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Untargeted Metabolomics Analysis of Liquid Endosperm of *Cocos nucifera* L. at Three Stages of Maturation Evidenced Differences in Metabolic Regulation

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Abstract: *Cocos nucifera* L. is one of the most cultivated palm trees in the world since it is used to obtain both raw materials and food. From a human point of view, the coconut fruit is a very valuable product, producing an aromatic and tasty liquid endosperm (coconut water) containing high levels of sugars, amino acids and other molecules of nutritional and nutraceutical value. Most of the chemical composition studies conducted on coconut to date have focused on the determination of fatty acid content in coconut oil and the extension of the shelf life of coconut water. Despite the economic importance of this species, the maturation of the coconut fruit is a complex biological process scarcely studied from the metabolic approach and biochemical changes occurring during fruit maturation are not well-known. The objective of this study is to investigate and elucidate the metabolic changes that occur during the maturation process of coconut (*Cocos nucifera* L.) fruits, specifically focusing on the liquid endosperm of the Yucatan green dwarf variety. In this study, the liquid endosperm of coconut fruits at the immature, intermediate and mature stages have been analyzed through an untargeted metabolomics approach by ultra performance liquid chromatography coupled to high resolution mass spectrometry (UPLC-HRMS). A total of 591 spectrometric features were detected and the corresponding identified compounds were classified into 24 chemical classes. The principal component analysis (PCA) showed segregation among the samples, according to their stage of maturation. Most of the metabolites detected were related to the metabolism of flavonoids, carbohydrates and organooxygen compounds. Pathway analysis showed that sphingolipid, starch and sucrose metabolisms were among the most over-accumulated during ripening, followed by the metabolism of glyoxylates and dicarboxylates and the metabolism of amino acids such as alanine, aspartate and glutamate, and others. This is the first study that focuses on elucidating the metabolic profiles of the liquid endosperm of coconut Yucatan green dwarf variety during three stages of maturation with an untargeted metabolomics approach through UPLC-MS.

Keywords: biochemical changes; coconut fruit; liquid endosperm; maturation; untargeted metabolomics; UPLC-HRMS

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1. Introduction

The coconut palm (*Cocos nucifera* L.), the only species in the genus *Cocos*, is one of the most widely cultivated palms in the world; it is a source of vegetable oil, foods and

drinks, construction materials, household products, etc.; practically, every part of the plant is useful [1,2]. This species is intensively cultivated in the tropical regions of the world; the estimated cultivation area is about 12 million ha with a world production of approximately 61 million tons annually. The main coconut producers are Indonesia, the Philippines and India; in Latin America, Brazil and Mexico lead the export market and rank fourth and eighth, respectively [3]. Within this species, two main groups of coconut varieties are described: tall and dwarf. Dwarf varieties are self-pollinating and early flowering. They are precocious plants, producing numerous fruits four years after planting compared to tall varieties that produce fruits six to ten years after planting [4,5]. Additionally, dwarf varieties are resistant to lethal yellowing, one of the most devastating diseases of coconut crops [6]. The most valuable products of the two varieties of coconut palm are the solid and liquid endosperms; the former is mainly composed of highly methyl-esterified pectin and medium-chain fatty acids such as lauric acid (C:12) and myristic acid (C:14). The solid endosperm is also the source of coconut oil [7–9]. The liquid endosperm is a reservoir of water, sugars and phytohormones with important functions in the germination and development of the coconut embryo [10]. Glucose, sucrose and fructose constitute 80% of the sugars contained in the liquid endosperm; the dwarf varieties tend to have a highest sugar content than tall varieties, making them ideal for coconut water consumption [11]. Other components of the liquid endosperm are fatty acids, amino acids, organic acids, enzymes, phenolic compounds, vitamins and minerals. Altogether, these molecules confer great nutritional and functional value to coconut water [5,12], which generates significant income for the economies of several countries, mainly in Southeast Asia [13]. Among the other uses of liquid coconut endosperm, it is can be used as a component in plant tissue culture media [14–16]; as an alternative to intravenous rehydration in critical situations [17]; as a dietary supplement [18]; and has recently been promoted as an important source of molecules with nutraceutical potential [19,20]. However, despite the nutraceutical and economic importance of coconut fruit, very few studies have been conducted to understand the biochemical and molecular basis of the ripening process.

Fruit development and ripening are complex, genetically programmed processes that occur in a species-specific manner [21]. Seed development involves a series of metabolic processes aimed at the accumulation of nutrients, mainly carbohydrates, lipids, amino acids, storage proteins and secondary metabolites, and many metabolic pathways are involved, e.g., amino acid metabolism, starch and sucrose metabolisms, fatty acid and flavonoid biosynthesis [22–24]. The ripening of the coconut is a complex biological process scarcely studied from the biochemical point of view; it is a non-climacteric fruit and they are unique in the fact that they contain solid and liquid endosperms throughout development [25]. In this era of omics, the emergence of metabolomics is presented as an alternative to help understand the physiological processes and biochemical changes that occur during the development of fruits and seeds [26]. Untargeted metabolomics is based on the comparison of patterns of compounds obtained from different biological samples, using univariate and multivariate statistical tools. This approach allows us to understand the complexity of these matrices, and contributes to the identification of metabolites that could have a more relevant role in various processes, such as postharvest storage, ripening or stress [27].

Regarding coconut metabolomics studies, Kumar et al. [28], used Gas Chromatography-Flame Ionization Detector (GC-FID) to analyze the fatty acid profile in the coconut oil extracted from fruits of tall, dwarf and hybrid varieties. They found differences among the fruits of the varieties analyzed, the fruits of the hybrids have less unsaturated fatty acids and lauric acid than in the tall and dwarf varieties. Chen et al. [29], reported that the analysis of pathway enrichment showed that the tricarboxylic acid pathway and protein hydrolysis were enriched probably responding to taurine metabolism. More recently, Zhang et al. [30], studied the deterioration process of coconut water since coconut water has a short shelf life, and their analysis led them to conclude that cysteine, methionine, glycine, serine and threonine metabolisms are the main metabolic pathways whose changes may be responsible for the deterioration of the organoleptic properties of coconut water.

Kumar et al. [4], analyzed the liquid endosperm of "Chowghat Orange Dwarf" (COD) and "Malayan Yellow Dwarf" (MYD) by targeted metabolomics using GC-MS and UPLC-MS to characterize the nutrients present at four maturity stages and to gain a brief understanding of the chemical profiles. They found great similarity in the profiles of amino acids, proteins, carbohydrates and organic acids, but differences in the mineral composition of the liquid endosperm of both varieties. On the other hand, they found eight metabolites, mainly organic acids, with ripening stage-specific accumulation, and they proposed them as biomarkers for distinguishing the ripening stages in coconut fruit.

Overall, most chemical composition and metabolomics studies currently conducted in coconut have focused on determining the nutrients and fatty acid content of coconut oil and shelf life of coconut water as well as other postharvest aspects. Therefore, in light of the lack of biochemical information on the coconut fruit ripening process, we addressed this gap by investigating the metabolic pathways that are regulated during ripening using an untargeted metabolomic approach by ultra performance liquid chromatography coupled to electrospray ionization and quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOF-MS). The liquid endosperm of coconut fruits during different stages of maturity of the Yucatan green dwarf cultivar was analyzed. The objective of this report was to identify the metabolic pathways that accumulate (up-accumulated and down-accumulated) during maturation of Yucatan green dwarf coconut. Principal component analysis (PCA) grouped, with little dispersion, samples according their stage of maturation, supporting that fruits were well classified. In addition, the over-accumulated and down-accumulated metabolic pathways were identified to better understand the ripening processes in coconut fruit. This is the first report of biochemical changes in the ripening of coconut fruit with a cultivar of economic importance for Mexico and the Caribbean, contributing to the existing basic knowledge of the coconut maturation process. Moreover, it is a worthwhile contribution to the field of metabolomics and the ontogenetic knowledge of this species.

2. Materials and Methods

2.1. Collection and Storage of Samples

Coconut fruits cultivar Yucatan green dwarf were collected in a plantation located in San Crisanto, Yucatan, Mexico (21°20′53.5″ N 89°12′08.6″ W). Maturity stages were classified according to [31–33]. This method consists of identifying the unopened inflorescence of the plant, to be considered as stage zero. The next open inflorescences (from top to bottom) are designated as stage 1, 2, 3, etc. Fruits at each stage of ripening have particular phenotypical characteristics. In this study, we selected three stages of fruit ripening: immature stages (6–8 inflorescence), intermediate stages (9–10 inflorescence) and mature stages (11–14 inflorescence). For each stage, liquid endosperm for 4 fruits were pooled and then 200 mL of the composite samples were stored at −80 °C. For each stage, four biological replicates were prepared, whit a total of 16 fruits per stage.

2.2. Extraction and Sample Preparation for LC-MS Analyses

Pooled samples were thawed at room temperature (25 ± 2 °C). Per composite sample, 200 µL was transferred to a 2 mL Eppendorf tube containing 600 µL of methanol HPLC grade (Sigma-Aldrich, St. Louis, MO, USA), ultrasonicated for 15 min at 25 °C and centrifuged at 10,500× *g* at 25 °C for 15 min. The supernatant was transferred to a new 1.5 mL centrifuge tube, and excess solvent was removed in a Centrivap Concentrator system (LABCONCO, Kansas City MO, USA). Then, samples were lyophilized to obtain the dry extracts. For each pool sample, 50 mg of dry extract were recovered, e.g., four replicates with 50 mg each were obtained per stage. The samples were sent to the Metabolomics core facility of the Instituto de Ecología A. C. (Xalapa, Veracruz, Mexico) for UPLC-HRMS analyses. Samples were resuspended in 1 mL of 0.1% formic acid in methanol, filtered through a 0.22 µm PTFE membrane, and the concentration was adjusted to 50 mg/mL.

2.3. Metabolomic Analysis on UPLC-ESI-MS-QTOF

A chromatographic system UPLC Class I coupled to a Synapt G2Si-HDMi mass spectrometer (Waters™, Milford, MA, USA), was used in this study. Chromatography was performed out on an Acquity BEH column (1.7 µm, 2.1 × 50 mm), with column and sample temperatures of 40 °C and 15 °C, respectively. The mobile phases comprised of water (A) and acetonitrile (B), both with 0.1% of formic acid (Sigma-Aldrich, St. Louis, MO, USA). The gradient condition of the mobile phase was 0–20 min linear gradient 1–99% B, 20–24 min 99% B isocratic, 24–25 min linear gradient 90–1% B (total run time 30 min); 5 µL of extract was injected and the flow rate was 0.3 mL/min. Mass spectrometric analysis was performed with an electrospray ionization (ESI) source in negative and positive mode with a capillary, sampling cone and source offset voltages were 3000, 40 and 80 V, respectively. The source temperature was 120 °C and the desolvation temperature was 20 °C. The desolvation gas flow was 600 L/h and the nebulizer pressure was 6.5 Bar. Leucine-enkephalin was used as the lock mass (556.2771, $[M+H]^+$; 554.2615, $[M-H]^-$). The conditions used for MS analysis were in the mass range 50–1200 Da; Function 1 CE of 6 V; function 2 CER of 10–30 V; scan time 0.5 s.

2.4. Data Analysis

Data were acquired and processed with MassLynx (version 4.1, Waters, Milford, MA, USA) and MarkerLynx (version 4.1, Waters, Milford, MA, USA) software. The retention times and the protonated masses were generated at a noise threshold of 1000 counts and peak smoothing was applied. The raw data were exported to Excel (Microsoft Software) tables for statistical analysis; MetaboAnalyst platform (V. 5.0; [34]; https://www.metaboanalyst.ca/), through its different modules, was used for the functional analysis of untargeted metabolomics data. Using the statistical analysis module, multivariate methods were used to compare the samples. Principal component analysis (PCA) was performed to determine samples similarity based on their chemical composition and fold changes analyses were performed to identify over-accumulated and down-accumulated metabolites [Fold Change (FC) values $\geq$1.50 or $\leq$0.67, respectively] in the samples; for each set of differentially accumulated metabolites, both ionization modes were linked. The spectrometric features [retention time-mass/charge (rt-m/z)] signals were tentatively identified using the using the functional analysis module and analyzed using the pathway analysis module and pathway analysis modules considering the Arabidopsis metabolome as refence. The ClassyFire platform was used to classify the identified molecular ions [35]; http://classyfire.wishartlab.com/). This application is powered by SMILES identifiers and uses a chemotaxonomic rule-based approach, providing a hierarchical chemical classification of chemical entities.

For the processing and visualization of metabolomics data, multivariate methods were used, such as the unsupervised method principal component analysis (PCA), and volcano plot (univariate method) was constructed to identify potentially discriminatory variables. PCA, Volcano plot, heatmap and hierarchical clustering (HCA) were generated with the Statistical Analysis of MetaboAnalyst. The Venn diagram was generated with the Venny Platform (V. 2.1; https://bioinfogp.cnb.csic.es/tools/venny/index.html).

3. Results

3.1. Cluster Analysis and PCA

Liquid endosperm samples from immature, intermediate and mature fruits were analyzed from four biological replicates (pooled samples), each one by UPLC-HRMS. Mass spectrometry analysis detected 238 rt m/z signals in positive ionization mode, and 353 rt m/z signals in the negative ionization mode. The intensity of the rt m/z signals was transformed (Log10) and normalized by Pareto scale. The heatmap with hierarchical clustering (HCA) and the principal component analysis (PCA) were carried out by combining the dataset matrix of both ionization modes (ESI+ and ESI−). The heatmap-HCA shows three specific clusters (Figure 1A), corresponding to each maturity stage; this indicates that

the liquid endosperm at each stage of maturity has particular chemical profiles. In the heatmap, the intensity of each rt m/z signal is indicated by the z-score using a red/blue scale. In most cases, a trend of decreasing intensities is observed from the immature to the mature fruits. For example, the ions (m/z) 272.9824, 344.9143 and 464.8038 had high intensities in the immature stage, medium intensities in the intermediate stage and lower intensities in the mature stage. This trend suggests that in the immature stage there was a greater presence of these compounds, which may be transforming into more complex molecules to supply the physiological requirements of the fruit. In some cases, an opposite trend is observed, e.g., the intensity of ions (m/z) 302.3057, 348.0608 and 146.1170 increased throughout maturity. The changes in the intensity of the detected signals reflected the dynamic accumulation of the compounds due to the activation and inactivation of various metabolic pathways. The PCA score plot (Figure 1B) shows that each biological replicate was grouped according to the stage of maturity, but with separation between clusters of the maturity stages, supporting the previous observation of chemical differences between the different stages of maturity. The principal components (PCs) explained 85.3% of the total variance. In summary, PCA and HCA showed that the overall profile of the UPLC-MS analysis was affected by the stage of fruit maturity.

A Venn diagram was created to depict the number of statistically significant rt m/z signals that were shared/unshared between the maturity stages (Figure 1C). The core metabolome consists of 176 rt m/z signals; the immature stage contained the highest number of unique rt m/z signals (76), while the stage with the lowest number of unique rt m/z signals was the mature stage (12). Signals unique to the intermediate stage numbered 20. Between the immature and intermediate stages 64 rt m/z signals are shared, 9 rt m/z signals were shared between the intermediate and mature stages, and 40 rt m/z signals between the immature and mature stages. Figure 1D shows how the liquid endosperms are observed at different stages of maturity. Although the metabolomic profile is more complex in the water of immature fruits, the liquid endosperm was less turbid at this stage.

3.2. Enrichment of Metabolic Pathways during Ripening

Statistical analysis of the signals detected at the different stages of ripening was performed and the results were presented by plotting p-values against the log (10) of the fold-change value on a volcano plot for each signal detected as statistically differential for each stage of maturation (e.g., immature vs. intermediate or mature). No similar distribution was observed between liquid endosperms of the fruits in different stages of ripening. The comparison of liquid endosperms from immature fruits vs. intermediate stage fruits revealed 79 signals were down-accumulated, 11 over-accumulated, and 88 did not show any change. In the immature stage vs. mature stage, 56 signals were down-accumulated, 34 over-accumulated, and 105 had no significant changes (Figure S1). Based on the differential ion intensities at each maturity stage, pathway enrichment analysis was performed.

First, the enrichment of metabolic pathways between the immature and intermediate stages was compared using the Mummichog and GSEA algorithm of MetaboAnalyst platform. In total, 11 metabolic pathways were over-accumulated, mainly sphingolipid metabolism, anthocyanin biosynthesis and arginine and proline metabolism (Figure 2A; Table S1), while 25 metabolic pathways were down-accumulated, mainly the biosynthesis of fatty acids, steroids and flavonoids (Figure 2B; Table S1) during the transition from the immature to the intermediate stage.

In the comparison of the intermediate and mature stages, 17 metabolic pathways were over-accumulated; the main ones were: glyoxylate and dicarboxylate metabolism, alanine, aspartate and glutamate metabolism and steroid biosynthesis (Figure 3A; Table S2). Conversely, 32 metabolic pathways were down-accumulated, the main ones being flavone and flavonol biosynthesis, purine metabolism, riboflavin metabolism and amino sugar and nucleotide sugar metabolism (Figure 3B; Table S2). Table S3 presents the main predicted

compounds annotated in silico and the metabolic pathway to which they belong in the comparisons performed in this section.

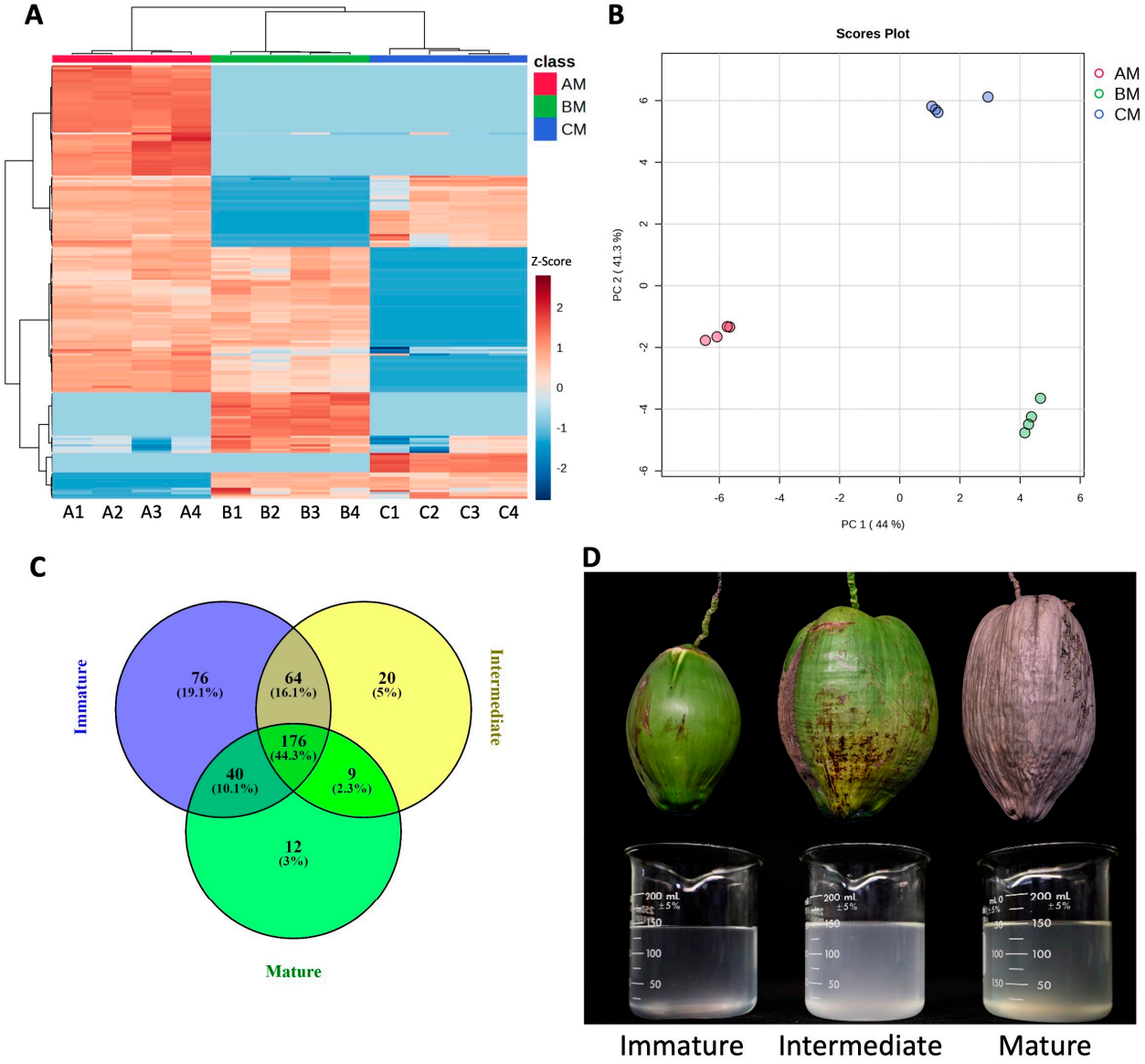

Figure 1. Untargeted metabolomic analysis and physical appearance of fruits and liquid coconut endosperm. (**A**) Heatmap with hierarchical ordering of the total ions detected by UPLC-MS-QTOF (ESI^+ and ESI^-). (**B**) Principal component analysis (PCA) of ions detected by UPLC-MS-QTOF (ESI^+ and ESI^-). (**C**) Venn diagram with the core metabolome of the liquid endosperm at three stages of maturity. (**D**) Physical appearance of liquid endosperm in three stages of maturity. AM/red dots (liquid endosperm from immature fruits), BM/green dots (liquid endosperm from intermediate fruits), CM/red dots (liquid endosperm from mature fruits).

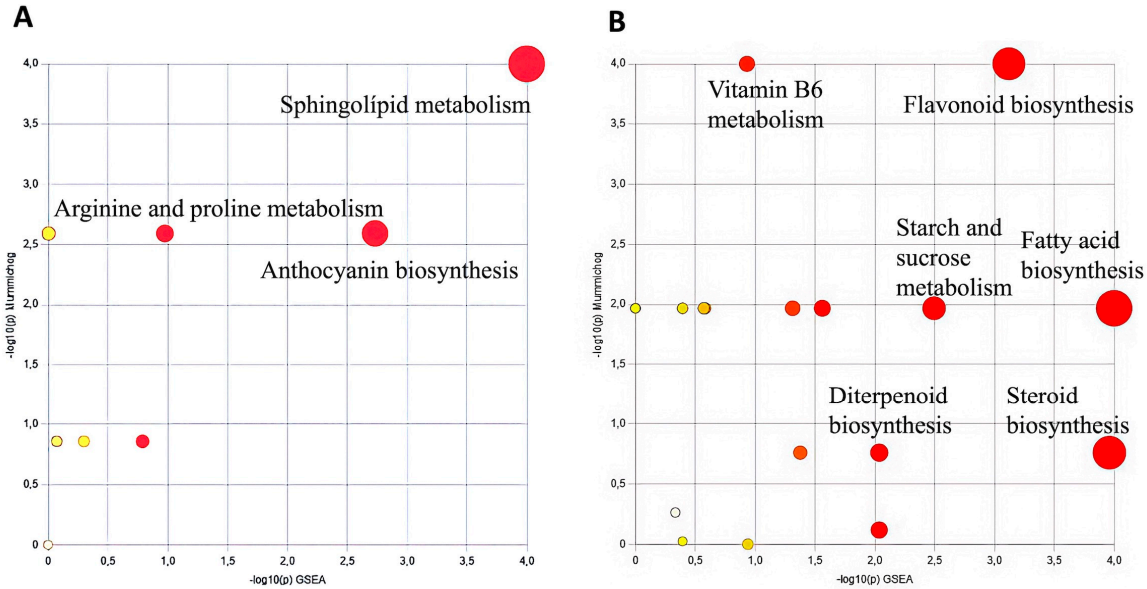

Figure 2. Comparison of pathway enrichment in immature vs. intermediate stages. (**A**) Metabolic pathways with over-accumulated metabolites. (**B**) Metabolic pathways with down-accumulated metabolites. The dots represent the enrichment factor of each metabolic pathway; the size of the circles indicate the pathway impact score and yellow-red color range describes the significance of the rt m/z signal in the corresponding pathway.

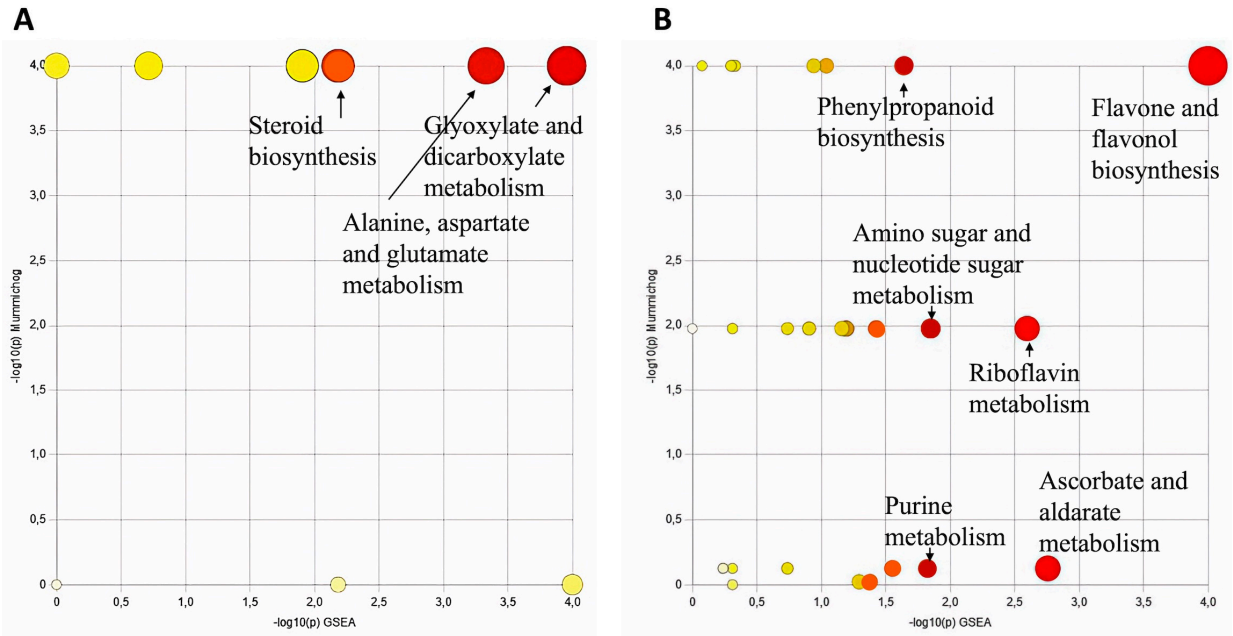

Figure 3. Comparison of pathways enrichment in intermediate vs. mature stages. (**A**) Metabolic pathways with over-accumulated metabolites. (**B**) Metabolic pathways with down-accumulated metabolites. The dots represent the enrichment factor of each metabolic pathway; the size of the circles indicate the pathway impact score and yellow–red color range describes the significance of the rt m/z signal in the corresponding pathway.

3.3. Annotated Chemical Classes

The most abundant chemical classes were identified based on in silico annotation, using Classyfire. In total 24 classes were annotated according to their chemical classes. Figure 4A,B show the over-accumulated and down-accumulated chemical classes in the comparison of immature liquid endosperm vs. intermediate liquid endosperm, respec-

tively. Figure 4C,D show the over-accumulated and down-accumulated chemical classes in the comparison of intermediate liquid endosperm vs. mature liquid endosperm, respectively. The main groups were carbohydrates and derivatives, lipids, flavonoids and organooxygen compounds.

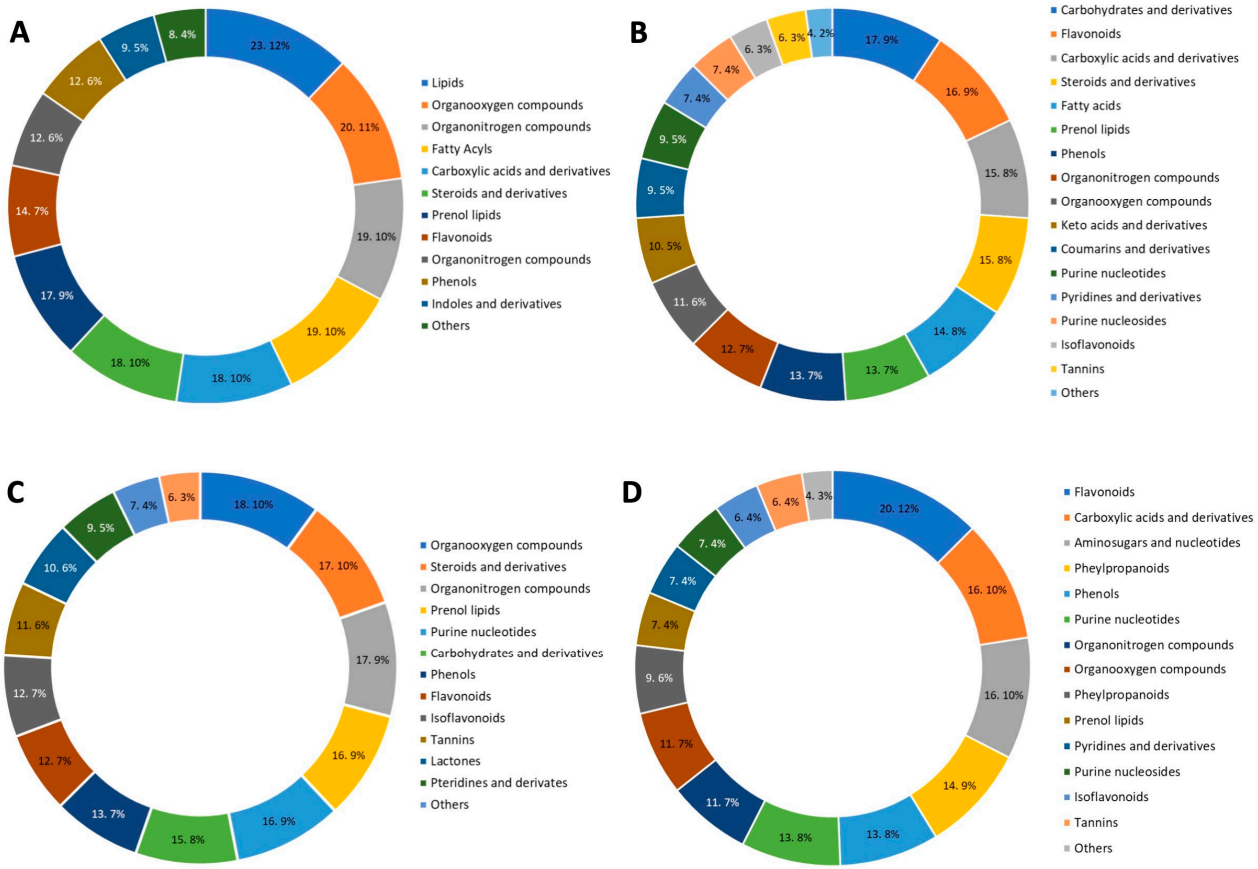

Figure 4. Classification of the accumulated metabolites in the liquid endosperm of *C. nucifera* L. during ripening. (**A**) Classes of over-accumulated compounds in the comparison of immature vs. intermediate stages. (**B**) Classes of down-accumulated compounds in the comparison of immature vs. intermediate stages. (**C**) Classes of over-accumulated compounds in the comparison of intermediate vs. mature stages. (**D**) Classes of down-accumulated compounds in the comparison intermediate vs. mature stages. Numbers within the colored spaces indicate the percentage of m/z signals classified in each category.

4. Discussion

Fruit development and ripening are complex genetically-programmed processes, that occur in a species-specific manner; the physiological changes that occur during ripening have an impact on organoleptic properties and fruit quality [21]. During fruit ripening, a dynamic and complex series of metabolic processes occur that are reflected in the production of metabolites [36,37]. Primary and secondary metabolites are the end products resulting from different cellular regulatory mechanisms [38]. In the development of most seeds, nutrients such as amino acids, soluble proteins, lipids, soluble sugars and starch are transported to the endosperm and stored as nutritional components [39]. Despite the complexity of fruit development and ripening, fruits are classified simply as climacteric and non-climacteric [29]. *Cocos nucifera* is a non-climacteric fruit that during early development presents mainly liquid endosperm (coconut water), and as it matures, the formation of solid endosperm (meat) occurs, reaching up to 30% of the total endosperm volume in mature fruits [40]. Coconut is the only fruit with well-differentiated liquid and solid endosperm in the intermediate and mature stages of ripening [11].

Untargeted metabolomics permits functional analyses of positively or negatively regulated pathways based on metabolite annotations [41]. Here, PCAs for both modes of ionization grouped the samples according to the state of maturation (immature, intermediate or mature). This supports an acceptable classification of the fruits according to their phenotypic characteristics, and fosters reproducibility in the extractions of metabolites and the LC-MS analyses. Similar results were reported by Kumar et al. [4], whose study found that the metabolome of the coconut liquid endosperm could be grouped according to the stage of physiological maturity which is based on phenotypic characteristics and months after pollination. The volcano plot is a powerful graphical tool for high-throughput analyses which aids in the identification of significantly differentially expressed genes or signals across two or more conditions [42]. The volcano plot supported (statistically with *p*-values) the significance of the fold-change values of the differentially accumulated signals in the liquid endosperm during coconut ripening (e.g., immature vs. intermediate stages). The volcano plots showed that the liquid endosperm was largely different among the different stages during fruit maturation, meaning that the composition and abundance of specific molecules or compounds vary while the fruit is ripening (blue and red dots) (Figure S1). Variation in the chemical composition of fruits during ripening is congruent with existing literature; there are similar reports for several fruits such as *Fragaria* × *ananassa* [43], *Citrus reticulata* [44] and *Rosa roxburghii* [45].

Due to the nutraceutical potential of coconut water, the nutrient profile has been analyzed by targeted metabolomics in fruits with different degrees of maturity. A study carried out on COD and MYD cultivars (common cultivars in India) found significant differences in the regulation of the metabolic pathways between the different maturation stages in each variety, but there were no significant differences when comparing both varieties [4]. In the Yucatan green dwarf coconut cultivar, starch and sucrose are among the main metabolic pathways regulated in the immature stage, and they showed similar regulation to that previously reported in COD and MYD cultivars. This pathway is more active in intermediate fruits since a higher accumulation of the compounds involved in this metabolic pathway is observed at this stage. Some annotations of compounds in the Yucatan green dwarf cultivar, based on their m/z ratios consulted in databases such as MassBank, match with compounds identified most of them coincide in the carbohydrate profile, for example, fructose, glucose, sucrose and mannitol.

During ripening of the Yucatan green dwarf coconut cultivar, changes in over-accumulation were observed for flavonoid biosynthesis, steroid biosynthesis, diterpenoid biosynthesis, anthocyanin biosynthesis, metabolism of ascorbate and aldarate, and metabolism of the amino acids: alanine, aspartate and glutamate. Although it cannot be ruled out that these results reflect biochemical differences between these cultivars, the differences are most probably due to methodological factors [46,47]. In the cultivars COD and MYD, up-regulation in the metabolism of phenylalanine, tyrosine and tryptophan was observed. This could be because the precursor chemicals of these amino acids have an affinity for the ethyl acetate used [48]. Conversely, the precursor chemicals of alanine, aspartate and glutamate are polar with an affinity for methanol, which was used in this present study.

The classification of fruits in different stages of maturity is based on phenotypic characteristics; although this classification has worked quite well overall, some scatter in the PCA analyses show that there is some bias in the classification of the samples [4,49]. Kumar et al. [4] proposed eight biomarkers for the different stages of maturity which were identified when they conducted targeted nutritional metabolomics. Here, the untargeted method employed was able to identify sucrose, succinic acid and fumaric acid, in semi-quantitative analysis. The advantages of our procedure include a significantly shorter analysis time in the preparation of the samples, since a small volume of solvent was used (200 µL methanol vs. 200 mL ethyl acetate in the literature). Furthermore, concentration by rotary evaporator was not required; there was no derivatization of compounds in our case. Consequently, the lower cost of analysis of our method may be more attractive for routine applications in the classification of samples for research or industry.

Sucrose and starch play key roles in photosynthesis and are synthesized from triose-phosphate during plant CO_2 fixation in the cytosol and the chloroplasts, respectively [50,51]. In the endosperm of the fruits in the intermediate ripening stage, intermediate precursors and end-products of these pathways were highly accumulated. Sucrose 6-phosphate, trehalose 6-phosphate, D-Fructose and others were identified in the liquid endosperms, which are also intermediates of glycolysis, the pentose phosphate cycle and the tricarboxylic acid cycle (TCA), all of which are related to carbon metabolism [52]. In the seed of *Styrax tonkinensis*, these metabolites are accumulated in fruits in the intermediate stages of maturation as a preliminary step for lipid synthesis [22]. These major sugars confer the sweet flavor of coconut water, mainly in the immature stage of ripening, supported by results from Kumar et al. [4]. Total soluble sugars in coconut liquid endosperm changes throughout ripening; in the intermediate stage, they are in high concentration and then decrease in the mature stage [4,53]. Reducing sugars also participate in the metabolism of reactive oxygen species (ROS), contributing to the generation of energy through the oxidative pentose phosphate pathway [54]. Additionally, they play a key role in osmoprotection and cell membrane stabilization [55].

Seed germination requires a great reserve of energy; the glyoxylate cycle, the TCA cycle and gluconeogenesis are key processes which provide the energy needed for germination [56]. Glyoxylate and dicarboxylate metabolisms are over-accumulated during the ripening of coconut fruits in the mature stage. A key enzyme in the glyoxylate cycle is isocitrate lyase (ICL), which catalyzes the cleavage of isocitrate into glyoxylate and succinate; succinate enters the mitochondria for subsequent reactions [57]. Félix et al. [49] recently demonstrated that glyoxylate is significantly regulated during the ripening of coconut fruits, following an inverted bell curve, in agreement with the regulation of glycolysis. Glyoxylate and dicarboxylate metabolisms are also related to abiotic stress, providing a balance in metabolic changes to improve tolerance to drought stress [37]. The metabolism of ascorbate and aldarate was found here to be one of the most over-accumulated, suggesting an important role of this metabolism in the detoxification of glyoxal and methylglyoxal in coconut, to overcome the oxidative stress imposed by highly active glycolysis.

Organic acids are involved in various metabolic pathways such as the synthesis of amino acids, auxins, gibberellins, salicylic acid, fatty acids, phenolic compounds and cell wall compounds [58]. The high concentration of aconitic acid, malic acid and succinic acid, and the low concentration of sugars in coconut water from immature fruits are determinants of its flavor [59]. Unexpectedly, a high accumulation of some ions related to organic acids in coconut water was also observed in mature fruit, and this may be related to metabolic activity in the tricarboxylic acid cycle, as well as gluconeogenesis and amino acid interconversion, prior to embryo germination and haustorium development [30].

The metabolism of alanine, aspartate and glutamate is a short catabolic pathway, where alanine is converted to pyruvate [60]. This metabolic pathway is intricately connected to various biochemical processes, and also influences cellular energy balance and signal transduction [61]. In the mitochondria, different multi-enzyme complexes are involved in various metabolic branches, such as for the synthesis of isoleucine, methionine or threonine, which are important nutrients and precursors for the synthesis of essential amino acids [62].

Other metabolic pathways that may play critical roles in the biochemical transformation of coconut fruit during ripening are: sphingolipid metabolism, anthocyanin biosynthesis, flavone and flavonol biosynthesis, riboflavin metabolism and amino sugar and nucleotide sugar metabolism. (A) Sphingolipid metabolism: Coconut liquid endosperm is described as a fat-free drink, however, in immature and intermediate stages of the green dwarf coconut water, the presence of sphingolipids was detected. They are likely to be involved in the transition from the immature to the intermediate stage, since these lipids are key elements in many cellular processes, including cell signaling, membrane structure, and apoptosis [63] and fruit maturity processes [64]. Likewise, Fonseca et al. [65] and Cunha et al. [66] reported in green dwarf coconut water the presence of long-chain fatty acids such as palmitic, myristic and stearic acids. In line with these reports, the biosynthesis

of fatty acids was found to be up-regulated in our study. Fatty acids play critical roles in energy storage and membrane structure. Over-representation of this pathway is congruent with the need for lipid-building blocks during fruit growth and maturation [67]. Another lipid metabolism that was found to be important was steroid biosynthesis. Steroids contribute to the synthesis of hormones, structural components of membranes, and signaling molecules. Changes in steroid biosynthesis are related with a shift in hormonal regulation [68], which is expected during fruit development. The content of these lipids, although important for fruit physiology, may be lower in coconut water compared with oily fruits, classifying this drink as a low-caloric product, suitable for hyperglycaemic, hyperlipidimic and nephropathy patients [69]. (B) Anthocyanin biosynthesis: anthocyanins are pigments responsible for the red, purple and blue colors in many fruits, flowers, and leaves [70], and they have been previously reported in coconut water [71,72]. For this reason, coconut water is considered to be useful for human health, against cancer and heart diseases. The coconut kernel may contain 10 times more anthocyanin compared with coconut water [71], and the over-accumulation of these products contribute to the change of color in the exocarp during fruit maturation. (C) Flavone and flavonol biosynthesis: Flavonoids are a group of plant secondary metabolites involved in pigmentation, UV protection, defense against pathogens, and as signaling molecules [63]. Changes in flavonoid biosynthesis during fruit ripening can affect fruit color and nutritional quality. Flavonoids are important antioxidants against reactive oxygen species (ROS), oxidases (e.g., xanthine oxidase [XO], and phosphoinositide 3-kinase [PI3K]), and they also activate antioxidant enzymes [73]. (D) Riboflavin metabolism: Riboflavin (vitamin B2) is a vital coenzyme involved in various redox reactions and energy metabolism. It is essential for plant growth, development, and stress response [74]; therefore, regulation of riboflavin metabolism is consistent with the expected biochemistry of coconut ripening. (E) Amino sugar and nucleotide sugar metabolism: these pathways are crucial for the synthesis of glycoproteins, glycolipids, and other important cellular components. Changes in these pathways can influence cell wall composition and other aspects of fruit development [75].

The identification of all these metabolisms have been related with other oilseeds such as cashew nut (*Anacardium occidentale* L.) [76] and sesame (*Sesamum indicum* L.) [77], showing similar metabolic profiles associated with simple and complex sugars, organo-oxygenated compounds, flavonoids and phenolic compounds, amino acids, nucleosides, nucleotides and organic acids during maturation. These compounds are an important fraction of the nutrients in most oilseeds, and they are directly associated with appearance, texture and flavor [78].

Although the liquid endosperm metabolomes of yellow dwarf and orange dwarf cultivars were previously reported, here we present, for the first time, information on the liquid endosperm metabolome of the Yucatan green dwarf; these three dwarf cultivars are the predominant cultivars used to obtain coconut water worldwide. Differences in sample preparation and analytical platforms make metabolomics results complementary [79,80]; therefore, the information obtained for Yucatan green dwarf coconut expands the knowledge of the metabolome of dwarf coconut cultivars.

The metabolome of the Yucatan green dwarf coconut is highly conserved with metabolomes reported for COD and MYD dwarf varieties from India, with respect to sugars, organic acids, and amino acid accumulation [4], and is consistent with the recently reported proteomics study for green dwarf coconut [49], although some discrepancies were also noted.

5. Conclusions

The metabolomic differences of Yucatan green dwarf with dwarf coconut varieties COD and MYD likely arise from differences in methodologies and not biological differences, since different solvents were used for the extraction and different approaches to MS analyses were taken. We identified potential biomarkers for the classification of coconut fruits at different stages of maturity. The protocol followed here is simpler, faster and cheaper, compared to previous metabolomics analyses in coconut, making it suitable for routine

classification of coconut for research projects or industry. Metabolomics profiles are different among the coconut fruits with different maturity. It will be interesting to further evaluate whether better selection of fruits with the same degree of maturity using biomarkers improve the nutraceutical properties of the food products. In terms of the biological results, the present metabolomics results suggest that the metabolism of ascorbate and aldarate is an important player in coconut development. They most likely play a role in overcoming oxidative stress, probably by detoxifying glyoxal and methylglyoxal to overcome the oxidative stress imposed by highly active glycolysis. Taken together, these results contribute to the current knowledge of coconut biochemistry to understand the molecular basis of fruit maturation. Further multi-omics analyses will be suitable to deeply uncover new insights of coconut ripening.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9080866/s1. Figure S1: Volcano plot for each signal detected with statistically significant differences; Table S1: Pathways enrichment in immature vs. intermediate stage comparison; Table S2: Pathways enrichment in intermediate vs. mature stage comparison; Table S3: Mummichog matched compounds.

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