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Metabolic profiles analysis and DPPH radical-scavenging assay of 'Nam Dok Mai' mango wine during fermentation

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Abstract

The 'Nam Dok Mai' mango, a famous fruits known worldwide, is produced and consumed extensively. However, mango is a climacteric fruit with a short shelf-life. Therefore, wine production is an alternative procedure, which can be used to process low-quality mangoes. The metabolic profiles of mango wine during fermentation would present a broadening of low molecular compositions, which might improve the production and quality of mango wine. Using the metabolomic approach, fifty-six peaks could be identified, accounting for approximately 40% of all metabolites. Based on the metabolites found in mango wine, Principal Component Analysis (PCA) coupled with Agglomerative Hierarchical Clustering Analysis (AHC) analysis was processed to differentiate all samples. Oleic acid, linoleic acid (ω-6), and α-linolenic acid (ω-3) were significantly decreased during the fermentation periods. The most abundant phytosterols, found in 'Nam Dok Mai' mango wine, were β-sitosterol, campesterol, and stigmasterol. A decrease in all phytosterols was also found during fermentation. Regarding the polar metabolites, most of the sugars, including sucrose, glucose, and fructose, were depleted by wine yeast during their alcoholic metabolism. Conversely, organic acids, such as malic acid and citric acid, were slightly increased during the fermentation process due to aerobic glycolysis channels. The antioxidant activity in mango wine during fermentation periods was not different.

Keywords: Mango wine, Metabolomics, Metabolic profiles, Phytosterols, Antioxidant

1. Introduction

Mango (*Mangifera indica* Linn.) is one of the most eminent fruits worldwide, which is produced and consumed extensively. Moreover, not only is 'Nam Dok Mai' a popular mango variety for Thai people, but it is also popular among international consumers because of its special flavor and taste. However, being a climacteric fruit, mangoes, therefore, have short shelf-life and become easily spoiled [1]. Mango wine is one of the novel products that can be used to produce added value for excess and low-quality mangoes [2]. Nonetheless, the quality of mango wine production could be improved by studying the changes in metabolite profiles together with examining the antioxidant activity during mango wine fermentation through the process of utilizing metabolomics techniques and DPPH radical-scavenging assay. This would lead to obtaining a better understanding of the broadening of low molecular compositions.

Metabolomics is one of the omics techniques that focuses on the changes of almost all metabolites from cell to organism. Metabolites, which are essential to sustain the lives of every organism, are called primary metabolites and those, which are not-essential, but are necessary for survival in an inferior environment, are considered

secondary metabolites [3]. Metabolomes could reflect proteomes. Therefore, changes of metabolites would be relative to alterations in the proteome, transcriptome, and genome. These are advantages of the metabolomics technique, which have made it become popularly applied in many fields, including food science, nutrition, and biomedical science. Therefore, the aim of this study was to investigate changes in the metabolites and the antioxidant activity during mango wine fermentation.

2. Materials and methods

2.1 Chemicals

High performance liquid chromatography (HPLC) and chemicals of analytical grade were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and from RCI Labscan (RCI Labscan, Pathumwan, Bangkok, Thailand). All standards were standard reagent grade purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA).

2.2 Selection of 'Nam Dok Mai' mango

'Nam Dok Mai' mangoes were all cultivated in the summer (May-June) in 2017. After 90-100 days of the fruit-set stage, mangoes with the weights between 400 and 420 g, lengths of 14 to 16 cm, and widths of 7 to 9 cm were selected. Floatation was used to establish their densities by floating them in a 3% NaCl solution and sinking in 1% NaCl to determine their uniform maturity index using a specific gravity value [4]. A controlled temperature of 30±2 °C and relative humidity of 75±2% were sustained for the conditions of ripening. After 8 days of ripening, samples were taken. Mangoes were cleaned and cut into small pieces before being stored at -20 °C for further use.

2.3 Mango wine production

The mango wine production was carried out by making a slight modification to the previous study [5]. Five liters of 30% mango juice called "must" was accommodated to 22.0 °Brix by adding sugar. To that, 50% w/w citric acid solution was added to adjust pH of the mango juice to 4.0. Potassium metabisulfite (KMS) with a concentration of 0.2 mg/ml and 0.5 mg/ml of diammonium phosphate (DAP) were added. The mixture was left in fermentation tanks for 1 day at 25 °C. Five grams of dried commercial wine yeast, *Saccharomyces bayanus* Lalvin EC 1118 (Lallemand, Inc., Montreal, Canada) was actuated in 20.0 ml of drinking water at 35 °C for 15 min and then 4.0 ml of yeast solution were inoculated into the "must". Fermentation was performed in 4 batches at 20±2 °C for 18 days. One hundred milliliters of the samples were taken every 2 days.

2.4 Metabolomics

2.4.1 Sample extraction and phase separation

The metabolomic methodology was followed from the previous study [6]. Four ml of dichloromethane and 10.0 ml of methanol/water (80:20 v/v) were added to 10.0 ml of mango wine for extraction. For lipid fraction, 100.0 µl of tetracosane (1 mg/ml) and 100.0 µl of 5α-cholestane-3β-ol (1 mg/ml) were added. Two hundred and fifty µl of phenyl-β-D-glucopyranoside (1 mg/ml) and 250.0 µl of ρ-chloro-L-phenylalanine (1 mg/ml) were then added as an internal standard for sugar and acid fractions. The sample was mixed and sonicated for 15 min at room temperature before the two phases were separated.

2.4.2 Lipid fractionation

At the beginning, 1.5 ml of lipid extract was evaporated to dryness and re-dissolved with 500.0 µl of methyl tertiary-butyl ether (MTBE), 300.0 µl of methanol, and 50.0 µl of 5.4 M sodium-methylate in methanol to test the transesterification reaction. The addition of 1 ml of dichloromethane and 2.0 ml of 0.35 M hydrochloric acid solution was performed for selective hydrolysis. The upper phase was then discarded. The lower phase containing transmethylated lipid was collected and evaporated to dryness, and was then re-dissolved with 250.0 µl of dichloromethane. Transmethylated lipids, including fatty acid methyl esters (Fraction 1) and polar lipids (Fraction 2), were eluted by fractionation with different concentrations of hexane-MTBE solution in a solid-phase microextraction C18-LP cartridge. All lipid fractions were immediately stored at -20 °C to await gas chromatography (GC) analysis.

2.4.3 Polar fractionation

Two fractions of polar extract were fractionated. For the sugar fraction, 200.0 µl of the polar extract was silylated by trimethylsilylimidazole. The sample was then hydrolyzed, and the phase was separated. The upper phase containing silylated sugar and sugar alcohol was collected for gas chromatography with flame ionization detector (GC-FID) analysis. In the case of an acid fraction, 300.0 µl of hydroxy ammonium chloride in pyridine (2 mg/ml) was added into 200.0 µl of the polar extract for oximation. After oximation, silylation was performed by adding 100.0 µl of N-methyl-N-(trimethylsilyl) fluoroacetamide (MSTFA). Three hundred microliters of hexane and 300.0 µl of water were then added for selective hydrolysis. The upper phase was discarded and the lower phase containing amino acids and organic acids were applied for the second silylation by adding 50.0 µl of MSTFA and 200.0 µl of acetonitrile. The solution was then ready for GC analysis.

2.5 GC-FID analysis

Gas chromatography 6890 plus, coupled with the flame ionization detector (Hewlett Packard, Palo Alto, CA, USA), was used for sample (GC) analysis. The GC condition was also followed as previously described by Na Jom et al., 2011 [6]. A DB-1 capillary column (60.0 m×0.32 mm×0.25 µm film thickness) with 100% dimethylpolysiloxane stationary phase was selected for separation. One microliter of each sample was injected into the GC-Flame ionization detector (FID) with spitless mode and helium was used as a mobile phase at a constant flow rate of 1.8 ml/min. The inlet temperature was 280 °C. The oven temperature program was first started at 100 °C, was then increased to 320 °C with a rate of 4 °C/min, and was finally held at 320 °C for 25 min. The detector temperature was 320 °C.

2.6 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay was modified from Chanput et al. (2016) [7]. Two hundred microliters of mango wine were reacted with 400.0 µl of DPPH solution (25 mg/ml in methanol) in darkness for 30 min. Then, the absorbance (Abs) was measured at 517 nm. Deionized water was used as a blank. The %DPPH inhibition was calculated in accordance with the following equation:

$$\%DPPH\ inhibition = \left[\frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}}\right] \times 100 \quad (1)$$

The lower absorbance of the mixture indicated a higher radical-scavenging activity.

2.7 Statistical data analysis

Chromatographic areas of each metabolite were acquired and integrated by the HP-ChemStation A.06.03 program (Hewlett Packard, Palo Alto, CA). They were then identified by using an analytical standard comparison technique and were semi-quantified by using a relative investigation with an area of the internal standard for each fraction. Principal Component Analysis (PCA) and Agglomerative Hierarchical Clustering Analysis (AHC) were accomplished by using the XLSTAT-base version 2018.3 (Addinsoft, NY, USA) to discriminate all samples based on the metabolites found in mango wine.

3. Results and discussion

For the metabolomics, the total chromatography peaks numbered more than 150, denoting both non-identified and identified compounds. However, there were 56 peaks, which constituted approximately 40% of all metabolites in mango wine and could be identified. All metabolite compounds from chromatograms were subject to principal component analysis (PCA). It was found that PCA biplot (Figure 1) showed metabolic changes by shifts in fermentation percentages along the PC, which represented approximately 65% of all total variables. Furthermore, the PCA biplot could be used to explain the grouping of samples based on every chemical constituent, including all of the 56 compounds found in mango wine. To confirm the grouping of mango wine samples from PCA, the similarity mode of AHC was performed. Mango wine samples were divided into 3 groups; 1) the group of pre-fermentation (0% fermentation = day 0), 2) the group of during-fermentation (25-75% fermentation = days 4-10), and 3) the group of post-fermentation of the "finished mango wine" (100% fermentation = day 18). Fatty acid methyl esters, free fatty acids, and phytosterols, including oleic acid (C18:1), linoleic acid (C18:2), α-linolenic acid (C18:3), β-sitosterol, campesterol, and stigmasterol were used to indicate the pre-fermentation group. The dominant compounds, indicating the during-fermentation group, were glucose and fructose. The "finished mango

wine" was separated from other groups by the acid components, which included malic acid, citric acid, glutamine, glycine, proline, and isoleucine.

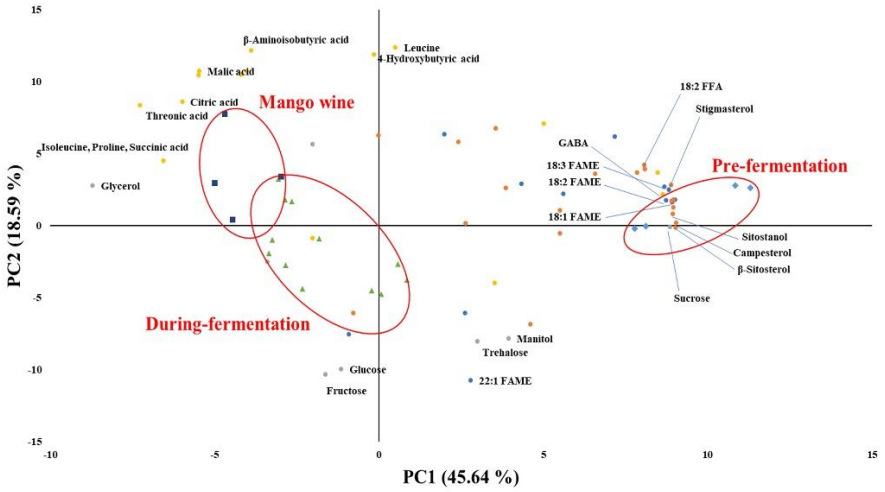

Figure 1 Biplot of principal component analysis (PCA) from all identified metabolites (●) in 'Nam Dok Mai' mango wine during the three wine fermentation periods: the pre-fermentation group (♦), the during-fermentation group (▲), and the finished mango wine group (■).

The progressive changes of the metabolite compounds obtained from fatty acid methyl ester fraction are shown in Figure 2. The most important compounds were oleic acid, linoleic acid (ω-6), and α-linolenic acid (ω-3), which were found in high amounts in mango pulp and juice. In terms of essential nutrients, these fatty acids play an important role in human health, which must be received from the daily intake of food. However, it was found that these unsaturated long-chain fatty acids had significantly decreased during fermentation periods, especially at the 0-25% fermentation. Then until the end of fermentation, they continued to slightly decrease. Linoleic acid decreased from 3.66 µg/ml at 0% fermentation to 1.15 µg/ml at 25% fermentation and then slightly decreased to 0.43 µg/ml at 100% fermentation. Furthermore, α-linolenic acid decreased from 3.36 µg/ml at 0% fermentation to 0.64 µg/ml at 25% fermentation. Then it slightly decreased again to 0.09 µg/ml at the end of fermentation. Given that fatty acids are necessary for the maintenance of yeast cell membrane under anaerobic conditions [8]. Fatty acids also act as anaerobic fermentation activators for the *Saccharomyces cerevisiae* yeast strain at the beginning of fermentation. The most important of these are oleic acid and linoleic acid [9]. Furthermore, they are active and react with alcohol to form ester compounds in a process called esterification. The formation of esters continues throughout the aging process due to the presence of many different acids in the wine, along with large quantities of ethanol (11% v/v of 'Nam Dok Mai' mango wine).

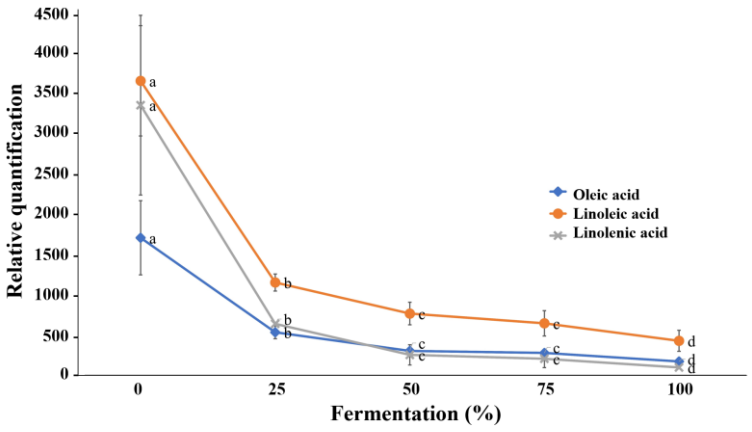

Figure 2 Changes in the relative quantification of imperative metabolite compounds in fatty acid methyl esters fraction (Fraction 1) during mango wine fermentation. Values are means ± standard deviation (SD). Means in the same line are indicated by different letters and were found to be significantly different at a confidence level of 95% ($p \leq 0.05$).

Phytosterols are one of the major lipophilic components, which found in mango juice and wine. Human health benefits are provided by a sufficient intake of phytosterols, including the decrease of cholesterol accumulation in blood vessels. The most abundant phytosterols found in 'Nam Dok Mai' mango wine were β-sitosterol, campesterol, and stigmasterol, respectively. High amounts of those 3 phytosterols can also be found in grapes, berries, and other mango cultivars, such as Rosa and OTT [10]. However, decreases in these phytosterols were also found during the fermentation periods (Figure 3). There is still a lack of information about changes in the phytosterols during wine processing. In this case, it might be due to yeast metabolism because phytosterols can be synthesized in aerobic conditions. Plant sterols might be used as substrates to produce some secondary metabolites to protect themselves from the apprehensive conditions, which include anaerobic conditions, high alcohol contents, and sulfur contents. In the same way, these results were also reported to be found in grape wine [11]. Generally, the average daily intake of phytosterol is 150.0-400.0 mg/day. However, to reduce blood cholesterol, the effective phytosterol range is approximately 1.5-2.0 g/day. Therefore, the addition of phytosterols to mango wine could be an alternative option to improve the nutritional value of mango wine.

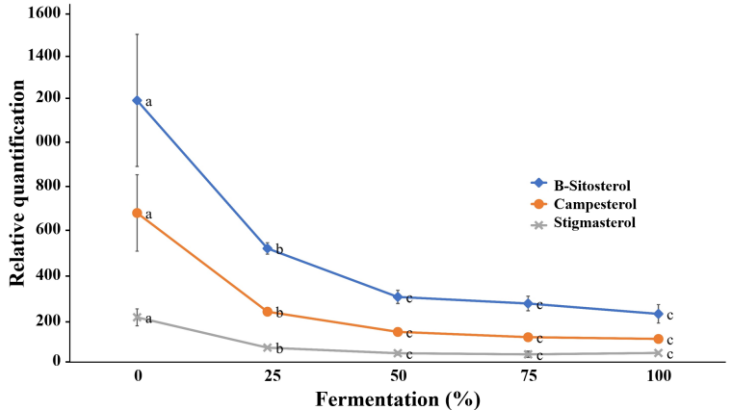

Figure 3 Changes in the relative quantification of major phytosterols during mango wine fermentation. Values are means ± standard deviation (SD). Means in the same line are indicated by different letters and were found to be significantly different at a confidence level of 95% ($p \leq 0.05$).

Regarding the polar metabolites, most of the sugars, including sucrose, glucose, and fructose, were dissipated by wine yeast during their ethanol metabolism. The sucrose content continuously decreased from 0-50% fermentation and remained stable until the end of fermentation. Conversely, when focusing on changes to reduce sugar contents (glucose and fructose), the reducing sugar concentrations significantly increased at 0-25% fermentation, and then decreased slightly until the end of fermentation as shown in Figure 4. This is a result of yeast metabolism, in which yeast would hydrolyze the sucrose making it become glucose and fructose at the initial stage. In the following stage, the yeast utilized the glucose and fructose as substrates to produce alcohol [12].

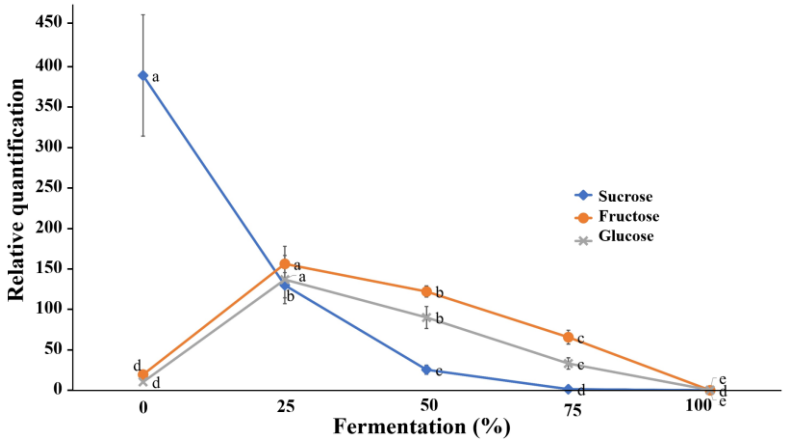

Figure 4 Changes in the relative quantification of imperative metabolite compounds in sugar fraction (Fraction 3) during mango wine fermentation. Means are in the same line indicated by different letters and were found to be significantly different at a confidence level of 95% ($p \leq 0.05$).0

Conversely, organic acids, such as malic acid and citric acid, are the main acids in mangoes and were found in high amounts in the mango wine. During the fermentation process, these slightly increased (Figure 5) due to aerobic glycolysis respiration. Lactic acid was not detected during the wine-making process. From this, it can be interpreted that no malolactic fermentation had occurred. In addition, glycerol was increased during fermentation because it is the end-product of glyceropyruvic fermentation. However, glycerol formation could have occurred rapidly at the beginning of wine fermentation, and then more slowly relevant to the amounts of substrates (e.g. glucose and fructose). The amounts of succinic acid slightly increased in this wine fermentation process. This might be due to the fact that succinic acid could be synthesized from pyruvate or aspartate by yeast during anaerobic fermentation via the reductive pathway [13]. Proline and isoleucine were the main amino acids found in mango wine. It was found that the proline and isoleucine contents had gradually increased during fermentation due to the yeast autolysis. Proline could be also produced from the degradation of arginine [14].

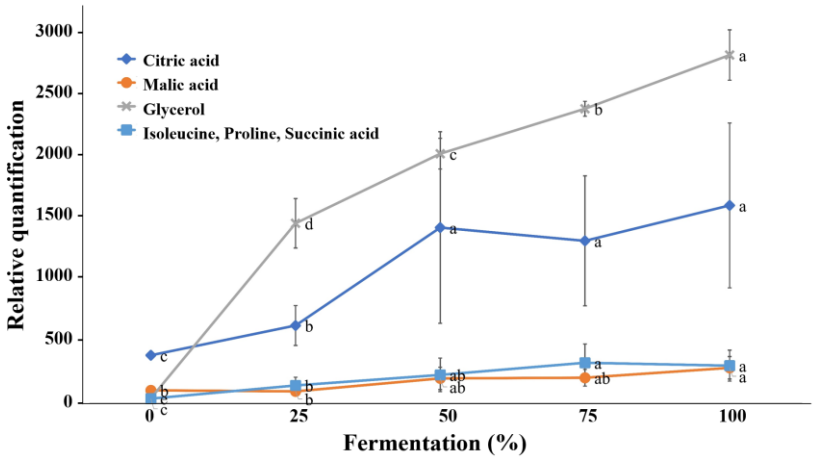

Figure 5 Changes of relative quantification of imperative metabolite compounds in organic acids and amino acids fraction (fraction 4) during mango wine fermentation. Values are means ± standard deviation (SD). Means in the same line are indicated by different letters and were found to be significantly different at a confidence level of 95% ($p \leq 0.05$).

The antioxidant activity of mango wine did not differ significantly during the fermentation process (Figure 6). It ranged from 60.98 to 59.32% gallic acid equivalents. However, during the processes of making and aging Indian mango wine, the depletion of xanthophyll (78.7-93.9%), neoxanthin (26.8-83.3%), violaxanthin (50.0-74.3%), and β-carotene (17.9-60.7%) was also reported [15]. Furthermore, the degradation of carotenoids during the fermentation of carrot juice was also described [16]. In addition, carotenoids could be substrates for the formation of norisoprenoids, which are the aroma compounds in mango wine [17].

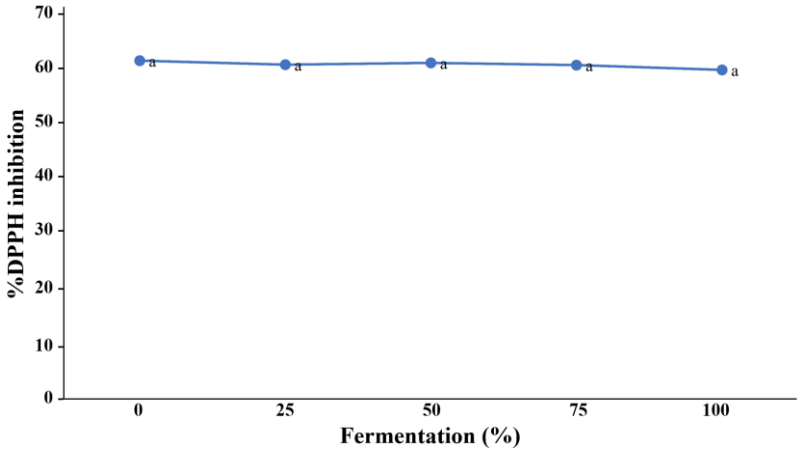

Figure 6 The Antioxidative value of 'Nam Dok Mai' mango wine during fermentation expressed as %DPPH inhibition. Results are means ± SD of four independent samples with three technical replicants (n=4). Means in the same line are indicated by different letters and were found to be significantly different at the confidence level of 95% ($p \leq 0.05$).

4. Conclusions

Sharing the same characteristics as a dry white wine (residual sugar contents <1.0 g/L), Mango wine production from 'Nam Dok Mai', in which the acid compounds of malic acid, citric acid, isoleucine, proline, and succinic acid could be used as possible biomarkers indicating the finished product of mango wine, has the potential to be successful. The antioxidant activity was not significantly different during fermentation. Therefore, utilizing the metabolomics approach and combining it with measuring antioxidant activity, could be effective in monitoring the changes in mango wine fermentation in order to improve the quality of Thai mango wine in the future.

5. Acknowledgments

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