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Lemon Peel and Juice: Metabolomic Differentiation

Pablo Melgarejo, Dámaris Núñez-Gómez, Francisca Hernández, Rafael Martínez-Font, Vicente Lidón Noguera, Juan José Martínez-Nicolás * and Pilar Legua

Centro de Investigación e Innovación Agroalimentaria y Agroambiental (CIAGRO-UMH), Miguel Hernandez University, Ctra. Beniel km 3.2, 03312 Orihuela, Spain

* Correspondence: juanjose.martinez@umh.es

Abstract: Lemon is one of the most significant crops globally, with annual production exceeding 20.8 million tons in 2021. Spain leads the production in Europe with over 62% of lemon production (1.17 million tons in 2021). This study evaluated the real impact of cultivation conditions (rootstock and culture medium) on the compositional characteristics of 'Verna' lemons (peel and juice) using ^{1}H-MNR metabolomic identification techniques and multivariate analyses. Twenty metabolites were identified in both the peel and juice samples. Arginine, phenylalanine, ethanol, and trigonelline were absent in the peel samples but present in all the juice. On the other hand, the metabolites asparagine, glutamate, formate, and malate were present in the peel samples but absent in the juice. The analysis of the results indicates that the rootstock had a significant impact on the metabolites related to the energy metabolism of the plant, which directly affects the development of fruits and the influence of the culture conditions (rootstock and culture medium) on the plant's adaptive response and modification of metabolic pathways.

Keywords: *Citrus limon* (L.) Burm. F; rootstock; culture media; 'Verna' cultivar; ^{1}H-MNR; multivariate analysis

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1. Introduction

Citrus limon (L.) Burm. F. is an evergreen tree from the Rutaceae family [1]. The yellow fruit of *C. limon*, commonly known as lemon, is its main raw material. Lemon is one of the most significant crops globally, with annual production exceeding 20.8 million tons in 2021, ranking second only to orange and tangerine [2]. According to the latest FAOSTAT report [2], India, Mexico, Turkey, and Spain are the largest lemon-producing countries, accounting for 17, 14, 7, and 4% of world production, respectively. However, Spain leads the production in Europe with over 62% of lemon production (1.17 million tons in 2021), followed by Italy (28%), Greece (5%), and Portugal (1%).

Lemons are grown for fresh fruit markets or processing into pectin, juice, and essential oil [3]. The size and peel color are significant characteristics of fresh market fruits, while for processing, soluble solids, juice, pectin, and essential oil content are critical [4]. Therefore, understanding the composition of lemons, both the peel and the juice, as well as the handling parameters that may affect its composition, is vital.

In citriculture, rootstock selection is one of the most important factors for crop management as lemon trees respond differently to growth, fruit quality, and nutrient accumulation when grown on various rootstocks [5]. Good lemon production largely depends on selecting compatible and adequate rootstocks, which provide better adaptability and response to the edaphoclimatic conditions of the trees [6]. This better adaptability translates into better fruit quality [6]. Despite advances in understanding rootstock–scion interactions, there is relatively little knowledge about their effects on the overall fruit metabolite composition [7].

Metabolomics is an analytical technique used to study the complete profile of metabolites present in an organism or tissue at a specific moment [8]. This technique has found a broad range of applications in fruit research, as the metabolic profile of fruits can vary

depending on the variety, maturity stage, and environmental and/or cultivation conditions [9]. One of the most widely used techniques in fruit metabolomic studies is proton nuclear magnetic resonance spectroscopy (^{1}H-MNR), which allows for the identification and quantification of different metabolites present in the sample [10]. This technique is based on the detection of the resonance signal of metabolite protons in the presence of a magnetic field. The signal of each metabolite is characterized by its chemical shift, which is unique and specific to each compound [10,11].

Combining ^{1}H-MNR with multivariate statistical techniques, such as principal component analysis (PCA) and partial least squares (PLS) regression, among others, has enabled the analysis of large datasets and the identification of the most relevant metabolites [12]. These statistical methods allow for the identification of patterns and correlations between different metabolites, which facilitates the interpretation of results and the identification of biomarkers for different biological and pathological processes [13].

The use of the ^{1}H-MNR technique combined with multivariate statistical techniques in fruit metabolomic studies has proven to be a potent approach for analyzing the metabolic composition of fruits and identifying valuable biomarkers. This approach is particularly useful in evaluating the quality and adaptive responses to agronomic modifications, such as changes in cultivation patterns or mediums.

This study aimed to evaluate the real impact of cultivation conditions (rootstock and culture medium) on the compositional characteristics of 'Verna' lemons using metabolomic identification techniques. The economic and industrial importance of lemons necessitated the evaluation of both the peel and the juice of the fruits. The present work builds on previous work carried out by the authors [6,14–16] and aims to provide clear answers and increase knowledge about cultivation techniques in citriculture and their impacts while maintaining an agronomic perspective and prioritizing fruit quality factors.

Note that, despite the extensive research on lemon fruit characterization [17–19], to our knowledge, no previous studies have specifically examined the influence of culture media and rootstock on lemon fruit metabolites, as the majority of studies that use a metabolomic approach in lemon fruits evaluate the impact of postharvest treatments on fruit quality [20–22]. Therefore, the present study intends to address this research gap and provide new insights into the impact of cultivation practices on lemon fruit metabolites.

2. Materials and Methods

2.1. Plant Material and Experimental Design

In this study, the metabolomic characteristics of *Citrus limon* (L.) Burm variety 'Verna' lemons obtained in nine different treatments (Table 1) were evaluated. The evaluated treatments respond to the modification of two controlled variables: the rootstock (n = 3) and the culture medium (n = 3). The most common rootstocks used in commercial citriculture were evaluated: (*i*) *Citrus macrophylla*; (*ii*) *Citrus aurantium*; and (*iii*) the combination between *Citrus aurantium* and *Citrus sinensis*.

Related to the culture medium, three substrates composed of the mixture of peat and phytoremediated marine sediment in different proportions were evaluated: (*i*) 25% sediment + 75% peat; (*ii*) 50% mix of peat and sediment; and (*iii*) 75% sediment + 25% peat. The marine sediment used comes from the port of Livorno (Italy) and was previously phytoremediated for three years and successfully used in other ornamental and food crops [23–27].

For each of the nine treatments (1 cultivar × 3 rootstocks × 3 substrates), a total of 10 trees were evaluated with an experimental design of random distribution by blocks (n = 5) and 2 trees of each combination per block. In total, the fruits obtained from 90 lemon trees (3 substrates × 3 rootstocks × 2 trees × 5 blocks) of 2 years of age cultivated in an experimental plot of the Miguel Hernandez University (Orihuela, Spain) were evaluated. Both the growing conditions and the management of the crop remained homogeneous throughout the trial in order to minimize external influences on the parameters evalu-

ated and study the morphological and nutritional variations/differences of the lemons objectively.

Table 1. Specifications of the rootstock and the culture medium of the lemon fruits evaluated in this study, with emphasis on the acronym used.

Rootstock	Culture Media		Acronym
	Peat Content (%)	Port Sediment Content (%)	
Citrus macrophylla	75	25	25 M
	50	50	50 M
	25	75	75 M
Citrus aurantium	75	25	25 A
	50	50	50 A
	25	75	75 A
Citrus aurantium/Citrus sinensis	75	25	25 AS
	50	50	50 AS
	25	75	75 AS

In all cases, the lemons were harvested manually once the fruit reached commercial maturity [28]. Once the lemons were collected, they were immediately transported to the laboratory, and their processing began. The morphological, pomological, and compositional characteristics of the lemons confirmed the adequacy of the experimental test and the quality of the fruits obtained. These results have already been published by the same authors and can be consulted at [6].

2.2. Metabolomic Profile of Lemons

For each combination studied (n = 9), 5 fruits were taken per replicate, totaling 25 fruits per sample (5 fruits × 5 blocks). Once in the laboratory, the surface of the lemons was cleaned manually with distilled water in order to remove possible dust and dirt residues. Lemon juice was carefully obtained using a manual commercial juicer (Citromatric Deluxe, MPZ-22, Braum), while the peel (albedo + flavedo) was cut into small pieces. In both cases, the samples were stored in sterile polypropylene containers with 50 mL maximum capacity screw-top buffer (Deltalab, Barcelona, Spain) and kept at −80 °C until lyophilization for 48 h (Christ ^{Alpha} 2–4, LSCplus, Martin Christ). The lyophilized samples were stored in sterile polypropylene tubes (Deltalab, Barcelona, Spain) at −20 °C until metabolomic analysis was performed. Both the extraction of the lyophilized samples and the determination of the metabolites using nuclear magnetic resonance (^{1} H-NMR) were performed according to the methodology described by Van der Sar et al. [29] with the modifications specified in [14,30]. In this sense, the following protocol was used for sample preparation: 0.5 mg of lyophilized sample was mixed with a hydromethanolic mixture (1:1, MeOH: H_2O) in Eppendorf tubes of 2 mL maximum capacity. The mixture was sonicated for 3 min at 1 min intervals and left at 4 °C for 30 min. After centrifugation at 11,000 rpm for 20 min at 4 °C, the recovered supernatant was subjected to Speed-Vaccum at a maximum temperature of 27 °C until all the liquid phase had evaporated overnight. The soluble solid obtained was then resuspended in 800 µL of 100 mM potassium phosphate buffer (KH_2PO_4) at pH = 6.0 (dissolved in 100% D_2O) + 0.58 mM of TPS (internal standard) and filtered using 0.45 µm nylon filters. Finally, 600 µL aliquots of the filtered volume were placed in 5 mm NMR tubes for quantification using ^{1}H-NMR.

2.3. Multivariate Statistical Analysis

^{1}H-NMR results of the samples were analyzed using the MestReNova Software (Mestrelab Research, Santiago de Compostela, Spain). Spectral intensities were pooled

(δ 0.04) considering the region of δ 0.5–9.0. The regions corresponding to the solvent D_2O (δ 4.70–4.9) and water (δ 3.09–3.15) were not considered in the analysis [31]. Subsequent statistical analysis was performed using MetaboAnalyst 5.0 (Wishart Research Group, University of Alberta, Edmonton, Canada), which allowed the identification and definition of spectral intensities, as well as principal component analysis (PCA) and partial least squares discriminant analysis (PLSD-DA). Loading plots, variable Importance in projection (VIP), and *t*-tests (*p*-values < 0.05) were used to determine metabolites contributing to significant between-group differences in PLS-DA score plots [32].

2.4. Metabolic Pathway and Network Analysis

Additionally, debiased sparse partial correlation algorithm (DSPC) network analysis was performed. The metabolic pathway was predefined with pathway impact values greater than 0.02 and a *p*-value less than 0.05. Each estimated metabolite in both lemon peel and lemon juice was compared with metabolites belonging to different metabolic pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Its statistical *p*-value was estimated, as well as the threshold for those with values less than 0.02 [33,34].

3. Results and Discussion

3.1. Metabolomic Profile of Lemon Fruits

The ^{1}H-NMR spectra analysis of the 27 lemon peel and juice samples (3 substrates × 3 rootstocks × 3 repetitions for each of the analyzed parts) revealed significant compositional differences. While 20 metabolites were identified in both the peel and the juice, the lemon peel contained 10 amino acids, 5 organic acids, 4 sugars, and 1 intermediate metabolite, while the lemon juice had 10 amino acids, 3 organic acids, 4 sugars, and 3 secondary metabolites (Table 2). The identified metabolites were consistent with previously reported values for both peel and juice in the literature [35–37].

Table 2. Concentration of the metabolites identified for the different parts of the lemon fruit. The results correspond to the mean values (*n* = 27) expressed in mM.

Metabolites	Samples	
	Peel	**Juice**
Amino acids (mM)		
GABA	0.44	1.31
Alanine	0.64	2.30
Arginine	ND	0.39
Asparagine	7.13	ND
Aspartate	0.27	23.55
Glutamate	0.25	ND
Glutamine	0.43	1.68
Isoleucine	0.02	0.05
Leucine	0.02	0.04
Phenylalanine	ND	0.08
Proline	3.05	4.47
Valine	0.03	0.13

Table 2. *Cont.*

Metabolites	Samples	
	Peel	**Juice**
Organic acids (mM)		
Ascorbate	0.75	2.01
Citrate	3.03	327.46
Format	0.02	ND
Lactate	0.06	0.17
Malate	0.67	ND
Sugars (mM)		
Fructose	17.15	32.41
Glucose	36.64	28.10
Myo-inositol	2.68	2.01
Sucrose	10.92	7.91
Other metabolites (mM)		
Choline	0.26	0.11
Ethanol	ND	0.75
Trigonelline	ND	0.08

ND: not detected.

At the qualitative level (the type of metabolites), certain metabolites, such as arginine, phenylalanine, ethanol, and trigonelline were absent in the peel samples but present in all the juice samples. Arginine is known for its diverse functional role in regulating the growth and development of plants, particularly in their fruits [38–40], and phenylalanine is linked to a range of enzymes involved in the biosynthesis of aromatic amino acids [41].

On the other hand, the metabolites asparagine, glutamate, formate, and malate were present in the peel samples but absent in the juice samples (Table 2). Studies on plants have established the relationship of these metabolites with biosynthetic pathways and energy metabolism [42–44]. Notably, there were concentration differences in some metabolites, such as aspartate in the juice or glucose in the peel, between the samples.

3.2. Lemon Peel Samples

Lemon peel is a valuable source of bioactive compounds; therefore, the primary compositional characterization of this part is particularly relevant for its application in the food and pharmaceutical industries [37,45]. To optimize its use in these sectors, it is important to identify the agronomic parameters that have a direct impact on its composition.

The analysis of variance (ANOVA), as shown in Figure 1 and Table 3, revealed that 7 out of the 20 metabolites identified in the lemon peel samples exhibited significant differences ($p < 0.05$) based on the Tukey test. These metabolites were proline, glucose, fructose, lactate, myo-inositol, choline, and aspartate, which were affected by the rootstock (Figure 1A). Glutamate was the only metabolite that showed significant differences depending on the substrate (Figure 1B). The remaining metabolites did not show significant differences among the treatments studied.

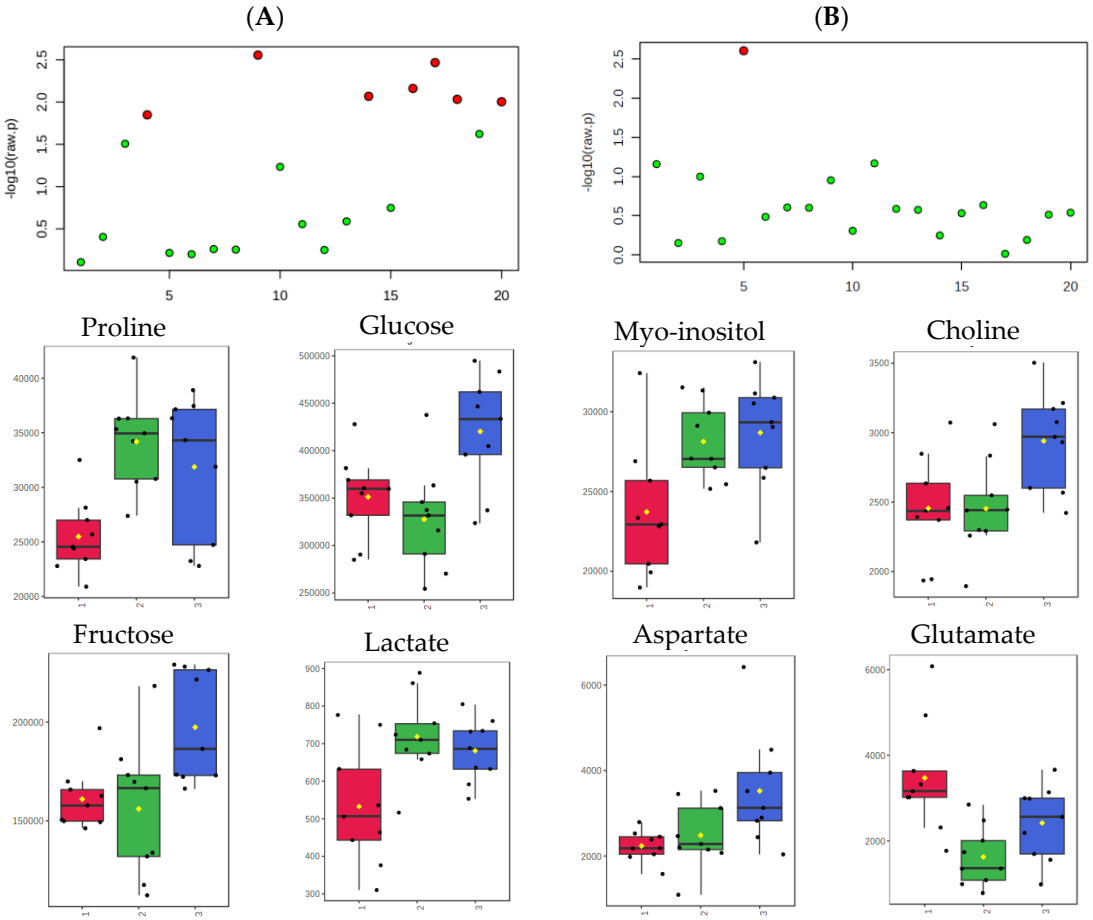

Figure 1. Important features selected by ANOVA plot with *p*-value threshold 0.05 for lemon peel cultivated under 9 different treatments (3 rootstocks × 3 substrates), highlighting (**A**) differences based on the substrate employed and (**B**) differences based on the rootstock.

Table 3. Important features identified by one-way ANOVA and post hoc analysis for the lemon peel related to the rootstock and the culture medium used, where 1 to 3 corresponds to the rootstock type as (1) *Citrus macrophylla*; (2) *Citrus aurantium*; and (3) *Citrus aurantium/Citrus sinensis*; and 4 to 5 is related to the culture media: (4) 50% peat + 50% port sediment; and (5) 75% peat + 25% port sediment.

Compound	f-Value	*p*-Value	−log 10 (*p*)	FDR	Tukey's HSD
Rootstock					
Proline	7.598	0.0027773	2.5564	0.032998	2-1; 3-1
Glucose	7.2669	0.0034074	2.4676	0.032998	3-1; 3-2
Fructose	6.1678	0.006895	2.1615	0.032998	3-1; 3-2
Lactate	5.8468	0.0085401	2.0685	0.032998	2-1; 3-1
Myo-inositol	5.7254	0.0092688	2033	0.032998	2-1; 3-1
Choline	5.6285	0.0098993	2.0044	0.032998	3-1; 3-2
Aspartate	5.1105	0.014158	1849	0.040453	3-1
Culture media					
Glutamate	7.7853	0.002477	2.6059	0.04955	4–5

3.2.1. Multivariate Analysis

Multivariate data analyses were employed to identify significant compounds. Principal component analysis (PCA) was performed initially to classify the samples and analyze

the metabolites responsible for the data variation. The PCA score graph displays a grouping of the data based on the rootstock or culture medium. Regarding the substrate (Figure 2A), the samples cultivated with the substrate containing 75% peat and 25% port sediment tended to be separate from those grown with the mixture of 50% peat and 50% port sediment. The samples grown with the substrate containing 25% peat and 75% port sediment showed some overlap with the other groups. For the rootstock (Figure 2B), while the groups were distinguishable, they shared common interactions. The PCA results for both the rootstock and culture medium demonstrate that the first three principal components (PCs) accounted for 93% of the total variance. To gain a better understanding of the variables responsible for the grouping observed in the PCA score plot, loading plots were generated. The loading plots showed that the sugars, including glucose, fructose, sucrose, myo-inositol, and proline, contributed most to the separation observed in PC1 (69.8% total variance). Meanwhile, organic acids, such as citrate, ascorbate, malate, and asparagine, were the main variables responsible for the separation observed in PC2 (16.1% total variance).

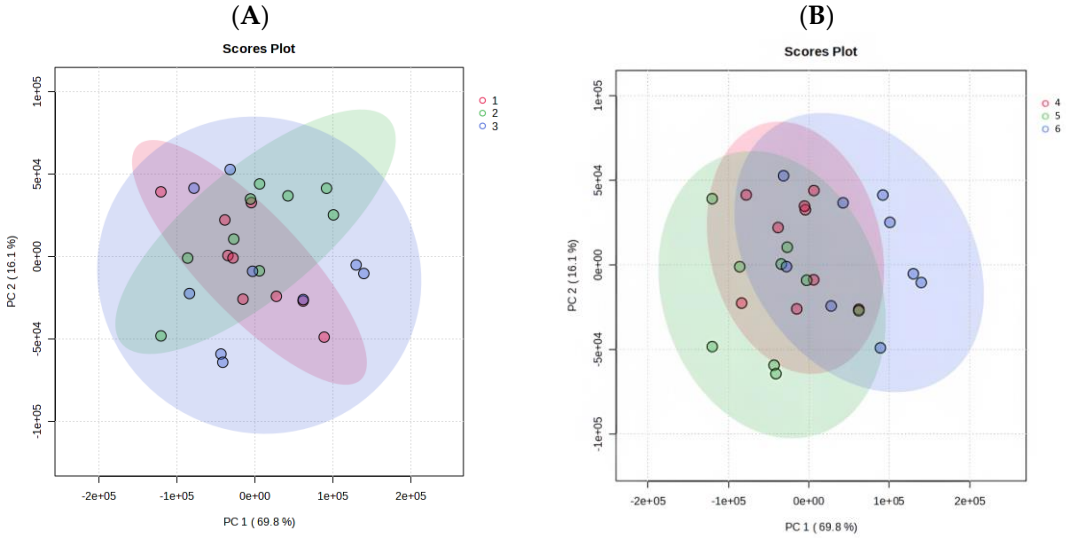

Figure 2. PCA score graph of the metabolomic analysis of lemon peel cultivated under nine different treatments (three rootstocks × three substrates), highlighting (**A**) differences based on the substrate employed and (**B**) differences based on the rootstock, where 1 to 3 corresponds to the rootstock type as (1) *Citrus macrophylla*; (2) *Citrus aurantium*; and (3) *Citrus aurantium*/*Citrus sinensis*; and 4 to 6 is related to the culture media: (4) 75% peat + 25% port sediment; (5) 50% peat + 50% port sediment; and (6) 25% peat + 75% port sediment.

Sugars play a crucial role as the primary energy source for plants and function as signaling molecules during biotic and abiotic stresses, as reported by several authors [46,47]. While there was some overlap between the groups, the grouping observed in the PCA score plot suggests that both the substrate and the rootstock influence the metabolites produced by the plants.

To further investigate the relationship between treatments and significant metabolites, a PLS-DA regression was conducted [48,49]. The results of the PLS-DA model and the variable importance in projection (VIP) reveal that glucose and fructose were significant and differentiating metabolites between the rootstocks (Figure 3A). Additionally, fructose, asparagine, sucrose, and citrate were significant metabolites between the substrates (Figure 3B). However, the remaining metabolites did not show significant differences between the samples and the variables, as indicated by the VIP values of less than 1.

To provide a more intuitive visualization, a hierarchical clustering heatmap was generated (Figure 4) [50]. Differences in relative metabolite levels between the samples were observed depending on the variables studied. Specifically, the fruits cultivated with the *Citrus aurantium/Citrus sinensis* rootstock showed higher relative concentrations

of organic acids (malate, ascorbate, and citrate), sugars (glucose and fructose), amino acids (aspartate and asparagine), and the secondary metabolite choline compared to other rootstocks. In contrast, sugar sucrose and myo-inositol showed medium to high relative levels in the peel of lemons grown with *Citrus aurantium* (Figure 4A).

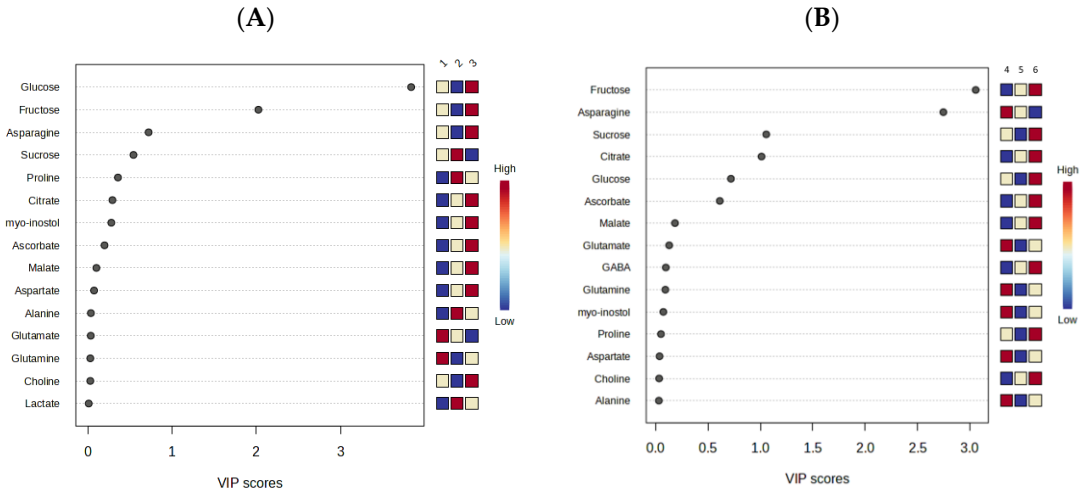

Figure 3. VIP scores (variable importance in projection) plot, derived from the partial least squares discriminant analysis (PLS-DA), is shown along with the corresponding heat map in which red and blue colors indicate the level of metabolites. The analysis is performed for (**A**) the rootstock used (n = 3) and (**B**) the substrate used (n = 3), where 1 to 3 corresponds to the rootstock type as (1) *Citrus macrophylla*; (2) *Citrus aurantium*; and (3) *Citrus aurantium/Citrus sinensis*; and 4 to 6 is related to the culture (4) 75% peat + 25% port sediment; (5) 50% peat + 50% port sediment; and (6) 25% peat + 75% port sediment.

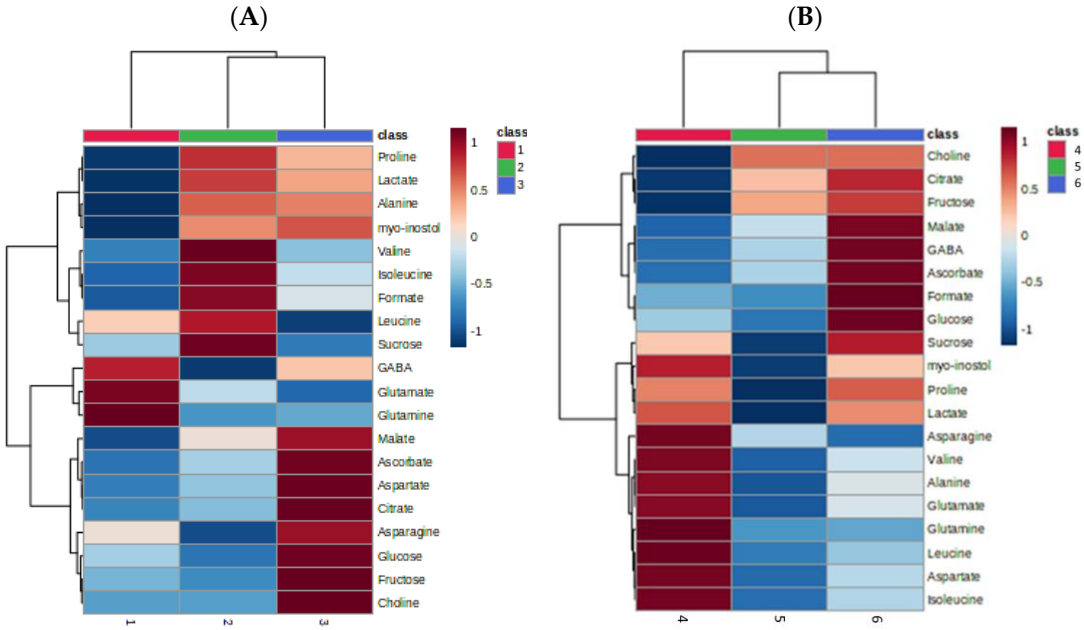

Figure 4. Visual representation of the metabolomic study of lemon peel grown in 9 different treatments (3 rootstocks × 3 substrates) using hierarchical clustering heatmaps. The heatmaps are analyzed based on (**A**) the rootstock used (n = 3) and (**B**) the substrate (n = 3), where 1 to 3 corresponds to the rootstock type as (1) *Citrus macrophylla*; (2) *Citrus aurantium*; and (3) *Citrus aurantium/Citrus sinensis*; and 4 to 6 is related to the culture media: (4) 75% peat + 25% port sediment; (5) 50% peat + 50% port sediment; and (6) 25% peat + 75% port sediment.

Regarding the substrate used and its content in port sediment, two different behaviors were observed in the most distant samples. Specifically, the peel of lemons grown with the substrate containing the highest peat content (75%) presented higher relative levels for most of the identified amino acids. On the other hand, the samples of cultivated lemon peel with the highest proportion of port sediment (75%) showed higher relative levels of organic acids and sugars (Figure 4B). The results could confirm that the metabolism of organic acids and sugars plays an important role in the response of plants to stress, such as an unfavorable substrate such as port sediment [6,14]. This is in contrast to the promotion of more structural metabolic pathways, such as amino acids, which occur when the culture media is ideal, as in the case of peat.

3.2.2. Debiased Sparse Partial Correlation (DSPC)

The metabolic pathway analysis was performed based on the ^{1}H-NMR data, with the goal of identifying significant modulations of metabolites based on the variables of interest, namely the rootstock and culture medium. To achieve this, the deviated scattered partial correlation (DSPC) algorithm was employed, which utilizes a deparsified graphical loop modeling procedure proposed by Jankova and Van De Geer [49] and consolidated by Basu et al. [51]. The DSPC algorithm allowed for the construction of a graphical model, providing partial correlation coefficients and *p*-values for each pair of metabolites in the dataset and determining the connectivity between all the identified metabolites, visualized as a weighted network with nodes representing input metabolites and connections representing measures of association (Figure 5).

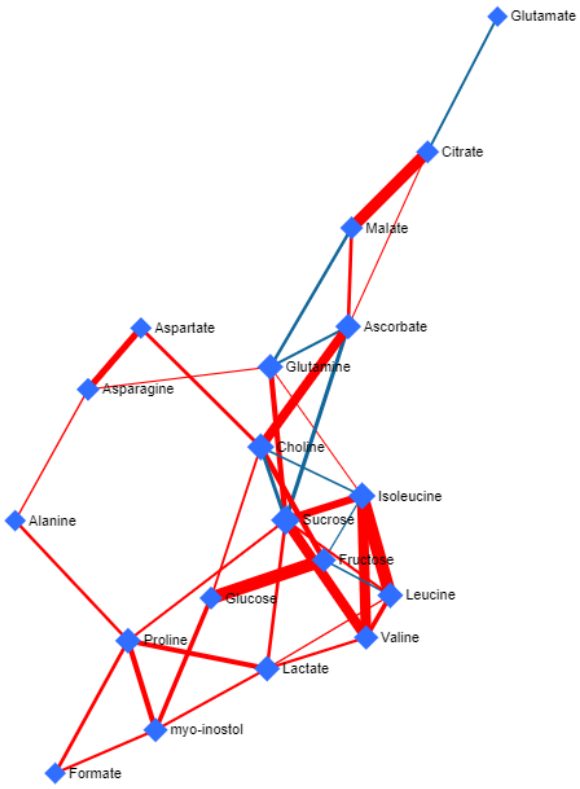

Figure 5. Partial correlation network constructed using the 20 identified metabolites in lemon peel. The size of the nodes represents the direction of change, and colored borders indicate a *p*-value < 0.05 and a false discovery rate (FDR)-adjusted *p*-value < 0.2. Red and blue borders indicate positive and negative correlations, respectively.

The analysis of the metabolic pathway based on the variables of interest (rootstock and culture medium) using the deviated scattered partial correlation (DSPC) algorithm revealed sucrose as the only variable with a grade of 8 and an interaction of 44.78, indicating its

significance in the system's metabolite relationships. Sucrose showed a positive correlation with glutamine, proline, lactate, leucine, valine, and isoleucine, indicating that an increase in its concentration would result in an increase in the other metabolites [52]. However, sucrose had a negative correlation with choline and ascorbate (Partial Coeff. -1). According to Tasseca et al. [53], choline increases in response to plant stress.

Isoleucine and choline presented a grade of 6 and betweenness of 8.88 and 27.33, respectively, both showing positive (red border) and negative (blue border) correlations with other metabolites. Grade 5 was identified for glutamine, leucine, ascorbate, proline, and lactate, with betweenness values ranging from 3.22 to 33.68, most of which had positive and negative interactions except for lactate and proline, which had minimal positive correlations. Glutamate was the metabolite with the lowest grade (1) and was only negatively correlated with citrate (a grade of 3 and betweenness of 17).

The DSPC analysis identified three matching pathways according to *p*-values and impact values (impact > 0.2) based on the pathway typology (Figure 6A). The identification of six metabolites in the metabolic pathway of alanine, aspartate, and glutamate indicates the impact of the variables studied (rootstock and culture medium) on this pathway. Alanine, a non-protein amino acid, protects plants from extreme temperatures, drought, and hypoxia by transforming them into osmoprotective compounds, such as alanine-betaine and the antioxidant homoglutathione [54]. Aspartate is a precursor of asparagine biosynthesis and is one of the primary nitrogen transporters in plants [55]. The next pathway with the highest coincidences (three) was the metabolism of arginine and proline, including L-glutamate, L-aspartate, and L-Glutamine. The affected pathway plays a role in plant responses to biotic and abiotic stress, mainly due to arginine, which is an essential precursor of proline and polyamine biosynthesis. These results are coherent with the modifications/impacts potentially caused mainly by changes in the culture medium, but the variation of the rootstock will also affect soil–plant interactions.

Finally, the enrichment analysis confirmed the results obtained, with a high enrichment ratio (>3.0) for fatty acyls and organic acids, supporting the suitability of the evaluated pathways (Figure 6B).

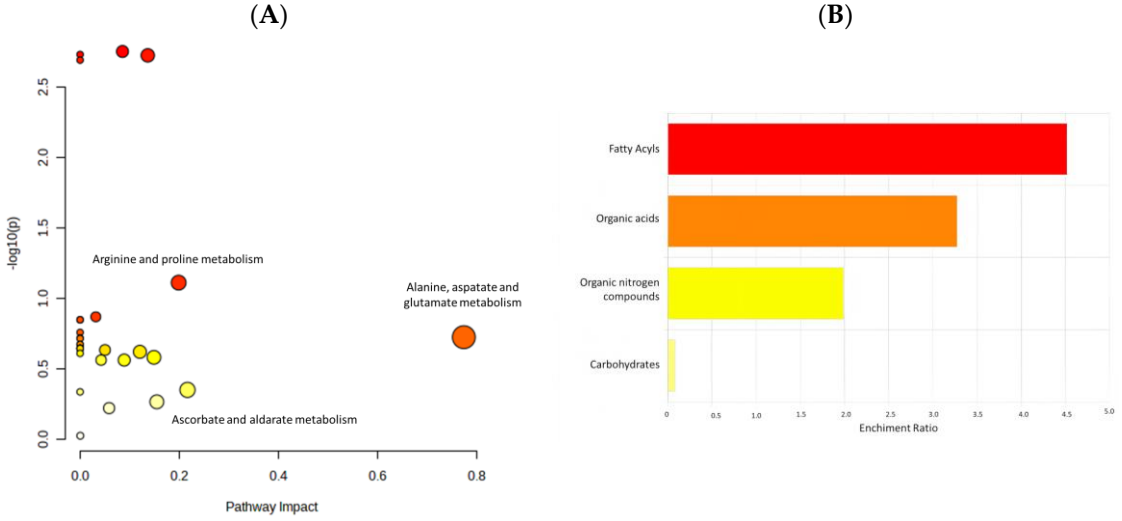

Figure 6. Pathway analysis. (**A**) Identification of the metabolic routes of the lemon peel altered by the variables studied (rootstock and culture medium). Pathways were considered significant when they presented a *p*-value < 0.05 and an impact factor > 0.2. (**B**) Bar graph resulting from the enrichment analysis of metabolites identified for the lemon peel samples.

3.3. Lemon Juice Samples

Lemon juice is a highly versatile ingredient that finds applications in various industries due to the presence of its unique bioactive compounds [56,57]. The analysis of variance (ANOVA) revealed that none of the identified metabolites in the lemon juice samples

showed significant differences according to the Tukey test ($p < 0.05$) based on the rootstock (Figure 7A) or substrate (Figure 7B). These results indicate the homogeneity of the samples irrespective of the studied variables, i.e., the rootstock and culture medium.

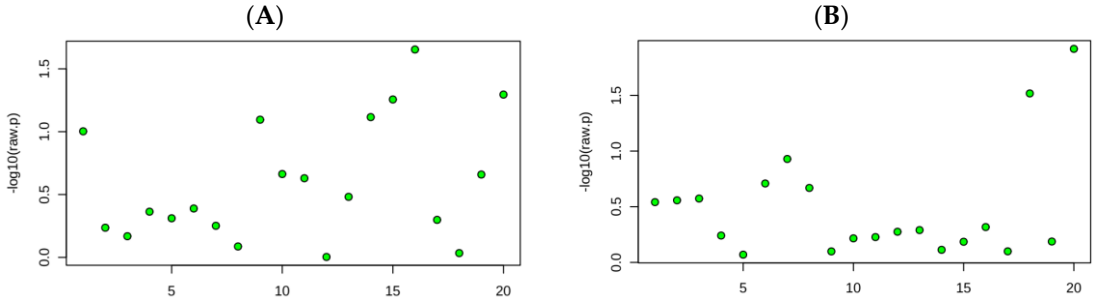

Figure 7. Important features selected by ANOVA plot with *p*-value threshold 0.05 for the lemon juice samples obtained in 9 different treatments (3 substrates × 3 rootstocks) related to (**A**) rootstock and (**B**) culture media.

3.3.1. Multivariate Analysis

The PCA score plot for lemon juice samples confirmed that the results for both variables overlapped and were similar (Figure 8). However, lemon juice grown using the highest percentage of port sediment (25% peat + 75% port sediment) showed greater dispersion and 95% confidence regions when differentiating by rootstock (Figure 8A). For both rootstock and substrate, the first two principal components (PC) explained 98.3% of the total variance. In all the juices, PC1, which accounted for 93.7%, was mainly related to amino acids, such as citrate, aspartate, proline, alanine, and glutamine. PC2, which represented 4.6% of the total variance, was correlated with sugars such as fructose, glucose, sucrose, and myo-inositol.

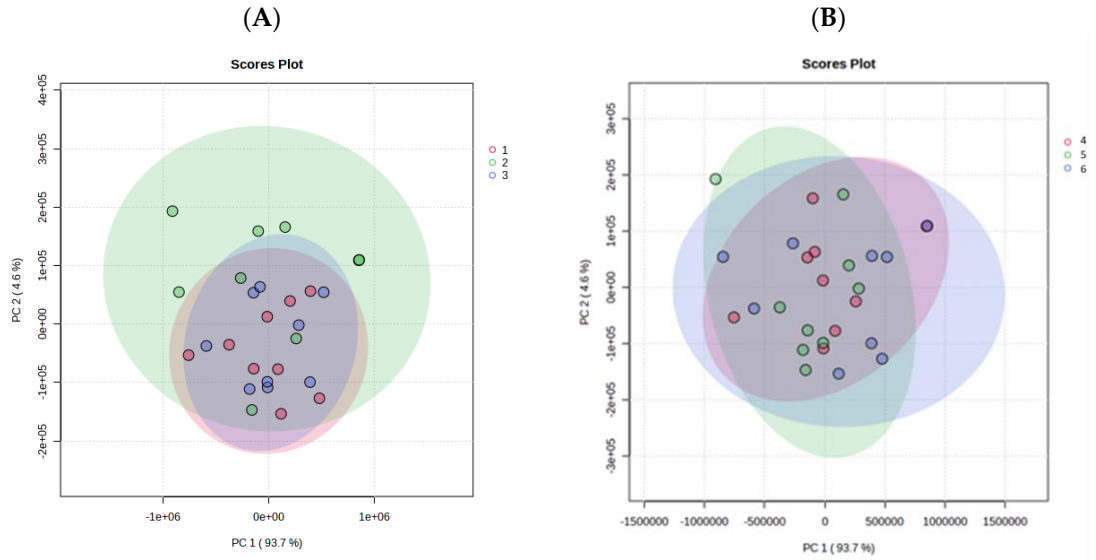

Figure 8. PCA score graph of the metabolomic study of lemon juice cultivated in 9 different treatments (3 rootstocks × 3 substrates) differentiating (**A**) depending on the substrate used and (**B**) depending on the rootstock, where 1 to 3 corresponds to the rootstock type as (1) *Citrus macrophylla*; (2) *Citrus aurantium*; and (3) *Citrus aurantium/Citrus sinensis*; and 4 to 6 is related to the culture media: (4) 75% peat + 25% port sediment; (5) 50% peat + 50% port sediment; and (6) 25% peat + 75% port sediment.

A PLS-DA regression was employed to establish correlations between the studied treatments and identified metabolites in lemon peel. The variable importance in projection (VIP) was also determined to assess the significance of the metabolites [58]. As for both

variables (rootstock and substrate), the aspartate, citrate, and fructose metabolites showed significance (VIP > 1). However, the degree of importance varied, with aspartate > citrate > fructose being more important for rootstock (Figure 9A), while citrate followed by glucose and aspartate had the highest VIP for substrate (Figure 9B). These metabolites are likely related to the final flavor of the juice, indicating their practical significance [59].

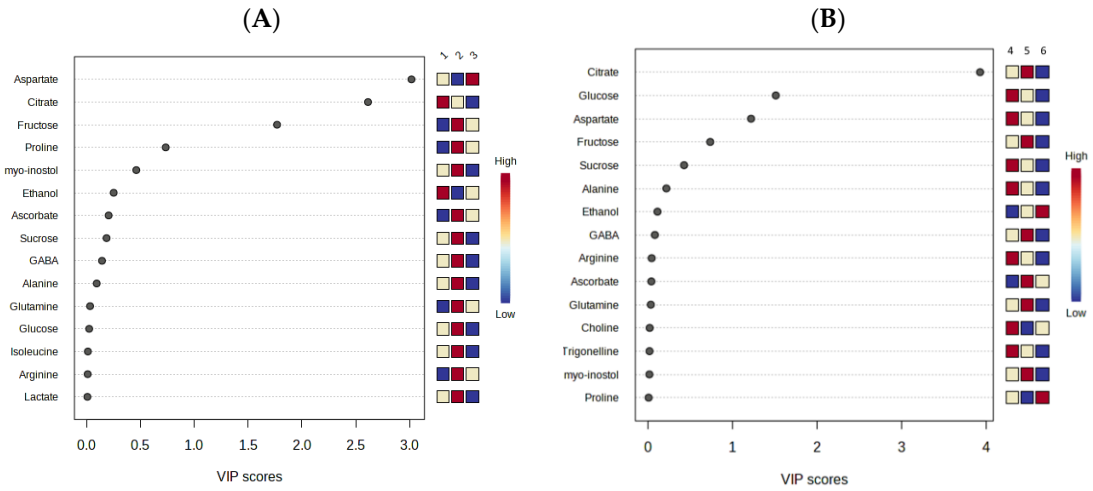

Figure 9. Graph of VIP scores (variable importance in projection), derived from the partial least squares discriminant analysis (PLS-DA), with the corresponding heat map where red and blue indicate the level of metabolites. The results are analyzed according to (**A**) the rootstock used (n = 3) and (**B**) the substrate (n = 3), where 1 to 3 corresponds to the rootstock type as (1) *Citrus macrophylla*; (2) *Citrus aurantium*; and (3) *Citrus aurantium/Citrus sinensis*; and 4 to 6 is related to the culture media: (4) 75% peat + 25% port sediment; (5) 50% peat + 50% port sediment; and (6) 25% peat + 75% port sediment.

The use of hierarchical clustering heatmaps allowed for a more detailed analysis and intuitive visualization of the mean concentration values of the identified metabolites and their differentiation between the studied variables (Figure 10). Specifically, a clear quantitative difference in the concentration of all metabolites was observed in the juice samples from lemons cultivated with *Citrus aurantium* rootstock, which generally had higher concentrations compared to those obtained from lemons cultivated with other rootstocks (Figure 10A). Moreover, the results based on the substrate showed a general decrease in metabolite content, with the exception of ethanol, in juices from lemons grown using a substrate with the highest percentage of port sediment (25% peat + 75% sediment) (Figure 10B).

3.3.2. Debiased Sparse Partial Correlation (DSPC)

The connectivity of all identified metabolites was defined based on the graphic model generated from the DSPC network (see Figure 11). Alanine had the highest grade (10) and betweenness value (20.13) among all variables, followed by valine and lactate, which had grades of 9 and betweenness values of 6.85. Gandolfi et al. [60] associated bactericidal activity in different fruit juices with alanine, which is highly relevant for preservation. Additionally, glutamine and sucrose had grades of 7 and 6, respectively. The lowest degree identified was 2 for the metabolites aspartate, isoleucine, and glucose.

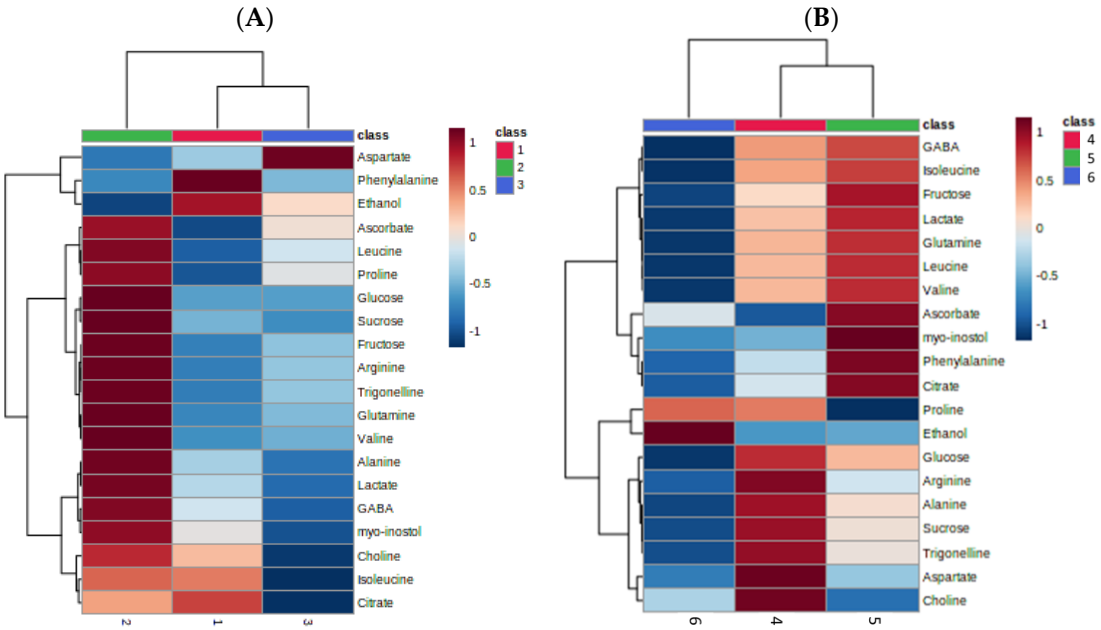

Figure 10. Hierarchical clustering heatmaps of the metabolomic study of lemon juice grown in 9 different treatments (3 rootstocks × 3 substrates). The results are analyzed according to (**A**) the rootstock used (n = 3) and (**B**) the substrate (n = 3), where 1 to 3 corresponds to the rootstock type as (1) *Citrus macrophylla*; (2) *Citrus aurantium*; and (3) *Citrus aurantium/Citrus sinensis*; and 4 to 6 is related to the culture media: (4) 75% peat + 25% port sediment; (5) 50% peat + 50% port sediment; and (6) 25% peat + 75% port sediment.

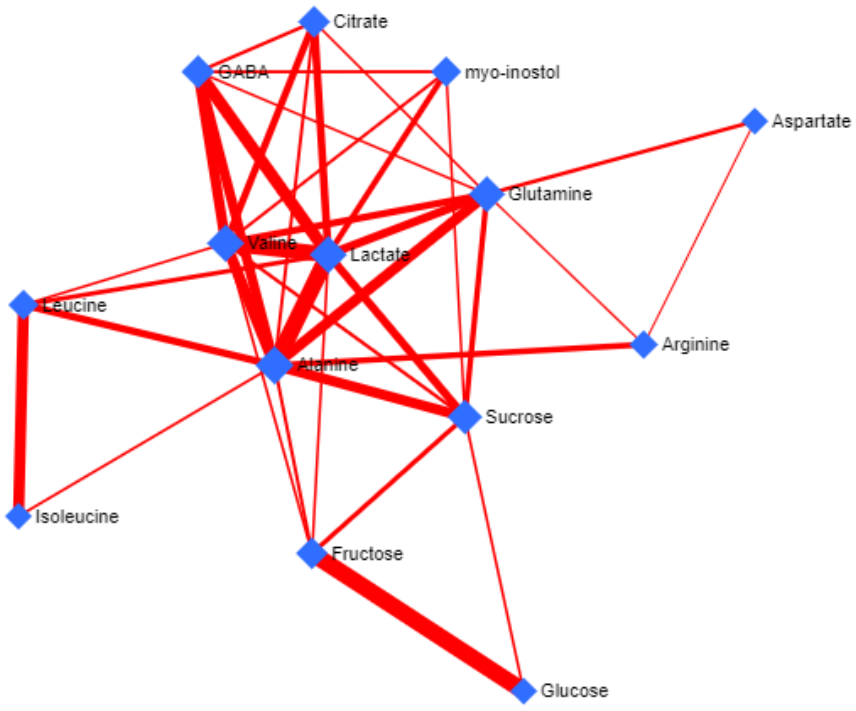

Figure 11. Partial correlation network of the metabolites identified in lemon juice. The size of the node indicates the direction of change. The colored borders have a p-value < 0.05 and the false discovery rate (FDR)-adjusted p-value < 0.2. The red borders show positive correlations.

Out of the 34 metabolic pathways determined based on the results of the juice DSPC analysis, 3 were found to be significant (Impact > 0.2). Similar to the lemon peel samples, the metabolism of alanine, aspartate, and glutamate had the highest number of metabo-

lites, once again confirming its relevance in the plant's response to stress caused by the studied variables (rootstock and culture medium). Despite the ascorbate, aldarate, and phenylalanine pathways having few metabolomic coincidences, their overall impact was still significant (see Figure 12A). Finally, the enrichment analysis, which calculated the ratio between detected compounds and those expected based on the identified metabolic pathways/nodes, revealed that alkaloids were the largest set of metabolites (enrichment ratio of >6), followed by nitrogenous organic compounds (enrichment ratio of >2) and fatty acyls (enrichment ratio of >1) (Figure 12).

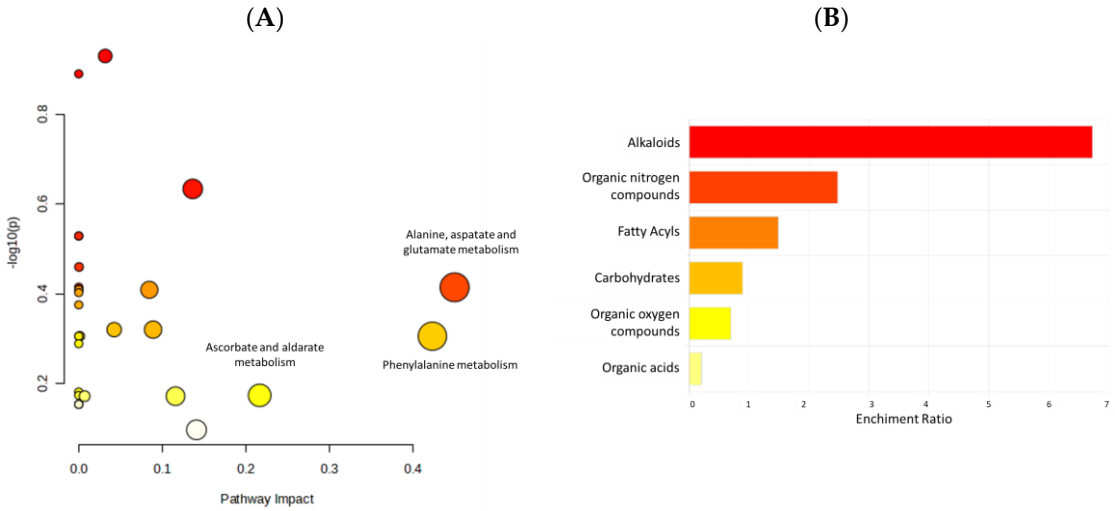

Figure 12. Pathway analysis. (**A**) Identification of the metabolic pathways of lemon juice samples altered by the variables studied (rootstock and culture medium). Pathways were considered significant when they presented a p-value < 0.05 and an impact factor > 0.2. (**B**) Bar graph resulting from the enrichment analysis of metabolites identified for the lemon juice samples.

4. Conclusions

In this study, the impact of rootstock and culture medium on different parts of lemon, namely the peel and juice, was investigated. The results revealed that the rootstock had a significant effect on the metabolites related to the plant's energy metabolism in the lemon peel samples. This finding suggests that the rootstock can influence the development of fruits and the plant itself. Furthermore, the study showed that the culture conditions, including both the rootstock and the culture medium, have an impact on the plant's adaptive response and the modification of metabolic pathways. Interestingly, sucrose was identified as the most important metabolic pathway in all samples. In contrast, the homogeneity of the results indicated that the rootstock and culture medium had a limited influence on the juice metabolites, which were mainly related to the sensory perception of flavor. The study also revealed that the lemon juice samples obtained with *Citrus macrophylla* rootstock had the highest concentrations of metabolites, indicating the rootstock's vigor. Conversely, the juices of lemons cultivated with the highest percentage of port sediment (75%) had the highest total content, suggesting the plant's response to abiotic stress conditions. Overall, these findings demonstrate the differentiated impact of both rootstock and culture medium on different parts of the lemon and provide valuable insights into the metabolic pathways involved in the production of this important fruit.

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