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HPLC/ESI-TOF-MS Identification and Quantification of Phenolic Compounds in Fermented/Non-Fermented Jaboticaba Fruit (*Myrciaria jaboticaba* (Vell.) O. Berg)

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Abstract

The phenolic composition of non-fermented jaboticaba pomace (solids derived from the skin, seeds and pulp after juice extraction) and fermented pomace (solids remaining after wine production) were compared using HPLC/ESI-TOF-MS. Fermented pomace contained higher concentrations of quercetin and myricetin while non-fermented pomace contained higher levels of ellagic acid, cyanidin 3-*O*-glucoside and delphinidin 3-*O*-glucoside. Fermented pomace retained a dark purple color due to the retention of moderate levels of cyanidin 3-*O*-glucoside (0.18 mg/g dwb) and delphinidin 3-*O*-glucoside (0.207 mg/g dwb). Fermented pomace may be added to food products to provide color (anthocyanins) and important biological activities such as such as antimicrobial, antidiabetes and controlling obesity and chronic obstructive pulmonary disease (COPD).

Keywords: jaboticaba, HPLC, phenolics, anthocyanins, pomace

Introduction

Jaboticaba (*Myrciaria jaboticaba* (Vell.) O. Berg) is a fruit native to Brazil that is popularly consumed as food or fermented beverages. In Brazil, fresh jaboticaba fruits are widely consumed and their popularity has been compared to that of grapes in the United States [1]. The fruit are round, about 2 to 3 cm in diameter with a dark purple pericarp covering a jelly-like white pulp [2]. The fruit have a sweet pleasant taste and low acidity probably due to its sugar, organic acid and terpene contents [3]. Jaboticaba spoils easily and thus is also consumed in the forms of juices, jams, liqueurs, distillates, wine and ice cream, as alternatives to reduce post-harvest losses [4]. It is thus critical to determine compositional changes occurring as a result of processing and storage. Jaboticaba is known as a rich source of antioxidant molecules such as aromatic acids and polyphenols, among others [4]. Leite *et al.* [5] reported that the addition of 1 and 2% freeze-dried jaboticaba peel to normal diets improved the antioxidant potential of rat plasma. Addition of 1, 2 and 4% freeze-dried jaboticaba peel (FJP) to high-fat diets reduced serum insulin (47, 57 and 52%, respectively) and homeostatic model assessment of insulin resistance (HOMA-IR; 40, 54 and 48%, respectively) in Sprague-Dawley rats [6]. The addition of 2% FJP to the high-fat diet elevated serum levels of HDL-cholesterol levels by 41.65% compared to the high-fat diet control, thus demonstrating an important cardioprotective effect [6]. The polar extract of jaboticaba peel has been shown to have antiproliferative effects against leukemia cells (K-562) while the non-polar extract of jaboticaba peel was active against prostate cancer cells (PC-3) [1]. These authors also reported that the micronucleus test in mice showed that the polar extract of jaboticaba peel induced no DNA damage and caused no mutagenic effects. Recently, *in vivo* study has shown positive effects of jaboticaba fruit peel extracts on wound healing in mice fibroblasts likely due to activity of antioxidant phytochemicals [7]. Similarly, fermented jaboticaba fruit beverages possess significant vasorelaxant effect that appears to be associated with antioxidant potential of phenolic compounds present in the fruit [8]. Principle component analysis of the fruit is important considering its growing popularity and the limited amount of studies done on physiological effects. Thus, this study primarily focuses on comparative analysis of total phenolic content in fermented/non-fermented jaboticaba fruit powder (JFP) produced from the species *Myrciaria jaboticaba* (Vell.) O. Berg. Detection and quantification was done utilizing high-performance liquid chromatography (HPLC) coupled with dual-electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS). The HPLC/MS protocols were developed and optimized for Type-C Silica™ based stationary phases [9, 10] that provide a new analytical approach for characterizing food products.

2. Materials and Methods

2.1 Experimental design and treatments

2.1.1 Jaboticaba samples

Fruit of the cultivar *Myrciaria jaboticaba* (Vell.) O. Berg (Jabuticaba sabará in local language) was used. The fruit was grown in the region of the Serra do Goiano specifically in the Fazenda Jabuticabal (Municipality of Hidrolândia-Goiás) 16 ° 55'32.35 "S and 49 ° 21'39.76" W. Rodovia GO-319, KM 18, District of Nova Fátima, Hidrolândia – GO. The whole fruit (skin, seeds and pulp) was used. After extraction of the juice the pomace was dried in a dryer with temperature and

controlled air up to 50 ºC for 24 h until a moisture content of 6 to 10 % was reached. The dried pomace was ground with a knife mill.

2.1.2 Jaboticaba fermentation

After crushing, the juice and pomace possessed the following properties: pH 3.3, acidity (9 g/L tartaric acid), 28° Brix, density (1.056), temperature (23 °C). *Saccharomyces cerevisiae* was added to the mixture. Fermentation continued for 5 days. At the end of the fermentation the liquid had an alcohol content of 12 degrees Gay-Lussac and contained 2 to 5° Brix. After removal of the wine the residue was dried and ground with a knife mill.

2.1.3 LC/MS Sample Preparation

Five hundred mg of each sample was extracted with a total of 250 mL MeOH + 0.5% HCl until colorless. At each cycle of extraction (10-15 mL), aqueous samples were sonicated for 5 min prior to centrifugation at 3,200 rpm for 15 min. Supernatant was combined and vacuum filtered through a 0.45 µm Nylon membrane filter (MicroSolv – Cat.#58545-N47) to remove residual JFP. The extract was dried with rotary evaporator (40°C water bath) followed by vacuum oven (VWR1410 - 25 kPa at room temperature). Dried extract was dissolved in 4 mL of MeOH + 0.1% formic acid (final concentration JFP: 125 mg/mL eq.) and filtered through a 0.45 µm Nylon membrane syringe filter (MicroSolv - Cat.#: 58045-N25-C) prior to HPLC/MS analysis. Quantification of phenolic compounds were done by standard addition measurement (concentration ≈ 0.1mg/mL).

2.1.4 Instrumentation

The HPLC column used in this study was a Cogent Phenyl Hydride™ packed with Type-C Silica™ material of 4 µm particle size, in a 2.1 x 100 mm column (Cat. No. 69020-10P-2; MicroSolv Technology Corp., Leland, NC, USA). The HPLC system used in this study was an Agilent (Little Falls, DE, USA) 1100 Series LC system, degasser, binary pump, thermostat autosampler and column oven. The mass spectrometer system was an Agilent (Santa Clara, CA, USA) Model 6210 MSD Time-of-flight (TOF) with a dual-sprayer electrospray ionization (ESI) source. MassHunter™ Workstation Software from the instrument manufacturer was used for qualitative analysis of MS data and its acquisition control (Agilent Technologies, Santa Clara, CA, USA). Some preliminary screening experiments were also performed with HP1050/1090 HPLC-DAD system as well as with a Flexar (Perkin Elmer, Waltham, MA, USA) UHPLC system, UV/Vis detector, and Flexar SQ 300 MS detector. Reagents used in this study are the following: HCl (JT Baker – Cat. No. 9535-01) Milli-Q water (Millipore Corporation, Billerica, MA, USA), 0.2 micron filtered acetonitrile (GFS Chemicals, Columbus, OH, USA), 0.2 micron filtered methanol (Fisher Scientific, Fair Lawn, NJ, USA; Cat. No. A452-4), formic acid (Sigma-Aldrich, Inc., St. Louis, MO, USA), and analytical standards (Sigma-Aldrich, Inc., MP Biomedicals, HWI Analytik GmbH). The mobile phase is composed of: Milli-Q water + 0.1% formic acid (A – aqueous phase) and acetonitrile + 0.1% formic acid (B – organic phase). The column flow rate was 0.4 mL/min. The column oven temperature was kept at 20ºC. The reverse phase gradient method used in this study is shown in Table 1A. MS detection was operated in positive and negative ion modes using the following *m/z* parameters as shown in Table 1B.

3. Results and Discussion

In this study, the analytical protocol was optimized for a set of phenolic compounds and jaboticaba pomace powders by using the Type-C Silica™ stationary phases (Figure 1). As observed it was not possible to detect all of the target compounds in either the positive or negative ion modes. However, between the two ionization modes all of the target analytes could be detected in at least one, and in some cases both as positive and negative ions. Because of exact mass TOF, only one analyte pair, quercetin and ellagic acid with similar *m/z* values, did not in reality require chromatographic separation. However, with the protocol used, partial separation was achieved. Further, comparative analyses were done on fermented/non-fermented JFP extracts with LC-MS for identification and quantification of tested phenolic compounds. Examples of the quantitative determinations through spiking of the samples is shown in Figure 2 with a list of the values determined for representative compounds given in Table 2. It was expected that highly polar phenolics would dissolve in the juice and thus not be detected in the remaining solids. For example, gallic acid, previously reported in jaboticaba whole fruit [11, 12], was not detected in the extracts obtained in this study. During fermentation the extraction of phenolics into the juice depends on the contact time with the solids and the ethanol content of the wine. Fermented JFP is estimated to have a significantly higher content of quercetin and myricetin than non-fermented JFP. Myricetin was previously reported in jaboticaba with the highest concentrations found in the skin [11]. Phenolics can exist in insoluble forms that are covalently bound to cell wall structural components such as cellulose, hemicellulose, lignin, pectin and structural proteins [11]. These insoluble phenolics are not extracted by solvents but can be liberated by acid and alkaline hydrolysis. Inada and co-workers [11] demonstrated that phenolics such as myricetin are released by acid and alkaline hydrolysis of jaboticaba. Concentrations of quercetin and myricetin increased during verification of Monastrell wines indicating the enzymatic release of these phenolics [13]. A previous study on whole jaboticaba fruit reported that quercetin occurred exclusively in the soluble form while myricetin was equally distributed as soluble and insoluble forms [11]. It is not known if the higher concentrations of quercetin and myricetin in fermented JFP is due to their release from glycosides and/or cell wall structural components. Meanwhile, ellagic acid and overall anthocyanin concentration are higher in non-fermented JFP. It is likely that these phenolics dissolved in the wine thus lowering their concentration in the solids. The concentration of these constituents could also decrease in the fermented solids due to fixation of these phenolics on yeast [14]. The ellagic acid concentration found in the samples tested in this study (5.532 mg/g dwb) was considerably higher than previously reported levels which ranged from 0.154 mg/g dwb to 0.52 mg/g dwb [11, 12, 15]. Ellagic acid was not detected in the solids derived after fermentation. Mena *et al.* [16] reported rapid decreases in ellagic acid concentration during fermentation of pomegranate

juice. Oxidation and/or precipitation may be responsible for losses in ellagic acid during fermentation [17].

Cyanidin 3-*O*-glucoside and delphindin 3-*O*-glucoside are the two major anthocyanins found in jaboticaba fruit. Reported concentrations of cyanidin 3-*O*-glucoside range from 2.80 mg/g dwb to 4.33 mg/g dwb while reported concentrations of dephinidin 3-*O*-glucoside range from 0.48 mg/g dwb to 0.81 mg/g dwb [11, 12, 15]. The value reported here for delphindin 3-*O*-glucoside concentration (0.549 mg/g dwb) was in good agreement with previously reported levels. However, the measured concentration of cyanidin 3-*O*-glucoside (0.952 mg/g dwb) was slightly lower than previously reported concentrations. The value was determined in solids obtained after juice removal so lower levels were expected. Fortes and co-workers [18] reported that the concentration of monomeric anthocyanins decreased from 0.20 g/L at day 1 to 0.06 g/L at day 5 during the fermentation of jaboticaba fruit (*Myrciaria cauliflora*). There is a striking difference between the concentrations of extracted phenols in grape and jaboticaba fermentation. During vinification of Vranec grapes (*Vitis vinifera* L.) total anthocyanins and total phenols reached maximum concentrations of about 0.85 g/L and 5.0 g/L, respectively [19] while maximum levels of total anthocyanins and total phenols only reached 0.20 g/L and 2.64 g/L, respectively, during vinification of jaboticaba [18]. The low extractability of anthocyanins from jaboticaba during fermentation may be due to the rigid and thick structure of the skins which contain high concentrations of cellulose and hemicellulose (340 g/kg and 80 g/kg [20]). Lower concentrations of cyanidin 3-*O*-glucoside (0.18 mg/g dwb) and delphinidin 3-*O*-glucoside (0.207 mg/g dwb) were found in solids after fermentation. This was expected since anthocyanins are transferred from the solids into the wine. However, the concentration of anthocyanins can also decrease during fermentation due to the following processes: (1) direct oxidation of anthocyanins with O_2, (2) polymerization reactions involving the condensation of anthocyanins with acetaldehyde that is generated by yeast metabolic activity and (3) the occurrence of enzymes degrading anthocyanins such as microbial β-glucosidases [21, 22].

4. Conclusions

Jaboticaba is a highly perishable fruit that is often processed into juices, jams, wine and liqueurs. Monitoring jaboticaba pomace after fermentation revealed important changes in phenolic composition. Pomace obtained after wine production contained higher levels of quercetin and myricetin compared to pomace obtained after juice extraction. Due to the thick and rigid structure of jaboticaba skin there is good retention of anthocyanins in the pomace derived after fermentation. Jaboticaba pomace, a by-product of wine production, may have a similar value as grape wine pomace in uses such as antioxidants, fortifying, coloring and antimicrobial agents.

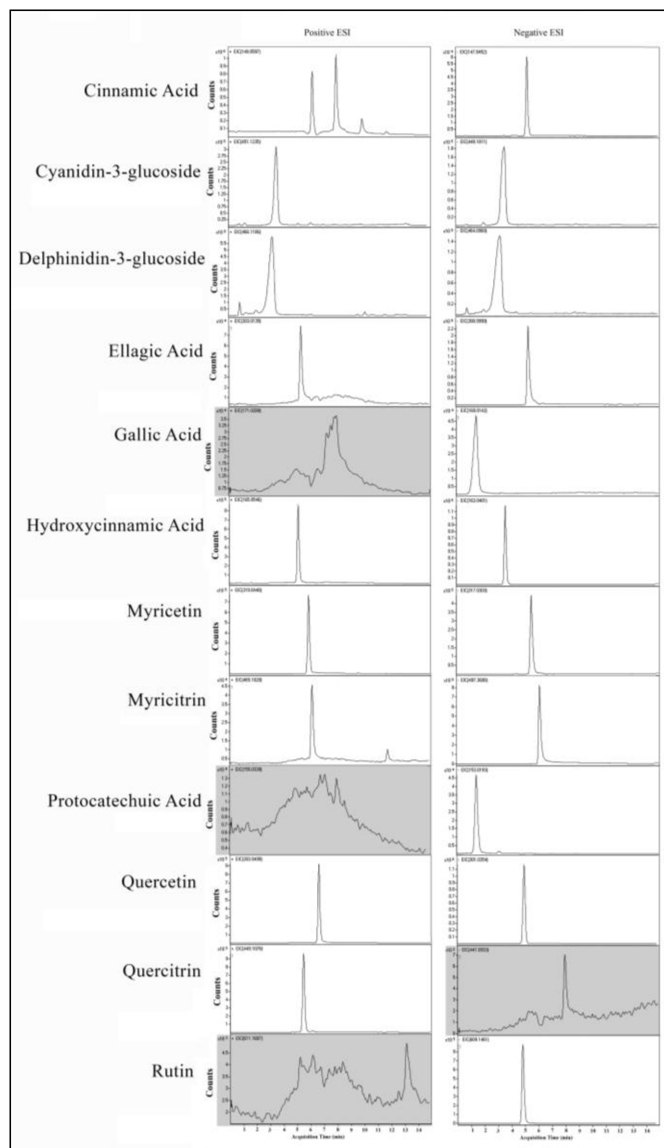

Fig 1: Extracted ion chromatograms of representative phenolic compounds evaluated in jaboticaba fruit samples.

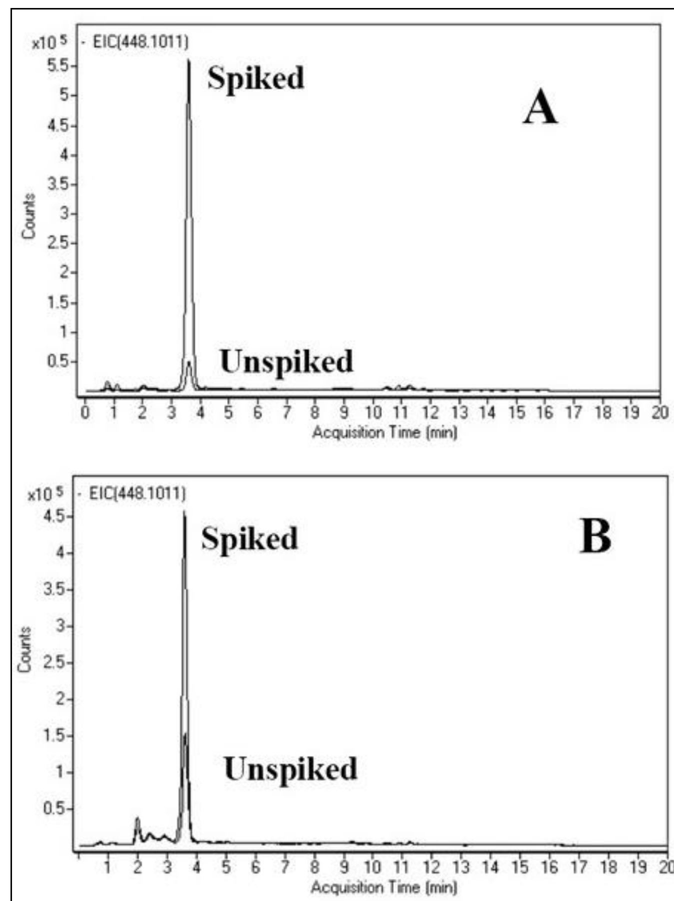

Fig 2: Overlaid extracted ion chromatograms of cyanidin 3-*O*-glucoside in (A) fermented JFP and spiked fermented JFP and (B) non-fermented JFP and spiked non-fermented JFP.

Table 1: HPLC reverse phase gradient

Time (min)	%B	Flow Rate (mL/min)
0	5	0.4
1	10	0.4
12	90	0.4
14	90	0.4
16	5	0.4

Table 2: M/Z parameters for MS detection

Name	Formula	Monoisotopic Mass	$[M+H]^+$	$[M-H]^-$
Cinnamic Acid	$C_9H_8O_2$	148.0524	149.0597	147.0452
Hydroxycinnamic acid	$C_9H_8O_3$	164.0473	165.0546	163.0401
Quercetin	$C_{15}H_{10}O_7$	302.0427	303.0499	301.0354
Protocatechuic Acid	$C_7H_6O_4$	154.0266	155.0339	153.0193
Myricetin	$C_{15}H_{10}O_8$	318.0376	319.0448	317.0303
Gallic Acid	$C_7H_6O_5$	170.0215	171.0288	169.0142
Myricitrin	$C_{21}H_{20}O_{12}$	464.0955	465.1028	463.0882
Ellagic Acid	$C_{14}H_6O_8$	302.0063	303.0135	300.9990
Rutin	$C_{27}H_{30}O_{16}$	610.1534	611.1607	609.1461
Quercitrin	$C_{21}H_{20}O_{11}$	448.1006	449.1078	447.0933
Cyanidin-3-glucoside	$C_{21}H_{21}ClO_{11}$	484.0772	485.0845	483.0699
Delphinidin-3-glucoside	$C_{21}H_{21}O_{12}^+$	465.1033	466.1106	464.0960

Table 2: Quantified phenolic contents in fermented/non-fermented JFP. Amounts are measured in milligrams per gram of JFP, and estimated by using standard addition method.

Compounds	Fermented JFP (mg/g)	Non-fermented JFP (mg/g)
Quercetin	0.264	0.009
Myricetin	0.029	-
Ellagic Acid	-	5.532
Cyanidin-3-O-Glucoside	0.180	0.952
Delphinidin-3-O-Glucoside	0.207	0.549

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