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Original Research Article

Nutritional and metabolomics characterization of the coconut water at different nut developmental stages

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ABSTRACT

Coconut water possesses remarkable nutritional value due to the presence of health-protective chemical constituents in it. Despite its commercial uses, there is lack of information on changes in the nutritional profile of coconut water along with nut developmental stages. In the current study, we investigated the major physicochemical, nutritional, and metabolic changes occurring in two coconut varieties 'Chowghat Orange Dwarf ' (COD) and 'Malayan Yellow Dwarf' (MYD) during nut development by following four different maturity stages. Gas chromatography-mass spectrometry (GC–MS)-based metabolomics approach was used to identify the metabolites responsible for discriminating the nutrients present in coconut water according to different nut maturity stages. Significant changes in the physicochemical properties, nutritional (free amino acid, protein, sugar, and ascorbic acid), and metabolite profile were observed within each variety during the process of nut maturation. Additionally, we observed development-associated changes in the mineral composition of coconut water. Ultra-performance liquid chromatography (UPLC) analyses identified 17 amino acids from coconut water of both 'COD' and 'MYD' varieties. Using partial least squares discriminant analysis, we identified 8 metabolites that could be used as biomarkers to distinguish between the nut maturity stages. In conclusion, the highly informative current data sets can be used in developing nutritionally balanced customized formulations from coconut water.

1. Introduction

Coconut (*Cocos nucifera* L.) is one of the most important nut-bearing palm crops, which belongs to the Arecaceae family. This plant is grown throughout the world with intensive cultivation in the tropical world. According to the data of the Food and Agriculture Organization (FAOSTAT, 2020), the total world production of coconut is approximately 60.7 million tons (MT). Among coconut-producing countries, India ranks as the third largest producer after Indonesia and Philippines, with a total production of 11.4 million tons (MT) nuts per year (FAOSTAT, 2020). Southern Indian states, namely Kerala, Tamil Nadu, Karnataka, and Andhra Pradesh account for approximately 80–90 % of the total production of Indian coconut (Mandal and Mandal, 2011). Both tall and dwarf coconut varieties are grown throughout the world. Tall varieties (approximately 95 % of total coconut is grown worldwide) are slow-growing, cross-pollinated, and usually take 6–10 years to fruit after planting whereas the dwarf varieties (approximately 5 % of total coconut grown worldwide) are relatively fast-growing, mostly self-pollinated and fruits usually within 4–5 years after planting (Bourdeix et al., 2014).

Various parts of coconut such as outer coconut husk (mesocarp), middle stony hard brown shell (endocarp), and inner soft white edible endosperm are used for different commercial purposes (Chandrasekharan et al., 2004; Manivannan et al., 2018). The inner part of the coconut, commonly known as endosperm constitutes the edible part of this nut, which is either edible in form of a solid white kernel or as a clear nutritious liquid (coconut water) (Prades et al., 2012a). Among all these coconut-derived products, coconut water is emerging as a very popular refreshing drink in the Indian and international market because of its excellent sweet aroma, high nutritional values, and proven health-protective properties (Pummer et al., 2001). Currently, the market for canned coconut water is increasing considerably (Prades et al., 2012b). Tender coconut water is known to possess an array of

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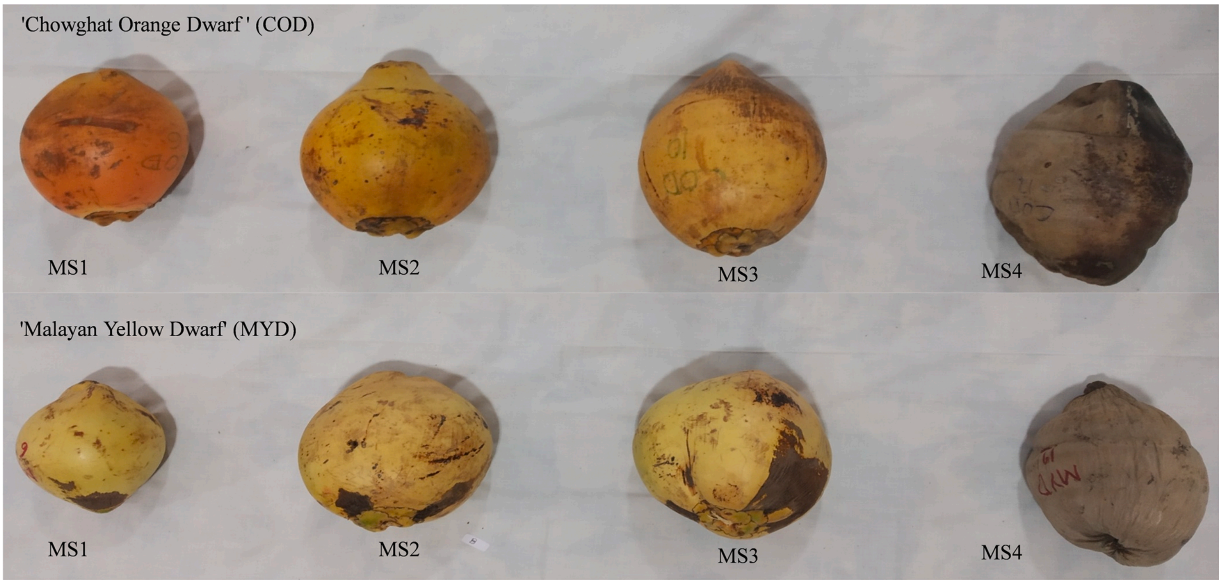

Fig. 1. Developmental stages of coconut variety ’Chowghat Orange Dwarf ’ (COD) and ’Malayan Yellow Dwarf’ (MYD). MS: maturity stage in terms of months after pollination. MS1: 5–6; MS2: 7–8; MS3: 9–10; MS4: 11–12.

health-friendly nutrients. It has been reported that coconut water is composed of 5–9 % of total soluble solids (TSS), of which, more than 80 % is constituted by soluble sugars dominated by glucose, sucrose, and fructose (Mahayothee et al., 2016). Other important constituents are minerals, amino acids, enzymes, organic acids, fatty acids, vitamins, and few phenolic compounds. In terms of the number of constituents in coconut water, minerals come after the sugars. Minerals constitute 0.4–1 % (w/v) of coconut water (Prades et al., 2012a) with potassium as the most predominant mineral. Besides, considerable amounts of sodium, calcium, chloride, magnesium, and phosphorus are also reported from coconut water, however, the amount of these minerals greatly varies with the nut age and cultivar type (Santoso et al., 1996; Prades et al., 2012a). Free amino acids such as alanine, cysteine, and serine and enzymes such as polyphenol oxidase, peroxidase, and catalase have been reported from coconut water (Yong et al., 2009). Presence of minor amount of organic acids (such as malic-, citric-, succinic- and tartaric acids), fatty acids (such as palmitic and oleic acids) and vitamins (such as thiamin (B1), riboflavin (B2), pantothenic acid (B5), and ascorbic acid) have been reported from the water of few coconut cultivars (Santoso et al., 1996; Unagul et al., 2007). Although phenolic compounds are known to exhibit important bioactive properties, the phenolic composition of coconut water is, however, scantly reported so far. The presence of catechin, salicylic-, 4-hydroxybenzoic-, syringic-, o-coumaric-, p-coumaric-, gallic-, and caffeic acids have been reported from coconut water (Chang and Wu, 2011; Mahayothee et al., 2016).

Although, both young tender and mature coconut water have nutritional values mostly fresh tender coconut water from young nuts is preferred by the consumers as health drinks, either directly consumed after collecting from the tender nuts or consumed after processing into various packed beverages (Mahayothee et al., 2016). Notably, matured coconut water is often not preferred as a refreshing drink due to its unpleasant taste. Among consumers, there is a general belief that tender coconut water is nutritionally rich and bears mostly health-protective nutrients. As a result, more studies are focused on analyzing the nutritional properties and health benefits of tender coconut water than on matured coconut water (Zhang et al., 2020).

It is quite likely that a series of physiological and metabolic changes occur in the coconut water during the on-tree maturation process through different developmental stages. However, what are those changes in the metabolite profile and how such changes influence the nutritional status of coconut water have not yet been studied in detail? Metabolomics coupled with multivariate data analyses offers a powerful tool to rapidly and precisely analyze the developmental associated alteration in the metabolite profile of many fruits and plant-based products (Cuthbertson et al., 2012). Recently, UPLC-MS-based metabolomics has been used successfully to assess metabolic changes in coconut water during post-harvest storage (Chen et al., 2018). Moreover, coconuts have vast nutritional diversity because of variety, geographical location, and developmental stages. In this study, the changes in the chemical composition of coconut water during different nut maturation stages were evaluated from the Chowghat Orange Dwarf (COD) and ’Malayan Yellow Dwarf’ (MYD) coconut varieties. We applied gas chromatography-mass spectrometry (GC–MS)-based metabolomics along with the with multivariate data analysis to identify metabolic alterations happening in the coconut water during nut maturation through four different developmental stages. Overall outcome from the present study advocates that metabolomics-based nutritional characterization of coconut water can be a valuable tool for selecting coconuts with appropriate maturity stages bearing coconut water with optimum nutrients with respect to consumer dietary requirements. This study will pave a solid theoretical basis for formulating coconut water-based designer drinks or food supplements with known nutritional constituents.

2. Materials and methods

2.1. *Chemicals and reagents*

Phenolic acid standards (shikimic acid, quinic acid, ferulic acid, caffeic acid, chlorogenic acid), catechin, amino acid standards, fatty acids (lauric acid, myristic acid, palmitic acid, oleic acid, and stearic acid), ascorbic acid, N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), methoxyamine hydrochloride, pyridine were purchased from Sigma-Aldrich Chemical Co. Ltd. (India). AccQ Tag Ultra eluent™ concentrate and AccQ Tag Ultra derivatization kit™ from Waters Corporation (Milford, MA, USA) were used for amino acid analyses. Methanol (High-performance liquid chromatography grade), ethyl acetate, and hexane were purchased from SRL chemicals (Mumbai, India).

2.2. *Coconut sample collection*

Two coconut cultivars ’Chowghat Orange Dwarf ’ (COD) and

'Malayan Yellow Dwarf' (MYD) were used in this study. Coconut samples were obtained from demonstration-cum-seed production (DSP) farms, Pitapally, Post-Kumarbasta, District- Khurda - 752 055 Odisha, India. The nut maturity stages (MS) selected were of approximately 5–6 months (MS1), 7–8 months (MS2), 9–10 months (MS3), and 11–12 months (MS4) after pollination (MAP), respectively (Fig. 1).

2.3. Coconut water collection and sample preparation

Coconuts were manually dehusked and then manually cracked to collect coconut water from each cultivar at four defined maturity stages (MS1-4). The collected water was either used fleshly or stored in a dark bottle at minus 20 °C. The collected coconut water was processed in different ways for determining physicochemical properties, nutritional analyses, and metabolomics analyses. Coconut water from each nut was collected carefully and passed through a muslin cloth to remove debris. Clear nut water (100 mL) was again passed through a 0.45 μm polyethersulfone (PES) membrane filter (Merck-Millipore, Germany). Filtered water was directly taken for determination of pH, titratable acidity, total sugar, reducing sugar, and ascorbic acid content.

2.4. Analyses of physicochemical properties of coconut water

Changes in the physicochemical properties of coconut water, such as pH, titratable acidity, turbidity, and total soluble solids (TSS) were evaluated. Coconut water pH was determined using an electronic processor-based pH meter (CyberScan pH Tutor; Eutech Instrument, India). Titratable acidity (TA) was calculated using 0.1 N sodium hydroxide solution until a faint pink colour was obtained at the endpoint (pH 8.1) and TA was expressed as malic acid (%). TSS was measured by a handheld pocket refractometer (PAL-1, Atago, Tokyo, Japan) and TSS value was expressed as °Brix. Freshly collected coconut water (1 mL) was directly used for turbidity analyses by using a spectrophotometer at 600 nm using pure water as blank.

2.5. Analyses of nutritional properties of coconut water

2.5.1. Estimation of sugar contents

The total soluble sugar and reducing sugar present in coconut water was estimated using standard protocols (Supplementary method S1 and S2). Sugar content was expressed as gram glucose equivalent per 100 mL water (g GE/100 mL water).

2.5.2. Estimation of protein contents

The total soluble protein content of coconut water was estimated using standard protocol (Supplementary method S3). Protein content was expressed as milligram BSA equivalent per 100 mL water (mg BSA/100 mL water).

2.5.3. Estimation of ascorbic acid content

Coconut water (1 mL) was centrifuged at 12,000 x g for 10 min to remove debris and then the supernatant was directly analyzed by high-performance liquid chromatography (HPLC) method for ascorbic acid estimation. Waters (Milford, MA, USA) HPLC system consisting of a 1525 binary pump and 2998 photodiode array detector (PDA) was used. Chromatographic separation was achieved on Waters C18 reversed-phase Xbridge column (150 × 4.6 mm, 5 μm) with a 20 μL sample injection. The isocratic mobile phase consisting of methanol and acidified water (15:85; v/v; water was acidified to pH 2.5 using *o*-phosphoric acid) was used at a flow rate of 0.9 mL/min. Data acquisition and analysis were performed by Empower 3 Software from Waters. All measurements were carried out at room temperature (∼ 25 °C). Ascorbic acid in the sample was identified by matching the retention time and uv–vis spectral characteristics with authentic standard. Ascorbic acid calibration curve was constructed using a concentration range of 162.5–5000 ng injected into HPLC (20 μL sample injection). Limit of detection (LOD) and limit of quantification (LOQ) values for ascorbic acid was calculated using the equation LOD = 3.3 x SD/S and LOQ = 10 x SD/S, where SD = standard deviation of peak areas and S = slope of the calibration curve. Ascorbic acid content of coconut water was expressed as mg/100 mL coconut water.

2.6. Determination of the mineral composition of coconut water using inductively coupled plasma mass spectrometry (ICP-MS)

Filtered coconut water (as described under section 2.3) was used for mineral composition analyses. Coconut water (10 mL, 50 % diluted with Milli-Q water) was acidified with 0.2 % (v/v) nitric acid. Acidified samples were directly used for mineral composition estimation by Perkin Elmer ELAN DRC-e ICP-MS (SCIEX, USA) available to the Institute Instrumentation Centre of IIT Roorkee. ICP-MS was coupled with a dynamic reaction cell (DRC) to eliminate polyatomic interferences hindering the sensitivity and specificity of the method. ICP-MS is used to measure the concentration ranging from ng/mL to mg/mL of inorganic elements from nitric acid-based liquid samples. ICP-MS working conditions were given in Supplementary Table S1.

2.7. Estimation of amino acid contents of coconut water

Amino acid content of coconut water was determined by using ultra-performance liquid chromatography (UPLC). Coconut water (1 mL) was acidified by adding 8.5 μL of concentrated hydrochloric acid (HCl) to get a final concentration of 0.1 M HCl. Standard amino acids were prepared in 0.1 M HCl. Prior to injection into UPLC, both amino acid mixture and acidified coconut water was derivatized with Waters AccQ Tag™ amino acid reagent. Derivatization reaction was set up where 70 μL of AccQ-Tag Ultra borate buffer was mixed with 10 μL of the amino acid mixture or acidified coconut water, followed by the addition of 20 μL of AccQ-Tag reagent (dissolved in 1 mL of AccQ-Tag Ultra reagent diluent). The resulting 100 μL mixture was then incubated at 55 °C for 10 min before UPLC analysis. UPLC analyses were carried out on the Waters Acquity™ UPLC system, equipped with a binary pump, an auto-sampler, and a PDA detector. A Waters AccQ-Tag Ultra C_{18} column (2.1 mm i.d. × 100 mm, 1.7 μm particles) was used for amino acid separation. Mobile phases used were eluent A (10 % AccQ-Tag Ultra concentrate solvent A), and eluent B (100 % AccQ-Tag Ultra solvent B). Separation was achieved with gradient flow program of 0 - 0.54 min (99.9 % A), 5.74 min (90.0 % A), 7.74 min (78.8 % A), 8.04–8.64 min (40.4 % A), 8.73−10 min (99.9 % A) with flow rate of 0.7 mL/min, as described before Armenta et al. (2010). One microliter of samples was injected for analysis. The PDA detector was set at 260 nm, with a sampling rate of 20 points/sec. Amino acid mixture was run in UPLC in a concentration range of 0.1–200 μM and peak areas were plotted against the corresponding concentrations of each amino acid to obtain the calibration curve. LOD and LOQ values were calculated using the formula LOD = 3.3 x SD/S and LOQ = 10 x SD/S, where SD = standard deviation of peak areas and S = slope of the calibration curve (Alquadeib, 2019).

2.8. Metabolomics analysis of coconut water using GC–MS

2.8.1. Sample preparation and instrument run conditions

GC–MS analysis was performed to identify sugars, sugar alcohols, organic acids, fatty acids, and phenolics present in the coconut water at different maturity stages. Briefly, 100 mL of freshly collected coconut water was centrifuged at 6000 x g for 15 min, then the supernatant was collected and a few drops of concentrated HCl was added to bring pH 2.0. Acidified water was then extracted twice with equal volumes of ethyl acetate. Ethyl acetate fraction was combined (∼ 200 mL) and then concentrated to 2 mL in a rotary evaporator under reduced pressure. This concentrated ethyl acetate fraction was processed in two different ways for GC–MS analyses. In method (a) 100 μL of ethyl acetate fraction was collected in a 1.5 mL microcentrifuge tube and then dried using a

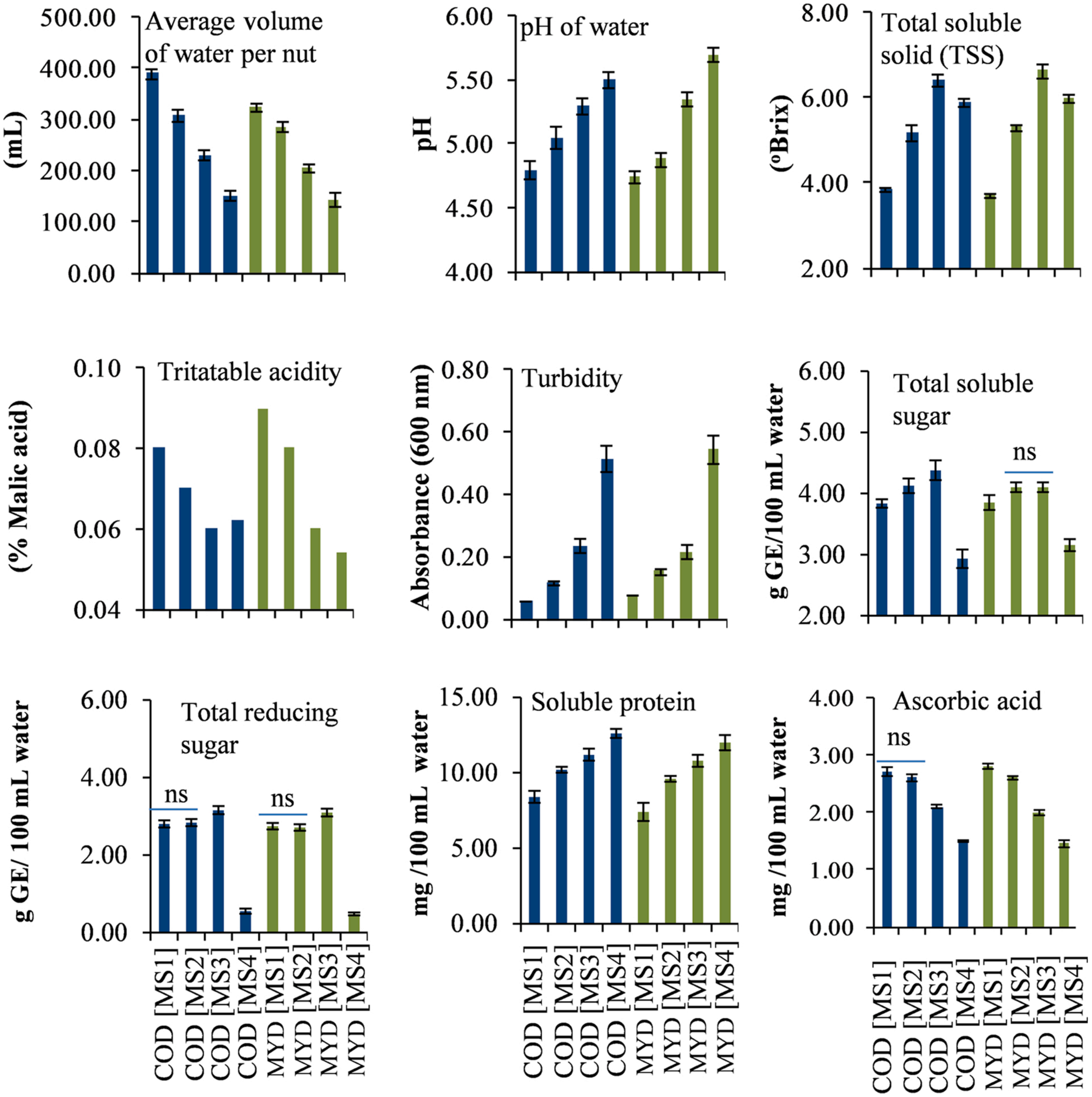

Fig. 2. Physicochemical and nutritional properties of coconut water from two varieties (cv. 'Chowghat Orange Dwarf' (COD) and 'Malayan Yellow Dwarf' (MYD)] at four developmental stages [maturity stage (MS) 1: 5–6 MAP; MS2: 7–8 MAP; MS3: 9–10 MAP MS4: 11–12 MAP]. MAP = months after pollination. GE: glucose equivalent. Data are mean ± standard deviation; n=6. ns: non-significant ($p < 0.05$).

vacuum concentrator (Eppendorf Concentrator plus, Germany). The dried mass was directly derivatized with 70 μL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) for 60 min at 37 °C, followed by centrifugation at 13,000 x g for 10 min. The resulting supernatant was taken out in a GC glass vial and then immediately analyzed by GC–MS for the detection of organic acids, fatty acids, and phenolics. In another method (b), the dried ethyl acetate fraction in 1.5 mL micro-centrifuge tube was double derivatized, first with 35 μL MeOX (20 mg/mL methoxyamine hydrochloride (MeOX) dissolved in pyridine) for 120 min at 37 °C and then the second derivatization with 50 μL of MSTFA for 30 min at 37 °C. Finally, after the double derivatization, the mixture was centrifuged at 13,000 x g for 10 min, the supernatant was taken out in a GC glass vial and then analyzed by GC–MS for detection of sugars and sugar alcohol.

GC–MS analysis (method a) was carried out in an Agilent 7890B gas chromatograph coupled with an Agilent 5977B mass detector (Agilent Technologies, CA, USA). An HP-5MS column (5 % phenyl methyl polysiloxane: 30 m length x 0.25 mm i.d. x 0.25 μm thickness; Agilent technologies, CA, USA) was used for the separation of compounds with helium as a carrier gas with a flow rate of 1 mL/min. The injection volume was 1 μL with split-less mode. The injector temperature was set at 280 °C. The oven temperature was initially set at 80 °C for 2 min, then ramped to 220 °C at a rate of 10 °C/min without any hold and further increased to 310 °C at the rate of 20 °C/min and then hold for 10 min. A five min solvent delay was used. The column flow rate was 1 mL/min. The mass spectrometer (MS) operation parameters were set as follows: ion source temperature 230 °C, MS quad temperature 150 °C, electron energy (70 eV), and mass spectra were collected using a range of 50−1000 m/z. The metabolites were identified by matching with retention indices (RI) and fragmentation pattern with standard metabolites (when available) or by matching their calculated retention indices (RI) with those reported in the literature and by library search (Wiley; National Institute of Standards & Technology library – NIST 17).

Table 1
Minerals composition of coconut water at four different developmental stages (MS1-MS4) from the 'Chowghat Orange Dwarf ' (COD) and 'Malayan Yellow Dwarf' (MYD) varieties.

		'COD' variety				'MYD' variety.			
		Minerals (mg/100 mL)				Minerals (mg/100 mL)			
S.No	Mineral	MS1	MS2	MS3	MS4	MS1	MS2	MS3	MS4
1	Potassium, K	202.0 ± 7.2	222.0 ± 8.0	244.6 ± 7.5	192.0 ± 8.0	196.0.0 ± 7.2	222.6 ± 11.0	240.0 ± 9.0	208.0 ± 10.0
2	Sodium, Na	8.0 ± 0.6	16.2 ± 1.1	22.0 ± 1.8	28.0 ± 1.1	9.2 ± 0.6	16. ± 0.9	23.0 ± 1.9	29.4 ± 2.0
3	Calcium, Ca	4.2 ± 0.3	8.4 ± 0.6	14.2 ± 0.5	18.4 ± 0.7	3.8 ± 0.2	8.6 ± 0.8	13.4 ± 0.8	19.9 ± 1.4
4	Zinc, Zn	0.28 ± 0.07	0.21 ± 0.03	0.14 ± 0.02	0.08 ± 0.02	0.26 ± 0.05	0.18 ± 0.02	0.12 ± 0.03	0.07 ± 0.03
5	Iron, Fe	^{a}2.2 ± 0.3	^{a}2.4 ± 0.4	^{a}2.5 ± 0.2	^{a}2.0 ± 0.1	^{b}2.6 ± 0.4	^{b}2.9 ± 0.3	^{b}2.2 ± 0.1	^{b}2.2 ± 0.2
6	Magnesium, Mg	4.2 ± 0.4	5.6 ± 0.5	8.2 ± 0.5	12.4 ± 0.7	4.0 ± 0.3	5.9 ± 0.5	8.8 ± 0.6	13.2 ± 0.8

Mean within each row under 'COD' and 'MYD' followed by the same letter ('a' for 'COD' and 'b' for 'MYD') are not significantly different ($p > 0.05$). Data are mean ± SD (*n;* 6).

2.8.2. Processing of GC–MS data and multivariate statistical analysis

Raw GC—MS data files were deconvoluted by using Automated Mass Spectral Deconvolution and Identification System (AMDIS) tools available with WsearchPro (www.wsearch.com.au). Resulting peak area data were converted into comma separated values (.csv) format and then uploaded in Metaboanalyst 4.0 (http://www.metaboanalyst.ca) for processing. Data were normalized using log transformation and auto scaling. To detect the differences in metabolite profiling between stage MS1-MS4 of 'COD' and 'MYD' samples, a multivariate method of Partial Least Squares Discriminant Analysis (PLS-DA) was conducted. The PLS-DA is a supervised method that was used to maximize the difference of metabolites between the stages to detect those metabolites which were playing a key role in discriminating the groups. The output for PLSDA (latent variables, LV1 vs LV2) was presented as 2D score plots. Variable importance in projection (VIP) scores were used to screen PLS-DA data for important features. Heatmaps were created using Metaboanalyst 4.0 by applying Euclidean distance measure, the Ward clustering algorithm, autoscale feature under standardization tool, and using normalized data source.

3. Results and discussion

3.1. Changes in the physicochemical properties of coconut water during maturity stages

The changes in the physicochemical properties of coconut water (variety 'COD' and 'MYD') at four different maturity stages (MS1-4) are shown in Fig. 2. In both the varieties, the volume of coconut water initially decreased slowly from MS1-MS2 and then decreased rapidly from stage MS3 to MS4. This finding agrees with those of Appaiah et al. (2015) and Santoso et al. (1996), where coconut water volume also decreased with maturity. This trend was not observed in the study of Jackson et al. (2004), where coconut water volume initially increased with maturity up to nine-month and thereafter decreased. The reduction in nut water volume with maturity occurs due to transformation of liquid endosperm into white coconut meat (jelly formation). The stage at which this jelly formation starts depends on the coconut variety. This is also plausible that the differences in the reduction rate of coconut water with maturity could be due to the effect of soil and climate conditions. For example, the MYD variety cultivated in Kasaragod, India, has an average water volume of 238 mL, whereas the same MYD variety obtained from Karnataka, India has a nut water volume of 480 mL (Prades et al., 2012a).

The pH and turbidity of coconut water increased as the coconut matures from MS1 to MS4 (Fig. 2), whereas the titratable acidity decreased with the nut maturation. These findings agree with those of Mahayothee et al. (2016) and Jackson et al. (2004). Increase in the pH with nut maturity correlates well with the concurrent decrease in the titratable acidity in both 'COD' and 'MYD' varieties. Decrease in the titratable acidity could be due to the reduction of the amount of organic acids and ascorbic acid present in the nut water with maturity. For both the varieties, initially, TSS initially increased as the coconuts matured from MS1 to MS3, thereafter TSS slightly decreased at MS4. This is in good agreement with the previous findings (Jackson et al., 2004). An increase in TSS value might be associated with the reduction of nut water followed by concurrent increase in the sugar and ion concentrations (Pue et al., 1992; Jackson et al., 2004). The increase in turbidity with coconut maturity might be associated with the reduction of nut water volume and a subsequent increase in the TSS.

3.2. Changes in the nutritional properties of coconut water during maturity stages

Changes in the nutritional composition of coconut water of 'COD' and 'MYD' at four maturity stages are shown in Fig. 2. In both 'COD' and 'MYD', the contents of total sugar increased from MS1 to MS3 and then decreased between MS3 to MS4. There were no significant differences observed in the reducing sugar contents between the MS1 and MS2. Similar changes in the sugar content of coconut water with maturity have been reported earlier by many researchers (Jackson et al., 2004; Appaiah et al., 2015; Mahayothee et al., 2016). Moreover, changes in the sugar profile of coconut water with maturity correlated well with the TSS data, which also initially increased and then decreased. There were no significant differences observed in the sugar content between the 'COD' and 'MYD' varieties. In both COD' and 'MYD', protein contents significantly increased during nut maturation from MS1 to MS4, which might be associated with the enhanced synthesis of storage proteins during the transformation of liquid watery endosperm into solid white coconut meat. A similar observation was noted earlier in other coconut varieties (Santoso et al., 1996; Jackson et al., 2004). Ascorbic acid present in the coconut water was separated and quantified by HPLC method. LOD and LOQ for this established HPLC method was found to be 71.7 and 217.4 ng, respectively. Notably, obtained LOD value for the ascorbic acid was lower than the lowest concentration of ascorbic acid used for plotting calibration curve. Ascorbic acid content of coconut water also decreased from MS1 to MS4 in both the 'COD' and 'MYD' varieties. Similar trend was observed in other coconut verities where ascorbic acid content of coconut water also decreased with maturation (Santoso et al., 1996). The ascorbic acid content of coconut water reported in this study was slightly higher than those reported by Mahayothee et al., 2016 (0.7 mg/100 g) but significantly lower than the value reported by the Santoso et al., 1996 (7.4 mg/100 g of young coconut water). This variation may be related to the coconut varieties used in this study. The results on the changes in the nutritional level of coconut water through different maturity stages will provide required information for the formulation of coconut water-based drinks or food supplements with known nutritional constituents.

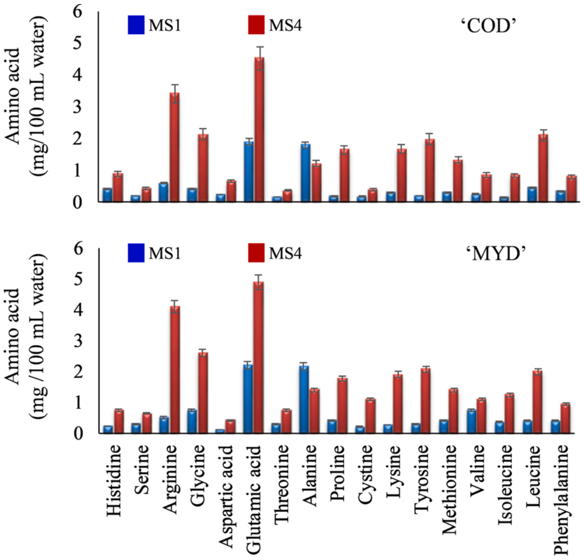

Fig. 3. UPLC based amino acid of coconut water from the cv. 'Chowghat Orange Dwarf ' (COD) and 'Malayan Yellow Dwarf' (MYD) at two developmental stages [maturity stage (MS) 1: 5–6 MAP; MS4: 11–12 MAP]. MAP = months after pollination. Data are mean ± standard deviation; n = 6.

3.3. Changes in the mineral compositions of coconut water during maturity stages

Changes in the mineral compositions of coconut water from 'COD' and 'MYD' variety are shown in Table 1. ICP-MS analyses of coconut water showed the presence of six major minerals potassium (K), sodium (Na), calcium (Ca), zinc (Zn), Iron (Fe), and Magnesium (Mg). Potassium was the most abundant mineral in the coconut water followed by sodium, calcium, and magnesium. Iron and zinc were present in relatively low concentrations. In both 'COD' and 'MYD' potassium content gradually increased from MS1 to MS3 and then slightly decreased at MS4. Sodium, calcium and magnesium concentration consistently increased from MS1 to MS4. In contrast, zinc concentration gradually decreased from MS1 to MS4 whereas there were no significant changes observed in the iron concentration during the nut maturation. There were no significant differences observed in the mineral composition between 'COD' and 'MYD' varieties. Similar data for the mineral composition of coconut water from other varieties have been reported earlier by other researchers (Appaiah et al., 2015; Kannangara et al., 2018). These results will help in selecting the appropriate coconut maturity stage for the formulation of coconut water-based food supplements with known mineral constituents.

Table 2
GC–MS based metabolite profile of coconut water at four different developmental maturity stages (MS1-MS4) from two coconut varieties 'Chowghat Orange Dwarf ' (COD) and 'Malayan Yellow Dwarf' (MYD). LRI: Linear retention index obtained on HP5-MS GC column. Metabolites were identified by matching LRI (linear retention index) and mass spectrum with the standard reference compound.

					Quantity of metabolite in 'COD' variety.				Quantity of metabolite in 'MYD' variety.			
S. No	Metabolite	RT (min)	LRI (cal)	Q-ion	MS1	MS2	MS3	MS4	MS1	MS2	MS3	MS4
1	Succinic acid^{1}	8.8	977.9	262,248,247	^{a}152.0 ± 7.6	^{a}140.2 ± 11.2	224.6 ± 13.4	245.4 ± 12.2	151.2 ± 7.5	138.8 ± 5.5	304.6 ± 15.2	385.6 ± 11.5
2	Fumaric acid^{1}	9.2	1243.6	260,247,246	133.6 ± 10.7	185.6 ± 9.3	^{a}108.5 ± 4.3	^{a}101.0 ± 4.5	^{b}180.9 ± 14.4	^{b}190.4 ± 10.4	217.1 ± 11.9	272.9 ± 13.6
3	Malic acid^{2}	11.2	1487.5	350,335,319	^{a}3.6 ± 0.2	^{a}3.5 ± 0.1	^{a}3.4 ± 0.2	3.2 ± 0.1	^{b}3.4 ± 0.1	^{b}3.4 ± 0.1	^{b}3.4 ± 0.1	3.1 ± 0.1
4	Citric acid^{1}	15.0	1638.3	480,466,465	0.0	0.0	484.6 ± 19.3	769.5 ± 46.1	0.0	0.0	298.7 ± 11.8	770.4 ± 41.6
5	Lauric acid^{1}	13.0	1439.1	272,257,145	32.0 ± 1.6	46.9 ± 2.3	50.0 ± 2.5	157.2 ± 7.8	23.8 ± 0.7	63.4 ± 3.8	73.4 ± 2.9	90.8 ± 4.0
6	Myristic acid^{1}	15.1	1684.8	300,285,145	28.7 ± 1.4	34.6 ± 1.6	69.1 ± 2.4	524.0 ± 26.2	0.0	0.0	0.0	0.0
7	Palmitic Acid^{1}	16.9	2329.8	328,314,313	85.5 ± 3.4	139.6 ± 8.4	254.6 ± 12.7	585.9 ± 23.4	64.4 ± 1.6	106.2 ± 6.9	124.5 ± 6.2	161.5 ± 9.6
8	Oleic Acid^{1}	18.1	2515.0	354,339,264	34.0 ± 1.7	110.0 ± 5.5	616.5 ± 24.6	979.4 ± 48.9	45.0 ± 2.2	134.7 ± 6.0	190.5 ± 12.3	244.9 ± 17.1
9	Stearic acid^{1}	18.2	2405.5	356,341,313	54.0 ± 2.7	91.3 ± 3.7	^{a}240.3 ± 12.0	^{a}257.8 ± 12.8	44.2 ± 2.6	90.2 ± 4.5	119.9 ± 5.9	158.5 ± 5.5
10	Shikimic acid^{1}	14.8	1538.5	462,372,357	41.5 ± 2.1	12.2 ± 1.0	Trace	Trace	13.3 ± 0.7	12.5 ± 1.0	Trace	Trace
11	Quinic acid^{1}	15.4	1809.4	552,537,345	0.0	0.0	40.6 ± 1.9	24.2 ± 1.2	0.0	0.0	0.0	25.4 ± 0.5
12	Ferulic acid^{1}	17.4	2590.2	338,323,308	9.1 ± 0.5	12.1 ± 0.6	Trace	Trace	2.5 ± 0.0	13.8 ± 0.7	Trace	Trace
13	Caffeic acid^{1}	17.6	2318.8	396,381,307	17.6 ± 0.9	8.5 ± 0.4	Trace	Trace	5.1 ± 0.2	7.9 ± 0.1	^{b}8.7 ± 0.2	^{b}8.4 ± 0.4
14	Catechin^{1}	21.3	3162.7	650,576,368	^{a}192.0 ± 9.6	^{a}185.0 ± 7.4	47.0 ± 2.3	Trace	^{b}29.3 ± 1.2	^{b}28.6 ± 0.8	^{b}19.7 ± 0.9	Trace
15	Chlorogenic acid^{1}	22.7	3228.7	786,419,397	158.0 ± 7.9	90.7 ± 4.5	33.2 ± 2.3	Trace	^{b}64.7 ± 2.9	^{b}62.0 ± 1.5	24.5 ± 1.2	Trace
16	D-Fructose^{3}	15.48	1805.7	569,554,364	1.40 ± 0.04	^{a}1.60 ± 0.10	^{a}1.66 ± 0.08	0.50 ± 0.04	1.28 ± 0.07	1.62 ± 0.06	1.87 ± 0.07	0.54 ± 0.02
17	D-Glucose^{3}	15.76	1877.5	569,466,364	1.36 ± 0.05	^{a}1.54 ± 0.04	^{a}1.58 ± 0.05	0.52 ± 0.03	^{a}1.35 ± 0.08	1.64 ± 0.08	1.84 ± 0.08	0.56 ± 0.03
18	Sucrose^{2}	20.36	2711.2	918,451,437	59.5 ± 2.7	136.6 ± 6.8	320.0 ± 16.0	527.0 ± 14.2	135.0 ± 5.4	180.0 ± 6.3	213.0 ± 7.6	694.0 ± 18.4
19	D-Mannitol^{2}	16.14	1949.7	614,421,319	0.0	0.0	0.8 ± 0.00	1.7 ± 0.01	0.0	0.0	0.15 ± 0.00	1.34 ± 0.08

RT: retention time in minutes; LRI cal: linear retention indices calculated from the RT in HP5-MS column using a series of n-alkanes standards (C6–C40); Q-ion: qualifying ion in mass spectra. Values are the mean of six independent run (*n*, 6) ± SD. Mean within each row followed by the same letter ('a' for 'COD' and 'b' for 'MYD) are not significantly different ($p > 0.05$). Metabolite concentrations were expressed either as ^{1} μg/ 100 mL or ^{2} mg/100 mL or ^{3} g/100 mL or Trace < 0.01 μg/ 100 mL.

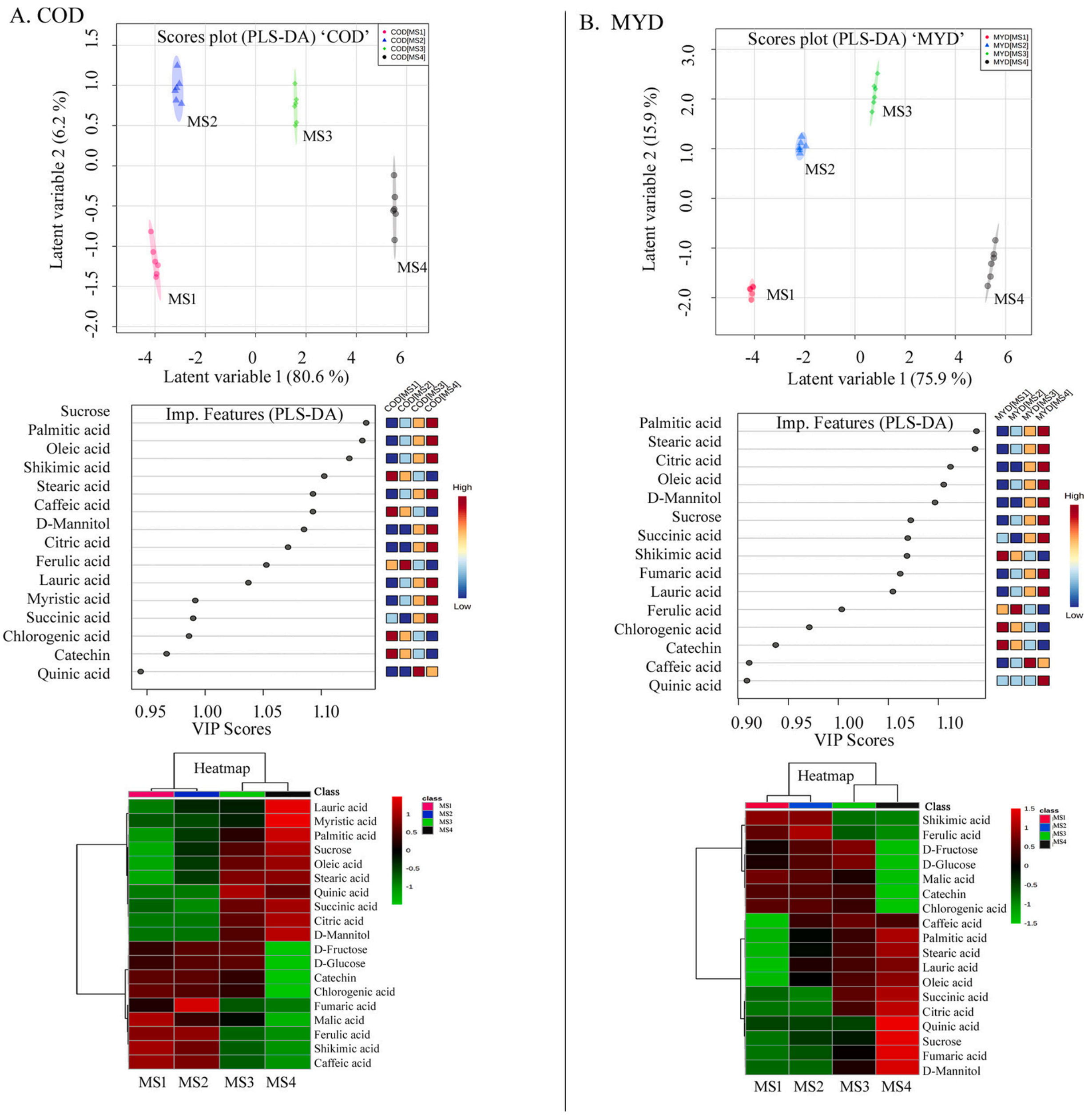

Fig. 4. 2D scores plot (PLS-DA), Important features (PLS-DA, VIP scores) and heatmap analyses of GC–MS data by using Metaboanalyst 4.0. (A) 'COD' variety (B) 'MYD' Variety. In scores plot, metabolites from four developmental stages didn't overlap with each other, indicating an altered state of metabolite levels.

3.4. Changes in the amino acid contents of coconut water during maturity stages

Both UPLC and GC–MS are well-established techniques for amino acid analyses from plant samples. In this study we used UPLC for amino acid profiling from coconut water because of certain advantages. Amino acid analyses in GC–MS is time consuming and aqueous samples cannot be analyzed directly in GC–MS. Aqueous samples need to be dried followed by complex chemical derivatization process to make samples thermostable, prior to GC–MS analyses. In contrast, amino acids present in aqueous samples can be analyzed directly in UPLC with simple AccQ Tag derivatization process. Moreover, UPLC-AccQ.Tag technology has very good sensitivity for the detection and quantification of amino acids in relatively shorter run time (Ferchaud Roucher et al., 2013). The UPLC amino acid profile of 'COD' and 'MYD' coconut varities at two commercially important stages (MS1 and MS4) are shown in Fig. 3. Total of 17 amino acids were recorded from both 'COD' and 'MYD' using UPLC method. The LOD and LOQ values of each amino acid detected by this UPLC method was presented in the Supplementary Table S2. The LOD and LOQ of the amino acids was found in the range of 0.04 - 0.65 μM and 0.12–1.97 μM, respectively. The LOD values obtained in this method are within the range reported earlier (Dahl-Lassen et al., 2018). Except for alanine, the contents of all other amino acids increase during maturation from MS1 to MS4. Any significant difference in amino acid profile was not observed between the 'COD' and 'MYD' varieties. The content of total amino acids in both the young and mature stages of 'MYD' was slightly higher than that of 'COD'. Previously, Tulecke et al. (1961) and Santoso et al. (1996) reported a similar trend of amino acid accumulation in young and mature coconut water, however, amino acid content observed in their study was higher than that of the values reported here. These differences might be due to varietal differences or due to the different units used for expressing amino acid content (mg/g defatted sample) by Santoso et al. (1996). This increase in amino acid content may be associated with the higher synthesis of storage proteins during

Table 3
A list of 10 metabolites as the identified biomarkers in GC–MS-based analysis of coconut water to distinguish between the four different developmental maturity stages (MS1-MS4) of coconuts from the 'Chowghat Orange Dwarf ' (COD) and 'Malayan Yellow Dwarf' (MYD) verities. Biomarker 1–8 are common in both 'COD' and 'MYD'. Biomarkers are identified based on having VIP sore ≥1 in PLS-DA.

S.No	Metabolite	KEGG ID	VIP Score	Log2 ˆFold Change			(-) Log10(*p*)	(-) Log10(FDR)
				MS2/MS1	MS3/MS1	MS4/MS1		
'COD' variety								
1	Chlorogenic acid	C00852	1.0	−0.84	−2.30	−13.75	34.64	33.97
2	Ferulic acid	C01494	1.07	0.38	−10.05	−11.83	35.66	34.38
3	Lauric acid	C02679	1.05	0.57	0.65	2.32	17.94	17.87
4	Oleic acid	C00712	1.12	1.69	4.18	4.84	25.60	25.23
5	Palmitic acid	C00249	1.13	0.72	1.57	2.79	20.17	19.96
6	Shikimic acid	C00493	1.1	−1.76	−10.06	−11.98	35.10	34.20
7	Succinic acid	C00042	1.0	−0.10	0.56	0.70	11.10	11.09
8	Sucrose	C00089	1.16	1.20	2.43	3.14	22.74	22.42
9	Caffeic acid	C01197	1.09	−1.05	−9.65	−11.77	34.50	33.97
10	Myristic acid	C06424	1.03	0.14	0.77	4.18	25.40	25.07
'MYD' variety								
1	Chlorogenic acid	C00852	0.99	−0.08	−1.38	−12.93	36.81	36.03
2	Ferulic acid	C01494	1.00	2.43	−7.29	−8.96	38.08	37.13
3	Lauric acid	C02679	1.05	1.40	1.62	1.90	18.30	18.23
4	Oleic acid	C00712	1.11	1.54	2.08	2.47	20.23	20.09
5	Palmitic acid	C00249	1.14	0.70	0.94	1.33	18.39	18.28
6	Shikimic acid	C00493	1.07	−0.11	−9.03	−9.62	38.91	37.66
7	Succinic acid	C00042	1.07	−0.13	1.00	1.36	21.25	21.04
8	Sucrose	C00089	1.07	0.42	0.68	2.36	23.63	23.28
9	Fumaric acid	C00122	1.06	0.05	0.23	0.59	10.48	10.46
10	Stearic acid	C01530	1.14	1.00	1.43	1.84	20.72	20.55

the conversion of liquid endosperm into solid coconut meat. An increase in the amino acid contents correlated well with the increase in the total protein contents during maturation stages. These amino acid composition data will help consumers or coconut industry to select nuts of appropriate maturity stage with known composition and quantity of amino acids, which could be used as fresh drink or in making coconut-water based food supplements.

3.5. Metabolic profiling analysis of coconut water during maturity stages

GC–MS metabolite profiling of coconut water identified a total of 19 metabolites from 'COD' and 18 metabolites from 'MYD' variety that included organic acids, fatty acids, phenolics, flavonoids, sugars, and sugar alcohol (Supplementary Fig. S1-S3). GC–MS is an excellent technique for the separation and quantification of both primary metabolites (organic acids, fatty acids, amino acids, sugars, and sugar alcohols) and secondary metabolites (phenolics and flavonoids) from plant samples with excellent sensitivity (Jorge et al., 2016). However, in this study, we were able to detect only three amino acids (glycine, 5-oxo-proline, and alanine) by GC–MS profiling of coconut water. Less abundance of amino acids in the coconut water could be a reason for the limited detection of amino acids by the GC–MS or derivatization reaction used for GC–MS analyses of amino acids were not sensitive enough. Therefore, we analyzed the amino acid profile separately by UPLC, and amino acids detected in the GC–MS run were excluded from the metabolomics analyses. Metabolite names, their retention time, retention index, and mode of identification, are shown in Table 2. The one-way ANOVA p-value < 0.05 and false discovery rate (FDR) multiple tests were conducted to compare the metabolites from MS1-MS4 stages of each variety of coconuts. The supervised partial least squares discriminant analysis (PLS-DA) has shown the contribution of these metabolites in distinguishing the coconut maturity stages. The 2D score plots obtained from the PLS-DA test showed that the metabolites of MS1, MS2, MS3, and MS4 stages did not overlap with each other, indicating an altered state of metabolite levels (Fig. 4A and B). The separation of four nut maturity stages in PLS-DA showed the differences in the metabolite

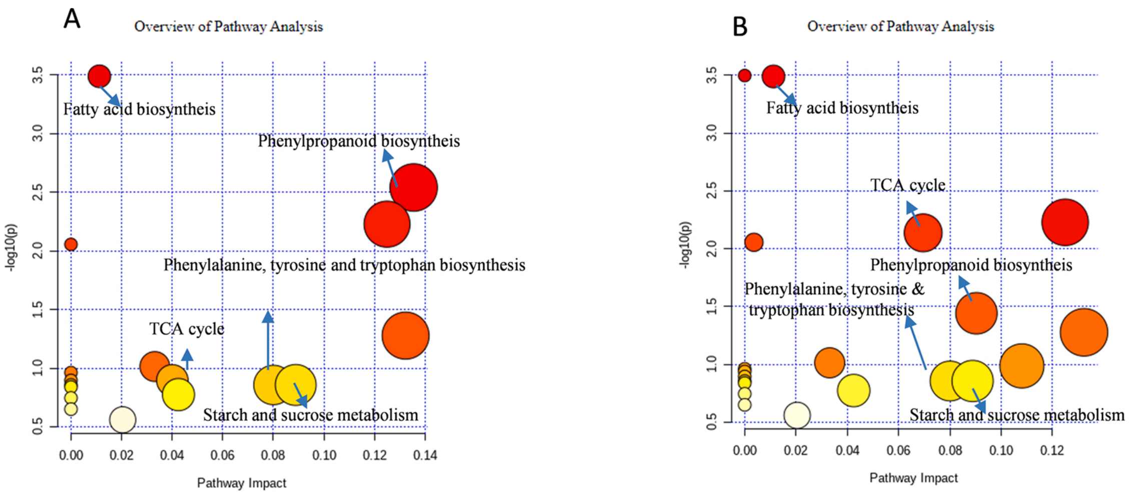

Fig. 5. Pathway analysis based on metabolite biomarkers detected from both 'COD' (A) and 'MYD' (B) coconut varieties.

levels at each nut maturity stage. The first two latent variables (LV1 and LV2) of PLS-DA of ’COD’ samples exhibited 80.6 % and 6.2 % variance, whereas ’MYD’ samples exhibited 75.9 % and 15.9 % variance in PLS-DA. The PLS-DA model was validated by using the cross-validation tool in the Metaboanalyst 4.0. We used the leave-one-out cross--validation (LOOCV) algorithm for the validation of the model and Q2 was chosen as the "performance measure" (Supplementary Fig S4). In both the models, R2/Q2 $>$ 0.7, which showed good predictive ability (Chiu et al., 2016). VIP scores highlight the influence of the independent variable (metabolites) in explaining the dependent variable (maturity stage). We consider metabolites as biomarkers for distinguishing the nut maturity stages if its VIP score ($\geq$1) and is present in all the four maturity stages (MS-MS4).

Eight differential biomarkers, namely, chlorogenic acid, ferulic acid, lauric acid, oleic acid, palmitic acid, shikimic acid, succinic acid, and sucrose have been detected in both ‘COD’ and ‘MYD’. The ‘COD’ variety bears two additional biomarkers, the caffeic- and myristic acids, whereas the fumaric- and stearic acid was present only in the ‘MYD’ variety (Table 3). The fold change in biomarker values are reported in Table 3. The heatmaps for the differential biomarkers combined with hierarchical cluster analysis are shown in Fig. 4. Pathway analyses (Fig. 5A-B) by Metaboanalyst 4.0 using KEGG pathway and plant Arabidopsis databases, the differential biomarkers of developmental stages of coconut water from both ‘COD’ and ‘MYD’ mainly altered the following metabolic pathways: (1) alteration in fatty acid metabolism induce increase level of fatty acids (2) alteration in phenylpropanopid metabolism reduces biosynthesis of phenylpropanoids and phenolic acids (3) alteration in phenylalanine, tyrosine, and tryptophan biosynthesis reduces biosynthesis of shikimic acid and related metabolites (4) alteration in tricarboxylic acid cycle (TCA) cycle metabolism which induces organic acid biosynthesis (5) alteration in sucrose metabolism that induce sucrose synthesis. By using LC–MS, recently Chen et al. (2018) identified 14 metabolites (L-isoleucine, L-leucine, L-valine, L-threonine, 5-oxo-D-proline, L-proline, gamma-aminobutyric acid, taurine, dibutyl phthalate, 4-hydroxyphenylacetic acid) from mature coconut water that can serve as biomarkers to distinguish between the stages of mature coconut water under storage conditions. Our results added more novel biomarkers to discriminate between coconut maturity stages during nut maturation. In future research, one can use these biomarkers from a large number of coconut samples to develop a predictive model that can track the maturity stages of coconut.

4. Conclusions

A series of physicochemical, nutritional, and metabolic changes occur in the composition of coconut water during the nut maturation process, which affects its nutritional value. Harvesting of coconuts at the appropriate maturity stage bearing a high nutrient level in terms of contents of sugar, amino acids, phenolic acids, flavonoids, protein, fatty acids, and minerals is crucial to gain optimum nutritional value of coconut water. Multivariate PLS-DA analyses identified eight metabolites from coconut water of both COD and MYD variety, which serve as differential biomarkers to discriminate between the maturity stages. The level of these biomarkers would help in determining the maturity stages of coconut. Based on these findings, consumers or the coconut industry can select coconut of appropriate maturity stages depending on their customized nutrition requirements. Our study showed that coconuts with a maturity of 9–10 months after pollination (MS3) hold reasonably good amounts of water along with the other essential dietary nutritional components.

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CRediT authorship contribution statement

Mukund Kumar: Investigation, Methodology, Data curation. **Shashank Sagar Saini:** Visualization, Data curation, Software. **Pawan Kumar Agrawal:** Visualization, Writing - review & editing. **Partha Roy:** Formal analysis, Writing - review & editing. **Debabrata Sircar:** Conceptualization, Investigation, Resources, Methodology, Supervision, Funding acquisition, Data curation, Writing - original draft, 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.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2020.103738.

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