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Original Research Article

The phenolic compounds, tocopherols, and phytosterols in the edible oil of guava (*Psidium guava*) seeds obtained by supercritical CO_2 extraction

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ABSTRACT

An edible oil was obtained from guava seeds by supercritical CO_2 extraction. The oil was characterized by its fatty acid composition, physicochemical properties, and the contents of phenolic, miscellaneous, phytosterol, and tocopherol compounds. The oil, obtained with a yield of 8.6 ± 1.2 g oil/100 g guava seeds, had a high content of linoleic acid (78.5 %, *w/w*), followed by that of oleic acid (13.8 %, *w/w*). The guava seed oil had physicochemical properties comparable to those published in previous research, except for the low stability to oxidation. The chromatographic profile of the phenolic and miscellaneous compounds was dominated by vanillin (9.6 ± 0.3 mg/100 g oil) and cinnamaldehyde (9.4 ± 0.2 mg/100 g oil), followed by vanillic acid (3.9 ± 0.4 mg/100 g oil), cinnamic acid (2.4 ± 0.1 mg/100 g oil), and minor amounts of other phenolic aldehydes. Among the phytosterols and tocopherols, β-sitosterol (1048.9 ± 48.4 mg/100 g oil) and γ-tocopherol (82.6 ± 3.7 mg/100 g oil) were the most abundant. The low oxidative stability of the oil compared to that published in previous reports might reflect the high complexity of this matrix. This oil might have applications, directly or after blending with more stable edible oils.

1. Introduction

Guava (*Psidium guava*) is a perennial tree native to South America, and its leaves, bark, and roots are used in traditional medicine (Barbalho et al., 2012). The fruit has considerable amounts of vitamin C, carotenoids, and phenolic compounds (Medina and Herrero, 2016). The fruit is consumed fresh and used in the food industry to produce nectars, jams, jellies, and syrups. In the last ten years, Colombia has produced more than 80,000 tons/year of guava fruit with nearly 10,000 ha dedicated to this crop. As projected by the Colombian government, in the near future, it will be necessary to produce 200,000 tons/year of guava fruit because of guava jelly production (Agronet, 2019). During guava fruit processing, seeds are a by-product that account for up to 30 % (*w/w*) of the fruit weight (Iha et al., 2018), representing an environmental and phytosanitary problem. Guava seeds, however, have potential use as a source of edible oil and bioactive compounds (Da Silva and Jorge, 2014).

The oil extracted from guava seeds with organic solvents contains bioactive compounds, including phenolic compounds (e.g., 4.8 mg *p*-coumaric acid/100 g oil), phytosterols (e.g., up to 437.6 mg β-sitosterol/100 g oil), and tocopherols (e.g., up to 10.7 mg α-tocopherol/100 g oil) (Da Silva and Jorge, 2014; Malacrida and Jorge, 2013; Piombo et al., 2006). Furthermore, when the oil was extracted with either organic solvents or supercritical CO_2 (with ethanol as a co-solvent), polyunsaturated fatty acids were reported to account for at least 52 % (*w/w*) of the total fatty acid profile (Arain et al., 2017; Castro-Vargas et al., 2011; Cerón et al., 2016; Da Silva and Jorge, 2014; Habib, 1986; Iha et al., 2018; Malacrida and Jorge, 2013; Opute, 1978; Prasad and Azeemoddin, 1994). Concentrations of phenolic compounds, phytosterols, and tocopherols as well as those of polyunsaturated fatty acids in guava seed oil are higher or comparable to those found in other fruit seed oils, including those extracted from the seeds of the Isabela grape (*Vitis labrusca* L.), melon (*Cucumis melo* var. inodorus Naudin), passion fruit (*Passiflora edulis* Sims), pumpkin (*Cucurbita moschata*), soursop (*Annona muricata* L.), and tomato (*Solanum lycopersicum*) (Da Silva and Jorge, 2014).

Oil extraction by supercritical CO_2 is considered to be fast and efficient, with low or no use of organic solvents, yielding a product in

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which degradation of components, such as fatty acids and bioactive compounds, is lower compared to that of other extraction techniques (Sahena et al., 2009). Oil has been obtained from guava seeds by supercritical CO_2 extraction under different conditions of pressure and temperature to find the optimum extraction conditions, and its fatty acid profile was evaluated (Castro-Vargas et al., 2011; Cerón et al., 2016). Because supercritical fluid extraction has been shown to have an important effect on the type and concentration of bioactive compounds present in an extract compared to that obtained by organic solvents, e.g., the Soxhlet method (Mezzomo et al., 2010), it was hypothesized that a different composition might be obtained when extracting oil from guava seeds by supercritical CO_2 extraction. To the best of our knowledge, nevertheless, no information on the composition of bio-active compounds, such as phenolic compounds, phytosterols, and tocopherols, in guava seed oil obtained by supercritical CO_2 extraction is available. The aim of this research was, therefore, to characterize an edible oil obtained from guava seeds by supercritical CO_2 extraction on such features.

2. Materials and methods

2.1. Plant material

Guava seeds were obtained from three different batches from a food company located at the Department of Nariño, southwest Colombia, Andina de conservas LTDA. Once in the lab, guava seeds (12.0 kg) were washed with distilled water and dried in an oven at 50 °C for 48 h to yield 5.1 kg of dried seeds. Seeds were ground, sieved, and stored in hermetically sealed plastic bags at room temperature in a desiccator containing silica gel until extraction with supercritical CO_2. The ground and sieved guava seeds had a moisture content of 8.05 ± 0.52 %, an average particle diameter (dap) of 490.02 ± 1.2 μm, true (ρt) and apparent densities (ρa) of 1,020.27 ± 10.61 and 616.17 ± 8.64 kg/m^3, respectively, and a porosity (ε) of 0.3960. The seeds had a crude fat content of 9.1 ± 0.3 g oil/100 g seeds as evaluated by the Soxhlet method with *n*-hexane as the extraction solvent.

2.2. Reagents and standards

Ultra-high-performance liquid chromatography (UHPLC)/mass spectrometry (MS)-grade acetonitrile containing 0.1 % (*v/v*) formic acid and UHPLC/MS-grade water containing 0.1 % (*v/v*) formic acid were obtained from Biosolve BV (Valkenswaard, The Netherlands). CO_2 (99.8 %) was obtained from Oxígenos del sur LTDA (Pasto, Nariño, Colombia). Helium (99.99 %) was purchased from Linde (EKITEC, Pasto, Colombia). A fatty acid methyl ester standard was obtained from RESTEK (Benner Circle, Bellefonte, USA) and was a mixture of 37 components, from C4:0 to C24:1. Gallic acid, vanillin, 4-hydroxy-3-methoxycinnamaldehyde (coniferyl aldehyde), *trans*-3,5-dimethoxy-4-hydroxycinnamaldehyde (sinapaldehyde), *trans*-cinnamic acid, and *trans*-cinnamaldehyde were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Vanillic acid and 3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde) were obtained from Roth (Karlsruhe, Germany), and 4-hydroxybenzaldehyde was obtained from Alfa Aesar (Karlsruhe, Germany). Sodium carbonate, Folin–Ciocalteu reagent, and other solvents were purchased from Merck® (Darmstadt, Germany). Cholesterol was obtained from Sigma Aldrich (St. Louis, MO, United States).

2.3. Supercritical CO_2 extraction

A Waters SFE-500 (Milford, Connecticut, USA) equipped with a 500 mL extraction cell was used. The pressure and temperature of the system and CO_2 flow were controlled by *Process Suite* software (version 5.9, Milford, Connecticut, USA). Supercritical CO_2 extraction was performed on 250.0 g of ground and dried guava seeds at 52 °C and 35.7 MPa (density of CO_2: 895 kg/m^3) at a constant flow of 30 g CO_2/min for 150 min according to an optimization process extraction evaluated by Cerón et al. (2016). Extraction was performed in triplicate using three different samples. After supercritical CO_2 extraction, an oil was obtained (21.4 ± 0.3 g). The extracted oil was aliquoted into ~2 g aliquots in amber glass vials, flushed with N_2 gas, and stored at -20 °C until analyses were completed.

2.4. Fatty acid composition of guava seed oil

The fatty acid composition was evaluated after interesterification with methanol under acidic conditions, and analysis of the fatty acid methyl esters was conducted with gas chromatography (GC) with a flame ionization detector (FID) as described elsewhere (Hurtado-Benavides et al., 2016). Esterification with methanol was performed by incubating 200 μL of oil with 5 mL of 5% (*v/v*) HCl in methanol at 50 °C for 8 h. Next, the fatty acid methyl esters were extracted twice with 2 mL *n*-hexane. Then, the *n*-hexane extract was analysed by GC-FID. A Shimadzu GC 17A version 3 instrument (Shimadzu, Kyoto, Japan) equipped with a split/splitless injector, a DB-WAX column (30 m × 0.25 mm I.D., and 0.25 μm df, J&W Agilent Scientific, Folsom, CA, USA), and a FID was used. The *n*-hexane extract (1 μL) was injected in split mode (split ratio 1:10) at 250 °C. Helium was used as the carrier gas at 1 mL/min. Separation of compounds was performed by using an oven temperature programme. The temperature of the oven was held at 40 °C for 5 min and then increased to 250 °C at a rate of 5 °C/min. Next, the temperature was dropped to starting conditions and equilibrated for at least 5 min. The FID was operated at 280 °C with 300 mL/min hydrogen and 30 mL/min air. The system was controlled by using Shimadzu Class VP 4.3 software (Kyoto, Japon). The identification of fatty acid methyl esters was performed by comparing their retention indexes against those of authentic standards. Quantification was performed by calculating the relative chromatographic area.

2.5. Physicochemical properties of guava seed oil

The density at 20 °C (AOCS Cc 10c-95), refractive index at 25 °C (AOCS Cc 7–25), saponification number (AOAC 920.160), iodine value (AOAC 993.20), peroxide value (AOAC 965.33), and acid value (AOCS Cd 3d-63) were evaluated for the extracted oil (AOAC, 1995; AOCS and Firestone, 2009).

The oxidative stability of the oil was evaluated in a Metrohm Professional Rancimat model 892 (Herisau, Switzerland), controlled with StabNet 1.0 Full software (Herisau, Switzerland). The sample (3.0 g oil) was oxidized at 100 °C with an air flow rate of 20 L/min. After oxidation, the induction time was evaluated.

2.6. Extraction of phenolic compounds from the oil

A methanolic extract was obtained from the oil (Da Silva and Jorge, 2014) and used for both identification and quantitation of individual phenolic compounds and for the determination of the total phenolic content. Oil (400 mg) was extracted with 1.2 mL 100 % (*v/v* methanol by vortexing 1 min, 20 °C followed by shaking in a Thermomixer comfort shaker 1000 s^{-1}; 10 min; 20 °C) (Eppendorf AG, Hamburg, Germany). Next, the oil-methanol mixture was centrifuged (18,000 × g; 5 min; 20 °C), and the upper layer (methanolic layer) was collected and stored at 10 °C. The remaining oil was extracted as described above, four times in total. After four extractions, 95 % of the polyphenols were extracted, as measured at 290 and 310 nm. The four supernatants were pooled together to obtain a methanolic extract (nonconcentrated extract). This nonconcentrated extract was analysed for the identification and quantitation of individual phenolic compounds as well as for the total phenolic content. An aliquot of the nonconcentrated extract was concentrated to 10 % of the initial volume in a SpeedVac Savant ISS110 concentrator (Thermo Scientific, San Jose, CA, USA), which was used

for the identification and quantitation of individual phenolic compounds.

2.7. Identification of individual phenolic and miscellaneous compounds in the methanolic extract from guava seed oil by RP-UHPLC-DAD-HESI-MS/MS and RP-UHPLC-HESI-IT-FTMS

Tentative annotation of compounds was performed by two strategies: reversed phase (RP)-ultra-high-performance liquid chromatography (UHPLC) with diode array detection (DAD) and heated electrospray ionization (HESI) tandem mass spectrometry (MS/MS) detection, by using a linear triple quadrupole, and by RP-UHPLC-HESI-ion trap (IT)-Fourier transformed mass spectrometry (FTMS). Special attention was paid to the analysis of phenolic compounds.

2.7.1. RP-UHPLC-DAD-HESI-MS/MS

UV spectra, precursor ions, and product ions of chromatographic peaks were obtained. A Thermo Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, an autosampler, a degasser, and both in-line DAD and HESI-MS/MS detectors was used. Extracts (concentrated and nonconcentrated, 2 µL) were injected into an Acquity UPLC BEH C18 column (150 mm × 2.1 mm, particle size 1.7 µm, Waters, Milford, MA, USA) coupled to a precolumn (10 mm × 2 mm, 3 μm, Waters, Milford, MA, USA) operated at 45 °C and eluted by using a gradient of two eluents. Eluent A was water/acetonitrile/formic acid (99:0.9:0.1, *v/v/v*), and eluent B was acetonitrile/formic acid (99.9:0.1, v/v). The elution programme was isocratic at 5% B for 1.5 min, a linear gradient from 15 to 45 % B from 1.5 to 20 min, a linear gradient from 45 to 100 % B from 20 to 21 min, isocratic at 100 % B from 21 to 24 min, a linear gradient from 100 to 5% B from 24 to 25 min, and isocratic at the starting conditions (5% B) from 25 to 30 min. The flow rate was 400 µL/min. The eluate was directed into the DAD, and the UV–vis spectrum of each chromatographic peak was recorded at wavelengths between 200 and 700 nm. After passing through the DAD, the flow was directed into the HESI (Thermo Scientific, San Jose, CA, USA) probe coupled to a Thermo Scientific LTQ-XL (Thermo Scientific, San Jose, CA, USA) mass spectrometer, operated in both positive and negative modes over the *m/z* range 120–2,000. Data-dependent MS/MS analysis was performed using collision-induced dissociation with a normalized collision energy of 35 %. After tuning with vanillin, the following conditions were set: heater temperature, 250 °C; source voltage, 4.5 kV; ion transfer tube temperature, 350 °C; sheath and auxiliary gas flow (both N_2), 40 arbitrary units and 20 arbitrary units, respectively. Data were processed using Xcalibur 2.2 (Thermo Scientific, San Jose, CA, USA).

2.7.2. RP-UHPLC-HESI-IT-FTMS

Using this strategy, the exact molecular mass of each chromatographic peak was assessed. A Vanquish UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, an autosampler, a degasser, an eluent preheater, a post-column cooler, and HESI-IT-FTMS was used. The nonconcentrated methanolic extract was diluted 10 times with methanol and injected into the system. The injection volume, characteristics of the column, gradient, and flow rate were the same as those described above for the **RP-UHPLC-DAD-HESI-MS/MS** analysis. The eluent preheater and the post-column cooler were set at 45 and 40 °C, respectively. Half of the flow was directed into a Thermo Q Exactive Focus hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a HESI probe. Full MS data were recorded in both negative and positive ionization modes over a range of *m/z* 100–1,500 at a resolution of 70,000. The heater temperature was 280 °C, the source voltage was 2.8 kV, the ion transfer tube temperature was 320 °C, and the S-lens RF level was 50. N_2 was used as the sheath gas and auxiliary gas at flow rates of 30 and 20 arbitrary units, respectively. Data processing was performed using Xcalibur 2.2 (Thermo Scientific, San Jose, CA, USA).

2.8. Quantification of individual phenolic and miscellaneous compounds by RP-UHPLC-DAD

Identified compounds were quantified by the external standard method with authentic standards under the conditions described under subtitle **RP-UHPLC-DAD-HESI-MS/MS**. To increase sensitivity, the absorbance of the eluate was recorded at several wavelengths, corresponding to the absorption maxima of chromatographic peaks: 276, 284, 290, 292, 308, 340, and 345 nm. For phenolic compound quantification, calibration curves were generated at five concentrations for vanillic acid at 292 nm (from 0.1 to 5.0 µg/mL), 4-hydroxybenzaldehyde at 284 nm (from 0.3 to 5.0 µg/mL), vanillin at 308 nm (from 0.3 to 20.0 µg/mL), syringaldehyde at 308 nm (from 0.2 to 5.0 µg/mL), coniferyl aldehyde at 340 nm (from 0.1 to 20.0 µg/mL), and sinapyl aldehyde at 345 nm (from 0.1 to 10.0 µg/mL). For miscellaneous compound quantification, calibration curves were generated with cinnamic acid at 276 nm (from 0.1 to 20.0 µg/mL) and cinnamaldehyde at 290 nm (from 0.1 to 20.0 µg/mL). In all cases, the linearity of the calibration curves was verified not only by the calculation of the R^2 values but also by checking for any change in slope in the lower and upper limits. The limit of detection (LOD) was visually evaluated by lowering the concentration of each analyte and evaluating the minimum concentration in which the response was different from the noise. The limit of quantification (LOQ) was evaluated by finding the minimum concentration at which the peak area response and the concentration of each analyte showed linearity. Information on the calibration curve, LOD, and LOQ of each compound is given in Supplementary Table S1. The concentration of each compound was expressed as mg/100 g oil. Each measurement was replicated three times.

2.9. Determination of the total phenolic content of guava seed oil

The total phenolic content was measured as described by Waterhouse (2002). Nonconcentrated extract 20 µL was placed in a 2 mL Eppendorf plastic tube and mixed with 1580 µL of water and 100 µL of undiluted Folin-Ciocalteu reagent. The mixture was vortexed for 20 s and held at 20 °C for 8 min. After adding 300 µL of 20 % (*w/v*) aqueous Na_2CO_3, the mixture was vortexed for 20 s and left to stand in darkness for 2 h. The absorbance of the solution was recorded at 765 nm on a spectrophotometer (Genesys 10 UV, Thermo Electron Corporation, Madison, WI, USA). A calibration curve was generated by using a standard solution of gallic acid with concentrations ranging from 50 to 500 µg/mL (six data points, $R^2 = 0.999$). The total phenolic content was determined in triplicate and expressed as mg gallic acid equivalents/100 g oil.

2.10. Phytosterols and tocopherols of guava seed oil

An extract prepared in dichloromethane was obtained from the oil and used for the identification and quantitation of phytosterols and tocopherols by GC–MS (Matthaus and Ozcan, 2011. The oil (100 µL) was extracted twice with dichloromethane (1000 µL) and analysed by GC–MS. Because no saponification was performed towards the extract, only the free phytosterol and tocopherol fractions were determined. A Shimadzu QP2010S (Shimadzu Scientific Corporation, Columbia, MD, USA) equipped with a split/splitless injector, a SHXRI-5MS column (Shimadzu, 30 m × 0.25 mm × 0.25 µm, Columbia, MD, USA) and a mass detector QP2010S with an electron impact ionization source operated at 70 eV in full scan mode was used. The dichloromethane extract (1 µL) was injected in split mode (split ratio 1:10) at 270 °C. Helium was used as the carrier gas at 1 mL/min. The separation of compounds was performed by using an oven temperature programme. The temperature of the oven was held at 100 °C for 2 min and then increased to 300 °C at a rate of 10 °C/min for 20 min. Next, the temperature was maintained at 300 °C for 15 min before being returned to

starting conditions and equilibrated for at least 5 min. The system was controlled by using Shimadzu LabSolutions GC–MS Ver 2.7 software (Columbia, MD, USA). Tentative identification of phytosterols and tocopherols was performed by comparing both their retention indexes and their MS spectra in the NIST database. The thresholds of a deviation in the retention index lower than 3 % and a mass spectral match higher than 85 % were applied. Quantification was performed by the internal standard method with cholesterol as the internal standard. The concentration of each compound was expressed as mg/100 g oil. Each measurement was replicated three times.

2.11. Statistical analysis

The results are reported as the average of three replicates (n = 3) and the standard deviation (SD). The yields of the extract obtained by CO_2 supercritical fluid extraction and by the Soxhlet method were compared by a two-tailed Student's *t*-test ($p < 0.05$) in Microsoft Excel.

3. Results

3.1. Extraction yield, fatty acid composition, and physicochemical properties of guava seed oil

Oil from guava seeds extracted with supercritical CO_2 was obtained with a yield of 8.6 ± 1.2 g oil/100 g guava seeds (Table 1). This yield was similar (no significant different, $p > 0.05$) to that obtained when the extraction was performed by the Soxhlet method (9.1 ± 0.3 g oil/100 g guava seeds). Linolenic acid was the predominant fatty acid in the extracted guava seed oil, followed by oleic acid (Table 1). Polyunsaturated fatty acids accounted for 78.5 % (*w/w*), with saturated fatty acids representing only 13.8 % (*w/w*). The physicochemical features of the obtained oil are presented in Table 1.

3.2. Identification and quantification of phenolic and miscellaneous compounds in the methanolic extract of guava seed oil

After analysing the methanolic extract of guava seed oil, twelve chromatographic peaks were observed (Fig. 1). These chromatographic peaks accounted for 98 % of the total area as evaluated at 284 nm. The other chromatographic peaks represented less than 1 % of the total area. Among those twelve peaks, one compound was annotated as a phenolic acid, five as phenolic aldehydes, three as miscellaneous compounds, and three as unidentified (Fig. 1, Tables 2,3).

3.2.1. Identification of phenolic compounds

On the one hand, compound **2** was identified as a phenolic acid. On the other hand, compounds **3** and **5–8** were annotated as phenolic aldehydes. Chromatographic (retention time), spectroscopic (UV–vis), and spectrometric (precursor ions and product ions in both positive and negative modes) information is given in Table 2.

Compound **2** with the precursor ion $[M-H]^-$ at *m/z* 167 yielded a product ion $[M-CO_2\text{-}H]^-$ at *m/z* 123 as the base peak in MS^2 as well as a product ion at *m/z* 108, corresponding to $[M-CO_2-CH_3\text{-}H]^-$. Furthermore, fragmentation in positive mode of compound **2** gave the product ion $[M-CO_2+H]^+$ as the base peak.

Fragmentation in positive mode of the precursor ions of compounds **3** and **5–8** yielded product ions at *m/z* 95, 125, 155, 151, and 181, respectively, corresponding, in all cases, to $[M-CO+H]^+$. The neutral loss of 28 a.m.u. (CO moiety) was also observed in negative mode after fragmentation of the precursor ions of compounds **5** and **7** to produce *m/z* 123 and 149 $[M-CO\text{-}H]^-$. The neutral loss of 15 a.m.u. observed after fragmentation of precursor ions in negative mode of compounds **5**, **7**, and **8** was attributed to a CH_3 moiety. The product ion in the negative mode of compound **7** at *m/z* 134 was assumed to correspond to $[M-CO-CH_3\text{-}H]^-$. Additionally, a neutral loss of 30 a.m.u. was observed when precursor ions of compounds **6** and **8** were fragmented in negative mode was attributed to the ion $[M\text{-}2CH_3\text{-}H]^-$. This ion was produced in both cases in the MS^2 spectra as the base peak.

The mentioned fragmentation pattern, together with the relatively low molecular weight and UV–vis spectra of compound **2**, was indicative of a phenolic acid, while such features for compounds **3** and **5–8** allowed for the annotation of them as phenolic aldehydes (Table 2). Accordingly, compound **2** was annotated as vanillic acid. Likewise, compound **3** was annotated as 4-hydroxybenzaldehyde, compound **5** as vanillin, compound **6** as syringaldehyde, compound **7** as coniferylaldehyde, and compound **8** as sinapaldehyde. Further evaluation of the retention time, UV–vis spectra, precursor ion, and product ions of authentic standards (Table 2) together with accurate mass evaluation (Table 3) confirmed this annotation.

3.2.2. Quantification of phenolic compounds

Two of the identified phenolic compounds accounted for 81 % (*w/w*) of the total concentration (Table 4). Within the phenolic compound class, vanillin (9.6 ± 0.3 mg/100 g oil) was the most abundant, followed by vanillic acid (3.9 ± 0.4 mg/100 g oil). The total amount of individual phenolic compounds (16.7 ± 0.3 mg/100 g oil) (Table 4) was lower than that found with the Folin-Ciocalteu method (263.1 ± 18.7 mg gallic acid equivalents/100 g oil).

3.2.3. Identification and quantification of miscellaneous compounds

Although not belonging to the family of phenolic compounds, spectroscopic and spectrometric data of chromatographic peaks **10**-**12** (Tables 2 and 3) revealed that these compounds were abscisic acid, cinnamic acid, and cinnamaldehyde, respectively. When assessing the phenolic profile, the non-phenolic compounds cinnamaldehyde and cinnamic acid were found at concentrations comparable to those of vanillin and vanillic acid, respectively (Table 4).

3.3. Identification and quantification of phytosterols and tocopherols

Phytosterols and tocopherols were tentatively identified based on the evaluation of retention index and MS spectra coincident with information from the NIST database. Among the identified phytosterols, β-sitosterol appeared to be the most abundant phytosterol (1048.9 ± 48.4 mg/100 g oil), followed by campesterol (23.9 ± 1.4 mg/100 g oil), and neither stigmasterol or stigmastanol were present (Table 5). When determining the tocopherol composition of the oil, γ-tocopherol was found to be the most abundant compound of the class (82.6 ± 3.7 mg/100 g oil), followed by α-tocopherol (19.6 ± 1.5 mg/100 g oil) (Table 5).

4. Discussion

4.1. Extraction yield, fatty acid composition, and physicochemical properties

The extraction yields obtained by either supercritical CO_2 or Soxhlet extraction were within those found in previous research with different extraction techniques (Arain et al., 2017; Cerón et al., 2016; Da Silva and Jorge, 2014; Habib, 1986; Iha et al., 2018; Opute, 1978; Prasad and Azeemoddin, 1994) but lower than those reported for supercritical CO_2 extraction with ethanol as a co-solvent (Castro-Vargas et al., 2011) (Table 1). Consistent with the abundance of linoleic acid found in this study, other oils obtained with supercritical CO_2 or organic solvents contained linoleic acid (52–79.4 %, *w/w*) as the most abundant fatty acid, followed by comparable quantities of oleic acid (7.8–14.0 %, *w/w*), palmitic acid (6.6–14.8 %, *w/w*), and stearic acid (3.4–16 %, *w/w*), with low contributions of linolenic acid (Arain et al., 2017; Cerón et al., 2016; Da Silva and Jorge, 2014; Habib, 1986; Iha et al., 2018; Opute, 1978; Prasad and Azeemoddin, 1994). In contrast to our results, linoleic acid was present in a similar proportion to stearic acid when the oil was

Table 1
Strategy of extraction, yield, fatty acid composition, and physical-chemical properties of oil extracted from *Psidium guava* seed. Data from other authors is included for comparative purpose.

	Present work	Cerón et al. (2017)	Castro et al. (2011)	Arain et al. (2017)	Iha et al. (2018)	Prasad and Azeemoddin (1994)	Malacrida and Jorge (2013)	Habib (1986)	Opute (1978)	Da Silva and Jorge (2014)
Strategy of extraction	$SC\text{-}CO_2$[a]	$SC\text{-}CO_2$[b]	$SC\text{-}CO_2$-ethanol[c]	*n*-hexane[d]	*n*-hexane[d]	*n*-hexane[e]	Petroleum ether[f]	$CHCl_3$:MeOH (2:1, *v/v*)[g]	$CHCl_3$:MeOH (2:1, *v/v*)[g]	$CHCl_3$:MeOH:water (2:1:0.8, *v/v/v*)[h]
Yield (g oil/100 g seed)	8.6 ± 1.2	2.3–13.4	17.3–19.8	11.1	9	16.0	14.0	9.1	9.4	10.5
Fatty acid composition (% *mol/mol*)										
Palmitic acid, C16:0	10.6 ± 0.6	9.8–11.8	4.2–6.3	14.8	7	6.6	6.9	13.3	9.7	7.6
Stearic acid, C18:0	3.2 ± 0.5	2.6–3.7	41.2–40.0	9.1	16	4.6	4.6	11.1	3.4	3.9
Oleic acid, C18:1	7.7 ± 0.8	7.0–8.5	2.8–2.8	12.6	9	10.8	9.7	14.0	7.8	8.3
Linoleic acid, C18:2	78.5 ± 1.5	76.3–79.8	51.8–50.8	60.0	52	76.4	78.4	52.1	79.1	79.4
Linolenic acid, C18:3	–	–	–	–	< 1	0.1	–	–	–	–
Other fatty acids	–	–	–	3.5	16	1.5	0.3	9.5	–	0.3
Total saturated fatty acids	13.8	13.1–15.3	45.4–42.8	26.4	23	11.8	11.8	32.3	13.1	11.8
Total monounsaturated fatty acids	7.7	7.0–8.5	2.8–2.8	13.4	9	10.8	9.7	15.6	7.8	8.3
Total polyunsaturated fatty acids	78.5	76.3–79.8	51.8–50.8	60.2	52	76.5	78.4	52.1	79.1	79.4
Total insaturated fatty acids	86.2	84.8–86.8	51.8 - 50.8	73.6	61	87.3	88.1	67.7	86.9	87.7
Physical-chemical properties										
Density at 20 °C (g/mL)	0.92 ± 0.01	–	–	–	0.92	0.92[i]	–	0.92[k]	–	–
Refractive index at 25 °C	1.482 ± 0.003	–	–	–	–	1.477[j]	1.468 at 40 °C	1.476[k]	–	–
Saponification number (mg KOH/g)	188 ± 8 (201)	–	–	191 (201)	–	196 (198)	195 (200)	191 (208)	–	–
Iodine value (g I_2/100 g)	143 ± 6 (149)	–	–	121 (121)	–	134 (148)	143 (151)	124 (109)	–	–
Peroxide value (meq O_2/Kg)	5.6 ± 0.1	–	–	4.1	–	–	2.2	–	–	–
Acidity index (mg KOH/g)	0.8 ± 0.1	–	–	3.7	1.2	1.7	0.7	0.5	–	–
Oxidative stability at 100 °C, 20 L/h (h)	1.0 ± 0.1	–	–	–	–	–	5.9	–	–	11.4

Saponification numbers and peroxide values given in brackets are those calculated from the fatty acid composition as described by Kalayasiri et al. (1996).

[a] Extraction with supercritical CO_2 at 35.7 MPa and 52 °C. Each value is the mean of three replications with the standard deviation.

[b] Extraction with supercritical CO_2. Interval for each parameter corresponds to results from different conditions of pressure (17–38 MPa) and temperature (36–64 °C) (Cerón et al., 2016).

[c] Extraction with supercritical CO_2 at 30 MPa and 40 °C and ethanol as co-solvent. Values separated by "-" are those obtained when either a home-made- or a commercial-supercritical fluid extraction system were used for extraction (Castro et al., 2011).

[d] Extraction with *n*-hexane by Soxhlet (Arain et al., 2017; Iha et al., 2018). In Iha et al. (2018) data are given without decimals as reported by the authors.

[e] Extraction with *n*-hexane at room temperature (Prasad and Azeemoddin, 1994).

[f] Extraction with petroleum ether by Soxhlet (Malacrida and Jorge, 2013).

[g] Extraction with cold $CHCl_3$:MeOH (2:1, *v/v*) (Habib, 1986; Opute, 1978).

[h] Extraction with $CHCl_3$:MeOH:water (2:1:0.8, *v/v/v*) at room temperature (Da Silva and Jorge, 2014).

[i] At 30 °C.

[j] At 40 °C.

[k] At 26 °C.

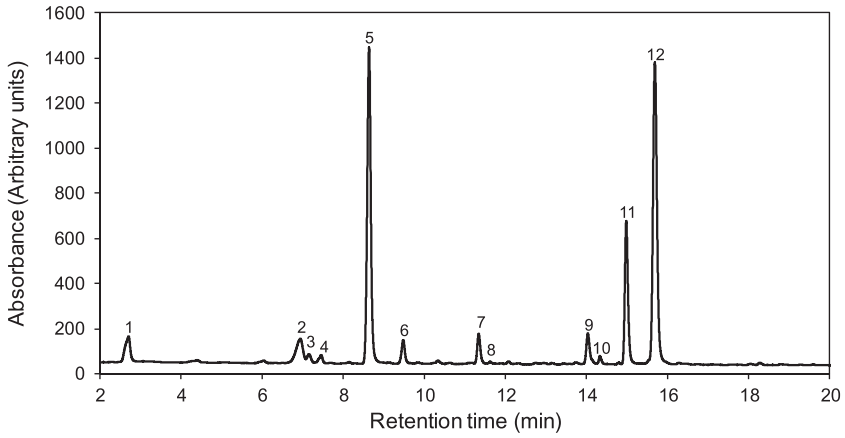

Fig. 1. RP-UHPLC chromatogram recorded at 284 nm of the guava seed oil, obtained by supercritical CO_2 extraction, after liquid-liquid partitioning with methanol. For given numbers of each chromatographic peak refer to either Table 2 or Table 3.

obtained by supercritical CO_2 extraction with ethanol as the co-solvent (Castro-Vargas et al., 2011). Despite the use of different extraction strategies, the density of guava seed oil obtained in the current research is similar to that reported in previous research, with refractive index and iodine values being higher than those reported previously (1.468–1.477, and 124–143 g I_2/100 g oil, respectively) (Table 1) (Arain et al., 2017; Iha et al., 2018; Habib, 1986; Malacrida and Jorge, 2013; Prasad and Azeemoddin, 1994). In agreement with our results, the saponification number and iodine value (Table 1) are similar to those previously reported (191.0 − 196 mg KOH/g oil and 121 − 143 g I_2/100 g, respectively) (Arain et al., 2017; Habib, 1986; Malacrida and Jorge, 2013; Prasad and Azeemoddin, 1994). The saponification number and iodine value of the obtained guava seed oil matched well with those calculated according to the fatty acid composition as described by Kalayasiri et al. (1996). The stability of lipids to the oxidation of guava seed oil as measured by the induction time by the Rancimat method was 5.9-fold lower than that reported when extracted by the Soxhlet method with petroleum ether (Malacrida and Jorge, 2013) and 11.4-fold lower than the value obtained when extracted with methanol:chloroform:water (2:1:0.8, *v/v/v*) (Da Silva and Jorge, 2014). Furthermore, the obtained oil complied with the peroxide value and acidity index (Table 1) recommended for virgin oils by Codex Alimentarius (1999), as these two parameters were lower than 15 meq O_2/kg oil and 4.0 mg KOH/g, respectively.

Differences observed in extraction yield, fatty acid composition, and physicochemical properties found between the current research and previous reports might be related not only to the marked differences in extraction strategies (as shown in Table 1) but also to the variety and agro-climatic conditions of cultivation. In this regard, oils obtained from 25 different varieties of pomegranate (*Punica granatum* L.) seeds had a 2.9-fold difference in yield and a large variability in fatty acid profiles. In this profile, linolenic acid was the most abundant fatty acid, followed by linoleic acid, exhibiting a 2.7-fold change and a 35-fold change, respectively (Fadavi et al., 2006). Agro-climatic conditions of cultivation might also have an impact on both the yield and relative abundance of fatty acids, as shown in oil extracted from chia seeds (*Salvia hispanica* L.); when cultivated in five agro-climatic conditions, there was a 1.2-fold change in yield and linoleic acid abundance, the most abundant fatty acid (Ayerza, 1995).

A differential effect on the type and concentration of individual phenolic compounds and the concentration of phytosterols and tocopherols in guava seed oil was observed when extracted by supercritical CO_2 extraction, which was used in the current research, compared to that involving organic solvents (Da Silva and Jorge, 2014; Malacrida and Jorge, 2013; Piombo, 2006). Despite higher concentrations of individual phenolic compounds, α-tocopherol, and γ-tocopherol were obtained in the oil extracted by supercritical CO_2 extraction than by organic solvents, a lower oxidation stability was observed (as evaluated by the Rancimat test). The lower stability of guava seed oil extracted with supercritical CO_2 might be related not only to the presence of pro-oxidants, such as chlorophylls and reducing compounds but also to the raw nature of such oil, where phenolic compounds can act as pro-oxidants depending on their concentration (Ospina et al., 2019).

Table 2
Identification of compounds present in a methanolic extract of guava seed oil obtained by supercritical CO_2 as determined by UHPLC-DAD-HESI-MS/MS.

Peak number	R_T (min)	Parent ion $[M-H]^-$ / $[M+H]^+$ (*m/z*)	MS^2 negative ion mode (*m/z*)[a]	MS^2 positive ion mode (*m/z*)[a]	UV λmax (nm)	Tentative annotation
1	2.71	–	–	–	284	Not identified
2	6.95	167/169	123, 108 (26)	125, 110 (2), 93 (22)	292	Vanillic acid[b]
3	7.16	121/123	121, 94 (4), 92 (2)	95 (31), 82	284	4-Hydroxybenzaldehyde[b]
4	7.46	–	–	–	276, 303	Not identified
5	8.64	151/153	151, 136 (2), 123(15), 107 (22)	125, 93(11)	280, 308	Vanillin[b]
6	9.48	181/183	181 (15), 151, 137 (19)	155,142 (4), 123 (22)	308	Syringaldehyde[b]
7	11.34	177/179	177, 162 (22), 149 (4), 134 (7), 106 (4)	151(4), 147,133 (26), 105 (7)	340	Coniferylaldehyde[b]
8	11.62	207/209	207 (26), 192 (2), 177, 163 (4)	181 (11), 177, 149 (19), 131 (11)	345	Sinapaldehyde[b]
9	14.03	–	–	–	282	Not identified
10	14.33	263/-	219 (54), 204 (10), 153	–	270	Abscisic acid
11	14.98	147/149	103, 147 (4)	149 (6), 131(12), 121(16), 103	276	Cinnamic acid[b]
12	15.68	-/133	–	134 (16), 105 (26), 91 (12), 55	289	Cinnamaldehyde[b]

[a] MS^2 base peak is shown in bold. Relative abundance is given in brackets.
[b] Retention time, parent ion, daughter ions (in negative and positive mode), and UV spectra matching those of authentic standards.

Table 3
Accurate mass data and empirical formula of compounds present in a methanolic extract of guava seed oil obtained by supercritical CO_2 as determined by UHPLC-ESI-IT-FTMS.

Peak number	Compound	Ionization	*m/z* observed	Molecular formula	*m/z* expected	Error (ppm)
1	Not identified					
2	Vanillic acid	$[M-H]^+$	169.0495	$C_8H_8O_4$	169.0501	−3.7
3	4-Hydroxybenzaldehyde	$[M-H]^-$	121.0296	$C_7H_6O_2$	121.0290	5.2
4	Not identified					
5	Vanillin	$[M-H]^-$	151.0400	$C_8H_8O_3$	151.0395	3.5
6	Syringaldehyde	$[M-H]^-$	181.0507	$C_9H_{10}O_4$	181.0501	3.3
7	Coniferylaldehyde	$[M-H]^-$	177.0557	$C_{10}H_{10}O_3$	177.0552	3.2
8	Sinapaldehyde	$[M+H]^+$	209.0807	$C_{11}H_{12}O_4$	209.0814	−3.2
9	Not identified					
10	Abscisic acid	$[M-H]^-$	263.1289	$C_{15}H_{20}O_4$	263.1283	2.3
11	Cinnamic acid	$[M+H]^+$	149.0597	$C_9H_8O_2$	149.0602	−3.6
12	Cinnamaldehyde	$[M+H]^+$	133.0648	C_9H_8O	133.0653	−4.3

Table 4
Quantification of individual compounds present in a methanolic extract of guava seed oil obtained by supercritical CO_2.

Compound	Content (mg/100 g oil)
Phenolic compounds	
Vanillic acid	3.9 ± 0.4
4-Hydroxybenzaldehyde	0.2 ± 0.0
Vanillin	9.6 ± 0.3
Syringaldehyde	1.2 ± 0.1
Coniferylaldehyde	1.5 ± 0.0
Sinapaldehyde	0.3 ± 0.0
Total amount of phenolics	16.7 ± 0.3
Miscellaneous compounds	
Cinnamic acid	2.4 ± 0.1
Cinnamaldehyde	9.4 ± 0.2

Each value is the mean of three replications with the standard deviation.

Table 5
Phytosterols and tocopherols present in oil extracted from *Psidium guava* seed (mg/100 g oil).

Compound	Present work[a]	Da Silva and Jorge (2014)[b]	Piombo et al. (2006)[c]	Malacrida and Jorge (2013)[d]
Phytosterols				
Campesterol	23.9 ± 1.4	–	9.9	–
β-sitosterol	1048.9 ± 48.4	437.6	traces	–
Stigmasterol	–	–	319.1	–
Stigmastanol	–	26.6	–	–
Tocopherols				
α-tocopherol	19.6 ± 1.5	4.6	10.7	7.1
ß-tocopherol	–	*	0.3	–
γ-tocopherol	82.6 ± 3.7	9.3	55.0	20.3
δ-tocopherol	–	*	0.5	–

[a] Extraction with supercritical CO_2 at 35.7 MPa and 52 °C. Each value is the mean of three replications with the standard deviation.
[b] Extraction with $CHCl_3$:MeOH:water (2:1:0.8, *v/v/v*) at room temperature (Da Silva and Jorge, 2014).
[c] Acelerated solvent extraction with petroleum ether at 10.0 MPa and 80 °C (Piombo et al., 2006).
[d] Extraction with petroleum ether by Soxhlet (Malacrida and Jorge, 2013).
* Authors report 0.3 mg ß-tocopherol + δ-tocopherol mg/100 g oil.

4.2. Diversity of phenolic compounds

The phenolic profile obtained in guava seed oil was found to be composed of phenolic aldehydes and a phenolic acid. Furthermore, chromatographic peaks **1**, **4**, and **9** had UVmax values that might correspond to phenolic compounds (Sakakibara et al., 2003); nevertheless, no further annotation could be done, as MS information could not be obtained. Composition of phenolic compounds found in the current work contrasts with the profile reported for other parts of the plant. These differences are evident in terms of the type and number of representatives. A total of 72 phenolic compounds were found in extracts prepared from guava leaves, including benzoic acids, flavonols, flavan-3-ols, flavanones, and proanthocyanidins (Díaz-de-Cerio et al., 2016). In the sub-class of benzoic acids reported in guava leaves, the only compound of this class that was found in the current research was gallic acid. Although less diverse in the number of compounds, in guava pulp, a total of 14 phenolic compounds were found, including anthocyanins, flavonoids, and proanthocyanidins (Flores et al., 2015). In the oil extracted from guava seeds, a simple phenolic profile composed of three compounds was reported (Da Silva and Jorge, 2014). That phenolic profile included salicylic acid, *p*-coumaric acid, and quercetin, which are representatives of the sub-classes of benzoic acids, hydroxycinnamic acids, and flavonoids, respectively.

The totally different phenolic profile found in this study compared to those described in guava leaves (Díaz-de-Cerio et al., 2016), guava pulp (Flores et al., 2015), and oil extracted from guava seeds (Da Silva and Jorge, 2014) might reflect differences in the extraction method, more so than differences in the analysed botanical parts, agro-climatic conditions of cultivation or variety. While guava leaves were extracted in an ultrasonic bath with ethanol:water (80:20, *v/v*) (Díaz-de-Cerio et al., 2016), extraction of guava pulp was performed with methanol:water:formic acid (70:25:5, *v:v:v*) at room temperature (Flores et al., 2015), and guava seeds were extracted by cold extraction with chloroform:methanol:water (2:1:0.8, *v/v/v*) (Da Silva and Jorge, 2014). The enormous influence of the nature of the solvents used for extraction on the phenolic composition (concentration and representatives) of different botanical parts of guava contrast with the similarity in the phenolic composition of different botanical parts of cherry plum (*Prunus* × *domestica* L.) (Khallouki et al., 2012).

The solubility of phenolic compounds depends on different thermodynamic parameters, with supercritical conditions playing an important role. Far below supercritical conditions, that is, near atmospheric pressure conditions, the enthalpy of fusion, melting temperature, and difference between the liquid and solid molar heat capacities of the solute are important thermodynamic features commanding the solubility of phenolic compounds (Queimada et al., 2009). In contrast, when above supercritical conditions, there are totally different parameters that are involved in the solubility of a compound. These parameters include critical temperature, critical pressure, molar volume, and acentric factor (Dadkhah et al., 2017). Therefore, differences in parameters governing the solubility of phenolic compounds might explain the differences observed in the chromatographic profile when oil was extracted by either organic solvents (Da Silva and Jorge, 2014) or by supercritical CO_2, as in the current research. In this regard, peach almond oil contained benzaldehyde (15 % of the total chromatographic area) when extraction was performed by the Soxhlet method with *n*-hexane:dichlorometane (1:1, *v:v*), while this compound was not

detected when extracted with supercritical CO_2 (either at 200 bar/40 °C, 300 bar/40 °C, or 300 bar/50 °C). Furthermore, a similar comparison shows that while 2,4-bis(1,1-dimethylethyl)-phenol was not detected when extraction was performed by the Soxhlet method or by supercritical CO_2 (either at 20.0 MPa/40 °C or 30.0 MPa/40 °C), it was found (0.4 % of the total chromatographic area) when extraction by supercritical CO_2 was conducted at 30.0 MPa/40 °C (Mezzomo et al., 2010). Vanillin and vanillic acid, relatively polar compounds found in guava seed oil extracted by supercritical CO_2, are soluble in supercritical CO_2, with increasing solubility as pressure and temperature are increased (Rojas-Ávila et al., 2016).

Interestingly, vanillin and cinnamaldehyde, found in comparable quantities in the extracted guava seed oil, have promising bio-activities. While vanillin improves insulin resistance in metabolic diseases, such as obesity, type 2 diabetes, and hyperlipidaemia (Park et al., 2011), cinnamaldehyde has hypoglycaemic and hypolipidaemic activities (Babu et al., 2007) as well as activity against the human gastric pathogen *Helicobacter pylori* (Ali et al., 2005).

The total amount of individual phenolic compounds (16.7 ± 0.3 mg/100 g oil) (Table 4) was higher than the value obtained after adding the individual contents of *p*-coumaric acid, salicylic acid, and quercetin (9.9 mg/100 g oil) together when extracted with organic solvents by cold extraction (Da Silva and Jorge, 2014). The total phenolic content in guava seed oil (263.1 ± 18.7 mg gallic acid equivalents/100 g oil) extracted by supercritical CO_2 was much higher than the values reported previously when the oil was obtained by cold extraction with chloroform:methanol:water (2:1:0.8 *v/v/v*) (27.2 ± 0.2 mg gallic acid equivalents/100 g oil) (Da Silva and Jorge, 2014) or with petroleum ether by Soxhlet (29.3 ± 0.3 mg gallic acid equivalents/100 g oil) (Malacrida and Jorge, 2013). The overestimation of phenolic compounds by the Folin-Ciocalteu method compared to the RP-UHPLC-DAD method has been reported previously in crude extracts and is related to the involved redox reaction itself. In such reactions, not only phenolic compounds but also other compounds with redox potentials high enough to reduce the Folin-Ciocalteu reagent, including reducing sugars, vitamin C, chlorophylls, amino acids, proteins, and carboxylic acids, are measured (Castro-Alves and Cordenunsi, 2015; Everette et al., 2010).

4.3. Diversity of phytosterols and tocopherols

In agreement with our results, β-sitosterol was the main phytosterol found in guava seed oil when extracted with chloroform:methanol:-water (2:1:0.8, *v/v/v*) at room temperature, but the concentration of this compound was 2.4-times higher in the current research than in the previous report (Da Silva and Jorge, 2014). In contrast to the results of this study, stigmasterol was the main phytosterol in oil extracted from guava seeds when extracted with petroleum ether by accelerated solvent extraction (Piombo et al., 2006). Furthermore, the high relative abundance of γ-tocopherol and α-tocopherol agrees with that reported in previous reports when guava seed oil was obtained with chloroform:methanol:water (2:1:0.8, *v/v/v*) (Da Silva and Jorge, 2014), petroleum ether by accelerated solvent extraction (Piombo et al., 2006) or petroleum ether by Soxhlet (Malacrida and Jorge, 2013).

The higher abundances of γ-tocopherol and β-sitosterol found in guava seed oil extracted by supercritical CO_2 than in guava seed oil obtained with organic solvents might be of interest from a health point of view. Beyond the vitamin E activity of tocopherols, γ-tocopherol has anti-inflammatory properties (Jiang and Ames, 2003), which might reduce the incidence of coronary heart disease (Lemcke-Norojärvi et al., 2001). Phytosterols, especially β-sitosterol, are known to have cholesterol-lowering effects as well as anti-inflammatory effects, which may offer protection against coronary artery disease (Othman and Moghadasian, 2011).

5. Conclusion

An edible oil obtained by supercritical CO_2 extraction was characterized and exhibited a unique phenolic and miscellaneous compound profile, a high proportion of β-sitosterol compared to that of the other phytosterols, a high contribution of γ-tocopherol to the tocopherol composition, a high contribution of linoleic acid to the fatty acid profile, and low stability to lipid oxidation. Within the phenolic and miscellaneous compound profile, vanillin and cinnamaldehyde were the most abundant, followed by vanillic acid and cinnamic acid. Overall, given the composition of bioactive compounds and the low oxidative stability, guava seed oil extracted by supercritical CO_2 might have applications in the food industry, directly or after blending with more stable edible oils, as a dressing for salads but not for cooking, e.g., frying.

CRediT authorship contribution statement

Carlos-Eduardo Narváez-Cuenca: Investigation, Writing - original draft, Writing - review & editing. **Mary-Lucía Inampues-Charfuelan:** Conceptualization, Investigation, Validation. **Andrés-Mauricio Hurtado-Benavides:** Conceptualization, Resources, Supervision, Funding acquisition. **Fabián Parada-Alfonso:** Conceptualization, Resources, Writing - review & editing. **Jean-Paul Vincken:** Conceptualization, Resources, Writing - review & editing.

Declaration of Competing Interest

None.

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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.2020.103467.

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