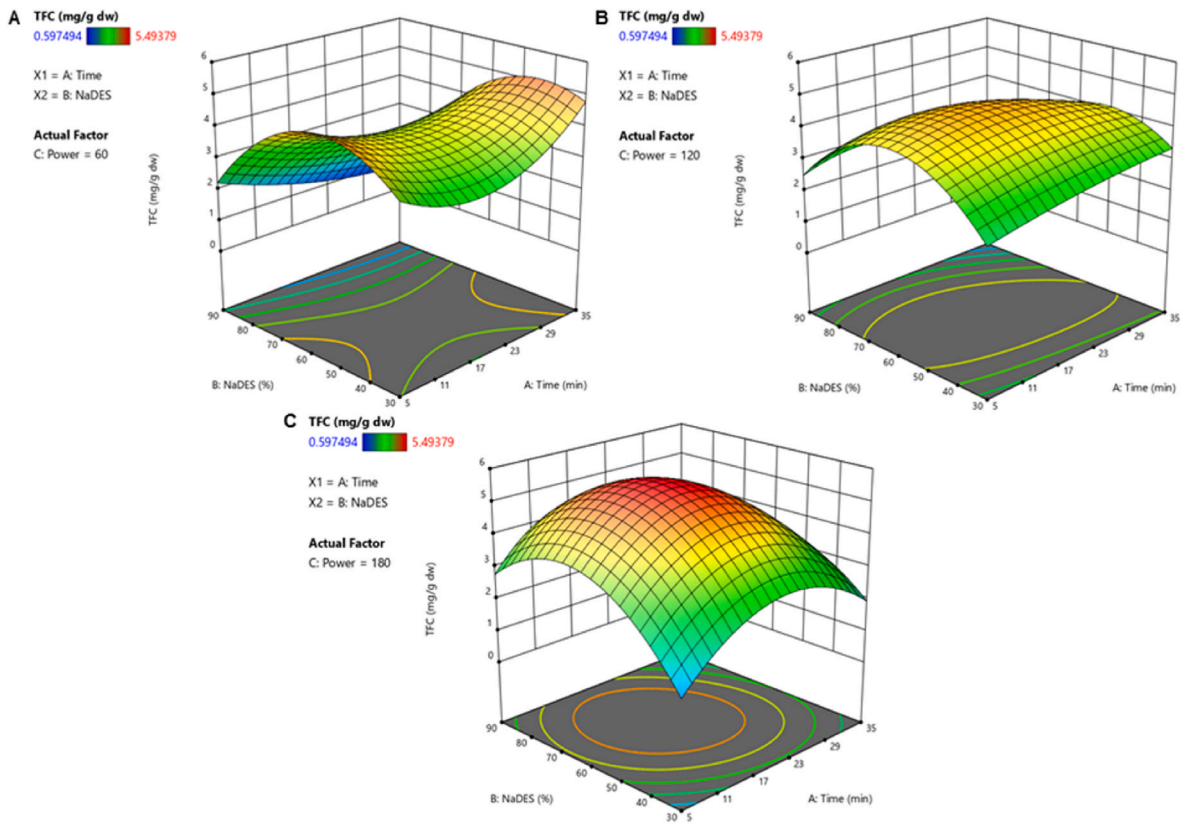

Fig. 4. Response surface plot representing the effects of time, temperature and NaDES solvent ratio on Total Flavonoid Content (TFC) from saffron floral by-products. (A) Power was kept constant at 60 W. (B) Power was kept constant at 120 W. (C) Power was kept constant at 180 W. Lower values are represented in blue and higher values in red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

other methods, such as microwave extraction.

3.2.2. *In vitro antioxidant properties*

As showed in Table 5, DPPH assay results revealed the antioxidant activities of saffron floral by-products of the optimal extracts, in terms of phenolic and flavonoid content. All the studied extracts showed remarkably high antioxidant power by DPPH assay which evaluates the *in vitro* radical scavenging ability of saffron extracts. The best DPPH radical scavenging ability was shown by extract 9 for saffron floral by-products (IC_{50} = 2.06 ± 0.15 mg/mL). Besides, Greek saffron stigmas extracts showed a good antioxidant activity (IC_{50} = 2.74 ± 0.47 mg/mL) that could be linked to their high bioactive content, such as total phenolic compounds and flavonoids.

Therefore, saffron flowers extracts presented a potential antioxidant activity that may be related to their bioactive content and could be used as natural antioxidant compounds to protect against the effects of oxidative stress, contributing to prevent several diseases. Furthermore, due to their high antioxidant power, these phenolic-enriched extracts from saffron stigmas and saffron floral by-products may be employed as ingredients to fortify food products, improving their functional properties, as well as to increase the preservation as natural additives, or having potential applications in the cosmetic industry, since previous studies have reported the potentiality of using phenols from oil mill wastewater as UV booster (Galanakis, 2018; Galanakis et al., 2018).

3.3. *Chitosan/alginate hydrogels*

Currently, chitosan/alginate hydrogels are novel economical candidates to use as delivery systems of different bioactive ingredients, since are safe for consumption, biocompatible, biodegradable and made with low-cost natural polymers (Ćorković et al., 2021; Detsi et al., 2020; Shewan and Stokes, 2013).

The optimal NaDES-extracts (extract 9 for saffron floral by-products, and saffron stigmas extracts), which were a natural rich source of bioactive ingredients with high antioxidant capacity, were incorporated into chitosan/alginate hydrogels to improve their stability and in order to study their potential use as a formulation for the development of functional food products or as efficient delivery systems of phenolic compounds.

In the Fig. S1, the surface of the different freeze-dried chitosan/alginate hydrogels incorporating saffron floral by-products or stigmas extracts is shown at different magnifications. The figure indicates the fibrous structure of chitosan/alginate hydrogels, and also a porous structure, as a result of the pores formed during the process of phase separation in the freeze-drying treatment (Baysal et al., 2013; Ehterami et al., 2019). Besides, in the Fig. S1B, saffron stigmas extracts incorporated into the structure of chitosan/alginate hydrogels can be distinguish due to their orange-red color.

3.3.1. *Water-uptake and water-retention capacity*

Water-uptake capacity and structural stability of hydrogels are important factors for their practical use in food products or for other applications such as hydrogel-based delivery systems, since a direct relationship could be between swelling properties of hydrogels and permeability of solute (Gehrke et al., 2006).

The data of the water-uptake capacity showed that all chitosan/alginate hydrogels had a maximum water-uptake at 20 min, which decreased by the time, especially for chitosan/alginate hydrogels incorporating the as-obtained NaDES-saffron floral by-products extracts, having a similar behavior with the blank samples (Fig. 5). However, chitosan/alginate hydrogels with NaDES-saffron stigmas extracts showed a different water-uptake capacity which could be owed to a

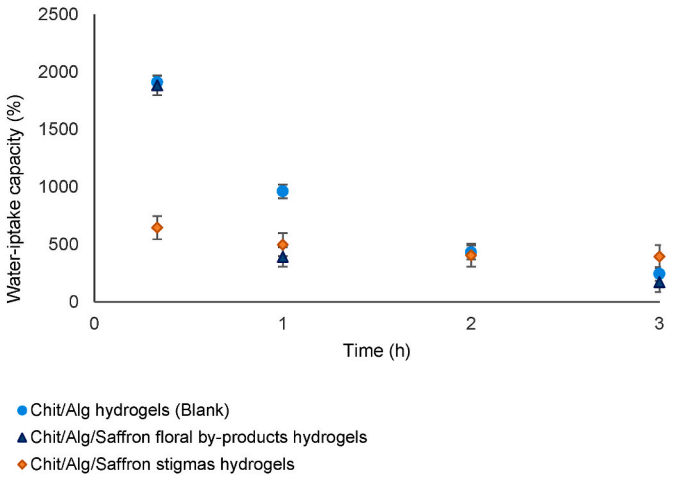

Fig. 5. Water-uptake capacity of chitosan/alginate hydrogels over time. The error bars represent the standard deviation (n = 3).

different interaction between the functional groups of extracts with gel matrices (Bera and Dutta, 2017).

In view of practical applications, water retention capacity is also an important parameter to develop hydrogels with a strong water absorption capacity, endowing them widespread applications such as drug-delivery systems, among others (Lv et al., 2019).

Regarding the data of water retention capacity measured when hydrogels reached the maximum water-uptake capacity (equilibrium) (Fig. 6), chitosan/alginate hydrogels containing the as-obtained NADES-saffron stigmas extracts presented the highest water retention capacity being around 80% after 1 h, compared with chitosan/alginate hydrogels containing the NaDES-saffron floral by-products extracts (20%). Besides, as it was expected, the water retention decreased by the time in the blank and chitosan/alginate hydrogels with NaDES-saffron floral by-products extract, being lower than 20% after 3 h. Nevertheless, the water retention capacity of chitosan/alginate hydrogels with saffron stigmas remained stable from 2 h, being around 60%, showing adequate properties to conserve large amounts of water over time.

3.3.2. *In vitro digestion*

Currently, several studies are focused into the development of hydrogels as a tool for the delivery of plant-derived phenolic compounds, since they present biocompatible properties, low toxicity and exceptional biological properties (Micale et al., 2020).

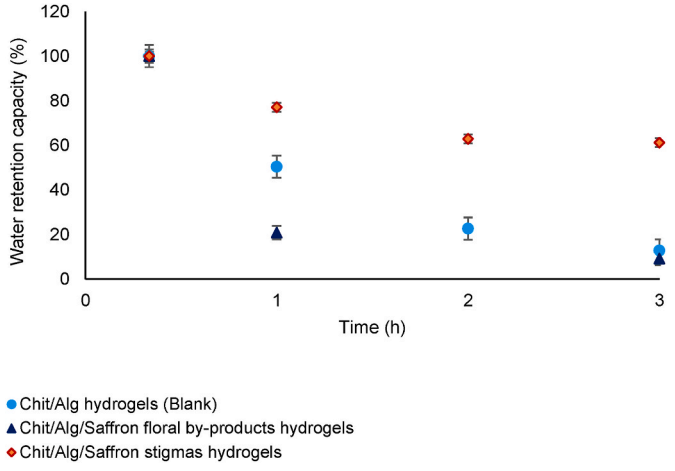

Fig. 6. Water-retention capacity of chitosan/alginate hydrogels over time. The error bars represent the standard deviation (n = 3).

Then, chitosan/alginate hydrogels could be promising candidates to retain and preserve high-value compounds. The impact of the *in vitro* digestion in the stability of the phenolic compounds of the saffron extracts incorporated in the hydrogels was evaluated.

In Fig. 7 is shown the TPC of the different hydrogels during the oral, gastric and intestinal (1 h and 2 h) *in vitro* digestion. The results showed that there is an increase of TPC after 1 h of intestinal digestion, which, for the Chit/Alg/Saffron stigmas hydrogels, remained practically unchanged during the 2 h of intestinal digestion. This fact could be due to intestinal conditions (neutral pH, presence of pancreatic enzymes and bile salts) that would lead to an improvement in the release of bioactive compounds (Ketnawa et al., 2022). In the case of Chit/Alg/Saffron floral by-products hydrogels a significant increase of TPC was observed from 1 h to 2 h, which can be attributed to the different phenolic compounds that are present in the NaDES extract of saffron floral by-products. Furthermore, the TPC released after 180 min of oral and gastrointestinal *in vitro* digestion, for Chit/Alg/Saffron stigmas and Chi/Alg/Saffron floral by-products hydrogels, was around 56–58%.

Under gastric conditions, no phenolic content could be detected for chitosan/alginate hydrogels with NaDES-saffron floral by-products extracts and the TPC for the hydrogels with NaDES-saffron stigmas extracts was very low, although it was higher than the TPC of the oral phase. This gradual increase may be due to the release of some phenolic compounds bound to carbohydrates in conditions of low pH and pepsin action (Cunha et al., 2018).

Therefore, after the *in vitro* gastrointestinal digestion, TPC values were very high, remaining stable into the hydrogel's matrices, but further research is necessary to identify these phenolic compounds and to study if these bioactive compounds were absorbed in the colon to exert their beneficial effects on human health.

4. Conclusions

This study evaluated the implementation and optimization of UAE using NaDES as green extracting solvents to obtain valuable compounds from saffron flowers, providing new information of an efficient environmental-friendly process. This process is characterized by the ease of NaDES preparation, the availability and low cost of the NaDES components as well as the scalability of the NaDES preparation. Taking everything into account one can envisage that this extraction process could be industrially exploited. Moreover, this method also implemented the valorization of saffron floral by-products, a high-value biomass that is currently unexploited, contributing to the improvement of the sustainability of the saffron spice production and

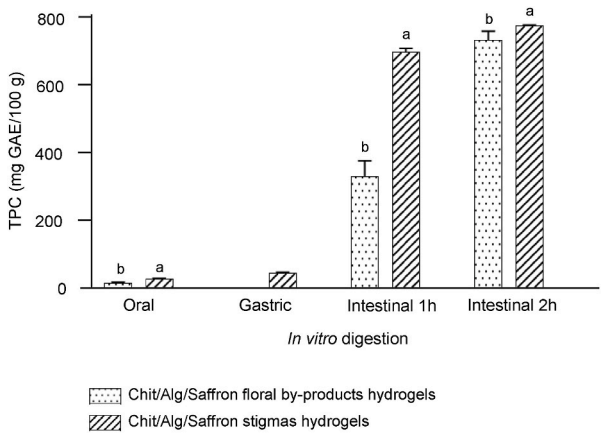

Fig. 7. Total phenolic content (TPC) of chitosan/alginate hydrogels during the oral and gastrointestinal *in vitro* digestion. The error bars represent the standard deviation and different lowercase letters indicate statistically significant differences ($p \leq 0.05$) for each sample in each phase (n = 3).

profitability of this industrial sector. According to the findings of this research, the novel chitosan/alginate hydrogels showed favorable properties and were suitable matrices to incorporate bioactive extracts which may be used as promising candidates for various applications like food or cosmetic, among others.

Funding

This article is supported by the Partnership for Research and Innovation in the Mediterranean Area (PRIMA) program, project SAFFROMFOOD. The PRIMA program is supported by the European Union. The research was also supported by the regional agency "Diputación de Alicante", Alicante, Spain.

CRediT authorship contribution statement

Débora Cerdá-Bernad: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Ioanna Pitterou:** Methodology. **Andromachi Tzani:** Conceptualization, Writing – review & editing, Supervision. **Anastasia Detsi:** Conceptualization, Writing – review & editing, Supervision. **María José Frutos:** Writing – review & editing, Supervision, Project administration, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments:

Thanks to Green Chemical Engineering Network towards upscaling sustainable processes (CA18224)—GREENERING funded by COST (European Cooperation in Science and Technology) for the STSM grant and thanks to the Spanish-Ministry of Education, Culture and Sport for the FPU-PhD fellowship (FPU18/02225).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2023.100469.

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Supplementary material

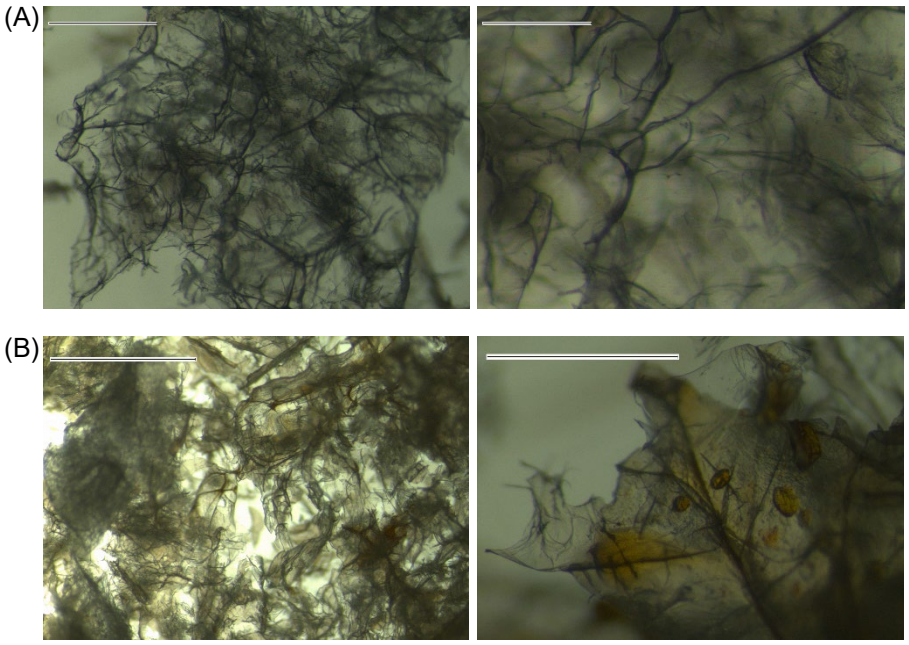

Figure S1. Stereo microscope images of chitosan/alginate hydrogels (A) incorporating saffron floral by-products extracts and (B) saffron stigmas extracts. Scale bars = 500 µm.

Publicación 6

Microencapsulated saffron floral waste extracts as functional ingredients for antioxidant fortification of yogurt: Stability during the storage

Autores	Débora Cerdá-Bernad, Estefanía Valero Cases, Joaquín Julián Pastor & María José Frutos
Revista	LWT – Food Science and Technology
DOI	10.1016/j.lwt.2023.114976
Editorial	Elsevier
Fecha de publicación	12 de Junio de 2023
Categoría JCR®	Food Science and Technology
Cuartil	Q1
Rango	24/142
Factor de impacto	6,000

LWT - Food Science and Technology 184 (2023) 114976

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Microencapsulated saffron floral waste extracts as functional ingredients for antioxidant fortification of yogurt: Stability during the storage

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ARTICLE INFO

Keywords:
Sustainability
Functional dairy products
Saffron floral bio-residues
Valorization
Encapsulation

ABSTRACT

Dairy industries are constantly seeking to develop innovative ingredients through the use of food by-products, since consumers' demand for healthier foodstuffs has increased over the last years. Therefore, the development of innovative dairy products enriched with saffron floral extracts could represent a novel environmentally-friendly technological solution. The present study aimed to determine the effect of adding alginate-based microencapsulated saffron floral by-products extracts (0.5 g/100 g, 1 g/100 g), or/and saffron stigmas extracts (0.05 g/100 g, 0.1 g/100 g) to homemade yogurts. For this purpose, physical-chemical properties, antioxidant capacity, total phenolic content (TPC), microbiological analysis, color and organic acids and soluble sugars profile were determined at 0, 7, 14 and 21 days of refrigerated storage. The results showed that these novel yogurt formulations allowed proper fermentation, being the microbiological profile and physicochemical parameters not affected by saffron extracts, compared to the control. The incorporation of microencapsulated saffron floral extracts improved the functional properties in terms of TPC and antioxidant properties which remained stable over 21 days of storage. Enriched yogurts also showed a good profile of organic acids and soluble sugars, mainly lactic acid and lactose. In conclusion, saffron floral by-products-alginate beads could be potential sustainable candidates to use as functional ingredients improving the nutritional and functional value of yogurts.

1. Introduction

Currently, efforts to valorize food by-products have become of interest to the food industry, since many of them contain valuable molecules (bioactive compounds, antioxidants, fibers, etc.) which can be reused as high value-added ingredients. The function of those ingredients within the food matrix is to provide biologically health-promoting compounds, through their applications in foods for the development of novel functional products, supplements, as well as in cosmetic, pharmaceutical and human health sectors, among others (Caponio, Piga, & Poiana, 2022).

For the production of saffron spice, the current system produces a large amount of waste since only the flower stigmas are used, but the rest of the parts of the *Crocus sativus* L. plant are discarded. To obtain ≈1 kg of saffron spice, a large number of flowers (≈165,000–230,000) is needed, generating a large quantity of waste product, mostly constituted by tepals (≈78%).

However, recent studies have shown that saffron floral bio-residues contain important amounts of biologically active compounds such as flavonoids, anthocyanins, or various volatile compounds which present beneficial effects due to their potential antioxidant activities (Cerdá-Bernad, Baixinho, Fernández, & Frutos, 2022; Cerdá-Bernad, Clemente-Villalba, Valero-Cases, Pastor, & Frutos, 2022; Stelluti, Caser, Demasi, & Scariot, 2021; Zeka et al., 2020). Therefore, these functional saffron floral by-products would represent an important and promising source of natural bioactive antioxidant compounds that could be incorporated into functional foods. At the same time, the valorization of saffron floral bio-residues would lead to the minimization of their environmental impact taking advantage of a high-value biomass that is currently unexploited. In addition, it could contribute to improve the sustainability of the saffron spice production and the profitability of this industrial sector, representing also a new economic income for saffron farmers.

In order to protect valuable sensitive compounds susceptible to oxidation and degradation in the food matrix and to ensure their stability, encapsulation technologies could be an effective approach (de

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https://doi.org/10.1016/j.lwt.2023.114976
Received 17 February 2023; Received in revised form 6 June 2023; Accepted 10 June 2023
Available online 12 June 2023

Oliveira, Fraceto, Bravo, & Polanczyk, 2021). Many polymers are employed as wall materials for encapsulation to protect the bioactive compounds, such as sodium alginate, chitosan, several gums, starch and cellulose, among others (da S. Pereira, Souza, Moraes, Fontes-Sant'Ana, & Amaral, 2021). One of the biopolymers most widely used for food applications is sodium alginate due to its properties, since shows a simple gelation with divalent cations such as Ca^{2+} and it is cheap, biocompatible, biodegradable and Generally Recognized as Safe (GRAS) (Cattelan et al., 2020; Zabot et al., 2022).

Additionally, industries, such as dairy industries, are constantly seeking to develop innovative products through the use of food by-products, since consumers' demand for healthier foodstuffs has increased over the last years (Faustino et al., 2019). Therefore, the development of innovative dairy products enriched with saffron and its floral by-products could be one of the novel technological and environmentally friendly solutions for food industries (Reguengo, Salgaço, Sivieri, & Maróstica Júnior, 2022). Yogurts are one of the most suitable traditional dairy matrices for the fortification and development of new functional products, because are among highly-consumed food in the world due to their versatility and beneficial properties (Šeregelj et al., 2021). Yogurts, fermented by *Lactobacillus delbrueckii* spp. *bulgaricus* and *Streptococcus thermophilus*, are an excellent source of proteins, essential minerals and vitamins, improving the immune system and their consumption reduces the pH in stomach, decreasing the risk of pathogen transit (O'Connell & Fox, 2001). Thus, fermented milk products could be used as vehicles to deliver nutritional and bioactive components into human diet, being fortification of yogurt a good approach to improve its functional properties (Hashemi Gahruie, Eskandari, Mesbahi, & Hanifpour, 2015).

Therefore, this research aimed to stabilize saffron and its floral by-products extracts, rich in polyphenols, through their encapsulation in alginate beads, as well as to explore the possibility of developing fortified yogurt by including them in a traditional recipe formulation. To our knowledge, there is no available research highlighting the use of saffron stigmas and saffron floral by-products as high value-added ingredients in yogurt formulations. Therefore, the enrichment of yogurts by incorporating different concentrations of saffron and its floral extracts and the study of their physicochemical and functional properties were evaluated during storage at 4 °C (0, 7, 14 and 21 days).

2. Materials and methods

2.1. Plant material

Saffron flowers were obtained from Castilla-La Mancha (Spain), during the 2020 harvest season and cultivated following the requirements established by the Protected Designation of Origin "La Mancha Saffron". Fresh saffron floral by-products (composed by tepals, stamens and styles) were frozen in liquid nitrogen and kept at −80 °C until drying in a vacuum oven at 50 °C for 36 h to constant weight. Then, they were crushed, sieved (500 μm mesh size) and stored at −20 °C until further use. Saffron stigmas were supplied by the Spanish company Verdú Cantó Saffron Spain, S.L. being the moisture lower than 11%, crushed, sieved (500 μm mesh size) and stored at 4 °C until further use.

2.2. Preparation of extracts

Saffron floral by-products and saffron stigmas extracts were prepared using ultrapure water, in a sample/water ratio 1:50 (w/v). The extracts were shaken for 30 min in dark at 400 rpm on a magnetic stirrer at room temperature (Ovan, mod. MultiMix Heat D-MMH30E, Barcelona, Spain) and then sonicated in an ultrasonic bath (Ultrasons J.P. Selecta®, Barcelona, Spain) for 15 min and centrifuged at 11200×*g* for 10 min at 4 °C (Eppendorf Centrifuge 5804/5804R, Sigma Aldrich, St. Louis, MO, USA). Then, supernatants were filtered (0.45 μm PTFE filter, Millipore, Spain) and stored at −20 °C. All extractions were done in triplicate.

2.3. Encapsulation of extracts

Microcapsules loaded with extracts of saffron floral by-products and stigmas were prepared as described by Vinceković, Jurić, Đermić and Topolovec-Pintarić (2017) with some modifications. Briefly, different aqueous extracts were dissolved into 1.5 g/100 mL of sodium alginate solution (Sigma Aldrich, St. Louis, MO, USA) and homogenized by mixing on a magnetic stirrer at room temperature for 60 min. Blank samples were also prepared using ultrapure water. The mixtures were dropped through a 21G x 5/8" (0.8 × 16 mm) needle into 1 mol/L $CaCl_2$ solution (Scharlau; Barcelona, Spain) under constant magnetic stirring. To allow microcapsules hardening and strengthening, they were kept at room temperature for 30 min. The microcapsules were washed three times with distilled water to remove $CaCl_2$ excess and stored at 4 °C for further analysis. Part of microcapsules were stored at −20 °C during 24 h and freeze-dried in a freeze-dryer Christ Alpha 2-4 (B. Braun Biotech International, Melsungen, Germany) for 24 h (initial temperature −25 ± 2 °C and pressure 0.220 mbar), for further use.

2.4. Characterization of encapsulated extracts

2.4.1. Total phenolic content (TPC) and encapsulation efficiency

TPC were determined using the Folin-Ciocalteu methodology as described by Cerdá-Bernad, Clemente-Villalba, et al. (2022). Briefly, 100 μL of the different extracts were mixed with 400 μL of phosphate buffer (50 mmol/L) at pH 7.8 and 2.5 mL of Folin-Ciocalteu reagent previously mixed with ultrapure water 1:10 (v/v). After 2 min, 2 mL of Na_2CO_3 (75 g/L) were added and kept at 50 °C for 10 min. The absorbance was measured at 760 nm (UV/Vis spectrophotometer T80, PG Instruments Limited, Lutterworth, UK). The results were expressed as mg Gallic Acid Equivalents (GAE) per g of sample.

The encapsulation efficiency (EE) was performed according to Pasukamonset, Kwon and Adisakwattana (2016) with slight modifications. In brief, aliquots of 50 mg of microcapsules were dissolved in 2 mL of sodium citrate (5 g/100 mL), sonicated in an ultrasonic bath for 30 min, and centrifuged at 11200×*g* for 10 min at 4 °C. The encapsulation efficiency was calculated using Equation (1):

$$EE\ (\%) = TPCe/TPCi \quad (1)$$

where TPCe is the total phenolic content encapsulated in the microcapsules, while TPCi the total phenolic content in the initial aqueous extract solution of saffron floral by-products and saffron stigmas used for the encapsulation process.

2.4.2. Swelling behavior

The equilibrium swelling ratio after freeze-drying was determined by a conventional gravimetric method. The weight of the dried sample was accurately weighed, and soaked in deionized water. The swelling behavior was measured in phosphate buffered saline (PBS) solution (pH = 7.4), at room temperature, using Equation (2):

$$\text{Swelling ratio}\ (\%) = [(m_1 - m_0)/m_0]\ \text{x}\ 100 \quad (2)$$

where m_0 is the initial weight and m_1 is the mass swollen weight at time t. After the predetermined time points samples were extracted from the solution and weighted quickly.

2.5. Elaboration of yogurt formulations

Yogurts were made with UHT (Ultra High Temperature) whole cow milk (carbohydrates 4.6 g/100 mL, protein 3.1 g/100 mL and fat 3.6 g/100 mL) with 4% skim milk powder was used. To prepare the different formulations, encapsulated extracts were added in different concentrations, and seven lots of yogurts were then prepared: control yogurt without additional ingredients (YC), yogurt with the addition of 0.5 g/100 g (YB0.5) and 1 g/100 g (YB1) of blank encapsulated extracts,

yogurt with the addition of 0.5 g/100 g (YSF0.5) and 1 g/100 g (YSF1) of saffron floral by-products encapsulated extracts, yogurt with the addition of 0.5 g/100 g of saffron floral by-products encapsulated extracts combined with 0.05 g/100 g of saffron stigmas encapsulated extracts (YSF0.5SS0.05), and yogurt with the addition of 1 g/100 g of saffron floral by-products encapsulated extracts combined with 0.10 g/100 g of saffron stigmas encapsulated extracts (YSF1SS0.1).

The different formulations were pasteurized at 80 °C for 30 min and cooled in an ice bath to 43 °C to aseptically inoculate the starter culture of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (CHOOZIT™ MY800 LYO 5 DCU, Danisco, France), following the manufacturer's recommendations. All yogurts were prepared in triplicate. Fermentation was carried out in sterile polypropylene containers in an incubator at 43 °C until a pH of 4.6 was reached. They were then stored at 4 °C for 21 days.

2.6. Characterization of yogurt formulations

2.6.1. Physical-chemical properties: pH, acidity, total soluble solids, aw, colour, syneresis

The pH and titratable acidity (expressed as % lactic acid) were measured using an automatic titrator (TitroMatic Crison pH-Matic 23, Barcelona, Spain). For the titratable acidity (%), based on Equation (3), the titrant NaOH 1 mol/L was employed:

$$\text{Titratable acidity (\% lactic acid)} = [(V \text{ x } N \text{ x } 0.09008)/W] \text{ x } 100 \quad (3)$$

where V is the volume used of NaOH, N is the normality of titrant, 0.09008 is the milliequivalent weight for lactic acid, and W is the weight of sample used.

The determination of total soluble solids (TSS) was carried out with a digital refractometer (Hanna® HI 96801, Bedfordshire, UK) and expressed as °Brix.

The water activity (aw) of the different samples was determined using a water activity meter (Novasina AW Sprint TH 500, Pfäffikon, Switzerland) at room temperature.

The colour was measured with a Minolta CR-300 Chroma Meter (Japan) colorimeter, using L*, a*, b* scale (CIELAB system). The results were expressed as luminosity L*, a* (greenness/redness), b* (blueness/yellowness), total colour difference (ΔE), Hue angle (h°), Chroma (C*) and browning index (BI) which were calculated according to the following Equations 4, 5, 6 and 7, respectively (Milovanovic et al., 2020):

$$\Delta E = \sqrt{(L*_0 - L*_1)^2 + (a*_0 - a*_1)^2 + (b*_0 - b*_1)^2} \quad (4)$$

where $L*_0$, $a*_0$, $b*_0$ are the values of the control sample (YC) at time *t* of storage, while $L*_1$, $a*_1$ and $b*_1$ are the corresponding values for each yogurt sample at time *t* of storage.

$$h^\circ = \left(\arctan\left(\frac{b^*}{a^*}\right)\right) x \frac{180}{\pi} \quad (5)$$

$$C^* = \left(a^{*2} + b^{*2}\right)^{1/2} \quad (6)$$

$$BI = \frac{[100(x - 0.31)]}{0.17}, \text{ where } x = \frac{(a* + 1.75L*)}{(5.645L* + a* - 3.012b*)} \quad (7)$$

The syneresis of the yogurts was determined according to Lima et al. (2021), centrifuging 10 g of yogurt at 176×*g* for 20 min at 4 °C. The syneresis (%) was estimated as the weight of the supernatant released over the weight of the initial yogurt x 100.

2.6.2. Preparation of yogurt extraction

To study the antioxidant properties, total phenolic content and organic acids and soluble sugars composition, yogurt extracts were prepared following the method described by Tizghadam, Roufegari-nejad, Asefi and Jafarian Asl (2021). Ten grams of each yogurt formulation were mixed with 2.5 mL of distilled water, and 0.1 mol/L HCl was used to adjust the pH to 4. During 45 min, the mixture was kept in a water bath (45 °C) and then centrifuged at 11200×*g* for 10 min at 4 °C. The supernatants were adjusted to pH 7 using 0.1 mol/L NaOH, filtered (0.45 μm PTFE filter, Millipore, Spain) and stored at −20 °C. All extractions were done in triplicate.

2.6.3. Antioxidant properties

The antioxidant capacity was determined by using the Ferric Reducing Antioxidant Power (FRAP) method, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging method and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method as described by Cerdá-Bernad, Clemente-Villalba, et al. (2022). Briefly, the FRAP reagent was prepared by mixing 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) solution in 40 mmol/L HCl, and 20 mmol/L $FeCl_3 \cdot 6H_2O$ solution in a volume ratio of 10:1:1, respectively. The absorbance was measured at 593 nm and Trolox (10 mmol/L) was used as standard solution (0.01–5.00 mmol/L). The ABTS radical was prepared mixing ABTS (7 mmol/L) with potassium persulfate (2.45 mmol/L), reacting for 16 h in dark at room temperature and diluting the solution with ultrapure water until its absorbance was adjusted to 0.70 ± 0.02 at 734 nm. Trolox (10 mmol/L) was used as a reference standard (0.20–3.00 mmol/L). The DPPH radical was prepared dissolving 0.0035 g with 10 mL of methanol, reacting for 30 min in dark at room temperature. The absorbance decrease was measured at 515 nm and Trolox (10 mmol/L) was used as a reference standard (0.50–4.00 mmol/L). The antioxidant capacity results were expressed as mmol of Trolox Equivalents (TE) per 100 g of sample. All the reagents used were purchased from Sigma Aldrich (St. Louis, MO, USA).

For TPC analysis in the different formulations of yogurts, the same methodology explained in 2.4.1 was followed. The results were expressed as mg GAE per 100 g of sample.

2.6.4. Organic acids, soluble sugars and inulin content

The identification and quantification of sugars, inulin and organic acids was made by high-performance liquid chromatography using a Hewlett-Packard HPLC series 1100 equipped with a Supelcogel C-610H column (30 cm x 7.8 mm) and a Supelcoguard C-610H pre-column (5 cm x 4.6 mm) (Sigma Aldrich, St. Louis, MO, USA). The organic acids were measured at 210 nm in UV–Vis with diode array detector (DAD G1315A). For sugars, a refractive index detector (G1362A RID) was used. As a mobile phase, 0.1 g/100 mL orthophosphoric acid was used with an injection volume of 20 μl and the flow rate of 0.5 mL/min under isocratic conditions, following the methodology described by Cerdá-Bernad, Valero-Cases, Pastor, Frutos and Pérez-Llamas (2020). The concentrations were calculated through calibration curves with the standards for sugars and organic acids (Sigma Aldrich, St. Louis, MO, USA). The results were expressed as mg/g of yogurt.

2.6.5. Microbiological evaluation

Yogurts were evaluated for microbiological analysis, weighting 1 g of each yogurt formulation individually, transferred to sterile polyethylene bags with 9 mL of peptone (Oxoid, Unipath Ltd., Basingstoke, UK) and homogenized for 1 min in a stomacher at room temperature. Then, appropriate dilutions were prepared for the following bacteriological determinations: (i) *Streptococcus thermophilus*, on spread plates of M17 agar and (ii) *L. delbrueckii* subsp. *lactis* y L. *delbrueckii* subsp. *bulgaricus* on spread plates of MRS Agar, both incubated at 42 °C and 37 °C, respectively, for 48 h in an anaerobic jar with the AnaeroGen system (Oxoid S.A., Madrid, Spain). The results were expressed as Log CFU (Colony-forming unit) per gram.

Moulds and yeasts were also determined in 3M™ Petrifilm™ yeast and mould count plates (3 M, Minnesota, MN, USA). The plates were incubated under aerobic conditions at 25 °C during 48–72 h for yeasts

and 72–140 h for moulds.

All these analyses were carried out on the day the yogurts were prepared (time 0) and after 7, 14, 21 days of storage at 4 °C.

2.7. Statistical analysis

Results were expressed as mean ± standard deviation. The mean comparisons were carried out using the one-way analysis of variance (one-way ANOVA) and by the Tukey's multiple range test, using SPSS version 21.0 software package (SPSS Inc., Chicago, IL). Principal Component Analysis (PCA) was conducted using XLSTAT (Microsoft Corp., Washington, DC). The significant differences were established as ($P \leq 0.05$).

3. Results and discussion

3.1. Characterization of beads containing saffron floral extracts

The encapsulation efficiency, defined by the concentration of the encapsulated material over the initial concentration used in the formulation, is one of the most critical parameters to characterize the quality of the developed microcapsules to ensure their functional properties in the final product. Then, for a successful encapsulation method, a high content and retention of the core materials must be achieved. The TPC in the encapsulated beads containing saffron floral by-products or saffron stigmas aqueous extracts were 1.243 mg GAE per g of dry beads and 1.065 mg GAE per g of dry beads, respectively. Furthermore, the EE was 55.66% in beads with saffron floral by-products and 67.55% in saffron stigmas encapsulates.

Furthermore, swelling behavior is a relevant factor of encapsulates for their practical use in food products. The swelling behavior of alginate beads as a function of time obtained is shown in Fig. 1. The results indicated that the swelling percentage of beads increased up to 3407%, 3050% and 2672 after 3 h, for blank, saffron floral by-products and saffron stigmas, respectively, presenting all the samples the same tendency regardless of the encapsulated extract. However, after 4 h, the swelling behavior could not be measured because all beads were disintegrated. This fact could be explained due to the presence of sodium ions in PBS which enter into the bead matrix and ionic exchange occurs with calcium ions, leading to increased swelling by relaxation of alginate chains. Then, the strong hydrophilicity of sodium alginate, in addition to the ion–exchange process between Na^{+} and Ca^{+2} ions, allow to a higher water-holding capacity and enhance the structural integrity of beads. Nevertheless, these ionic interactions influence in the subsequent degradation of beads, since the alginate chains begin to disintegrate and dissolve in the medium, because of the disruption of the 'egg-box' cavities (Bajpai & Tankhiwale, 2006; Hou & Wu, 2019).

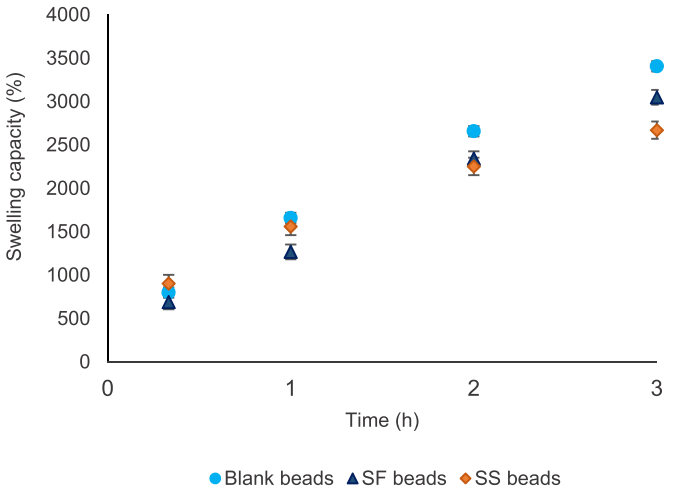

Fig. 1. Swelling behavior as a function of time of the developed beads in PBS at room temperature. The error bars represent the standard deviation (n = 3). SF: saffron floral by-products; SS: saffron stigmas.

3.2. Characterization of fortified yogurts formulations

3.2.1. Metabolism of organic acids, sugars, and physical-chemical properties of the different yogurt formulations during storage

Organic acids and soluble sugars have an effective role in terms of sensory properties and as preservatives in yogurt production. Table 1 shows organic acids, soluble sugars and inulin content in different yogurt formulations during the 21 days of refrigerated storage.

Important organic acids were present in the different yogurt formulations, as a result of the metabolism of lactic acid bacteria such as lactic acid, formic acid and propionic acid. Lactic acid was the main compound produced, and it was found in the highest concentration in all samples (1.51–2.73 mg/g of yogurt). Furthermore, in some yogurt formulations, such as YC, YB0.5, YB1 and YSF0.5, there was an increase of this organic acid over time, showing statistically significant higher amounts after 21 days compared to time 0. This increase of lactic acid was due to fermentation by LAB, using lactose to produce lactic acid. However, in YSF1, YSF0.5SS0.05 and YSF1SS0.1, the concentration of lactic acid remained stable during all the storage period, without statistically significant differences after 21 days, compared to the control yogurt (YC). Therefore, the encapsulated saffron and its floral by-products extracts did not affect this natural fermentation process in the novel developed yogurt formulations.

Formic acid was the second predominant organic acid in several yogurt formulations, such as YC, YB0.5, YB1, YSF0.5 and YSF0.5SS0.05 (0.74–1.96 mg/g of yogurt). In the fermentation process of yogurt, formic acid is produced by homofermentative metabolic pathway due to the action of starter cultures that metabolize carbohydrates present in milk (Vénica, Perotti, & Bergamini, 2014). However, in yogurt formulations with 1 g/100 g of encapsulated saffron floral by-products extracts (YSF1, YSF1SS0.1), there is a lower production of formic acid (0.35–0.76 mg/g of yogurt), which may be due to the fact that LAB, in addition to milk nutrients, could metabolize alternatively other molecules present in saffron flowers and produce different compounds. Besides, formic acid content fluctuated during the storage period in all samples, suggesting the production and consumption of this organic acid by the bacterial metabolism (da Costa, Frasao, Lima, Rodrigues, & Junior, 2016).

Propionic and citric acid were also identified in lower amounts. Citric acid, which is a predominant organic acid in milk, decreased during the storage period, and after 7 days, it was not detected in yogurt formulations, indicating its use by yogurt starter cultures. Regarding propionic acid, which was generated as the result of the metabolic activity of LAB, was not detected in some yogurt samples (YB0.5, YB1) after 21 days of storage at 4 °C, while in the rest of formulations, there was a fluctuation in its concentration, being statistically significantly lower after 21 days compared to time 0 in each sample. Therefore, these changes also suggest the generation and metabolization of propionic acid by LAB starter culture during the refrigerated storage period.

These results were in accordance to other studies that reported the presence of lactic, formic and citric acid in goat's milk yogurts, being lactic acid the most abundant one (4.75 mg/g) with varying formic and citric acid concentrations during fermentation (da Costa et al., 2016). Ndhlala, Kavaz Yüksel and Yüksel (2022) also indicated the content of lactic, citric and propionic acids in enriched yogurts with Jerusalem artichoke tubers, also changing their concentration during the storage time.

Therefore, these changes in organic acid content resulted in different pH and titratable acidity values during the storage period at 4 °C. Table 2 shows physicochemical parameters of the different yogurts incorporated with microencapsulated saffron floral extracts during the 21 days of the storage period. Statistical evaluations showed differences

Table 1
Content of soluble sugars, inulin and organic acids (mg/g) of different yogurt formulations developed during 21 days of storage at 4 °C.

	Time (days)	YC	YB0.5	YB1	YSF0.5	YSF1	YSF0.5SS0.05	YSF1SS0.1
Citric acid	0	0.17 ± 0.01^{b}	0.18 ± 0.00^{b}	0.16 ± 0.01^{b}	0.18 ± 0.00^{b}	0.18 ± 0.02^{b}	0.24 ± 0.00^{Aa}	0.18 ± 0.00^{Bb}
	7	n.d.	0.18 ± 0.03^{bc}	0.18 ± 0.00^{bc}	0.19 ± 0.00^{b}	0.17 ± 0.00^{c}	0.19 ± 0.00^{Bb}	0.30 ± 0.00^{Aa}
	14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lactic acid	0	1.76 ± 0.08^{Cb}	1.84 ± 0.02^{Cb}	1.51 ± 0.00^{Cb}	1.81 ± 0.00^{Db}	1.82 ± 0.35^{ab}	2.36 ± 0.04^{a}	1.90 ± 0.14^{Bab}
	7	2.71 ± 0.02^{Aa}	2.52 ± 0.07^{Bab}	2.13 ± 0.06^{Bde}	2.44 ± 0.01^{Abc}	2.08 ± 0.00^{e}	2.30 ± 0.01^{cd}	2.69 ± 0.07^{Aa}
	14	2.51 ± 0.11^{Aa}	2.58 ± 0.05^{Ba}	1.98 ± 0.00^{Bc}	2.07 ± 0.00^{Cbc}	1.92 ± 0.00^{c}	2.15 ± 0.00^{b}	1.72 ± 0.00^{B}
	21	2.23 ± 0.06^{Bbc}	3.19 ± 0.02^{Aa}	2.73 ± 0.07^{Aab}	2.20 ± 0.00^{Bbc}	1.96 ± 0.00^{c}	2.27 ± 0.00^{bc}	1.78 ± 0.00^{Bc}
Formic acid	0	1.48 ± 0.00^{Bb}	1.21 ± 0.03^{Bc}	0.74 ± 0.00^{Bd}	1.15 ± 0.02^{Bc}	0.40 ± 0.08^{Bf}	1.87 ± 0.03^{Aa}	0.56 ± 0.05^{e}
	7	1.96 ± 0.01^{Aa}	1.52 ± 0.03^{ABb}	0.92 ± 0.05^{Ad}	1.16 ± 0.02^{Ac}	0.35 ± 0.06^{Be}	1.48 ± 0.05^{Bb}	0.76 ± 0.10^{d}
	14	1.44 ± 0.05^{Ba}	1.37 ± 0.01^{ABa}	0.87 ± 0.03^{ABb}	0.99 ± 0.00^{Cb}	0.60 ± 0.04^{Ac}	1.46 ± 0.08^{Ba}	0.54 ± 0.04^{c}
	21	1.20 ± 0.00^{Cc}	1.62 ± 0.01^{Aa}	0.94 ± 0.03^{Ad}	0.82 ± 0.00^{De}	0.46 ± 0.01^{Bg}	1.42 ± 0.00^{Bb}	0.61 ± 0.05^{f}
Propionic acid	0	0.51 ± 0.12^{Ab}	0.58 ± 0.21^{b}	0.62 ± 0.07^{b}	0.54 ± 0.05^{ABb}	0.57 ± 0.04^{ABb}	0.62 ± 0.03^{Aab}	0.92 ± 0.06^{Aa}
	7	0.77 ± 0.18^{A}	0.78 ± 0.19	0.60 ± 0.02	0.86 ± 0.23^{A}	0.67 ± 0.09^{A}	0.55 ± 0.08^{AB}	0.87 ± 0.05^{A}
	14	0.55 ± 0.11^{Aab}	0.70 ± 0.20^{a}	0.39 ± 0.19^{b}	0.29 ± 0.00^{Bb}	0.39 ± 0.04^{BCb}	0.36 ± 0.00^{Cb}	0.43 ± 0.00^{Bab}
	21	0.14 ± 0.08^{Bb}	n.d.	n.d.	0.21 ± 0.00^{Bb}	0.31 ± 0.04^{Cab}	$0.47 \pm 0.06B^{Ca}$	0.42 ± 0.06^{Ba}
Lactose	0	12.86 ± 0.02^{Bb}	12.99 ± 0.00^{Cb}	13.51 ± 0.01^{Cb}	13.44 ± 0.00^{Bb}	16.08 ± 0.28^{Aa}	17.96 ± 0.08^{Aa}	16.39 ± 0.37^{Aa}
	7	18.15 ± 0.11^{Aa}	16.82 ± 0.28^{Bb}	17.18 ± 0.14^{Bb}	16.61 ± 0.04^{Ab}	16.32 ± 0.19^{Ab}	16.36 ± 0.09^{Bb}	16.46 ± 0.54^{Ab}
	14	17.08 ± 0.66^{Aa}	16.72 ± 0.17^{Ba}	13.53 ± 0.04^{Cb}	12.57 ± 0.01^{Cb}	13.58 ± 0.06^{Bb}	12.96 ± 0.22^{Cb}	14.10 ± 0.01^{Bb}
	21	12.79 ± 0.06^{Bb}	19.12 ± 0.88^{Aa}	18.85 ± 0.58^{Aa}	12.95 ± 0.06^{Cb}	13.40 ± 0.02^{Bb}	13.20 ± 0.03^{Cb}	13.60 ± 0.00^{Bb}
Glucose	0	0.28 ± 0.00^{B}	0.26 ± 0.01^{C}	0.30 ± 0.00^{D}	0.27 ± 0.00^{C}	0.34 ± 0.03	0.32 ± 0.00^{A}	0.36 ± 0.10^{B}
	7	0.33 ± 0.00^{Ad}	0.30 ± 0.00^{Be}	0.41 ± 0.00^{Bb}	0.31 ± 0.00^{Ade}	0.38 ± 0.00^{c}	0.31 ± 0.00^{Ade}	0.91 ± 0.01^{Aa}
	14	0.33 ± 0.00^{Ab}	0.30 ± 0.00^{Bd}	0.36 ± 0.01^{Cb}	0.28 ± 0.00^{Cd}	0.36 ± 0.00^{b}	0.29 ± 0.00^{Bd}	0.52 ± 0.00^{Ba}
	21	0.30 ± 0.00^{Bc}	0.35 ± 0.01^{Ac}	0.44 ± 0.01^{Aab}	0.29 ± 0.00^{Bc}	0.36 ± 0.00^{bc}	0.30 ± 0.00^{Bc}	0.50 ± 0.00^{Ba}
Galactose	0	1.95 ± 0.01^{Cb}	1.93 ± 0.00^{Cb}	1.67 ± 0.00^{Db}	1.98 ± 0.00^{Db}	2.08 ± 0.47^{ab}	2.76 ± 0.04^{Aa}	2.15 ± 0.27^{Bab}
	7	3.25 ± 0.05^{Aa}	2.90 ± 0.07^{Bb}	2.57 ± 0.04^{Bcd}	2.81 ± 0.02^{Ab}	2.46 ± 0.04^{d}	2.69 ± 0.00^{Abc}	3.25 ± 0.08^{Aa}
	14	3.02 ± 0.15^{aA}	2.94 ± 0.02^{Ba}	2.14 ± 0.02^{Cbc}	2.21 ± 0.00^{Cbc}	2.14 ± 0.00^{bc}	2.29 ± 0.00^{Cb}	2.52 ± 0.00^{Bc}
	21	2.42 ± 0.02^{Bb}	3.67 ± 0.23^{Aa}	3.20 ± 0.10^{Aa}	2.35 ± 0.00^{Bb}	2.17 ± 0.00^{bc}	2.39 ± 0.00^{Bb}	2.10 ± 0.00^{Bc}
Inulin	0	0.68 ± 0.00^{Bf}	1.14 ± 0.00^{Be}	1.64 ± 0.00^{Cbc}	1.17 ± 0.00^{Ade}	1.93 ± 0.30^{b}	1.54 ± 0.04^{Acd}	2.54 ± 0.05^{Ba}
	7	0.84 ± 0.02^{Ae}	1.24 ± 0.04^{ABd}	1.95 ± 0.03^{Bb}	1.17 ± 0.00^{Ad}	1.80 ± 0.03^{c}	1.24 ± 0.00^{Bd}	2.70 ± 0.04^{Aa}
	14	0.78 ± 0.03^{Ae}	1.21 ± 0.00^{ABc}	1.85 ± 0.03^{Bb}	1.09 ± 0.00^{Cd}	1.80 ± 0.00^{b}	1.16 ± 0.00^{Cc}	2.01 ± 0.00^{Ca}
	21	0.68 ± 0.00^{Bf}	1.29 ± 0.09^{Ad}	2.20 ± 0.05^{Aa}	1.15 ± 0.00^{Be}	1.80 ± 0.00^{c}	$1.21 \pm 0.01B^{Cde}$	1.97 ± 0.01^{Cb}

Means ± standard deviation in the same column followed by different uppercase letters indicate statistically significant differences at ($P \leq 0.05$) for each yogurt sample at different storage time (n = 3). Means ± standard deviation in the same row followed by different lowercase letters indicate statistically significant differences at ($P \leq 0.05$) between yogurt samples at the same storage time (n = 3); YC: control yogurt without additional ingredients; YB0.5 and YB1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of blank encapsulated extracts;YSF0.5 and YSF1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of saffron floral by-products encapsulated extracts; YSF0.5SS0.05: yogurt with the addition of 0.5 g/100 g of saffron floral by-products encapsulated extracts combined with 0.05 g/100 g of saffron stigmas encapsulated extracts; YSF1SS0.1: yogurt with the addition of 1 g/100 g of saffron floral by-products encapsulated extracts combined with 0.10 g/100 g of saffron stigmas encapsulated extracts; n.d.: not detected.

in the pH of each yogurt sample ($P < 0.05$) at time 0, respect to time 7, with decreasing the pH values. However, after 7 days of storage, no significant differences were observed until the end of the storage period. This pH decline is related to the lactose fermentation, leading to the production of organic acids, with subsequent lower pH values (Table 1). However, titratable acidity (%) did not show a statistical change within the different storage periods and between the different yogurt formulations, and values remained stable (0.26–0.41%). This fact may be due to the presence of some predominant organic acids formed in each storage period evaluated. Then, the continued metabolic activity of starter cultures led to deep changes in the chemical and physical attributes of yogurt formulations after the fermentation and during the refrigerated storage period, influencing on the decrease of the initial pH (4.50–4.61) to a more acidic values at day 7 (4.15–4.46). Other research observed similar results in yogurts supplemented with the maca plant (*Lepidium meyenii*) and propolis in which pH level decreased significantly from day 1–7 of storage (Korkmaz, Bilici, & Korkmaz, 2021).

As it is shown in Table 1, during the lactic fermentation process, the concentration of lactose decreased significantly over the storage time due to its conversion into lactic acid by LAB, in YSF0.5, YSF1, YSF0.5SS0.05 and YSF1SS0.1. However, lactose was not fully converted and remained stable in YC or significantly increased in blank samples (YB0.5, YB1). In addition to lactose, which was the most predominant soluble sugar in all yogurt samples (12.57–18.85 mg/g), glucose and galactose were also presented in lower concentrations (0.26–0.91 mg/g and 1.67–3.25 mg/g, respectively). Both monosaccharides were not detected in milk, but were identified in the yogurt formulations as a result of hydrolysis of lactose by the bacterial metabolic activity (Vénica et al., 2014). The concentration of both soluble monosaccharides remained stable after 21 days of storage and in some samples a slight increase was observed. Nevertheless, the concentration of galactose monosaccharide was higher than that of glucose which was very low, since galactose was not metabolized by the starter microorganisms due to the lack of essential enzymes involved in this carbohydrate metabolism.

It should be noted that these novel enriched yogurts with microencapsulated plant extracts are formulations without added sugar, since they present naturally milk sugars as lactose, as well as glucose and galactose. Besides, they are low in carbohydrates, because the most relevant sugar is lactose with levels lower than 2%. Therefore, these new yogurts with saffron flowers could be new alternatives for people with diabetes, which is the most common metabolic disease in the 21st century (Mostafai et al., 2018). In addition to the nutritional properties of yogurts, these new healthy alternatives contain saffron that could present anti-diabetic potential. Several researches reported that this natural product may exert hypoglycemic effects by different mechanisms of action (Sani et al., 2022; Yaribeygi, Zare, Butler, Barreto, & Sahebkar, 2019).

Moreover, the content of inulin as part of soluble dietary fiber was studied. The inulin concentration did not change in any of the yogurt

Table 2
Physicochemical parameters of different yogurt formulations developed during 21 days of storage at 4 °C.

	Time (days)	YC	YB0.5	YB1	YSF0.5	YSF1	YSF0.5SS0.05	YSF1SS0.1
pH	0	4.51 ± 0.06^{Ab}	4.50 ± 0.06^{Ab}	4.58 ± 0.07^{Aa}	4.59 ± 0.07^{Aa}	4.59 ± 0.07^{Aa}	4.59 ± 0.06^{Aa}	4.61 ± 0.07^{Aa}
	7	4.15 ± 0.06^{Bb}	4.14 ± 0.00^{Bb}	4.39 ± 0.01^{Ba}	4.16 ± 0.01^{Bb}	4.38 ± 0.01^{Ba}	4.18 ± 0.02^{Bb}	4.46 ± 0.03^{Ba}
	14	4.17 ± 0.08^{Bb}	4.17 ± 0.04^{Bb}	4.35 ± 0.03^{Ba}	4.20 ± 0.03^{Bb}	4.39 ± 0.01^{Ba}	4.22 ± 0.02^{Bb}	4.42 ± 0.10^{Ba}
	21	4.07 ± 0.04^{Bb}	4.02 ± 0.03^{Bb}	4.24 ± 0.01^{Ba}	4.08 ± 0.02^{Bb}	4.28 ± 0.04^{Ba}	4.06 ± 0.03^{Bb}	4.26 ± 0.07^{Ba}
TA (% Lactic acid)	0	0.30 ± 0.00^{c}	0.41 ± 0.01^{a}	0.35 ± 0.00^{b}	0.31 ± 0.00^{c}	0.30 ± 0.00^{c}	0.35 ± 0.00^{b}	0.26 ± 0.00^{d}
	7	0.31 ± 0.05	0.39 ± 0.05	0.37 ± 0.07	0.32 ± 0.07	0.34 ± 0.03	0.41 ± 0.07	0.31 ± 0.03
	14	0.33 ± 0.04	0.33 ± 0.09	0.35 ± 0.07	0.37 ± 0.07	0.32 ± 0.03	0.40 ± 0.09	0.29 ± 0.05
	21	0.31 ± 0.01	0.38 ± 0.03	0.34 ± 0.00	0.38 ± 0.04	0.37 ± 0.07	0.37 ± 0.05	0.31 ± 0.07
Water activity (aw)	0	0.89 ± 0.00	0.89 ± 0.02	0.89 ± 0.04^{B}	0.89 ± 0.05^{B}	0.89 ± 0.00	0.89 ± 0.04^{B}	0.89 ± 0.03^{B}
	7	0.90 ± 0.01^{b}	0.90 ± 0.01^{b}	0.98 ± 0.03^{Aa}	0.97 ± 0.01^{Aa}	0.90 ± 0.00^{b}	0.96 ± 0.01^{Aa}	0.97 ± 0.01^{Aa}
	14	0.89 ± 0.00	0.89 ± 0.00	0.90 ± 0.01^{B}	0.90 ± 0.02^{B}	0.89 ± 0.01	0.89 ± 0.02^{B}	0.89 ± 0.04^{B}
	21	0.89 ± 0.02	0.89 ± 0.01	0.90 ± 0.02^{B}	0.90 ± 0.01^{B}	0.89 ± 0.01	0.89 ± 0.05^{B}	0.89 ± 0.07^{B}
TSS (°Brix)	0	8.08 ± 0.11^{d}	8.38 ± 0.11^{Ad}	9.39 ± 0.13^{Ab}	8.89 ± 0.10^{Ac}	9.09 ± 0.23^{bc}	7.98 ± 0.33^{d}	9.90 ± 0.15^{Aa}
	7	8.05 ± 0.09^{c}	8.38 ± 0.11^{Abc}	8.35 ± 0.12^{Bbc}	8.01 ± 0.22^{Bc}	8.72 ± 0.05^{b}	8.35 ± 0.13^{bc}	9.26 ± 0.17^{Ba}
	14	8.05 ± 0.10^{c}	8.08 ± 0.09^{Bc}	8.38 ± 0.12^{Babc}	8.18 ± 0.06^{Bbc}	8.75 ± 0.08^{a}	8.21 ± 0.08^{bc}	8.59 ± 0.33^{Cab}
	21	8.01 ± 0.11^{b}	8.21 ± 0.10^{Ab}	8.82 ± 0.20^{Ba}	8.28 ± 0.08^{Bb}	8.85 ± 0.15^{a}	8.21 ± 0.11^{b}	9.02 ± 0.14^{Ca}
Syneresis (%)	0	17.27 ± 0.14^{Bc}	19.06 ± 0.27^{Ab}	20.93 ± 0.19^{Aa}	19.89 ± 0.18^{Aab}	20.69 ± 0.29^{Aa}	20.20 ± 0.43^{Aa}	20.76 ± 0.34^{Aa}
	7	18.83 ± 0.26^{Ab}	18.77 ± 0.26^{Ab}	19.08 ± 0.27^{Bb}	19.83 ± 0.15^{Aab}	19.58 ± 0.18^{Bab}	19.59 ± 0.13^{Aab}	20.47 ± 0.21^{Aa}
	14	19.02 ± 0.37^{A}	18.64 ± 0.26^{A}	18.52 ± 0.46^{B}	19.03 ± 0.20^{B}	18.93 ± 0.09^{C}	19.33 ± 0.23^{A}	19.32 ± 0.13^{B}
	21	18.43 ± 0.21^{Aa}	17.15 ± 0.24^{Bb}	17.80 ± 0.25^{Cab}	18.40 ± 0.26^{Ba}	18.22 ± 0.12^{Ca}	17.73 ± 0.07^{Bab}	18.71 ± 0.38^{Ba}

Means ± standard deviation in the same column followed by different uppercase letters indicate statistically significant differences at ($P \leq 0.05$) for each yogurt sample at different storage time (n = 3). Means ± standard deviation in the same row followed by different lowercase letters indicate statistically significant differences at ($P \leq 0.05$) between yogurt samples at the same storage time (n = 3); TA: titratable acidity; TSS: total soluble sugars; YC: control yogurt without additional ingredients; YB0.5 and YB1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of blank encapsulated extracts;YSF0.5 and YSF1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of saffron floral by-products encapsulated extracts; YSF0.5SS0.05: yogurt with the addition of 0.5 g/100 g of saffron floral by-products encapsulated extracts combined with 0.05 g/100 g of saffron stigmas encapsulated extracts; YSF1SS0.1: yogurt with the addition of 1 g/100 g of saffron floral by-products encapsulated extracts combined with 0.10 g/100 g of saffron stigmas encapsulated extracts.

formulations during storage because LAB metabolized monosaccharides as the main energy sources. Besides, at time 0 samples with saffron floral by-products extracts at 1 g/100 g presented the highest inulin content respect to the other formulations due to the rich fiber source of saffron flowers (1.93 and 2.54 mg/g, respectively). At the same time, inulin contributed to an increase in TSS, since yogurts with the highest inulin concentration presented higher TSS than control samples, such as YSF1 and YSF1SS0.1, with values around 9 °Brix (Table 2). This increase in TSS could be due to the presence of fructose in the composition of inulin.

These findings were also corroborated with a Principal Component Analysis (PCA) (Fig. 3 and Fig. S1). The PCA biplot graph (Fig. 3A) showed that the yogurt formulation YSF0.5SS0.05, at day 0, was associated to the content of citric and lactic acids, galactose and lactose. Yogurt formulations with 1 g/100 g of saffron floral by-products microencapsulated extracts, YSF1 and YSF1SS0.1, were mainly related to propionic acid and inulin content. However, after 21 days of storage (Fig. 3B), YB0.5 was the yogurt formulation associated to lactic acid, lactose and galactose, changing the composition of organic acids and soluble sugars in yogurt formulations during storage and together with the physical-chemical parameters, due to the continued metabolic activity of LAB starter cultures. Moreover, it can be also observed that yogurt formulations with 0.5 g/100 g of microencapsulated saffron floral by-products extracts YSF0.5 and YSF0.5SS0.05, had similar physicochemical characteristics and content of water-soluble compounds to the control sample YC, but they were not associated with any specific characteristic.

Regarding the physicochemical parameters, water activity and syneresis were also determined (Table 2). The water activity was not affected by either the storage period or by the yogurt formulation as interpreted statistically, since no significant differences were found between the different yogurt formulations at time 0, 14 and 21 days, nor between time 0 and 21 days of storage in each sample, being the values in the range 0.89–0.98. Similar values (0.99) were shown in previous research in yogurts incorporating preservative agents from sage and basil (Ueda et al., 2021). The syneresis ratio (serum release), a quality indicator of yogurts during storage, significantly increased in the control sample (YC) after 7 days of storage, but remained stable in YB0.5, YSF0.5 and YSF0.5SS0.05 and YSF1SS0.1 samples. After 21 days of storage, the syneresis ratio (%) significantly decreased in all samples compared to time 0, except for YC. However, at time 21 days no significant differences were found between all the yogurt formulations, with values between 17 and 18%. Initial syneresis of yogurts decreased over the time, and values were lower at the end of storage because of the pH reduction during the first 7 days, leading to a higher serum release due to the contracting effect on the casein micelle matrix (Akgün et al., 2020).

3.2.2. *Microbiological evaluation of the different yogurt formulations during storage*

In addition to carrying out a physicochemical stability evaluation to confirm that these novel yogurt formulations with microencapsulated saffron flower extracts remained with similar characteristics during storage, it was necessary to determine if, at the same time, they maintained suitable concentrations of *Lactobacillus* and *Streptococcus* starter cultures. The results of Fig. 2 showed that after 21 days of refrigerated storage, no significant differences were observed in the survival of *Lactobacillus* sp. and *Streptococcus* sp. in any of the yogurt formulations and in any of the storage periods, remaining the concentrations stable (5-6 Log CFU/g and 8-10 Log CFU/g, respectively). Therefore, yogurts fortified with saffron stigmas and saffron floral by-products were good matrices to reach a high concentration of LAB starter cultures, however, it was not possible to determine whether or not the saffron flowers can act as a prebiotic, given its encapsulated form, but they did not affect the survival of LAB. Other studies also reported that the supplementation of yogurt with chia seeds did not alter the viability of LAB, and in yogurts with the maca plant (*Lepidium meyenii*) the concentration of *Lactobacillus* remained stable during 7 days of storage (Korkmaz et al., 2021; Kowaleski et al., 2020).

Respect to the microbiological analysis of moulds and yeasts, they were not detected in any sample (<10 CFU/g) throughout the storage period, showing a good hygienic-sanitary quality during the storage for at least 21 days at 4 °C.

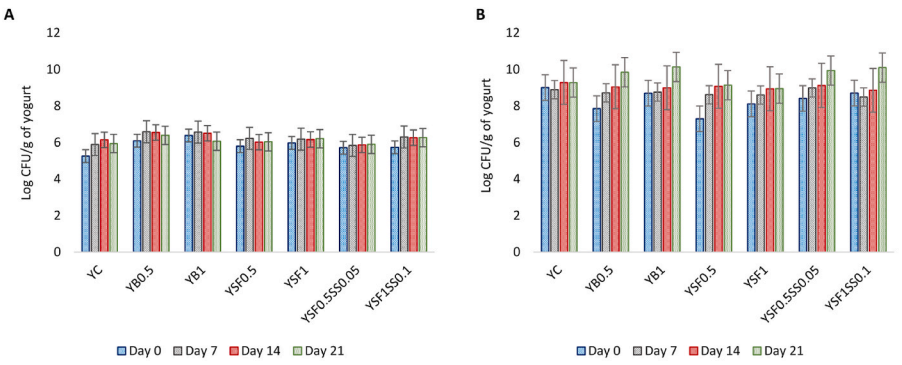

Fig. 2. **A** Viability of *Lactobacillus* sp. and **B** *Streptococcus* sp. (Log CFU/g) in different yogurt formulations during the refrigerated storage period. The error bars represent the standard deviation (n = 3). Non-labeling with different letters indicates statistically non-significant differences at ($P > 0.05$) for each yogurt sample at different storage time and between yogurt samples at the same storage time. YC: control yogurt without additional ingredients; YB0.5 and YB1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of blank encapsulated extracts;YSF0.5 and YSF1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of saffron floral by-products encapsulated extracts; YSF0.5SS0.05: yogurt with the addition of 0.5 g/100 g of saffron floral by-products encapsulated extracts combined with 0.05 g/100 g of saffron stigmas encapsulated extracts; YSF1SS0.1: yogurt with the addition of 1 g/100 g of saffron floral by-products encapsulated extracts combined with 0.10 g/100 g of saffron stigmas encapsulated extracts.

3.2.3. *Colour of the different yogurt formulations during storage*

Colour is also an important attribute to be evaluated in yogurts, since it is the first characteristic perceived by consumers. Table S1 shows the changes in colour parameters (L*, a*, b*, ΔE, Chroma, Hue angle and BI) of the different yogurt formulations during 21 days of storage period at 4 °C. Regarding the L* index, which indicates the brightness, no differences were found in yogurt samples with microencapsulated saffron extracts compared to YC, indicating that the addition of encapsulated saffron floral extracts did not change the lightness of yogurt samples. Conversely, yogurt formulations containing saffron stigmas extracts, YSF0.5SS0.05 and YSF1SS0.1, tended to show higher values for yellowness (b*) as expected during the storage period compared to the other yogurt formulations and this implied a change also in the chroma (C*), and thus a change in the color intensity. This fact is related to the orange-yellow color of saffron stigmas which act as natural colorant of yogurts and to the gradual release of saffron extracts from microencapsulates which protected the pigments over time (Carmona, Robert, Vergara, & Sáenz, 2021). Furthermore, due to the colorant properties of saffron, the values of total colour difference between YC and yogurt samples containing saffron stigmas (YSF0.5SS0.05 and YSF1SS0.1) were the highest at time 0, 7, 14 and 21 days of refrigerated storage ($\Delta E > 12$), being these changes visually perceptible (Milovanovic et al., 2020). The browning index, BI, an important quality parameter and one of the most common indicators of browning in food products, significantly decreased during the storage period in all the yogurt formulations, except for the control (YC). This fact could be related to the antioxidant properties of the encapsulates that effectively reduced BI and extended the shelf life of the end product. These novel yogurt formulations with microencapsulated saffron floral by-products and/or saffron stigmas extracts showed adequate pH, acidity and sugars and acids contents, acting as preservative to improve the shelf life, avoiding microbiological or chemical spoilage and maintaining high levels of LAB starter cultures during the 21 days of storage period. Therefore, saffron and its floral by-products could be potential high value-added ingredients to develop novel dairy functional food products as their addition could contribute to maintain the quality and improve the nutritional value of yogurts.

3.2.4. *Antioxidant properties and total phenolic content of the different yogurt formulations during storage*

Several bioactive peptides are produced during fermentation of milk, being yogurts a rich source of antioxidant compounds with beneficial effects for health, as well as for increasing the shelf life of food products, protecting against the lipid oxidation process (Nguyen & Hwang, 2016). However, the fortification of yogurts with microencapsulated saffron floral by-products extracts or/and with saffron stigmas extracts could increase their antioxidant properties and content in bioactive compounds. Thus, the bioactivity of yogurt formulations, in terms of antioxidant activities by DPPH, ABTS and FRAP assays, and total phenolic content was studied during 21 days of storage at 4 °C. Table 3 shows the antioxidant activities and total phenolic content in the yogurt formulations developed during the 21 days of refrigerated storage.

Regarding total phenolic content, the values after 7 days of refrigerated storage, in the samples YC, YB1, YSF0.5, YSF1 and YSF1SS0.1 showed a significant increase compared to time 0. Furthermore, TPC values remained stable in all samples until the end of the storage. This fact at the beginning (time 0) may be related to the interactions of phenolic compounds with milk proteins, being maximum at pH 4.6 when proteins reached their isoelectric point. During storage, the pH decreased and thus the interactions were reduced, leading to higher TPC values after 7 days of storage. These findings were in line with those reported by Akgün et al. (2020).

Yogurt fortification with 1 g/100 g saffron floral by-products increased its functionality, since YSF1 and YSF1SS0.1 showed the highest TPC values during 21 days of storage (3.20 and 3.51 mg GAE/100 g, respectively). The incorporation of saffron floral extracts to yogurts increased their bioactivity, given that saffron floral by-products represent rich source of polyphenols, being potential ingredients with high value-added to develop new functional food products (Cerdá-Bernad, Clemente-Villalba, et al., 2022).

As it is presented in Table 3, the interaction ability of phenolic compounds with milk did not affect the free radical scavenging activity of yogurts (DPPH and ABTS assays), since values remained stable in all yogurt formulations and no significant differences were observed in terms of storage time. In addition, the results of DPPH assay revealed the significant higher antioxidant power of YSF1SS0.1 respect to the other yogurt formulations, since this yogurt sample contained the highest concentrations of saffron floral by-products and saffron stigmas microencapsulated extracts which presented a strong antioxidant capacity (Cerdá-Bernad, Clemente-Villalba, et al., 2022). FRAP values also confirmed that the incorporation of 1 g/100 g of saffron floral by-products microencapsulated extracts may be a suitable alternative for increasing the antioxidant activity of yogurts, since YSF1 and YSF1SS0.1 had significant higher ferric reducing antioxidant power (1.65 and 1.66 mmol Trolox/100 g at time 21 days, respectively) than the rest of samples, thus presenting good chelating properties to avoid lipid oxidation. These results are in accordance with those reported by Lima et al. (2021), who found an increase in the antioxidant activity of yogurts with the addition of microencapsulated fish protein hydrolysate and no differences in antioxidant activity were observed during storage.

These findings agreed with the Principal Component Analysis (PCA) biplot graph (Fig. 4) which showed that yogurt formulations incorporating 1 g/100 g of saffron floral by-products microencapsulated extracts, YSF1 and YSF1SS0.1, might be related to a greater antioxidant power such as free radical scavenging ability, revealed by DPPH and

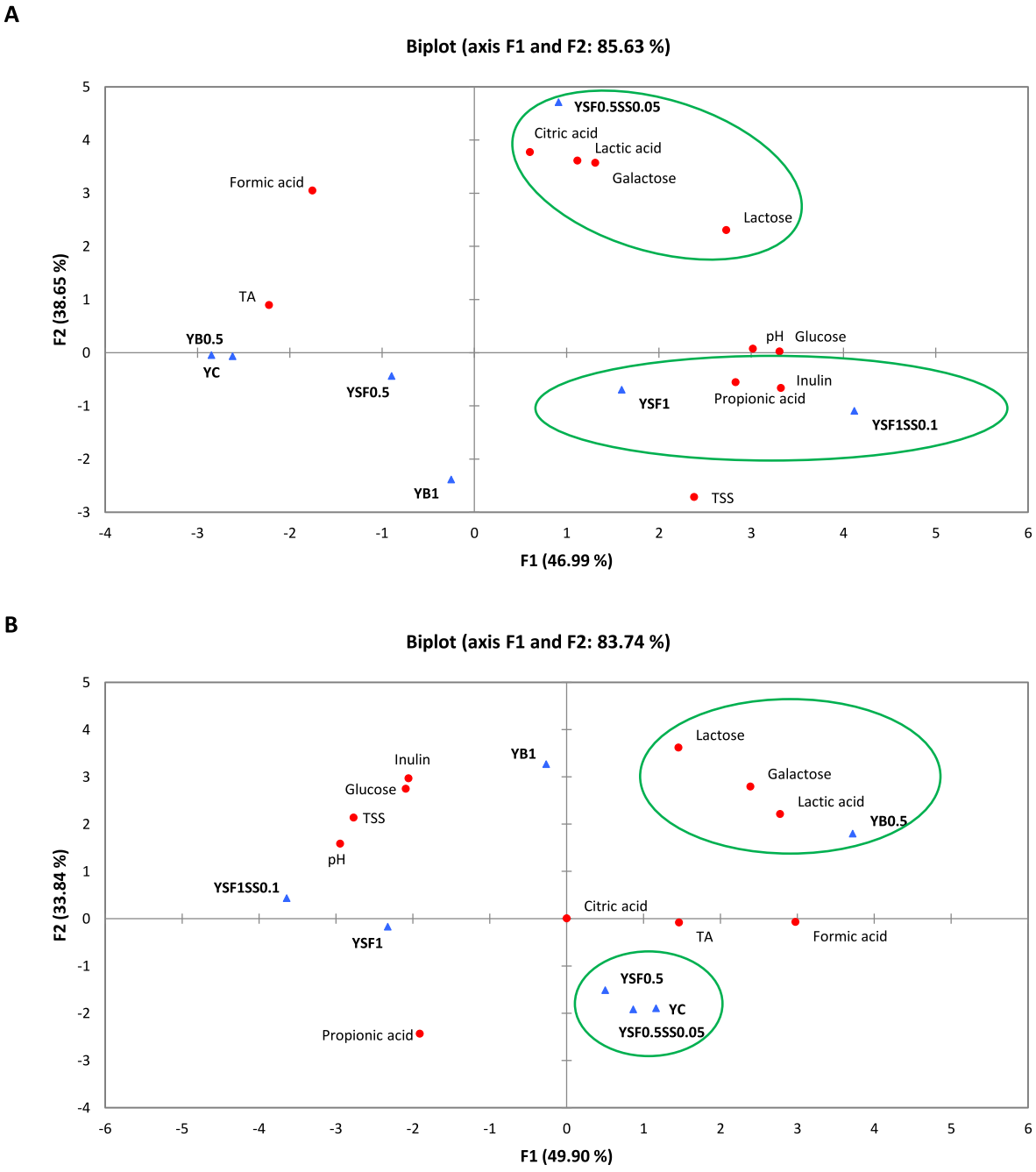

Fig. 3. PCA biplot graph at time 0 days (**A**) and 21 days (**B**) regarding physicochemical properties and organic acids and sugars content. TA: titratable acidity; TSS: total soluble sugars; YC: control yogurt without additional ingredients; YB0.5 and YB1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of blank encapsulated extracts;YSF0.5 and YSF1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of saffron floral by-products encapsulated extracts; YSF0.5SS0.05: yogurt with the addition of 0.5 g/100 g of saffron floral by-products encapsulated extracts combined with 0.05 g/100 g of saffron stigmas encapsulated extracts; YSF1SS0.1: yogurt with the addition of 1 g/100 g of saffron floral by-products encapsulated extracts combined with 0.10 g/100 g of saffron stigmas encapsulated extracts.

ABTS assays. In addition, it is also observed in the graph that the storage time (time 0, T0 and time 21 days of storage, T21) did not influence the antioxidant activity. Therefore, the extracts were protected by the microencapsulation from the direct contact with the food matrix environment, avoiding their oxidation and degradation, and maintaining their antioxidant activity throughout storage time. It should be noted that in other studies in which yogurts were enriched with mushroom extracts incorporated in free form, the antioxidant capacity decreased during storage due to their degradation when in contact with the yogurt matrix (Francisco et al., 2018).

Therefore, the incorporation of 1 g/100 g of microencapsulated saffron flower extracts could be a promising approach to improve the antioxidant properties and TPC of yogurts and to maintain their functionality during storage at 4 °C to exert potential beneficial effects in health after their intake.

4. Conclusions

Saffron floral by-products-alginate beads are potential candidates to be used as functional ingredients improving the bioactive potential of yogurts. The results demonstrated that alginate microencapsulation provided protection keeping the antioxidant properties of saffron

Table 3
Antioxidant properties and Total Phenolic Content (TPC) of different yogurt formulations developed during 21 days of storage at 4 °C.

	Time (days)	YC	YB0.5	YB1	YSF0.5	YSF1	YSF0.5SS0.05	YSF1SS0.1
TPC (mg GAE/100 g)	0	1.70 ± 0.08^{Bd}	1.99 ± 0.34^{Bcd}	1.71 ± 0.08^{Bd}	2.22 ± 0.18^{Bc}	2.88 ± 0.01^{Bab}	2.26 ± 0.14^{c}	3.20 ± 0.02^{Ca}
	7	2.49 ± 0.18^{Ac}	2.52 ± 0.05^{ABc}	2.75 ± 0.19^{Ac}	2.69 ± 0.23^{Ac}	3.29 ± 0.28^{Ab}	2.47 ± 0.05^{c}	4.02 ± 0.07^{Aa}
	14	2.38 ± 0.12^{Ac}	2.53 ± 0.22^{ABc}	2.45 ± 0.16^{Ac}	2.31 ± 0.16^{ABc}	3.05 ± 0.05^{ABab}	2.34 ± 0.24^{c}	3.75 ± 0.18^{ABa}
	21	2.47 ± 0.02^{Ac}	2.80 ± 0.25^{Abc}	2.60 ± 0.14^{Ac}	2.38 ± 0.02^{ABc}	3.20 ± 0.11^{ABab}	2.64 ± 0.10^{c}	3.51 ± 0.09^{Ba}
FRAP (mmol Trolox/100 g)	0	1.21 ± 0.06^{ABc}	1.65 ± 0.03^{Ab}	1.66 ± 0.09^{Ab}	1.58 ± 0.11^{b}	1.68 ± 0.09^{a}	1.56 ± 0.05^{b}	1.79 ± 0.01^{Aa}
	7	1.36 ± 0.10^{ABb}	1.46 ± 0.06^{Bb}	1.48 ± 0.07^{ABb}	1.44 ± 0.09^{b}	1.73 ± 0.07^{a}	1.50 ± 0.10^{b}	1.76 ± 0.00^{Aa}
	14	1.09 ± 0.18^{Bd}	1.24 ± 0.06^{Ccd}	1.18 ± 0.10^{Cd}	1.29 ± 0.17^{bd}	1.61 ± 0.09^{ab}	1.51 ± 0.08^{abc}	1.67 ± 0.02^{Ba}
	21	1.39 ± 0.02^{Ae}	1.49 ± 0.05^{Bbce}	1.27 ± 0.03^{BCd}	1.46 ± 0.07^{be}	1.65 ± 0.05^{a}	1.58 ± 0.02^{ac}	1.66 ± 0.03^{Ba}
ABTS (mmol Trolox/100 g)	0	0.79 ± 0.02^{ab}	0.83 ± 0.03^{ab}	0.77 ± 0.07^{b}	0.84 ± 0.04^{ab}	0.89 ± 0.05^{a}	0.84 ± 0.02^{ab}	0.89 ± 0.01^{a}
	7	0.80 ± 0.00^{b}	0.82 ± 0.04^{ab}	0.84 ± 0.02^{ab}	0.94 ± 0.09^{a}	0.94 ± 0.07^{a}	0.80 ± 0.04^{ab}	0.92 ± 0.04^{ab}
	14	0.61 ± 0.10^{c}	0.71 ± 0.10^{bc}	0.71 ± 0.11^{bc}	0.87 ± 0.02^{ab}	0.91 ± 0.06^{ab}	0.85 ± 0.00^{ab}	0.92 ± 0.02^{a}
	21	0.81 ± 0.03^{bc}	0.79 ± 0.03^{cd}	0.76 ± 0.04^{c}	0.88 ± 0.01^{ab}	0.91 ± 0.04^{a}	0.87 ± 0.03^{abd}	0.91 ± 0.00^{a}
DPPH (mmol Trolox/100 g)	0	0.27 ± 0.03^{b}	0.29 ± 0.06^{b}	0.33 ± 0.01^{ab}	0.24 ± 0.04^{b}	0.39 ± 0.08^{ab}	0.28 ± 0.09^{b}	0.48 ± 0.04^{Ba}
	7	0.26 ± 0.05^{b}	0.27 ± 0.02^{b}	0.24 ± 0.08^{b}	0.29 ± 0.03^{b}	0.32 ± 0.00^{b}	0.24 ± 0.07^{b}	0.52 ± 0.01^{Ba}
	14	0.27 ± 0.01^{c}	0.24 ± 0.01^{c}	0.28 ± 0.04^{c}	0.25 ± 0.02^{c}	0.36 ± 0.04^{b}	0.27 ± 0.03^{c}	0.66 ± 0.03^{Aa}
	21	0.24 ± 0.06^{b}	0.27 ± 0.03^{b}	0.24 ± 0.07^{b}	0.29 ± 0.06^{b}	0.32 ± 0.02^{ab}	0.29 ± 0.03^{b}	0.46 ± 0.07^{Ba}

Means ± standard deviation in the same column followed by different uppercase letters indicate statistically significant differences at ($P \leq 0.05$) for each yogurt sample at different storage time ($n = 3$). Means ± standard deviation in the same row followed by different lowercase letters indicate statistically significant differences at ($P \leq 0.05$) between yogurt samples at the same storage time ($n = 3$); YC: control yogurt without additional ingredients; YB0.5 and YB1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of blank encapsulated extracts;YSF0.5 and YSF1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of saffron floral by-products encapsulated extracts; YSF0.5SS0.05: yogurt with the addition of 0.5 g/100 g of saffron floral by-products encapsulated extracts combined with 0.05 g/100 g of saffron stigmas encapsulated extracts; YSF1SS0.1: yogurt with the addition of 1 g/100 g of saffron floral by-products encapsulated extracts combined with 0.10 g/100 g of saffron stigmas encapsulated extracts.

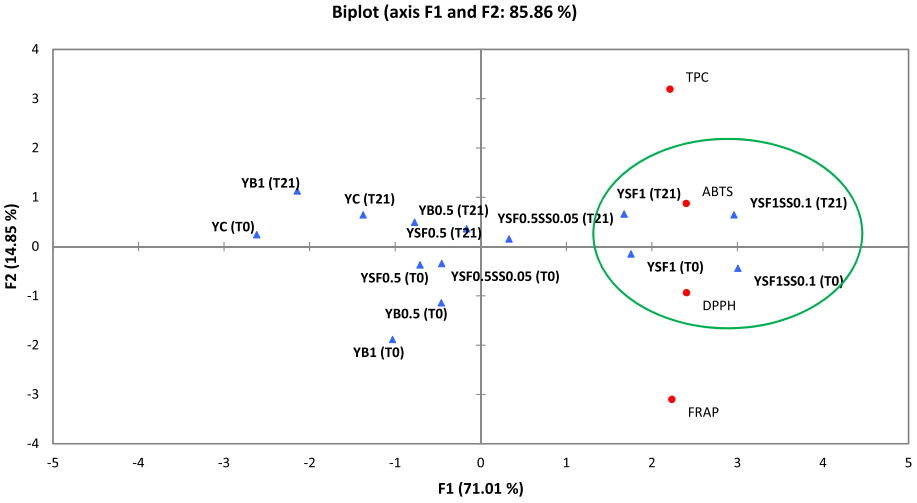

Fig. 4. PCA biplot graph at time 0 days (T0) and 21 days (T21), regarding antioxidant properties and total phenolic content (TPC). YC: control yogurt without additional ingredients; YB0.5 and YB1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of blank encapsulated extracts; YSF0.5 and YSF1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of saffron floral by-products encapsulated extracts; YSF0.5SS0.05: yogurt with the addition of 0.5 g/100 g of saffron floral by-products encapsulated extracts combined with 0.05 g/100 g of saffron stigmas encapsulated extracts; YSF1SS0.1: yogurt with the addition of 1 g/100 g of saffron floral by-products encapsulated extracts combined with 0.10 g/100 g of saffron stigmas encapsulated extracts.

flowers in the yogurt matrix over 21 days of the refrigerated storage period. Furthermore, the microbiological profile and physical-chemical parameters were not affected by the addition of saffron extracts in the novel yogurt formulations, and showed a good composition of organic acids and soluble sugars to improve the shelf life of the food product. Besides, the present study revalorizes saffron floral by-products through their application as sustainable ingredients in the production of novel enriched yogurts, improving their functionality. Hence, these novel yogurts after their ingestion can provide greater health benefits and, at the same time, the use of the saffron floral by-products extracts as ingredients in their production, taking advantage of an unexploited biomass, would lead to a reduction of the environmental impact.

Funding

This article is supported by the Partnership for Research and Innovation in the Mediterranean Area (PRIMA) program, project "SAFFROMFOOD." The PRIMA program is supported by the European Union. The research was also supported by the regional agency "Diputación de Alicante", Alicante, Spain.

CRediT authorship contribution statement

Débora Cerdá-Bernad: Conceptualization, Methodology, Writing – original draft, preparation, Writing – review & editing. **Estefanía Valero-Cases:** Supervision. **Joaquín Julián Pastor:** Supervision, All authors have read and agreed to the published version of the manuscript. **María-José Frutos:** Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgment

Thanks to the Spanish-Ministry of Education, Culture and Sport for the FPU-PhD fellowship (FPU18/02225) and thanks to the Spanish company Verdú Cantó Saffron Spain, S.L for the supply of samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.114976.

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SUPPLEMENTARY MATERIAL

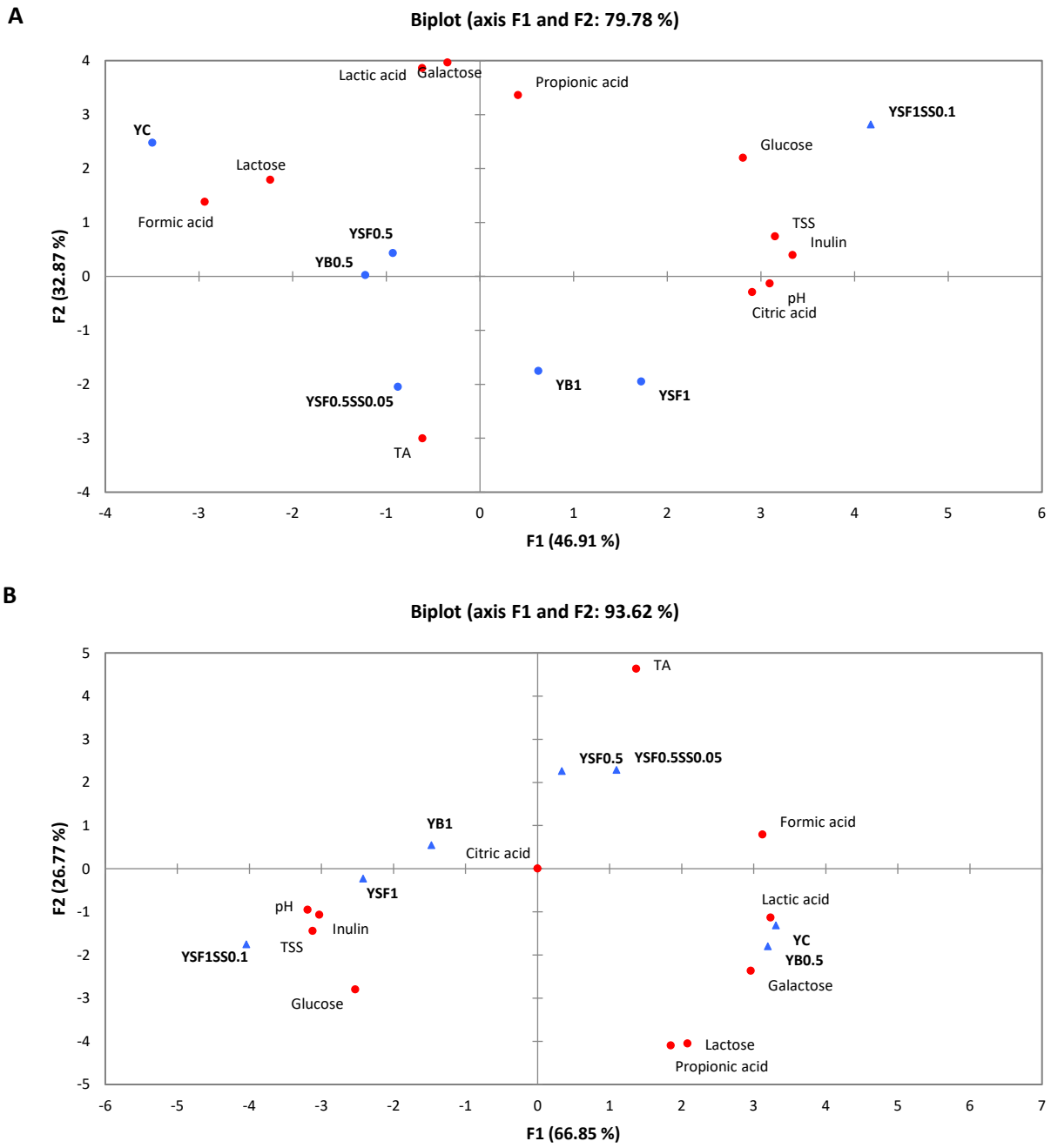

Figure S1 PCA biplot graph at time 7 days (A) and 14 days (B) regarding physicochemical properties and organic acids and sugars content. TA: titratable acidity; TSS: total soluble sugars; YC: control yogurt without additional ingredients; YB0.5 and YB1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of blank encapsulated extracts;YSF0.5 and YSF1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of saffron floral by-products encapsulated extracts; YSF0.5SS0.05: yogurt with the addition of 0.5 g/100 g of saffron floral by-products encapsulated extracts combined with 0.05 g/100 g of saffron stigmas encapsulated extracts; YSF1SS0.1: yogurt with the addition of 1 g/100 g of saffron floral by-products encapsulated extracts combined with 0.10 g/100 g of saffron stigmas encapsulated extracts.

Table S1 Colour parameters of different yogurt formulations developed during 21 days of storage at 4 °C.

	Time (days)	YC	YB0.5	YB1	YSF0.5	YSF1	YSF0.5SS0.05	YSF1SS0.1
L*	0	55.86 ± 0.78^{deC}	70.51 ± 0.99^{aAB}	64.25 ± 0.90^{bC}	61.93 ± 0.87^{bcC}	58.99 ± 0.83^{cdC}	57.17 ± 0.80^{dC}	53.09 ± 0.74^{eC}
	7	62.01 ± 0.87^{dB}	65.58 ± 0.92^{abC}	67.51 ± 0.95^{aB}	65.64 ± 0.92a^{bBC}	64.10 ± 0.90^{bcB}	63.08 ± 0.88^{cdB}	63.23 ± 0.89^{cdB}
	14	67.50 ± 0.95^{A}	67.10 ± 0.94^{BC}	67.98 ± 0.95^{B}	68.02 ± 0.85^{B}	69.82 ± 0.98^{A}	69.03 ± 0.97^{A}	67.87 ± 0.95^{A}
	21	68.43 ± 0.96^{bcA}	72.24 ± 1.01^{aA}	70.51 ± 0.99^{abA}	72.13 ± 1.01^{aA}	66.92 ± 0.94^{cAB}	72.39 ± 1.01^{aA}	68.36 ± 0.96^{bcA}
a*	0	-2.04 ± 0.03^{aA}	-3.38 ± 0.05^{cC}	-3.07 ± 0.04^{b}	-3.01 ± 0.04^{b}	-3.17 ± 0.04^{bB}	-5.84 ± 0.08^{dB}	-5.80 ± 0.08^{dB}
	7	-2.92 ± 0.04^{aBC}	-2.96 ± 0.04^{aA}	-3.19 ± 0.02^{b}	-3.18 ± 0.03^{b}	-3.15 ± 0.03^{bB}	-5.53 ± 0.06^{cA}	-5.87 ± 0.07^{dB}
	14	-3.03 ± 0.02^{aC}	-3.05 ± 0.03^{aAB}	-3.11 ± 0.03^{a}	-3.07 ± 0.03^{a}	-3.11 ± 0.04^{aB}	-5.38 ± 0.07^{bA}	-5.77 ± 0.08^{cB}
	21	-2.87 ± 0.04^{bB}	-3.20 ± 0.04^{cB}	-3.13 ± 0.04^{c}	-3.16 ± 0.04^{c}	-2.40 ± 0.03^{aA}	-5.42 ± 0.08^{dA}	-5.47 ± 0.05^{dA}
b*	0	3.05 ± 0.04^{eC}	5.13 ± 0.07^{dB}	5.88 ± 0.08^{cA}	4.67 ± 0.07^{dD}	5.08 ± 0.07^{dD}	14.53 ± 0.20^{bD}	16.79 ± 0.24^{aC}
	7	3.24 ± 0.05^{fC}	3.75 ± 0.05^{fC}	4.42 ± 0.06^{eC}	5.61 ± 0.08^{dC}	6.98 ± 0.10^{cB}	16.48 ± 0.23^{bC}	23.97 ± 0.34^{aB}
	14	5.57 ± 0.08^{eA}	5.09 ± 0.07^{eB}	5.17 ± 0.07^{eB}	6.89 ± 0.10^{dB}	8.15 ± 0.11^{cA}	19.43 ± 0.27^{bB}	25.79 ± 0.36^{aA}
	21	4.74 ± 0.07^{eB}	5.42 ± 0.08^{dA}	5.08 ± 0.07^{eB}	7.27 ± 0.10^{cA}	6.10 ± 0.09^{dC}	20.88 ± 0.29^{bA}	26.10 ± 0.37^{aA}
ΔE	0	-	14.85 ± 0.21^{aA}	8.90 ± 0.12^{cA}	6.36 ± 0.09^{dA}	3.90 ± 0.05^{eC}	12.16 ± 0.10^{bC}	14.50 ± 0.20^{aB}
	7	-	3.70 ± 0.05^{Be}	5.62 ± 0.08^{cB}	4.33 ± 0.06^{Bd}	4.29 ± 0.06^{Ade}	13.53 ± 0.06^{bB}	20.96 ± 0.29^{aA}
	14	-	0.62 ± 0.00^{eC}	0.63 ± 0.00^{eD}	1.42 ± 0.02^{dC}	3.48 ± 0.05^{cB}	14.14 ± 0.02^{bB}	20.41 ± 0.28^{aA}
	21	-	3.88 ± 0.05^{cD}	2.12 ± 0.03^{dC}	4.50 ± 0.06^{cB}	2.08 ± 0.03^{dD}	16.81 ± 0.06^{bA}	21.51 ± 0.30^{aA}
C*	0	3.67 ± 0.05^{fA}	6.15 ± 0.09^{cdBC}	6.63 ± 0.09^{cC}	5.55 ± 0.08^{eA}	5.99 ± 0.08^{deA}	15.66 ± 0.22^{bA}	17.76 ± 0.25^{aA}
	7	4.36 ± 0.06^{eB}	4.77 ± 0.07^{eA}	5.45 ± 0.08^{dA}	6.45 ± 0.09^{dB}	7.66 ± 0.11^{cC}	17.39 ± 0.24^{bB}	24.68 ± 0.35^{aB}
	14	6.34 ± 0.09^{eD}	5.93 ± 0.08^{eB}	6.03 ± 0.08^{eB}	7.54 ± 0.11^{dC}	8.74 ± 0.12^{cD}	20.16 ± 0.28^{bC}	26.42 ± 0.37^{aC}
	21	5.54 ± 0.08^{eC}	6.30 ± 0.09^{dC}	5.97 ± 0.07^{deB}	7.93 ± 0.10^{cC}	6.56 ± 0.09^{dB}	21.57 ± 0.30^{bD}	26.67 ± 0.40^{aC}
h°	0	307 ± 4^{a}	306 ± 4^{a}	301 ± 4^{ab}	306 ± 5^{a}	305 ± 4^{a}	295 ± 4^{b}	292 ± 4^{ab}
	7	315 ± 5^{a}	311 ± 6^{a}	309 ± 6^{a}	303 ± 6^{ab}	297 ± 3^{bc}	291 ± 5^{c}	287 ± 5^{c}
	14	302 ± 3^{a}	304 ± 5^{a}	304 ± 8^{a}	297 ± 4^{ab}	294 ± 5^{ab}	288 ± 4^{b}	285 ± 6^{b}
	21	304 ± 4^{ab}	303 ± 4^{ab}	305 ± 4^{a}	296 ± 2^{b}	294 ± 6^{b}	287 ± 3^{bc}	285 ± 5^{c}
BI	0	3.15 ± 0.00^{gD}	4.14 ± 0.02^{fA}	4.32 ± 0.03^{dA}	4.21 ± 0.01^{eB}	4.68 ± 0.04^{cA}	9.77 ± 0.10^{bA}	10.90 ± 0.12^{aA}
	7	3.88 ± 0.01^{fB}	3.78 ± 0.00^{gD}	4.02 ± 0.00^{eB}	4.30 ± 0.02^{dA}	4.58 ± 0.02^{cB}	8.84 ± 0.05^{bB}	10.41 ± 0.05^{aB}
	14	4.02 ± 0.01^{eA}	4.01 ± 0.01^{eB}	4.02 ± 0.01^{eB}	4.22 ± 0.01^{dB}	4.37 ± 0.01^{cC}	8.36 ± 0.02^{bC}	9.86 ± 0.00^{aC}
	21	3.68 ± 0.00^{eC}	3.91 ± 0.03^{dC}	3.88 ± 0.02^{dC}	4.13 ± 0.00^{cC}	3.47 ± 0.10^{fD}	8.21 ± 0.00^{bD}	9.54 ± 0.07^{aD}

Means ± standard deviation in the same column followed by different uppercase letters indicate statistically significant differences at ($P \leq 0.05$) for each yogurt sample at different storage time (n = 3). Means ± standard deviation in the same row followed by different lowercase letters indicate statistically significant differences at ($P \leq 0.05$) between yogurt samples at the same storage time (n = 3);YC: control yogurt without additional ingredients; YB0.5 and YB1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of blank encapsulated extracts;YSF0.5 and YSF1: yogurts with the addition of 0.5 and 1 g/100 g, respectively, of saffron floral by-products encapsulated extracts; YSF0.5SS0.05: yogurt with the addition of 0.5 g/100 g of saffron floral by-products encapsulated extracts combined with 0.05 g/100 g of saffron stigmas encapsulated extracts; YSF1SS0.1: yogurt with the addition of 1 g/100 g of saffron floral by-products encapsulated extracts combined with 0.10 g/100 g of saffron stigmas encapsulated extracts.

Publicación 7

Saffron floral by-products as novel sustainable vegan ingredients for the functional and nutritional improvement of traditional wheat and spelt breads

Autores	Débora Cerdá-Bernad & María José Frutos
Revista	Foods
DOI	10.3390/foods12122380
Editorial	MDPI
Fecha de publicación	15 de Junio de 2023
Categoría JCR®	Food Science and Technology
Cuartil	Q1
Rango	34/142
Factor de impacto	5,200

foods

MDPI

Article

Saffron Floral By-Products as Novel Sustainable Vegan Ingredients for the Functional and Nutritional Improvement of Traditional Wheat and Spelt Breads

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Abstract: Saffron (*Crocus sativus* L.) is a traditional Mediterranean plant whose stigmas are used to obtain the most expensive spice in the world. Nevertheless, there is a lack of sustainability in its production, since, to produce 1 kg of saffron, about 350 kg of tepals are discarded. Therefore, this study aimed to develop wheat and spelt breads enriched with saffron floral by-products at a ratio of 0, 2.5, 5, and 10% (*w*/*w*), respectively, and to evaluate their nutritional, physicochemical, functional, and sensory properties, as well as the stability of antioxidant compounds during the in vitro digestion. The results revealed that the addition of saffron floral by-products, especially at 10%, increased the dietary fiber content by 25–30% of traditional wheat and spelt breads; improved their mineral content (270–290 mg/100 g for K, 90–95 mg/100 g for Ca, 40–50 mg/100 g for Mg, and 15–18 mg/100 g for Fe); changed their textural properties; and significantly enhanced the phenolic content and antioxidant ability (at 5 and 10%), which remained stable throughout the in vitro oral and gastrointestinal digestion processes. From a sensory point of view, the addition of saffron flowers modified the organoleptic properties of breads. Thus, these novel vegan enriched breads could exert beneficial effects on human health after their intake, making saffron floral by-products suitable and sustainable ingredients to develop new functional foods such as healthier alternative vegan bakery products.

Keywords: saffron flowers; polyphenols; antioxidant capacity; bioactive compounds; in vitro digestion; vegan bakery foods; sensory analysis; food waste; upcycling

Citation: Cerdá-Bernad, D.; Frutos, M.J. Saffron Floral By-Products as Novel Sustainable Vegan Ingredients for the Functional and Nutritional Improvement of Traditional Wheat and Spelt Breads. *Foods* **2023**, *12*, 2380. https://doi.org/10.3390/foods12122380

Academic Editors: Barbara Simonato and Roberta Tolve

Received: 31 May 2023
Revised: 12 June 2023
Accepted: 13 June 2023
Published: 15 June 2023

1. Introduction

In recent years, consumers are more aware of the relationship between food and health, considering veganism as a suitable option to change their lifestyle and diet. Therefore, worldwide, vegetarian and vegan diets have become widespread in the population due to greater consumer attention to nutritional, environmental, and ethical aspects [1].

Current trends open up great opportunities for new alternative markets, so food industries are actively seeking the development of novel innovative and sustainable vegan products to meet the needs of consumers. Meat alternatives are dominated by soy derivatives since soy protein is abundant, cheap, and, after hydration, has a meat-like texture, but due to allergies issues, new vegetal sources must be sought [2]. Thus, the recovery and valorization of vegetable by-products from plants and vegetables have become of utmost interest to the food industry. These food by-products are considered high-added value ingredients whose function within the food matrix is to provide health-promoting bioactive compounds.

The production of saffron (*Crocus sativus* L.) spice generates large amounts of waste, since only flower stigmas are employed while the rest of the plant is discarded, generating around 350 kg of tepals to obtain 1 kg of the commercial product (Figure 1). However,

several studies have reported the potential of these saffron floral by-products at nutritional and biological levels as a source of interesting compounds, such as polyphenols, especially flavonoids (anthocyanins, flavonols), with high antioxidant activity. Their nutritional composition includes carbohydrates (~70%), proteins (~8%), lipids (~4%), fiber (~27%), macro and microminerals, soluble sugars, and vitamin C, among others [3–5]. Therefore, the valorization of these saffron floral by-products through their use as novel sustainable vegan ingredients, with high-added value for the production of new functional food products, would lead to the minimization of their environmental impact, in addition to ensuring sustainability and profitability of the saffron industrial sector, developing, at the same time, an alternative vegetal source that would represent a new income for saffron farmers.

Figure 1. *Crocus sativus* L. flower.

The selection of food matrices as carriers of these plant ingredients is an essential factor to ensure the stability of their functional properties in the final product. Bread, a natural and balanced staple food, which contains a large amount of essential nutrients—carbohydrates, proteins, vitamins A, B1, B2, niacin, folic acid, and minerals—could be considered as a promising carrier matrix of functional substances [6]. Bread has been considered the food par excellence by many civilizations since ancient times, being the result of baking a mixture of flour, water, yeast, and salt. Furthermore, the high consumption and availability of this traditional bakery product, especially wheat and spelt breads, together with its quick and easy production, makes it a suitable matrix for its enrichment with functional ingredients providing bioactive compounds in the diet with positive health effects through the prevention or reduction of the risk of certain diseases [7]. It should be noted that spelt flour was used for its nutritional quality, presenting a high content in protein, and is an excellent source of minerals and vitamin E and B-complex vitamins.

The main aim of this research was to develop different improved traditional formulations of wheat and spelt breads enriched with saffron floral by-products as a sustainable vegan ingredient to produce breads with improved nutritional and functional quality compared to their traditional counterparts. Their physicochemical properties—namely, texture, content of minerals, organic acids, and soluble sugars—and the stability of the antioxidant capacity by ABTS and FRAP assays and total phenolic compounds during the oral and gastrointestinal in vitro digestion, as well as their sensory evaluation, were evaluated. Therefore, this research contributes to the valorization of a currently unexploited biomass through its use as a high-added value ingredient to develop novel functional food products, providing new information regarding the suitability of saffron floral by-products as sustainable alternatives to obtain healthier vegan bakery products.

2. Materials and Methods

2.1. Raw Materials

Saffron floral by-products, mainly composed of tepals, were obtained from the Castilla-La Mancha region (Spain) after harvesting of saffron flowers and the manual removal of the stigmas during the 2021 harvest season. The fresh flowers were frozen in liquid nitrogen and kept at −80 °C until dehydration in a vacuum oven (VACIOTEM, JP SELECTA®, Barcelona, Spain) at 50 ± 3 °C, 28 mbar, for 36 h, and then crushed, sieved (500 µm mesh), and kept at −20 °C until further use.

For the elaboration of different formulations of bread, two types of flour were used: wheat flour (*Triticum aestivum*; W: 360, P: 100, L: 100, 14.5% of protein) or organic spelt flour (*Triticum spelta*; W:95, P:36, L:105, 13.9% of protein) (El Amasadero, Malaga, Spain). Baker's active dry yeast (Mauripan, Barcelona, Spain), common commercial salt, and water were also employed.

2.2. Bread Baking Procedure

The bread formulations were prepared according to a traditional recipe with wheat flour or spelt flour (100%) and adding—based on the weight of flour and following the procedure of Świeca, Gawlik-Dziki, Dziki and Baraniak [8] with slight modifications—water (65–70%), yeast (1%), salt (2%), and dried saffron floral by-products in different concentrations (0%, 2.5%, 5%, and 10%). Eight different formulations were made, four using wheat flour at 0 (WB0%), 2.5 (WB2.5%), 5 (WB5%), and 10% (WB10%) of dried saffron floral by-products; and four formulations using spelt flour at 0 (SB0%), 2.5 (SB2.5%), 5 (SB5%), and 10% (SB10%) of dried saffron floral by-products.

The ingredients were mixed and kneaded for 10 min. A first short fermentation (30 min) was carried out at 30 ± 2 °C and humidity of 40 ± 5%. Loaves were manually round-shaped and left for a second short fermentation (30 min) under the same controlled temperature and humidity conditions. Finally, different bread loaves were baked at 210 °C for 20 min in a domestic oven, the final weight being around 40 g per piece. All formulations were prepared in triplicate.

Once cooled, a group of breads was stored at −20 °C until further physicochemical analysis, texture, and in vitro digestion tests. Another group was freeze-dried (Christ Alpha 2–4, B. Braun Biotech International, Melsungen, Germany) for 24 h (−25 ± 2 °C, 0.220 mbar). Then, they were crushed, sieved (500 µm mesh), and stored in polyethylene bags at −20 °C until further analysis (minerals, organic acids, and sugar composition).

2.3. Physicochemical Characterization

2.3.1. Moisture and Ash

The moisture (%) of fresh samples was determined by keeping bread formulations in an oven at 105 ± 5 °C until they reached constant weight, according to the AOAC [9]. The ash content (%) was carried out by incineration of samples obtained after moisture in a muffle oven (Habersal PR 1300 PAD, Barcelona, Spain) at 550 ± 25 °C, for one hour [9].

2.3.2. pH, Acidity, TSS, aw, Colur

The pH, acidity, TSS, aw, and color were determined according to Cerdá-Bernad, Valero-Cases, Pastor, Frutos and Pérez-Llamas [10], with some modifications. The pH and titratable acidity (expressed as % citric acid) were measured using an automatic titrator (TitroMatic Crison pH-Matic 23, Barcelona, Spain). The determination of total soluble solids (TSS) was carried out with a digital refractometer (Hanna® HI 96801, Bedfordshire, UK) and expressed as °Brix. Fresh samples were previously mixed with distilled water (1:10, *w*/*v*) with an ULTRA-TURRAX® (IKA T18, Werke GmbH & Co, Staufen, Germany) at 5000 rpm for 10 s.

The water activity (aw) of the different fresh samples was determined using a water activity meter (Novasina AW Sprint TH 500, Pfäffikon, Switzerland) at room temperature.

The color was measured with a Minolta CR-300 Chroma Meter (Japan) colorimeter, using the L*, a*, b* scale (CIELAB system). The results were expressed as luminosity L*, a* (greenness/redness), and b* (blueness/yellowness).

2.4. Texture

Texture profile analysis was performed on bread slices (2.5 cm width) using a TA-XTPlus Texture Analyser (Stable Micro Systems Ltd., Godalming, UK) according to García-Segovia, Igual and Martínez-Monzó [11], a cylindrical aluminum probe (SMS P100, 10 cm in diameter), and a 50 kg load cell. The parameters of the assay were defined as crosshead speed 1.70 mm/s and 40% deformation of the original length. The textural parameters' hardness (N), cohesiveness, springiness (mm), gumminess (N), and chewiness (N) were determined.

2.5. Minerals

The minerals' composition was determined according to Serrano-Díaz, Sánchez, Martínez-Tomé, Winterhalter and Alonso [4], with slight modifications. Freeze-dried bread samples weighing 0.5 g were digested with 10 mL of 65% HNO_3 (v/v) using a microwave reactor digestor (CEM Mars one, Matthews, NC, USA) for 30 min with a temperature ramp whose final temperature was 200 °C. All samples were filtered (Whatman qualitative filter paper 90 mm), diluted with ultrapure deionized water 1:50 (v/v), and stored at 4 °C. Total concentrations of macronutrients (Ca, Mg, Na, and K) and micronutrients (Zn, Cu, Mn, and Fe) in the previously mineralized samples were quantified with an Inductively Coupled Plasma Mass Spectrometer (ICPMS-2030, Shimadzu, Kyoto, Japan). Internal standards included calcium (44Ca), magnesium (26Mg), sodium (23Na), potassium (39K), zinc (66Zn), copper (65Cu), manganese (55Mn), and iron (56Fe), and the calibration curves used for quantification showed good linearity ($R^2 \geq 0.998$). The results were expressed as mg/100 g of dw (dry weight).

2.6. Organic Acids, Soluble Sugars, and Inulin Content

The extractions were prepared using ultrapure water and a freeze-dried sample/water ratio 1:20 (w/v). The extracts were shaken for 5 min at 300 rpm on a magnetic stirrer at room temperature (Ovan, mod. MultiMix Heat D-MMH30E, Barcelona, Spain), sonicated for 15 min, and centrifuged at 11,200× g for 10 min at 4 °C. The supernatants were filtered (0.45 µm PTFE filter, Millipore, Spain) and stored at −20 °C.

The identification and quantification of sugars, inulin, and organic acids were carried out by high-performance liquid chromatography using Hewlett-Packard HPLC series 1100 equipment equipped with a Supelcogel C-610H column (30 cm × 7.8 mm) and a Supelcoguard C-610H pre-column (5 cm × 4.6 mm) (Sigma Aldrich, St. Louis, MO, USA). The organic acids were measured at 210 nm in UV-Vis with a diode array detector (DAD G1315A). For sugars, a refractive index detector (G1362A RID) was used. As a mobile phase, 0.1% orthophosphoric acid was used with an injection volume of 20 µL and the flow rate of 0.5 mL/min under isocratic conditions, following the methodology described by Cerdá-Bernad, Valero-Cases, Pastor, Frutos and Pérez-Llamas [10]. The results were expressed as mg/100 g of dw.

2.7. In Vitro Digestion

The oral phase of in vitro digestion was performed according to Gawlik-Dziki, Dziki, Baraniak and Lin [12] with slight modifications. Fresh bread samples (2 g) were homogenized in the presence of 30 mL of PBS (phosphate buffered saline) in a stomacher for 30 s to simulate mastication. The solution was previously adjusted to pH 6.75 and alpha-amylase (E.C. 3.2.1.1.) was added to obtain 100 U per mL of enzymatic activity.

Gastrointestinal digestion was carried out following the methodology described by Cerdá-Bernad, Valero-Cases, Pastor, Frutos and Pérez-Llamas [10] for 180 min at 37 °C under continuous stirring. Aliquots were taken after 60 min of gastric digestion (pH = 3), and at 60 min and 120 min of intestinal digestion (pH = 7). All samples were filtered (0.45 µm; Millipore, Spain) and stored at −20 °C until further analysis (antioxidant capacity and total phenolic content).

2.8. Antioxidant Properties and Total Phenolic Content (TPC)

The antioxidant capacity determined by using the Ferric Reducing Antioxidant Power (FRAP) method and the 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging method was carried out as described by Cerdá-Bernad, Clemente-Villalba, Valero-Cases, Pastor and Frutos [3]. Briefly, the FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution in a volume ratio of 10:1:1, respectively. The absorbance was measured at 593 nm and Trolox (10 mM) was used as the standard solution (0.01–5.00 mM). ABTS radical was prepared mixing ABTS (7 mM) with potassium persulfate (2.45 mM), reacting for 16 h in the dark at room temperature and diluting the solution with ultrapure water until its absorbance was adjusted to 0.70 ± 0.02 at 734 nm. Trolox (10 mM) was used as a reference standard (0.20–3.00 mM). The results were expressed as mmol Trolox/100 g of bread.

The TPC was determined using the Folin–Ciocalteu methodology as described by Cerdá-Bernad, Clemente-Villalba, Valero-Cases, Pastor and Frutos [3]. Briefly, 100 µL of the different digested samples were mixed with 400 µL of PBS (50 mM) at pH 7.8 and 2.5 mL of Folin–Ciocalteu reagent previously mixed with ultrapure water 1:10 (v/v). After 2 min, 2 mL of Na_2CO_3 (75 g/L) were added and kept at 50 °C for 10 min. The absorbance was measured at 760 nm and the results were expressed as mg of gallic acid equivalents/100 g of bread.

2.9. Sensory Analysis

A quantitative descriptive analysis (QDA) of selected bread formulations was performed by a sensory panel composed of 12 trained judges (five males and seven females) aged between 21 and 60 years, at the Miguel Hernández University (Spain), following the procedure of Sicari, Romeo, Mincione, Santacaterina, Tundis and Loizzo [13] with some modifications. All the panel members had neither allergies nor food intolerances and were regular consumers of bakery products. The samples were labeled with an alphanumeric code and distributed in a random order. A total number of 17 sensory descriptors were considered regarding the appearance, flavor, and texture, using a 10-point scale (Table S1).

2.10. Statistical Analysis

Results were expressed as mean ± standard deviation. All experiments were carried out in triplicate. The mean comparisons were undertaken using an analysis of variance (ANOVA) and by the Tukey multiple range test, using the SPSS version 21.0 software package (SPSS Inc., Chicago, IL, USA). Principal Component Analysis (PCA) was conducted using XLSTAT (Microsoft Corp., Washington, DC, USA). The significant differences were established as ($p \leq 0.05$).

3. Results and Discussion

3.1. Characterization of Bread Formulations Enriched with Saffron Floral By-Products: Physicochemical, Nutritional, and Technological Properties

3.1.1. Physicochemical Parameters and Minerals' Content

Physicochemical and technological properties of the novel bread formulations were evaluated in order to study the influence of adding different amounts of dried saffron floral by-products compared to the control sample.

Table 1 shows the results of the physicochemical parameters (moisture, ash, pH, acidity, aw, and TSS) of the experimental wheat and spelt bread formulations with saffron floral by-products. Moisture is a relevant factor in the quality of bread since it is related to the shelf life but also influences the texture of the final product. Moisture content regulates the firming rate of bread since its decrease accelerates the process of cross-linking of proteins and starch, which further accelerates the process of firming [14].

Table 1. Physicochemical parameters of the different wheat and spelt bread formulations enriched with saffron floral by-products (moisture, ash, pH, acidity, TSS, aw) [1].

	Moisture (%)	Ash (%)	pH	TA (% Citric Acid)	Aw	TSS (°Brix)
WB0%	31.12 ± 2.43 ^{ab}	2.75 ± 0.07 ^{c}	5.62 ± 0.01 ^{a}	0.09 ± 0.01 ^{d}	0.85 ± 0.03	0.77 ± 0.01 ^{d}
WB2.5%	25.47 ± 2.49 ^{b}	2.95 ± 0.17 ^{c}	5.50 ± 0.01 ^{b}	0.12 ± 0.01 ^{c}	0.84 ± 0.02	0.93 ± 0.02 ^{c}
WB5%	31.12 ± 2.40 ^{ab}	3.44 ± 0.10 ^{b}	5.31 ± 0.02 ^{c}	0.23 ± 0.02 ^{b}	0.84 ± 0.04	1.07 ± 0.02 ^{b}
WB10%	32.44 ± 2.44 ^{a}	3.79 ± 0.12 ^{a}	5.20 ± 0.01 ^{d}	0.28 ± 0.02 ^{a}	0.84 ± 0.02	1.30 ± 0.01 ^{a}
SB0%	32.71 ± 2.61	3.05 ± 0.15 ^{c}	5.54 ± 0.01 ^{a}	0.10 ± 0.02 ^{b}	0.86 ± 0.01	0.97 ± 0.01 ^{d}
SB2.5%	29.51 ± 2.46	3.37 ± 0.11 ^{b}	5.23 ± 0.00 ^{b}	0.21 ± 0.01 ^{a}	0.85 ± 0.00	1.03 ± 0.03 ^{c}
SB5%	31.89 ± 2.36	3.39 ± 0.08 ^{b}	5.21 ± 0.00 ^{c}	0.23 ± 0.02 ^{a}	0.85 ± 0.01	1.07 ± 0.02 ^{b}
SB10%	31.50 ± 2.33	3.95 ± 0.16 ^{a}	5.18 ± 0.01 ^{d}	0.24 ± 0.01 ^{a}	0.85 ± 0.03	1.30 ± 0.01 ^{a}

[1] Means ± standard deviation in the same column followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each wheat or spelt sample ($n = 3$); TA: titratable acidity; TSS: total soluble sugars; WB0%, WB2.5%, WB5%, and WB10%: wheat bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively; SB0%, SB2.5%, SB5%, and SB10%: spelt bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively.

Regarding the results, in the spelt bread formulations (SB), no statistically significant differences were found between the spelt samples with different concentrations of saffron floral by-products formulations, showing values around 30% of moisture. In the wheat bread samples (WB), moisture values were between 25 and 32%, indicating statistically significant differences between WB2.5% (25.47 ± 2.49%) and WB10% (32.44 ± 2.44%). Moreover, water activity (aw) is also related to the firming process in the bakery products. The addition of different concentrations of dried saffron flowers' powder did not affect aw compared with the control bread in both wheat and spelt bread formulations since no statistically significant differences were found. These results were similar to those reported in other studies, in which breads enriched with 5 and 10% of insect-based proteins (*Alphitobius diaperinus*) were developed, showing aw values between 0.877 and 0.894 [11]. Therefore, the addition of a new sustainable vegan ingredient to improve the functional properties of spelt and wheat bread did not change the moisture and water activity compared to the control samples.

As expected, the addition of saffron floral by-products in wheat and spelt bread increased the ash values which are related to the average level of mineral components, since saffron flowers are good sources of minerals [4]. For wheat formulations, the addition of 5% of saffron flower ingredient (3.44 ± 0.10%) significantly increased the ash content with respect to the control sample, WB0% (2.75 ± 0.07%). However, in spelt breads, the ash content (3.37 ± 0.11%) in the formulations with 2.5% of the saffron flower ingredient was significantly higher than the control, SB0% (3.05 ± 0.15%). Furthermore, as shown in Table 2, the macromineral and micromineral concentrations revealed that the enrichment process of wheat and spelt breads with saffron floral by-products had a nutritional positive effect due to an increase in the content of beneficial minerals.

Table 2. Composition of minerals (mg/100 g dw) of the different wheat and spelt bread formulations enriched with saffron floral by-products [1].

		WB0%	**WB2.5%**	**WB5%**	**WB10%**	**SB0%**	**SB2.5%**	**SB5%**	**SB10%**
Macrominerals	Ca	39.22 ± 1.24 ^{b}	56.18 ± 5.15 ^{b}	67.92 ± 8.02 ^{a}	91.63 ± 10.02 ^{a}	33.47 ± 6.00 ^{c}	52.74 ± 8.10 ^{bc}	65.98 ± 1.33 ^{b}	94.56 ± 3.14 ^{a}
	K	162 ± 3 ^{d}	196 ± 9 ^{c}	226 ± 8 ^{b}	277 ± 14 ^{a}	153 ± 10 ^{c}	178 ± 11 ^{b}	209 ± 16 ^{b}	289 ± 18 ^{a}
	Mg	31.21 ± 1.54^{c}	34.72 ± 1.82 ^{bc}	36.97 ± 1.43 ^{b}	41.61 ± 1.93 ^{a}	33.95 ± 3.01 ^{b}	34.46 ± 3.11 ^{b}	40.81 ± 1.04 ^{a}	48.46 ± 8.03 ^{a}
	Na	641 ± 18 ^{ab}	713 ± 32 ^{a}	682 ± 23 ^{ab}	605 ± 85 ^{b}	810 ± 55	713 ± 55	743 ± 13	739 ± 12
Microminerals	Fe	2.01 ± 0.10 ^{c}	6.01 ± 1.15 ^{b}	9.15 ± 1.96 ^{b}	15.87 ± 1.25 ^{a}	3.27 ± 1.83 ^{c}	6.27 ± 0.35 ^{c}	10.63 ± 0.39 ^{b}	17.85 ± 0.40 ^{a}
	Mn	1.03 ± 0.19 ^{b}	1.22 ± 0.03 ^{ab}	1.25 ± 0.05 ^{ab}	1.42 ± 0.21 ^{a}	0.74 ± 0.09 ^{b}	0.91 ± 0.10 ^{ab}	1.04 ± 0.01 ^{ab}	1.37 ± 0.20 ^{a}
	Zn	0.77 ± 0.11	0.97 ± 0.00	0.97 ± 0.01	1.03 ± 0.06	0.71 ± 0.02	0.78 ± 0.06	0.81 ± 0.10	0.96 ± 0.20

[1] Means ± standard deviation in the same row followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each wheat or spelt sample ($n = 3$); WB0%, WB2.5%, WB5%, and WB10%: wheat bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively; SB0%, SB2.5%, SB5%, and SB10%: spelt bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively.

The most abundant mineral that presented in all wheat and spelt breads was Na due to the salt used in the formulation (600–800 mg/100 g), and no significant differences were shown between bread formulations with different concentrations of saffron floral by-products. Potassium was the macromineral also found in high levels, with increasing concentrations as the amount of saffron floral by-products added was higher, showing that WB10% and SB10% had the highest K content (277 ± 14 and 289 ± 18 mg/100 g, respectively). These values represent 7.91% and 8.26%, approximately, of the Recommended Daily Intake (RDI) of potassium in adults (3500 mg per day) with a consumption of 100 g of WB10% or SB10%, respectively [15]. Moreover, calcium and magnesium were also present in significantly higher amounts than the control bread in those with 5 or 10% of saffron floral by-products added, representing a vegan ingredient that improves the nutritional value of traditional spelt and wheat breads because of its rich mineral composition. The WB10% and SB10% samples showed a concentration of Ca of 91.63 ± 10.02 and 94.56 ± 3.14 mg/100 g, respectively, representing 9.65% and 9.95%, approximately, of the RDI of calcium in adults (950 mg per day) with a consumption of 100 g of wheat or spelt bread with saffron floral by-products ingredient at 10% [15]. Regarding Mg content, WB10% and SB10% had 41.61 ± 1.93 and 48.46 ± 8.03 mg/100 g, respectively, indicating around 13.87% and 16.15% of the RDI of magnesium in adults (300 mg per day), respectively, with a consumption of 100 g wheat or spelt bread with 10% of saffron flowers [15]. These results were in accordance with Fahim, Sadat, Janati and Feizy [16] who reported a high content of K and Ca in saffron petals.

In addition to the macrominerals content, which is relevant for human physiological functions, the microminerals iron, zinc, and manganese were also present in the different bread formulations although in a lower amount compared to Ca, Mg, Na, and K (Table 2). The most abundant micromineral was Fe in all samples, followed by Mn and Zn. The WB10% and SB10% samples showed significantly higher levels of Fe (15.87 ± 1.25 and 17.85 ± 0.40 mg/100 g, respectively) with respect to bread formulations enriched with 2.5 or 5% of saffron floral by-products (WB5%, WB10%, SB5%, SB10%), and with respect to the control samples (WB0%, SB0%). The consumption of 100 g of WB10% and SB10% provides the RDI in adults (11 mg per day) [15]. The amount of Mn in the control samples (1.03 ± 0.19 mg/100 g for WB0% and 0.74 ± 0.09 mg/100 g for SB0%) was statistically significantly lower than WB10% and SB10%, at 1.42 ± 0.21 and 1.37 ± 0.20 mg/100 g, respectively. With respect to Zn content, no statistically significant differences were found in both the wheat and spelt bread formulations, showing concentrations ranging from 0.77 to 1.03 for WB and from 0.71 to 0.96 for SB.

Minerals are essential micronutrients for human health and wheat flour is an important source of these components, such as Ca (100–200 mg/100 g), Mg (100–200 mg/100 g), Fe (1–5 mg per 100 g), Zn (1–5 mg/100 g), and Cu (0.1–1 mg/100 g) [17]. In addition, saffron floral by-products can provide mineral enrichment through their use in these novel vegan bread formulations, especially at 10%, showing values around 270–290 mg/100 g for K, 90–95 mg/100g for Ca, 40–50 mg/100 g for Mg, and 15–18 mg/100 g for Fe.

The pH value and acidity are also relevant factors to determine the quality of breads and may influence the sensory properties since both could have an impact on the taste of the final product as well as on the texture, with the solubility of gluten constituent proteins being greater at acidic pH values [18]. Table 1 shows pH values between 5.20 and 5.62 for wheat breads and 5.18 and 5.54 for spelt breads, being significantly lower in breads with a higher concentration of saffron floral by-products with respect to the control samples. At the same time, acidity was higher in bread formulations with higher amount of flowers with respect to the control samples, which is related to the concentration of organic acids in the saffron floral ingredient. Acidity values ranged from 0.09 to 0.24% citric acid in all wheat and spelt bread formulations.

3.1.2. Organic Acids, Soluble Sugars, and Inulin Content

The study of the composition of organic acids is an important quality parameter since they have a considerable effect on the technological characteristics, relevant sensory properties, and shelf life of breads. Data on the organic acid composition of bread formulations are shown in Table 3. Important organic acids were found in the different bread formulations, not only due to their presence in wheat and spelt flour as well as in saffron floral by-products but also as a result of the fermentation process during bread making. In the course of the fermentation period, the *Saccharomyces cerevisiae* yeast used in the formulation, together with the microbiota (bacteria and yeasts) naturally present in wheat and spelt flour, played an important role in terms of bread characteristics [19]. The biochemical changes due to the effects of fermentation on the degradation of carbohydrates and proteins mainly by the yeast and lactic acid bacteria led to the production of organic acids and other metabolites. Lactic acid was one of the main organic acids present in all bread formulations, being significantly higher in breads with 10% of saffron floral by-products (595 ± 20 and 676 ± 11 mg/100 g for WB10% and SB10%, respectively) compared to the other samples. Malic and citric acids were also found in wheat and spelt formulations in high amounts, and previous studies have reported their presence in saffron tepals [4] and in wheat flour [20,21]. Statistically significant differences were found in the content of citric acid in wheat and spelt bread formulations, being higher in WB10% (1957 ± 19 mg/100 g) and SB10% (1934 ± 22 mg/100 g) compared to the control samples, WB0% (1878 ± 7 mg/100 g) and SB0% (1600 ± 24 mg/100 g). Oxalic and fumaric acids were also found in lower amounts in the wheat and spelt bread samples, showing values around 0.24–1.98 mg/100 g for fumaric acid and 9.77–14.24 mg/100 g for oxalic acid. These results were in accordance with other studies that reported the presence of lactic, malic, and fumaric acids in wheat and spelt flours [21].

Furthermore, propionic acid was identified in wheat and spelt bread samples enriched with saffron flowers; this may have been generated as a result of the metabolic activity of microbiota that could metabolize molecules present in saffron floral by-products, producing different compounds. Moreover, the differences in the composition of organic acids between spelt and wheat breads could be related to the wide diversity in the presence of diverse microbial strains leading to the production of different enzymes and metabolites [22]. Therefore, the presence of organic acids in the different bread formulations could act as natural preservatives to improve their shelf life, avoiding microbiological spoilage.

The evaluation of sugar content in bakery products such as bread is essential due to its functional role. The natural composition of sugars in the initial ingredients is a major contributor to flavor in the final product by interacting with other ingredients due to two processes which are involved: fermentation and crust browning (caramelization and the Maillard reaction) during baking. Then, the sugars remaining after fermentation also contribute to the overall color and texture of the final product, since the affinity of sugars to bind to water will delay the development of gluten, which is essential for maintaining a tender bread texture [23]. The results of the analysis of the individual sugars are shown in Table 3.

Glucose was the major component in all bread formulations, followed by fructose and maltose. The disaccharide maltose is the first product released by the digestion of flour starch by the amylase enzyme that is further hydrolyzed to release glucose. Therefore, maltose is found in lower concentrations than glucose in the final product, ranging from 30 to 37 mg/100 g in all wheat and spelt breads. However, the glucose content is very high, showing statistically significant differences between each wheat and spelt formulation. The highest concentrations of this monosaccharide were found for WB10% (1917 ± 34 mg/100 g) and for SB10% (1829 ± 35 mg/100 g) and the lowest concentrations were found for the control samples, WB0% and SB0% (1303 ± 12 and 1210 ± 62 mg/100 g, respectively). These results were in accordance with other studies that reported the presence of maltose and glucose in wheat and spelt flours [21].

Table 3. Content of organic acids, soluble sugars, and inulin (mg/100 g dw) of the different wheat and spelt bread formulations enriched with saffron floral by-products [1].

		WB0%	WB2.5%	WB5%	WB10%	SB0%	SB2.5%	SB5%	SB10%
Organic acids	Phytic acid	n.d.	n.d.	n.d.	n.d.	178 ± 27	165 ± 5	168 ± 11	172 ± 3
	Lactic acid	241 ± 60 ^{d}	384 ± 22 ^{c}	503 ± 19 ^{b}	595 ± 20 ^{a}	301 ± 33 ^{d}	385 ± 6 ^{c}	448 ± 5 ^{b}	676 ± 11 ^{a}
	Citric acid	1878 ± 7 ^{c}	1895 ± 6 ^{bc}	1922 ± 14 ^{b}	1957 ± 19 ^{a}	1600 ± 24 ^{c}	1885 ± 5 ^{b}	1910 ± 9 ^{b}	1934 ± 22 ^{a}
	Malic acid	132 ± 5 ^{d}	214 ± 11 ^{c}	335 ± 30 ^{b}	516 ± 30 ^{a}	102 ± 10 ^{d}	189 ± 16 ^{c}	292 ± 26 ^{b}	521 ± 32 ^{a}
	Oxalic acid	14.24 ± 1.90	13.91 ± 0.62	13.84 ± 0.93	13.79 ± 0.93	11.33 ± 0.76	11.75 ± 0.22	9.77 ± 3.73	10.98 ± 0.26
	Fumaric acid	0.24 ± 0.07 ^{c}	0.04 ± 0.00 ^{c}	1.05 ± 0.08 ^{b}	1.98 ± 0.13 ^{a}	n.d.	n.d.	n.d.	1.71 ± 0.20
	Propionic acid	n.d.	835 ± 71 ^{b}	933 ± 33 ^{a}	982 ± 48 ^{a}	n.d.	845 ± 9 ^{c}	910 ± 16 ^{b}	1051 ± 48 ^{a}
Soluble sugars	Glucose	1303 ± 12 ^{d}	1411 ± 16 ^{c}	1617 ± 13 ^{b}	1917 ± 34 ^{a}	1210 ± 62 ^{d}	1317 ± 25 ^{c}	1512 ± 82 ^{b}	1829 ± 35 ^{a}
	Maltose	37.70 ± 0.14 ^{a}	33.32 ± 0.10 ^{c}	36.75 ± 0.16 ^{b}	33.11 ± 0.33 ^{c}	33.19 ± 0.24 ^{a}	33.03 ± 0.41 ^{a}	31.19 ± 0.32 ^{b}	30.49 ± 0.13 ^{b}
	Fructose	n.d.	n.d.	480 ± 40 ^{b}	913 ± 82 ^{a}	n.d.	n.d.	307 ± 48 ^{b}	813 ± 12 ^{a}
	Inulin	5011 ± 70 ^{c}	5377 ± 95 ^{b}	6504 ± 58 ^{a}	6511 ± 223 ^{a}	5464 ± 80 ^{c}	5682 ± 112 ^{c}	6231 ± 211 ^{b}	6846 ± 48 ^{a}

[1] Means ± standard deviation in the same row followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each wheat or spelt sample ($n = 3$); WB0%, WB2.5%, WB5%, and WB10%: wheat bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively; SB0%, SB2.5%, SB5%, and SB10%: spelt bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively; n.d.: not detected.

Fructose could be also released by hydrolysis of fructans, but this compound was only present in wheat and spelt bread formulations with 5 and 10% of saffron floral by-products. Thus, the content of this monosaccharide could be due to its natural occurrence in the saffron flowers ingredient [4]. Furthermore, the content of inulin as part of soluble dietary fiber was studied. The inulin concentration significantly increased with the content of saffron floral by-products in wheat and spelt breads. The highest amounts of inulin were present in WB10% and SB10%, with values around 6511 and 6846 mg/100 g, respectively. However, the lowest concentrations were found in samples without saffron floral by-products, being these amounts those present in wheat and spelt flours (around 5011 and 5464 mg/100 g for WB0% and SB0%, respectively). Therefore, this vegan ingredient is a natural source of dietary fiber to develop new bakery products with improved nutritional properties since inulin can be used as a prebiotic, stimulating the proliferation of the intestinal microbiota [24] At the same time, inulin contributed to an increase in TSS, since bread formulations with the highest inulin concentration (WB10% and SB10%) presented significantly higher TSS than the control samples, with values around 1.30 °Brix compared to WB0% and SB0% (0.77–0.93 °Brix) (Table 1). Moreover, the increase in TSS could be due to the presence of fructose in the composition of inulin in the WB10% and SB10% samples.

3.1.3. Texture

The textural properties are one of the quality parameters that have a crucial influence on the bread quality since they determine the shelf life and consumers' acceptability. Table 4 shows the textural parameters (hardness, cohesiveness, springiness, gumminess, chewiness) of the different wheat and spelt bread formulations. The results show that the control wheat and spelt breads, WB0% and SB0%, were characterized by the lowest hardness, whereas the bread enrichment with saffron floral by-products resulted in a statistically significant increase in this parameter, being around 28% in WB10% and around 50% in SB10% with respect to the control samples. All wheat- and spelt-enriched bread formulations presented a statistically significant decrease in cohesiveness when compared to control breads. Thus, these results revealed that the addition of saffron floral by-products significantly affected the textural properties of breads, which could be related to the interruption of the gluten network that leads to a low cohesiveness and the crumb disintegration [7]. These findings agree with those obtained in previous studies in which bread was enriched by adding chickpea and soy flour, also observing an increase in hardness [25].

Table 4. Parameters of the texture of the different wheat and spelt bread formulations enriched with saffron floral by-products [1].

	Hardness (N)	Cohesiveness	Springiness (mm)	Gumminess (N)	Chewiness (N)
WB0%	42.24 ± 2.45 ^{d}	0.93 ± 0.02 ^{a}	1.59 ± 0.01 ^{a}	39.47 ± 0.41 ^{d}	37.73 ± 0.74 ^{c}
WB2.5%	48.36 ± 3.32 ^{c}	0.88 ± 0.00 ^{b}	1.50 ± 0.08 ^{b}	42.58 ± 0.67 ^{c}	40.00 ± 0.49 ^{b}
WB5%	51.12 ± 1.12 ^{b}	0.87 ± 0.01 ^{b}	1.49 ± 0.03 ^{b}	44.68 ± 0.48 ^{b}	41.51 ± 0.23 ^{b}
WB10%	54.17 ± 0.53 ^{a}	0.86 ± 0.00 ^{b}	1.47 ± 0.02 ^{c}	46.91± 0.82 ^{a}	43.21 ± 0.15 ^{a}
SB0%	34.65 ± 1.66 ^{c}	0.89 ± 0.01 ^{a}	1.53 ± 0.04 ^{a}	31.14 ± 1.11 ^{d}	28.64 ± 0.16 ^{c}
SB2.5%	41.31 ± 2.24 ^{b}	0.84 ± 0.02 ^{b}	1.43 ± 0.00 ^{b}	34.76 ± 0.53 ^{c}	31.93 ± 0.27 ^{b}
SB5%	43.75 ± 2.35 ^{b}	0.82 ± 0.02 ^{bc}	1.40 ± 0.00 ^{c}	36.06 ± 0.14 ^{b}	32.94 ± 0.98 ^{b}
SB10%	52.12 ± 3.87 ^{a}	0.80 ± 0.00 ^{c}	1.37 ± 0.01 ^{d}	41.91 ± 1.14 ^{a}	38.22 ± 0.54 ^{a}

[1] Means ± standard deviation in the same column followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each wheat or spelt sample ($n = 3$); WB0%, WB2.5%, WB5%, and WB10%: wheat bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively; SB0%, SB2.5%, SB5%, and SB10%: spelt bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively.

The springiness parameter is associated with freshness, and high values are desired to extend the bread shelf life. However, the enriched bread samples had a statistically significant decrease in springiness values when compared to the controls, being linked to a high crumb brittleness [26]. Chewiness and gumminess increased significantly with the saffron floral by products' incorporation in wheat and spelt breads when compared to control samples. Similar results were found by García-Segovia, Igual and Martínez-Monzó [11] using pea protein or insect powder in the formulation of breads. These values might be related to the dietary fiber content, like inulin, which results in a strong chewiness and high water-swelling capacity [26].

3.1.4. Color

Bread crumb and crust color were also evaluated since it is an important factor for the consumers' choice of bread (Table 5). The L* values describing the brightness of breads decreased in bread crust and crumb prepared with saffron floral by-products.

Table 5. Color of the crust and crumb of the different wheat and spelt bread formulations enriched with saffron floral by-products [1].

	Crust			Crumb		
	L*	**a***	**b***	**L***	**a***	**b***
WB0%	40.25 ± 3.15	12.49 ± 0.19 ^{a}	22.22 ± 2.19 ^{b}	61.86 ± 1.69 ^{a}	0.28 ± 0.10 ^{b}	18.45 ± 0.35 ^{b}
WB2.5%	37.21 ± 2.36	11.35 ± 0.63 ^{ab}	20.18 ± 2.51 ^{b}	51.72 ± 3.73 ^{b}	−0.38 ± 0.02 ^{c}	27.83 ± 0.60 ^{a}
WB5%	41.95 ± 2.37	10.72 ± 0.47 ^{b}	22.99 ± 1.56 ^{a}	47.72 ± 0.63 ^{b}	0.08 ± 0.04 ^{b}	27.44 ± 3.54 ^{a}
WB10%	34.78 ± 3.56	9.21 ± 0.53 ^{c}	17.05 ± 2.32 ^{b}	41.29 ± 1.47 ^{c}	1.52 ± 0.32 ^{a}	28.35 ± 1.89 ^{a}
SB0%	50.58 ± 1.71 ^{a}	11.05 ± 0.34 ^{a}	25.35 ± 1.16 ^{a}	63.15 ± 5.68 ^{a}	0.02 ± 0.00 ^{d}	19.08 ± 1.07 ^{b}
SB2.5%	43.54 ± 5.48 ^{ab}	10.60 ± 1.15 ^{a}	23.30 ± 2.87 ^{a}	58.45 ± 1.81 ^{ab}	0.30 ± 0.01 ^{c}	28.21 ± 1.40 ^{a}
SB5%	40.15 ± 0.42 ^{bc}	11.06 ± 0.56 ^{a}	21.42 ± 0.57 ^{a}	52.50 ± 2.54 ^{c}	0.59 ± 0.03 ^{b}	30.70 ± 0.81 ^{a}
SB10%	33.75 ± 1.42 ^{c}	8.73 ± 0.53 ^{b}	15.22 ± 1.14 ^{b}	47.01 ± 3.63 ^{c}	1.75 ± 0.09 ^{a}	30.57 ± 1.70 ^{a}

[1] Means ± standard deviation in the same column followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each wheat or spelt sample ($n = 3$); WB0%, WB2.5%, WB5%, and WB10%: wheat bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively; SB0%, SB2.5%, SB5%, and SB10%: spelt bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively.

Thus, the enrichment of the bread decreased crust and crumb L* values by about 13% and 33%, respectively, in WB10% compared to the control samples, WB0%. A similar tendency was followed by spelt breads, showing a darker color of the bread crumb and crust supplemented with saffron floral by-products, decreasing the crust and crumb L* values by about 33% and 25%, respectively, in SB10% when compared to SB0%.

These results suggested that the reduction of crust L* values could be related to an increase in Maillard browning reactions, and the decrease in L* crumb values may be explained by the intrinsic color of saffron floral by-products. Other authors reported similar observations in breads enriched with vegetal by-products [27]. Regarding the parameters, a* and b*, the crust bread showed similar values in enriched wheat and spelt bread formulations with respect to control samples. Nevertheless, the a* and b* values of the bread crumb increased in the wheat and spelt formulations with the addition of saffron flowers, resulting in redder-yellower loafs (Figure 2). Therefore, the color parameters of the crumb are related to those of the saffron flower by-products powder since the temperatures inside the loaves during baking did not exceed 100 °C [3,11].

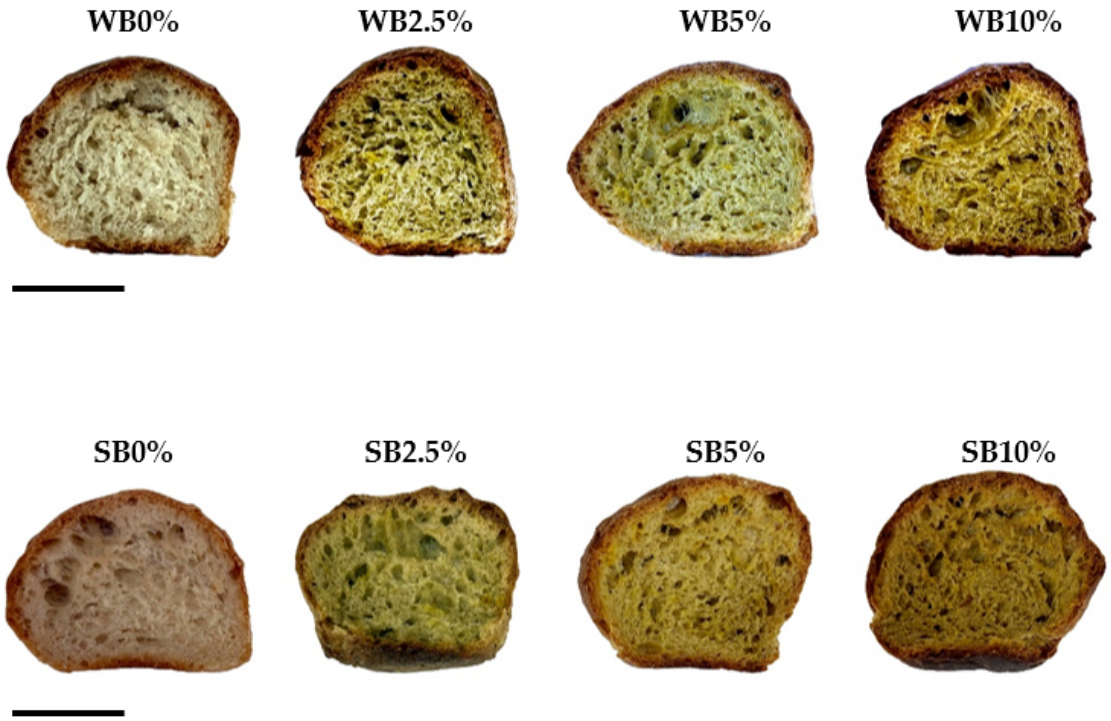

Figure 2. Wheat and spelt bread formulations obtained by adding different concentrations of dried saffron floral by-products. WB0%, WB2.5%, WB5%, and WB10%: wheat bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively; SB0%, SB2.5%, SB5%, and SB10%: spelt bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively. Scale bar: 2.5 cm.

3.2. Antioxidant Properties and Total Phenolic Content of Bread Formulations Enriched with Saffron Floral By-Products during Oral and Gastrointestinal In Vitro Digestion

The effect of the incorporation of saffron floral by-products into wheat and spelt bread matrices was also evaluated through the determination of the antioxidant activity and bioactive content at individual stages of simulated oral and gastrointestinal in vitro digestion. Plant bioactive compounds are very sensitive to several environmental factors, which means that their biological properties, such as the antioxidant power, could change significantly depending on the environmental conditions (pH, presence of digestive enzymes, temperature, etc.) [28]. Therefore, the changes in the antioxidant activity and in the total phenolic content at the different stages of in vitro digestion (oral, gastric, intestinal) of breads enriched with saffron floral by-products, as a rich source of phenolic compounds, were investigated.

The results of antioxidant activity ($ABTS^+$ and FRAP assays) and total phenolic compounds (TPCs) in the enriched wheat and spelt bread formulations at various stages of simulated in vitro digestion are presented in Table 6.

According to the values obtained for the ability to reduce free radicals by the $ABTS^+$ assay, Fe^{2+} ions by the FRAP assay, and TPC during the in vitro digestion, the addition of saffron floral by-products into wheat and spelt breads significantly improved the phenolic content and their antioxidant ability, especially in the formulations with 10% of the saffron floral ingredient. The enriched breads, WB10% and SB10%, exhibited a significantly higher antioxidant activity (ABTS and FRAP assays) compared to the control in all digestion phases. This could be due to a significant increase of phenolic compounds from saffron floral by-products with well-known antioxidant capacities. For the ABTS assay, the antioxidant capacity increased in all bread formulations in each digestion phase, reaching the highest values under intestinal digestion conditions, being the maximum values for WB10% and SB10% after 2 h of intestinal digestion (1576 ± 34 mmol Trolox/100 g and 1583 ± 83 mmol

Trolox/100 g, respectively). This increase could be due to intestinal conditions (neutral pH; presence of pancreatic enzymes and bile salts) which would facilitate an improvement in the release of bioactive compounds that may previously be bound or insolubilized due to their interaction with the bread matrix. These findings were in line with literature reports indicating that the free radical scavenging activity grew along with the increase of plant ingredients addition and with the progress of the in vitro digestion [12,28].

Table 6. Antioxidant properties (FRAP and ABTS assays) and Total Phenolic Content (TPC) of the different wheat and spelt bread formulations enriched with saffron floral by-products during the oral and gastrointestinal in vitro digestion process [1].

		In Vitro Digestion			
		Oral	**Gastric**	**Intestinal (1 h)**	**Intestinal (2 h)**
ABTS (mmol Trolox/100 g)	WB0%	3.47 ± 1.75	22.37 ± 1.99 ^{c}	421 ± 12 ^{a}	458 ± 6 ^{b}
	WB2.5%	3.94 ± 1.28	34.32 ± 8.47 ^{b}	437 ± 4 ^{a}	452 ± 10 ^{b}
	WB5%	3.61 ± 2.61	48.74 ± 2.14 ^{a}	435 ± 8 ^{a}	461 ± 5 ^{b}
	WB10%	4.94 ± 1.50	49.99 ± 0.98 ^{a}	399 ± 7 ^{b}	1576 ± 34 ^{a}
	SB0%	2.20 ± 0.16 ^{b}	30.74 ± 3.31 ^{b}	429 ± 11	437± 27 ^{b}
	SB2.5%	5.49 ± 0.67 ^{a}	43.34 ± 1.99 ^{a}	430 ± 12	1373 ± 59 ^{a}
	SB5%	4.91 ± 0.81 ^{a}	45.44 ± 6.40 ^{a}	426 ± 19	1458± 177 ^{a}
	SB10%	5.69 ± 0.20 ^{a}	50.54 ± 0.39 ^{a}	437 ± 27	1583 ± 83 ^{a}
FRAP (mmol Trolox/100 g)	WB0%	0.89 ± 0.50 ^{c}	8.07 ± 2.03 ^{c}	n.d.	n.d.
	WB2.5%	14.09 ± 4.08 ^{b}	17.12 ± 0.56 ^{bc}	1.40 ± 0.83 ^{b}	2.43 ± 0.73 ^{b}
	WB5%	28.05 ± 4.85 ^{a}	25.64 ± 6.04 ^{b}	9.83 ± 5.35 ^{ab}	15.00 ± 6.58 ^{ab}
	WB10%	38.77 ± 5.90 ^{a}	37.67 ± 4.67 ^{a}	20.71 ± 7.96 ^{a}	27.89 ± 6.99 ^{a}
	SB0%	6.99 ± 0.73 ^{c}	11.21 ± 1.01 ^{b}	n.d.	n.d.
	SB2.5%	18.08 ± 4.03 ^{bc}	21.56 ± 1.19 ^{b}	3.97 ± 2.81 ^{b}	7.60 ± 0.34 ^{c}
	SB5%	32.01 ± 9.90 ^{b}	26.69 ± 12.58 ^{ab}	14.26 ± 8.84 ^{b}	18.16 ± 4.89 ^{b}
	SB10%	61.86 ± 10.75 ^{a}	43.97 ± 6.96 ^{a}	31.79 ± 1.22 ^{a}	36.62 ± 3.130 ^{a}
TPC (mg GAE/100 g)	WB0%	76.99 ± 6.60 ^{d}	118 ± 8 ^{d}	109 ± 7 ^{d}	91.31 ± 5.25 ^{c}
	WB2.5%	111 ± 6 ^{c}	149 ± 16 ^{c}	140 ± 16 ^{c}	146 ± 13 ^{b}
	WB5%	198 ± 8 ^{b}	207 ± 1 ^{b}	222 ± 8 ^{b}	166 ± 14 ^{b}
	WB10%	221 ± 18 ^{a}	231 ± 11 ^{a}	272 ± 17 ^{a}	220 ± 17 ^{a}
	SB0%	102 ± 16 ^{c}	181 ± 92	137 ± 18 ^{c}	105 ± 15 ^{c}
	SB2.5%	124 ± 32 ^{bc}	236 ± 129	135 ± 10 ^{c}	135 ± 45 ^{bc}
	SB5%	175 ± 1 ^{b}	251 ± 71	187 ± 6 ^{b}	186 ± 5 ^{b}
	SB10%	261 ± 13 ^{a}	313 ± 63	258 ± 25 ^{a}	251 ± 17 ^{a}

[1] Means ± standard deviation in the same column followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each wheat or spelt sample ($n = 3$); WB0%, WB2.5%, WB5%, and WB10%: wheat bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively; SB0%, SB2.5%, SB5%, and SB10%: spelt bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively; n.d.: not detected.

For the FRAP assay, however, the maximum values were obtained after simulated oral digestion, which remained stable after simulated gastric digestion but decreased after the intestinal phase in all bread formulations. The lowest activity after the gastric digestion was detected in control samples and the highest in the samples obtained from wheat and spelt bread with 10% of saffron floral ingredients. Therefore, the influence of the bread food matrix and its interaction with bioactive compounds from saffron flower ingredients could have played an important role in the antioxidant activity of the bread along the oral and gastrointestinal in vitro digestion.

Regarding TPC values, in the wheat and spelt bread formulations, their concentrations remained stable in the oral, gastric, and intestinal digestion steps, reaching the statistically significant highest TPC content for the bread formulations, WB10% and SB10%. In spelt breads, a slight increase in TPC after gastric conditions was observed, which could be

related to an increase in the solubility of certain phenolic compounds under acidic pH conditions, which may have been previously chelated, or in reduced form [29]. It should be noted that the TPC values were high after 2 h of the intestinal stage (220 ± 17 and 251 ± 17 mg GAE/100 g for WB10% and SB10%, respectively), so that the matrix enables these bioactive compounds to reach the colon after digestion and contribute to the maintenance of intestinal health [30]. However, further research is necessary to understand their mechanism of action at the physiological level in human health, since the bioavailability of phenolic compounds through the diet depends on many factors [28].

According to the results, saffron floral by-products have a high content of phenolic compounds that remained stable in the cereal food matrix during the in vitro digestion, so they could be absorbed and exert their beneficial physiological effects. In addition, these functional breads have improved antioxidant properties, especially in the formulations with a higher content of saffron floral by-products (5 and 10%).

The Principal Component Analysis (PCA) biplot graph (Figure 3) showed two different groups, one related to wheat and spelt bread formulations incorporating 10% of saffron floral by-products, WB10%, and SB10%, associated with a greater antioxidant power, revealed by FRAP and ABTS assays, after the in vitro digestion process, and with a higher total phenolic content. Therefore, the cereal matrix protected the bioactive content of saffron floral by-products, avoiding their oxidation and degradation and maintaining the antioxidant activity throughout the oral and gastrointestinal in vitro digestion. It is also observed in the figure that these samples were related to a high concentration of organic acids, inulin, and minerals improving the nutritional value when compared to SB0% and SB2.5%, which were not associated with any specific characteristic. The other group, WB0% and WB2.5%, was associated with pH, cohesiveness, and springiness.

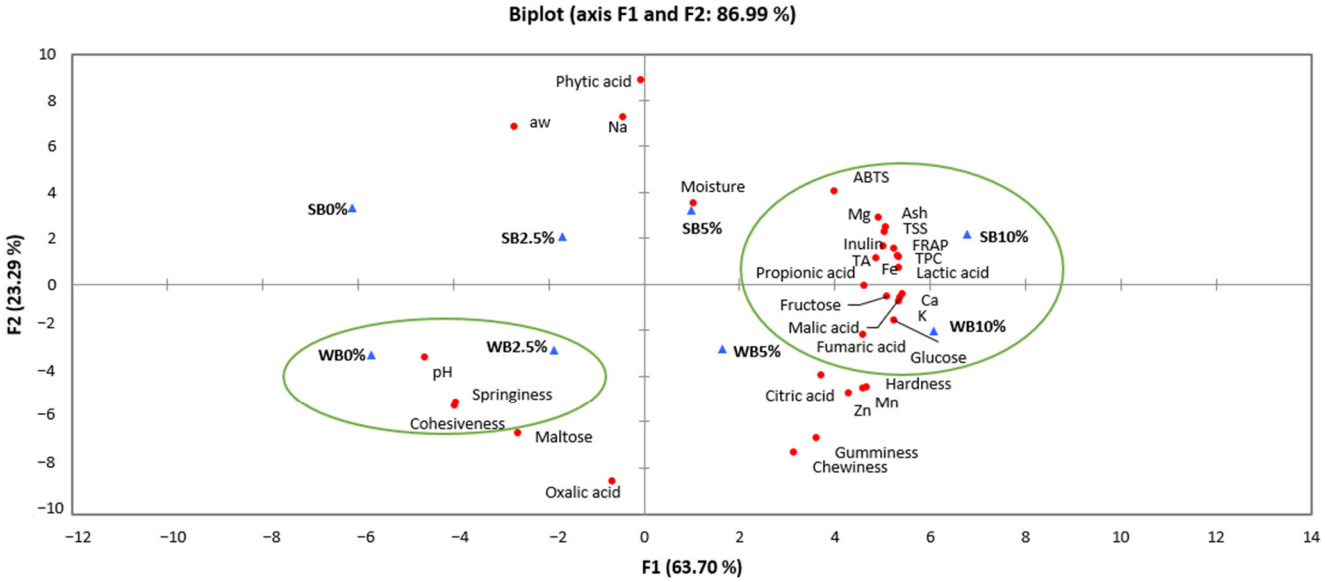

Figure 3. PCA biplot graph regarding physicochemical properties, minerals, organic acids, sugars content, texture, and antioxidant capacity (FRAP, ABTS) and Total Phenolic Content (TPC) after the in vitro digestion. TA: titratable acidity; TSS: total soluble sugars; WB0%, WB2.5%, WB5%, and WB10%: wheat bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively; SB0%, SB2.5%, SB5%, and SB10%: spelt bread formulations with the addition of 0%, 2.5%, 5%, and 10% of dried saffron floral by-products, respectively.

3.3. Sensory Analysis of Bread Formulations Enriched with Saffron Floral By-Products

Sensory evaluation plays an important role in the development of innovative and novel functional food products. Figure 4 summarizes the results of the sensory analysis of enriched bread formulations incorporating 5% of saffron floral by-products compared to the control samples. Appearance descriptors showed high scores regarding the uniformity

of shape in all the formulations, but for the evenness of color of crust, as expected, enriched formulations such as WB5% and SB5% exhibited lower values when compared to the control samples WB0% and SB0%, respectively, due to the incorporation of saffron flowers which had an impact on the color of the crust.

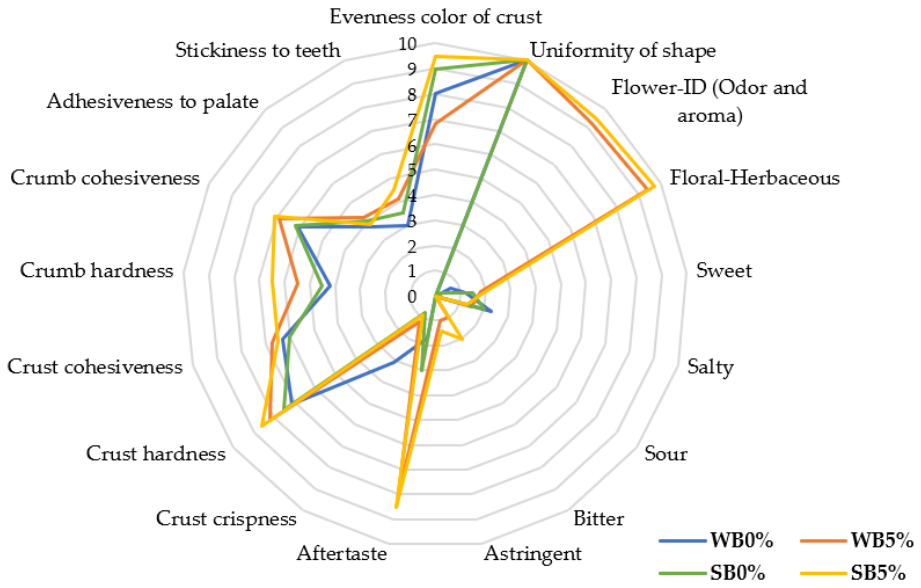

Figure 4. Results of the sensory analysis of selected bread formulations. Data are represented as means ± SD of three replicates. WB0% and WB5%: wheat bread formulations with the addition of 0% and 5% of dried saffron floral by-products, respectively; SB0% and SB5%: spelt bread formulations with the addition of 0% and 5% of dried saffron floral by-products, respectively.

As regards flavor evaluation, WB5% and SB5% had slightly higher values in terms of sweetness, probably due to the sugar concentration of saffron flowers. In the same way, flower-ID and floral–herbaceous attributes were related to the *Crocus sativus* L. flowers in enriched bread formulations, WB5% and SB5%. The intensity of astringency and bitterness, as expected, was higher in samples formulated with saffron floral by-products because of the content of polyphenolic compounds like anthocyanins. The astringency and bitterness could be derived from the precipitation of proline-rich salivary protein in the mouth caused by phenolic compounds [31,32]. Moreover, aftertaste may be linked to the high astringency of samples.

Moreover, the results of texture attributes were in line with the TPA analysis, according to which WB5% and SB5% had higher hardness than the control samples. The use of saffron floral by-products made breads softer than the control samples, reducing their crispness and affecting their cohesiveness, probably due to the addition of this vegetal ingredient that interrupts the gluten network, making breads more fragile [33]. However, with respect to adhesiveness to palate and stickiness to teeth, no important differences were observed between the studied bread formulations.

4. Conclusions

The present study aimed to evaluate the possibility of incorporating saffron floral by-products obtained from saffron spice production into wheat and spelt breads and to study their effect on physicochemical, functional, technological, and sensory properties. The enrichment of traditional breads with saffron flowers was an effective tool that allowed for obtaining functional food with significantly enhanced nutritional and functional potential, increasing the content of dietary fiber such as inulin, minerals, and organic acids, and showing a high antioxidant capacity and total phenolic content after the in vitro digestion process. Therefore, this study confirms that saffron floral by-products are a sustainable alternative for the development of novel bakery products suitable for vegans and vegetarians

which could exert beneficial effects on human health after their intake. It also encompasses the valorization of a biomass that is currently unexploited, minimizing, at the same time, its environmental impact. Furthermore, this research provided new information for further studies that focus on the effects of the saffron floral ingredient at a physiological level after the intake of these functional breads.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12122380/s1; Table S1: Lexicon for the sensory description of bread formulations.

Author Contributions: Conceptualization: D.C.-B. and M.J.F.; methodology: D.C.-B.; writing—original draft preparation: D.C.-B.; writing—review and editing: D.C.-B. and M.J.F.; supervision: M.J.F.; project administration: M.J.F. All authors have read and agreed to the published version of the manuscript.

Funding: This article is supported by the Partnership for Research and Innovation in the Mediterranean Area (PRIMA) program, project "SAFFROMFOOD". The PRIMA program is supported by the European Union.

Data Availability Statement: Data is contained within the article or supplementary material.

Acknowledgments: Thanks to the Spanish Ministry of Education, Culture, and Sport for the FPU-PhD fellowship (FPU18/02225) and thanks to the Spanish company Verdú Cantó Saffron, Spain, S.L for the supply of samples.

Conflicts of Interest: The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Lexicon for the sensory description of bread formulations.

	Attributes	Definition	References and intensities
Appearance	Evenness colour of crust	Distribution of colour	No uniform = 0.0; Uniform = 10.0
	Uniformity of shape	Homogeneity of the bread	No uniform = 0.0; Uniform = 10.0
Flavour	Flower *Crocus sativus* L.-ID (Odor and aroma)	Floral and herbaceous odor associated with *Crocus sativus* L. flowers	5 g of grinded dried flowers of *Crocus sativus* L. + 100 mL H_2O = 4; 20 g of grinded dried flowers of *Crocus sativus* L. + 25 mL H_2O = 10
	Floral-Herbaceous	Fresh, green, slightly sour aromatics associated with green vegetables	Kroger lima beans (canned) = 3.0; Small sprig fresh parsley = 7.0 (aroma); Fresh parsley = 10.0
	Sweet	The fundamental taste factor associated with a sucrose solution	Sucrose solution 4% = 2.5; Sucrose solution 8% = 5.0; Sucrose solution 16% = 9.5
	Salty	Fundamental taste sensation of which sodium chloride is typical	0.2% NaCl solution = 2.5; 0.35% NaCl solution = 5.0; 0.8% NaCl solution = 9.0
	Sour	The taste stimulated by acids, such as citric and malic acids	Tartaric acid solution 0.05% = 2.5; Tartaric acid solution 0.08% = 4.0; Tartaric acid solution 0.20% = 9.5
	Bitter	The taste stimulated by substances such as quinine or caffeine	Caffeine solution 0.05% = 2.5; Caffeine solution 0.08% = 4.0
	Astringent	Dry sensation on the surface of the tongue or mouth associated with alum solution	Alum solution 0.03% = 1.5; Alum solution 0.05% = 2.5; Alum solution 0.1% = 5.0
	Aftertaste	Time in which the specific flavour of bread remains in the mouth after swallowing the sample	10 s = 2.0; 30 s = 8.0
Texture	Crust crispness	The noise and force with which the sample breaks or fractured	Chewing gum (Wrigley) = 0.0; Graham cracker (Honey maid) = 5.0; Corn flakes (Kellogg's) = 10.0
	Crust hardness	Force required to bite through	Cream cheese (Philadelphia) = 1.0; Olives (Goya Foods) = 6.0; Fresh carrot = 10.0
	Crust cohesiveness	Amount of sample that deforms rather than ruptures	Corn muffin (Jiffy) = 1.0; Pretzel = 6.0; Chewing gum (Wrigley) = 10.0
	Crumb hardness	Force required to bite through	Cream cheese (Philadelphia) = 1.0; Olives (Goya Foods) = 6.0; Fresh carrot = 10.0
	Crumb cohesiveness	Amount of sample that deforms rather than ruptures	Corn muffin (Jiffy) = 1.0; Pretzel = 6.0; Chewing gum (Wrigley) = 10.0
	Adhesiveness to palate	Force required to remove sample from palate	Fresh cherry tomato = 0.0; Pretzel rod (Bachman) = 7.0; Rice Krispies (Kellogg's) = 10.0
	Stickiness to teeth	Amount of product that adheres to oral surface	Fresh cherry tomato = 0.0; Pretzel rod (Bachman) = 7.0; Rice Krispies (Kellogg's) = 10.0

Artículo 8

Unveiling the interaction between food thickeners and flavonoids on model beverages of saffron floral by-products

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Enviado a publicar

Unveiling the interaction between food thickeners and flavonoids on model beverages of saffron floral by-products

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ABSTRACT

Despite the rapid and dynamic evolvement of research into dietary polyphenols, there is still a knowledge gap regarding their bioaccessibility, since it could be influenced by the chemical and nutritional compositions of the food matrix. This study aimed to describe the impact of food thickeners (xanthan gum, guar gum, β-glucan, pectin) on the bioactivity of flavonoids from saffron floral by-products in model beverages before and after thermal processing. The different beverage formulas were characterized in terms of polyphenolic composition by HPLC-DAD-ESI-MS^n and rheological properties. The impact of food thickeners and thermal processing on the inhibition of digestive enzymes was also determined. The model beverages mainly presented glycosylated flavonols (of kaempferol, quercetin and isorhamnetin), with a reduced content in some heat-treated samples. The inhibitory effect on α-amylase was only detected in heat-treated beverages, showing the formulation without any thickener the greatest inhibitory effect. Finally, the presence of saffron floral by-products in the beverages showed a decreasing tendency in the flow consistency index (*K*) and increased in flow behavior index (*n*), most probably driven by the aggregation of phenolics with thickeners. Therefore, this research provides insights on the phenolic/thickeners interactions affecting the potential bioactivity of bioaccessible flavonoids in a complex food matrix.

Keywords: rheology; inhibition assay; phenolic compounds; polysaccharides; aggregation.

1. Introduction

Globally, significant amounts of agricultural by-products, side streams or harvest remains are treated as low-value material. Efforts to valorize them have become of interest and key for the sustainability of the agri-food system. In particular, they can be turned into high value-added ingredients for the development of new products, including functional foods (Ben-Othman, Jõudu, & Bhat, 2020). Valorization of saffron (*Crocus sativus* L.) tepals are archetypal of this opportunity since the industrial production of saffron spice only uses the flower's stigmas. Recent studies have reported that the utilization of saffron industrial by-products may represent an interesting source of bioactive nutrients such as flavonols and anthocyanins, and a significant source of income for saffron producers and processors (Cerdá-Bernad, Clemente-Villalba, Valero-Cases, Pastor, & Frutos, 2022; Cerdá-Bernad, Valero-Cases, Pérez-Llamas, Pastor, & Frutos, 2023). In this perspective, the water-soluble phenolic fraction of saffron tepals could be leveraged into functional beverages able to modulate glycemic response. Indeed, phenolic compounds have been shown to inhibit digestive amylases, slow down starch digestion and decrease the rate of intestinal glucose absorption *in vitro*, thereby providing a viable strategy to manage type 2 diabetes (Freitas & Le Feunteun, 2019; Moser et al., 2018; Moser et al., 2020; Nyambe-Silavwe et al., 2015).

The development and production of such beverages usually involve formulation with hydrocolloids such as pectins (E440), xanthan (E415) or guar gum (E412), or even cereal polysaccharides such as oat β-glucans, in order to adjust their stability and their sensory properties (BeMiller, 2019). However, it has been shown that hydrocolloids and phenolic compounds can from non-covalent complexes that affect their respective functionality. Indeed, whereas phenolic compounds are usually partially degraded through thermal processes, it has been shown that complexation with hydrocolloids can protect them from extensive degradation by shielding the phenolics' peripheral hydroxyl groups from oxidative, degradative reactions. Nevertheless, such complexes also sequester phenolic compounds and limit their ability to bind digestive enzyme and glucose transporters, thereby altering their impact on glycemic response (Moser et al., 2020). Finally, the formation of hydrocolloid-phenolics non-covalent complexes has also been shown to lead to extensive aggregation of the hydrocolloids, thereby decreasing their ability to build up viscosity and bind water, and preventing their adequate use as texture agent and stabilizers (Dridi & Bordenave, 2021).

Nonetheless, all these phenomena seem to be phenolic and hydrocolloid-dependent and the use of saffron petal extracts into functional beverages requires to

elucidate their behavior in realistic beverage models incorporating hydrocolloids as texture agents. With this in mind, the current study describes the impact of food thickeners on the bioactivity of flavonoids from saffron floral by-products and their ability to modulate starch digestion, before and after thermal processing (TP) (10 min, 80 °C). The specific objectives were settled as follows: (1) characterization of the phenolic composition of saffron floral by-products and saffron floral by-products-model beverages by HPLC-DAD-ESI-MS^n; (2) Determination of the impact of food thickeners and thermal processing on the inhibition of digestive enzyme (α-amylase); and (3) Characterization of the rheological properties of saffron floral-model beverages which could influence the bioaccessibility of polyphenols, and to provide in-depth information on the structural changes associated with processing of foods upon thermal treatment. Detailed understanding of such food matrix factors was explored for evaluating the positive health-promoting effects of the functional beverages.

2. Material and methods

2.1. Chemicals and reagents

The polysaccharides β-glucan (P-BGBM), guar gum (P-GGMMV) and xanthan gum (P-XANTH) were purchased from Cedarlane (Burlington, ON, Canada). Pectin from apple (poly-D-galacturonic acid methyl ester) was purchased from Sigma-Aldrich (St. Louis, MO, USA). D-(+)-Sucrose (purity 99%) was obtained from TCI America (Portland, OR, USA) and citric acid monohydrate from Fisher Chemical (Hampton, NH, USA). The enzyme α-amylase from porcine pancreas (PPA, type VI-B, 15 units/mg solid), amylopectin from maize (purity > 99%), maltose monohydrate, disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride, potassium sodium tartrate, 3,5-dinitrosalicylic acid and sodium hydroxide were from Millipore-Sigma (Oakville, ON, Canada). Standard compounds, methanol, acetonitrile and formic acid HPLC grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used were of analytical grade and all solutions were prepared with MilliQ water.

2.2. Plant material

Saffron floral by-products were obtained from Castilla-La Mancha region (Spain) and were composed of tepals, stamens and styles, being the stigmas detached manually. Saffron floral by-products were freeze-dried for 48 h, crushed, sieved (500 μm mesh size) and kept at -20 °C until further analysis.

2.3. Preparation of model beverages

The model beverages were prepared using potable water (> 90 % w/w), sucrose (7 % w/w), citric acid (0.20 % w/w), food thickener (0.25 % w/w) (xanthan gum, guar gum, β-glucan, pectin; no thickener as control for the effect of thickeners) and freeze-dried saffron floral by-products (1 % w/w). For each thickener, a model beverage was prepared and a control sample including all the ingredients except saffron flowers was also elaborated. The model beverages were prepared first mixing food thickeners with water under stirring, following dissolution instructions from suppliers. Then, the rest of ingredients were added under stirring until completely dissolved. Samples were centrifugated (11,200xg, 20 min) (Eppendorf Centrifuge 5804/5804R, Sigma Aldrich, St. Louis, MO, USA) to remove insoluble compounds. Furthermore, to study the effect before and after thermal processing, part of samples were thermally treated at 80 °C for 10 min in a water bath (Unitronic 200, J.P. Selecta, Barcelona, Spain) and cooled to 37 °C in an ice bath.

A total of 20 different beverages, including control samples were obtained:

- Control (water, sucrose, citric acid) without thickener non-heat-treated (C), model beverage without thickener non-heat treated (SF), control without thickener heat-treated (C80), model beverage without thickener heat-treated (SF80).
- Control (water, sucrose, citric acid) with xanthan gum non-heat-treated (XGC), model beverage with xanthan gum non-heat treated (XGSF), control with xanthan gum heat-treated (XGC80), model beverage with xanthan gum heat-treated (XGSF80).
- Control (water, sucrose, citric acid) with guar gum non-heat-treated (GGC), model beverage with guar gum non-heat treated (GGSF), control with guar gum heat-treated (GGC80), model beverage with guar gum heat-treated (GGSF80).
- Control (water, sucrose, citric acid) with β-glucan non-heat-treated (BGC), model beverage with β-glucan non-heat treated (BGSF), control with β-glucan heat-treated (BGC80), model beverage with β-glucan heat-treated (BGSF80).
- Control (water, sucrose, citric acid) with pectin non-heat-treated (PC), model beverage with pectin non-heat treated (PSF), control with pectin heat-treated (PC80), model beverage with pectin heat-treated (PSF80).

Each beverage formulation was made in triplicate.

2.4. Preparation of extracts for phenolics identification and quantification

Freeze-dried saffron floral by-products were prepared for HPLC analysis, 100 mg of dried material were weighed for extraction with 1 mL of 50% methanol acidified with

1% formic acid, mixed 30 s vortex, sonicated for 1 h (Branson 551021 Sigma-Aldrich, St. Louis, MO, USA), and subjected to a 24 h overnight at 4 ℃. Then, samples were centrifuged at 10000 rpm for 15 min (Hettich® EBA 21 Sigma-Aldrich, St. Louis, MO, USA). Supernatants were collected and filtered through a 0.22 μmØ polytetrafluoroethylene membrane (Millipore, Bedford, MA, USA) and stored at -20 ℃ until further analysis.

Beverages samples were directly used for HPLC analysis, being previously filtered through a 0.22 μmØ polytetrafluoroethylene membrane (Millipore, Bedford, MA, USA) and stored at -20 ℃ until further analysis.

2.5. Identification and quantification of phenolic compounds by HPLC-DAD-ESI/MS^n

Identification of the phenolic compounds was performed with an Agilent HPLC 1200 series model equipped with a photodiode array detector (model G1315B), a mass detector in series (Agilent Technologies, Waldbronn, Germany), a binary pump (model G1312A), a degasser (model G1322A) and an autosampler (model G1313A), based on a method described Gonçalves et al. (2021). Mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionization interface and was controlled by LC/MS software (Esquire Control Ver. 6.1. Build No. 534.1., Bruker Daltoniks GmbH, Bremen, Germany). A Nucleosil® 100–5 C18 column (25.0 cm x 0.46 cm; 5 μm particle size waters; Macherey-Nagel, Düren, Germany) was used. Mobile phase, pumped at a flow rate of 0.8 mL/min, consisted of 1% aqueous formic acid (solvent A) and acetonitrile (solvent B). The solvent system started with 8% of B, and reached 15% of B at 25 min, 22% at 55 min, and 40% at 60 min, with a washout period of 5 min and returned to initial conditions afterwards. Mass spectra were acquired with a scan range from m/z 100 to 1200 and MS parameters were set as follows: capillary temperature was 350 °C, capillary voltage was set at 4 kV, nebulizer pressure was 65.0 psi and nitrogen flow rate was 11 L/min. Collision-induced fragmentation experiments were performed in ion trap using helium as collision gas, with voltage ramping cycles from 0.3 to 2 V. For anthocyanins, mass spectrometry data were acquired in positive ionization mode while for non-colored phenolics, acquisition was done in a negative ionization mode. MS^n was carried out in automatic mode on more abundant fragment ion in MS$^{(n-1)}$. HPLC system was controlled by ChemStation for LC 3D Systems software Rev. B.01.03-SR2 (204) (Agilent Technologies Spain S.L., Madrid, Spain). Injections (20 μL) of each sample were performed in triplicate.

Spectral data from all peaks were accumulated in a range of 200–600 nm and chromatograms were recorded at 360 nm (flavonols) and 520 nm (anthocyanins), and

compounds in each sample were tentatively identified based on their elution order retention times, and ultraviolet-visible and mass spectra features as compared to authentic standards analyzed under same conditions and data available in literature. Identified compounds were finally quantified using calibration curves of the standard reference compounds, when available, or using the most structurally related reference compound, built in a concentration range from 0.06 to 1 mmol/L.

2.6. α-amylase in-vitro inhibition assay

The inhibition assay was carried out according to D'Costa and Bordenave (2021) with minor modifications, using 50 µL of PBS buffer (blank) or 50 µL of each sample. The model beverages were previously neutralized according to Freitas and Le Feunteun (2019), because the pH of the chime is quickly neutralized in the duodenum, so that it is unlikely that pancreatic enzymes are affected by the native pH of foods. The amount of maltose generated by this assay in the control beverage without thickener and without heat treatment (C) was used as a reference corresponding to 100 % PPA activity. In other beverage systems, the amount of maltose released through the assay was expressed as a percentage of this reference PPA activity, based on the amount of maltose released from the control beverage.

After the reaction, samples (250 µL) were transferred into a 96 well micro-plate and the absorbance was read at 540 nm at 25 °C (Tecan Spark multimode micro-plate reader, Baldwin Park, CA, USA). Absorbance reading was converted into a maltose concentration with a maltose standard curve (R^2 = 0.9982), with maltose concentration ranging from 0 to 1 mg/mL.

2.7. Rheology measurements

Rheological characterization of beverages was performed using a Discovery series Hybrid Rheometer (HR-2) from TA Instruments (New Castle, DE, USA) with measuring concentric cylinder geometry. For each test solution, shear stress (τ in Pa) vs. shear rate (γ in s^{-1}) profiles were measured for shear rate ranging 10^{-1} to 10^{3} s^{-1}.

These profiles were fitted to a Herschel-Bulkley power law, $\tau = \tau_0 + K\gamma^n$ by the least-square method using the Solver function on Microsoft Excel 365 (Microsoft, Redmond, WA, USA). Flow consistency index (*K*, in $Pa.s^{-n}$) and flow behavior index (*n*, dimensionless) of the solutions were extracted from this non-linear regression method (Dridi & Bordenave, 2021).

2.8. Statistical analysis

All experiments were carried out in triplicate. Results were expressed as mean ± standard deviation. The mean comparisons were done using two-way analysis of variance (ANOVA) and by the Tukey's multiple range test, using SPSS version 21.0 software package (SPSS Inc., Chicago, IL, USA). The significant differences were established as $p \leq 0.05$.

3. Results and discussion

3.1. Phenolic profile of saffron floral by-products and model beverages

Saffron floral by-products and the model beverages prepared without adding food thickeners (SF, SF80) were firstly characterized for their profiles in native polyphenols. As reported in Table 1 and Table 2, a total of 14 and 13 polyphenolic compounds were identified or tentatively identified and quantified in saffron floral by-products and in the model beverages, respectively. Regarding the flavonol fraction in saffron floral extracts (Table 1), it was composed by kaempferol, quercetin and isorhamnetin glycosides. Kaempferol-*O*-sophoroside resulted the most abundant polyphenolic compound (1058 ± 38 mg/100 g), followed by quercertin dihexoside (II) (222 ± 12 mg/100 g) and kaempferol-*O*-sophoroside-*O*-glucoside (204 ± 16 mg/100 g). Glycosides of isorhamnetin, like isorhamnetin dihexoside and isorhamnetin-*O*-rutinoside, were also presented but in lower concentrations (42-74 mg/100 g). Moreover, four anthocyanins were identified and quantified in the extracts of saffron floral by-products, including petunidin and delphinidin derivatives. Delphinidin-di-*O*-glucoside, followed by delphinidin-*O*-glucoside, were the main anthocyanins found in saffron floral extracts, showing a concentration of 145 ± 13 and 80.7 ± 6.8 mg/100 g, respectively. Petunidin-di-*O*-glucoside and petunidin-*O*-glucoside were presented in lower amounts (43.5 ± 3.7 and 23.9 ± 1.4 mg/100 g, respectively). These results were in accordance with previous studies that reported the highest content for kaempferol-*O*-sophoroside and delphinidin-di-*O*-glucoside in saffron floral bio-residues, being the flavonol fraction mainly composed by kaempferol derivatives (Gigliobianco et al., 2021; Goupy, Vian, Chemat, & Caris-Veyrat, 2013).

Concerning saffron floral model beverages, the same composition on polyphenols as saffron floral extracts was found, except quercetin hexoside, but with lower concentrations of each compound, since beverages contained 1% of saffron floral by-products (Table 2). Nevertheless, statistically significant differences were observed in the concentration of some compounds between the heat-treated (SF80) and the non-heat-treated (SF) model beverages. Both model beverages were mainly composed by kaempferol, quercetin and isorhamnetin glycosides, being kaempferol-*O*-sophoroside the major one found, showing SF statistically significant higher amounts (22.9 ± 0.6

mg/100 mL) compared to the heat-treated beverage SF80 (21.6 ± 0.4 mg/100 mL). Kaempferol-*O*-sophoroside-*O*-glucoside was also presented in significant higher concentrations in the model beverage non-heat-treated (5.57 ± 0.11 mg/100 mL) respect to the heat-treated sample (5.27 ± 0.06 mg/100 mL). Besides kaempferol sophoroside-derivatives, quercetin dihexoside (II) was detected in elevated amounts (4.1-4.6 mg/100 mL). Regarding anthocyanins composition, petunidin and delphinidin derivatives were also present in saffron floral model beverages, being delphinidin-di-*O*-glucoside and delphinidin-*O*-glucoside the major ones. The model beverage non-heat-treated showed statistically significant higher amounts of delphinidin-*O*-glucoside, petunidin-di-*O*-glucoside and petunidin-*O*-glucoside compared to the heat-treated beverage. These differences found in polyphenols' concentration of saffron floral by-products model beverages could be related to the degradation of polyphenols upon thermal processing (D'Archivio, Filesi, Varì, Scazzocchio, & Masella, 2010). These results exhibited trends similar to those of previous research in which the thermal processing significantly affected individual polyphenolic compounds (Xu & Chang, 2009).

3.2. Inhibitory effect of saffron floral by-products model beverages on the enzymatic activity of pancreatic α-amylase

Due to the polyphenolic composition of saffron floral by-products model beverages, their capacity to inhibit the activity of digestive amylases, such as the pancreatic α-amylase, was studied. Functional foods rich in polyphenols could be potential candidates for improving glucose homeostasis by reducing intestinal absorption of dietary glucose through inhibition of digestive enzymes and thereby slow down starch digestion (Nyambe-Silavwe et al., 2015). The inhibitory effect of the tested saffron floral by-products model beverages on the activity of PPA is presented in Figure 1, as a percentage of PPA activity in the control beverage.

In the non-heat-treated samples, no inhibitory effect was detected in any model beverage, thus only the results of samples after thermal processing are shown in Figure 1.

No PPA inhibitory activity was detected in heat-treated control beverages without saffron floral by-products, as expected. No statistically significant PPA inhibitory activity was detected either in BGSF80 and PSF80 model. The heat-treated model beverage without thickener SF80 showed the greatest inhibitory effect on PPA (~37%), since PPA activity was 62.46 ± 7.18%, followed by GGSF80 and XGSF80. The heat-treated model beverage with guar gum also presented similar inhibitory capacity on PPA (~29%), being the PPA activity 71.42 ± 5.38%, without statistically significant difference compared to

SF80. However, statistically significant differences were found between XGSF80 and SF80, presenting inhibitory levels about 20% on PPA, as the α-amylase activity in XGSF80 was 79.75 ± 4.59%.

All model beverages contained 1% of phenolic-rich saffron floral by-products, did not differ in their sugar composition, and although the model beverages differed in their pH, the PPA assays were all conducted after pH neutralization, reflecting *in vivo* pH neutralization of the chime once it is propelled into the duodenum (Freitas & Le Feunteun, 2019). Therefore, differences in PPA inhibition could only be due to the addition of food thickener added and the thermal processing.

Polyphenols can inhibit α-amylase through binding with it. However, the presence of polysaccharides, such as those naturally present in saffron flowers and the added food thickeners, could introduce competitive binding opportunities for the phenolic compounds and decrease polyphenol-enzyme binding, and consequently decrease the ability of polyphenols to inhibit the enzyme (Bordenave, Hamaker, & Ferruzzi, 2014; Sun, Warren, & Gidley, 2019). This has been shown in previous studies with soluble fibers such as β-glucan, pectins, arabinoxylans, starch itself as well as xanthan and guar gum (D'Costa & Bordenave, 2021; D'Costa & Bordenave, 2023; D'Costa, Golding, Raval, Rolland-Sabaté, & Bordenave, 2023; Northrop, D'Costa, Tosh, & Bordenave, 2022; Sun et al., 2019). Our results are consistent with these findings with the observation that the model beverage without thickener (therefore without polysaccharide to interfere with enzyme-polyphenol binding and inhibition) showed the greatest PPA inhibition.

However, temperature also played an important role as it appears that all samples that were heat-treated did not exhibit PPA inhibition. This observation suggests that in heat treated samples, polyphenols were available enough to bind (and inhibit) PPA as if polysaccharides were not present, although they were. The thermal processing could have affected the hydrogen-bonding driving polysaccharides-polyphenols interactions, and molecular thermal agitation may prevent polysaccharide-polyphenol binding, which is known to be weaker than polyphenol-protein binding (Dobson et al., 2019; Tudorache, McDonald, & Bordenave, 2020). This could explain why heat-treated model beverages, such as SF80, XGSF80 and GGSF80, showed an inhibitory effect on digestive enzymes. Therefore, polyphenol-polysaccharide complexation had a direct effect on the inhibition of digestive enzymes activity by phenolic compounds, but further research is needed in order to unveil the influence of other factors and how they impact on the functional properties of food products, altering the functionality of polyphenols.

3.3. Rheological properties of saffron floral by-products model beverages

Rheological properties play an important role in developing new functional food products, providing valuable information on the structural changes associated with food processing, and to understand the interaction of food components (Day & Golding, 2016).

Rheological properties of the saffron floral by-products model beverages were characterized from the fitting of stress vs. shear rheograms fitted with a Herschel-Bulkley power law and the results are reported in Table 3.

In these results, the flow behavior index, *n,* characterizes the behavior of a solution under shear (shear-thinning for n<1, Newtonian for n=1, shear-thickening for n>1). Flow consistency index, *K*, is reflective of the viscosity of a solution at comparable shear rate and flow behavior index.

Results reported in Table 3 for solutions without saffron extract show that control solutions were essentially Newtonian, as were beverages with β-glucans and pectins (n ≈ 1) which also exhibited similarly low viscosity (similar consistency index K). On the contrary, model beverages containing xanthan and guar gums exhibited marked shear-thinning behavior (n ≈ 0.4-0.9) and marked viscosity. The addition of saffron floral by-products rich in polyphenols to model beverages thickened with guar and xanthan gums led to a decrease flow consistency index *K* and an increase of flow behavior index *n*. This has been observed before and has been attributed to phenolic-driven aggregation of thickeners, leading the solution to behave more like water in terms of flow and of viscosity (Dridi & Bordenave, 2021; Tudorache & Bordenave, 2019; Tudorache et al., 2020). Specifically, in model beverages with xanthan gum, thermal processing (XGC80, XGSF80) tends to significantly decrease *K* and τ_0, which is reflective of the minimum stress to be applied to the liquid so it starts flowing. Thermal processing and saffron floral by-products (XGSF80) tend to significantly increase *n*. In model beverages thickened with guar gum, saffron floral by-products (GGSF, GGSF80) tend to significantly increase τ_0 and *n*, and combined with thermal processing tend to significantly decrease *K* (GGSF, GGC80, GGSF80). The results about model beverages with β-glucans were consistent with those of guar and xanthan gum but they had very limited sensitivity to the presence of saffron floral by-products. This fact may be related to a less binding of β-glucans with flavonols, the major polyphenols presented in the studied beverages (Jakobek, Ištuk, Matić, & Skendrović Babojelić, 2021).

As expected, model beverages with pectin showed opposite behavior as compared to xanthan gum, guar gum and β-glucans, since pectin was the only strongly ionic gum in this study, which may impact the nature of interactions with phenolic

compounds. Saffron floral by-products (PSF) tend to significantly increase K and τ_0 and decrease n. However, for model beverages without thickener no statistically significant variations were found for K and n, regarding the presence of saffron floral by-products and the thermal processing.

It must be noted that these observations about the effect of thermal processing-saffron floral by-products interactions could be confounded by paradoxical effects, with possible heat degradation of saffron phenolic compounds on the one hand (decreasing their effect of the rheological properties of the beverages), and potentially enhanced hydration/dispersion of food thickeners on the other hand (increasing potential binding with polyphenols). Nevertheless, further detailed examination is needed to bring additional insights into the binding and aggregation mechanisms. From the point of view of the impact of polyphenol-polysaccharide interactions on these model beverages, the reported observations are important as they may influence food processing and formulation choices depending on food thickener composition requirement and phenolic content of the formulation. Indeed, in these saffron floral by-products model beverages, aggregation may lead to drastically different effects depending on the type of food thickener chosen.

4. Conclusions

Detailed understanding of how food matrix factors interactions influenced the bioactivity of polyphenols from saffron floral model beverages was explored, providing new information on their functionality. This research demonstrated that the addition of food thickeners in beverages formulations played a key role in the bioactivity and bioaccessibility of flavonoids due to the polysaccharide-polyphenol interactions such as phenolic-driven aggregation of polysaccharides. These conclusions may be important to develop new functional food products rich in phenolic compounds where gums or fibers are used as thickening agents, in order to ensure that they exert the expected beneficial effects after their ingestion. Besides, the possibility of using dried saffron floral by-products without any additional processing could be an important issue for the food industry, contributing to minimize the environmental impact.

CRediT authorship contribution statement: Débora Cerdá-Bernad: Conceptualization, methodology, writing-original draft preparation, writing-review and editing. **Adrian S. D'costa:** Methodology. **Diego A. Moreno:** Methodology, writing-review and editing. **Nicolas Bordenave:** Conceptualization, writing-review and editing, supervision. **María José Frutos:** Writing—review and editing, supervision, project

administration. All authors have read and agreed to the published version of the manuscript.

Funding: This article is supported by the Partnership for Research and Innovation in the Mediterranean Area (PRIMA) program, project SAFFROMFOOD. The PRIMA program is supported by the European Union. The research was also supported by the regional agency “Diputación de Alicante”, Alicante, Spain.

Acknowledgments: Thanks to Green Chemical Engineering Network towards upscaling sustainable processes (CA18224)—GREENERING funded by COST (European Cooperation in Science and Technology) for the STSM grant and thanks to the Spanish-Ministry of Education, Culture and Sport for the FPU-PhD fellowship (FPU18/02225).

Conflicts of Interest: The authors declare no conflict of interest.

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Table 1. Chromatographic, mass spectra characteristics and quantification of native polyphenols identified in saffron floral by-product extracts. Data are expressed as mean ± SD (*n*=3).

Compound	RT (min)	$[M]^+$ (m/z)	$[M-H]^-$ (m/z)	MS^n (m/z)	Concentration (mg/100 g dw)
Flavonols					
Kaempferol-3-*O*-sophoroside-7-*O*-glucoside	7.50		771	609, 285, 429	204 ± 16
Isorhamnetin 3,7-*diO-hexoside* (I)	13.63		639/641	315, 271	44.9 ± 4.5
Quercetin 3,4'-*O*-diglucoside (I)	16.03		625	463, 301	70.6 ± 2.7
Quercetin 3,4'-*O*-diglucoside (II)	16.45		625	301, 463, 445	222 ± 12
Isorhamnetin 3,7-*diO-hexoside* (II)	17.30		639	315, 477, 300	74.3 ± 2.7
Kaempferol-3-*O*-sophoroside	18.95		609	285, 429	1058 ± 38
Quercetin 3-*O*-glucoside	21.51		463	301, 151	42.6 ± 4.2
Isorhamnetin-3-*O*-rutinoside	21.68		623	315, 459	71.5 ± 4.2
Kaempferol-3-*O*-(acetyl-glycoside)-7-*O*-glycoside	24.66		651	285, 489, 471	103 ± 5
Kaempferol 3-*O*-glucoside	24.88		447	285	151 ± 8
Anthocyanins					
Delphinidin-3,5-*O*-diglucoside	5.05	627		465, 303	145 ± 13
Petunidin-3,5-*O*-diglucoside	7.21	641		479, 317	43.5 ± 3.7
Delphinidin-3,5-*O*-diglucoside	8.96	465		303	80.7 ± 6.8
Petunidin-3-*O*-glucoside	12.02	479		317	23.9 ± 1.4

RT: retention time; m/z: mass to charge.

Table 2. Chromatographic, mass spectra characteristics and quantification of native polyphenols identified in saffron floral by-product model beverages without thickener.

Compound	RT (min)	$[M]^+$ (m/z)	$[M-H]^-$ (m/z)	MS^n (m/z)	Concentration (mg/100 mL of beverage)	
Flavonols					**Non-heat-treated (SF)**	**Heat-treated (SF80)**
Kaempferol-3-*O*-sophoroside-7-*O*-glucoside	7.50		771	609, 285, 429	5.57 ± 0.11[a]	5.27 ± 0.06[b]
Isorhamnetin 3,7-*diO*-*hexoside* (I)	13.63		639/641	315, 271	2.71 ± 0.12	2.74 ± 0.01
Quercetin 3,4'-*O*-diglucoside (I)	16.03		625	463, 301	2.85 ± 0.02	2.82 ± 0.02
Quercetin 3,4'-*O*-diglucoside (II)	16.45		625	301, 463, 445	4.64 ± 0.10[a]	4.17 ± 0.09[b]
Isorhamnetin 3,7-*diO*-*hexoside* (II)	17.30		639	315, 477, 300	2.93 ± 0.04[a]	2.85 ± 0.02[b]
Kaempferol-3-*O*-sophoroside	18.95		609	285, 429	22.9 ± 0.6[a]	21.6 ± 0.4[b]
Isorhamnetin-3-*O*-rutinoside	21.68		623	315, 459	2.85 ± 0.01[a]	2.81 ± 0.02[b]
Kaempferol-3-*O*-(acetyl-glycoside)-7-*O*-glycoside	24.66		651	285, 489, 471	3.08 ± 0.01[a]	3.02 ± 0.02[b]
Kaempferol 3-*O*-glucoside	24.88		447	285	3.37 ± 0.03	3.33 ± 0.04
Anthocyanins						
Delphinidin-3,5-*O*-diglucoside	5.05	627		465, 303	3.57 ± 0.37	3.44 ± 0.07
Petunidin-3,5-*O*-diglucoside	7.21	641		479, 317	1.29 ± 0.03[a]	1.23 ± 0.02[b]
Delphinidin-3-*O*-diglucoside	8.96	465		303	1.67 ± 0.03[a]	1.60 ± 0.02[b]
Petunidin-3-*O*-glucoside	12.02	479		317	0.88 ± 0.00[a]	0.86 ± 0.00[b]

Means ± standard deviation in the same row followed by different lowercase letters indicate statistically significant differences at $p \leq 0.05$ for each model beverage; RT: retention time; m/z: mass to charge.

Table 3. Flow parameters of saffron floral by-products model beverages fitted by the Herschel and Bulkley model.

	τ_0 (Pa)	K (Pa.s^{-n})	n
C	0.56 ± 0.62 [a]	0.19 ± 0.03	1.01 ± 0.01
SF	1.4 ± 1.6 [ab]	0.18 ± 0.02	1.02 ± 0.01
C80	0.78 ± 0.68 [ab]	0.20 ± 0.05	1.01 ± 0.02
SF80	0.0 ± 0.0 [b]	0.22 ± 0.04	1.00 ± 0.02
XGC	750 ± 70 [a]	294 ± 7 [b]	0.39 ± 0.00 [bc]
XGSF	615 ± 71 [b]	342 ± 33 [a]	0.38 ± 0.01 [c]
XGC80	115 ± 4 [c]	167 ± 45 [c]	0.42 ± 0.02 [b]
XGSF80	206 ± 11 [c]	1.52 ± 0.11 [d]	0.84 ± 0.01 [a]
GGC	0.0 ± 0.0 [c]	95.3 ± 5.7 [a]	0.48 ± 0.00 [c]
GGSF	121 ± 4 [a]	1.54 ± 0.29 [c]	0.87 ± 0.02 [a]
GGC80	0.0 ± 0.0 [c]	77.2 ± 4.0 [b]	0.50 ± 0.00 [c]
GGSF80	90.9 ± 2.6 [b]	9.42 ± 0.80 [c]	0.70 ± 0.01 [b]
BGC	38.0 ± 1.8 [a]	0.14 ± 0.02	1.09 ± 0.01 [a]
BGSF	12.8 ± 3.5 [b]	0.17 ± 0.02	1.05 ± 0.01 [b]
BGC80	39.3 ± 5.4 [a]	0.14 ± 0.02	1.09 ± 0.02 [a]
BGSF80	33.4 ± 1.7 [a]	0.17 ± 0.01	1.07 ± 0.01 [ab]
PC	8.2 ± 1.3 [a]	0.19 ± 0.02 [b]	1.03 ± 0.01 [a]
PSF	287 ± 256 [b]	1.06 ± 0.12 [a]	0.88 ± 0.01 [b]
PC80	10.0 ± 0.9 [a]	0.18 ± 0.01 [b]	1.03 ± 0.01 [a]
PSF80	8.3 ± 7.3 [a]	0.20 ± 0.02 [b]	1.02 ± 0.02 [a]

Means ± standard deviation in the same column followed by different lowercase letters indicate statistically significant differences at $p \leq 0.05$ for each model beverage; C: control without thickener non-heat-treated; SF: model beverage without thickener non-heat treated; C80: control without thickener heat-treated; SF80: model beverage without thickener heat-treated; XGC: control with xanthan gum non-heat-treated; XGSF: model beverage with xanthan gum non-heat treated; XGC80: control with xanthan gum heat-treated; XGSF80: model beverage with xanthan gum heat-treated; GGC: control with guar gum non-heat-treated; GGSF: model beverage with guar gum non-heat treated; GGC80: control with guar gum heat-treated; GGSF80: model beverage with guar gum heat-treated; BGC: control with β-glucan non-heat-treated; BGSF: model beverage with β-glucan non-heat treated; BGC80: control with β-glucan heat-treated; BGSF80: model beverage with β-glucan heat-treated; PC: control with pectin non-heat-treated; PSF: model beverage with pectin non-heat treated; PC80: control with pectin heat-treated; PSF80: model beverage with pectin heat-treated.

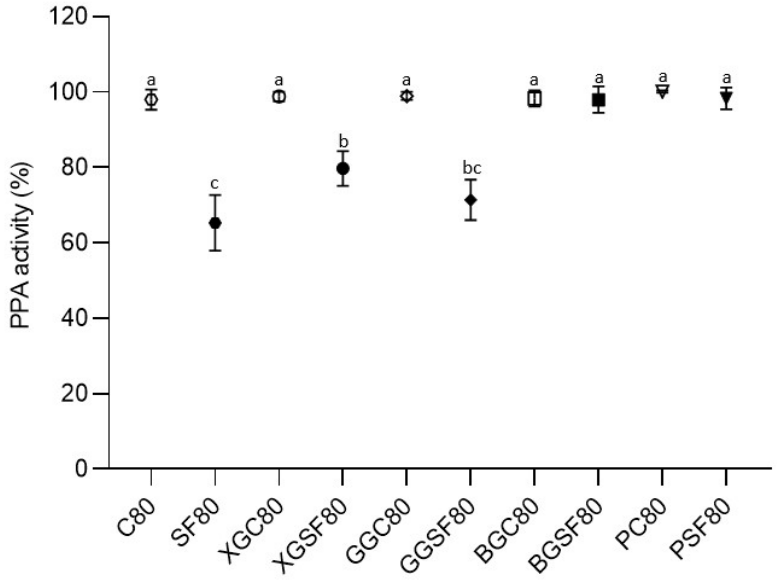

Figure 1. Impact of saffron floral by-products model beverages on the activity of pancreatin α-amylase. Data represent means ± standard deviation and different lowercase letters indicate statistically significant differences at $p \leq 0.05$ for each model beverage; C80: control without thickener heat-treated; SF80: model beverage without thickener heat-treated; XGC80: control with xanthan gum heat-treated; XGSF80: model beverage with xanthan gum heat-treated; GGC80: control with guar gum heat-treated; GGSF80: model beverage with guar gum heat-treated; BGC80: control with β-glucan heat-treated; BGSF80: model beverage with β-glucan heat-treated; PC80: control with pectin heat-treated; PSF80: model beverage with pectin heat-treated.