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Triacylglycerol Composition of Coffee Beans (*Coffea canephora* P.) by Reversed Phase High-Performance Liquid Chromatography and Positive Electrospray Tandem Mass Spectroscopy

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The triacylglycerol (TAG) composition of coffee beans (*Coffea canephora* P.) was determined by reversed phase liquid chromatography−mass spectrometry and tandem mass spectrometry. The TAGs were separated on a Microsorb RP C-18 column in series with a Supelcosil RP C-18 column using isocratic elution with acetonitrile/2-propanol/hexane (v/v/v, 57:38:5) as the mobile phase at a flow rate of 1 mL/min for 100 min. Under these conditions, 13 TAGs were identified (elution order): trilinoleyl-glycerol (LLL, 11.76%), dilinolenoyl-palmitoyl-glycerol (PLnLn, 2.94%), dilinoleyl-oleyl-glycerol (OLL, 7.77%), dilinoleyl-palmitoyl-glycerol (PLL, 25.90%), dipalmitoyl-linolenoyl-glycerol (PPLn, 1.66%), dioleoyl-linoleyl-glycerol (OOL, 1.68%), dilinoleyl-stearyl-glycerol (SLL, 8.28%), palmitoyl-oleyl-linoleyl-glycerol (POL, 8.76%), dipalmitoyl-linoleyl-glycerol (PPL, 13.74%), dilinoleyl-arachidyl-glycerol (ALL, 3.51%), trioleoyl-glycerol (OOO, 2.33%), palmitoyl -linoleyl-stearyl -glycerol (PLS, 8.73%), and distearoyl-linolenonyl-glycerol (SSLn, 2.91%). The relative composition (%) was obtained directly from the data system with the photodiode array detector.

KEYWORDS: ***Coffea canephora* P.; coffee beans; triacylglycerols; LC-MS; LC-MS/MS; coffee oil; lipids**

INTRODUCTION

The two most important coffee species are *Coffea arabica* L. and *Coffea canephora* P., the former accounting for three-quarters of the world production while the latter represents about one-quarter (*1*). Coffee quality is an important parameter, which determines largely its commercial value, and a number of factors such as poor harvesting and postharvesting practices, fungal contamination, and presence of undesired compounds (*2*) have been speculated to be involved in lowering coffee quality. Information on *C. arabica* L. coffee lipids is very limited (*3*−*5*) but suggests that the poor quality of coffee is also due to the hydrolysis of triacylglycerols (TAGs) with release of free fatty acids, which, in turn, are oxidized (*6*−*8*). However, there is a lack of information on the TAG composition of *C. canephora* in the literature.

As a part of a coffee-breeding program aiming to study the role of lipids on coffee quality, methods such as Ag-TLC (thin-layer chromatography) and RP-TLC (reversed phase TLC) were used to identify TAG molecular species of coffee (*C. arabica* L.) beans (*3*). However, those techniques, while giving reliable results, were found to be quite tedious and therefore not suitable for the analysis of a large number of coffee samples, as frequently necessary in many research/quality control programs. Reversed phase high-performance liquid chromatography (RP-HPLC) methods were developed for use with light scattering (LSD) (*9*) and refractive index (RI) detectors (*10*), but using those techniques, identification was based on literature comparison and some TAG could not be separated under the conditions used. Although TAG analysis using "universal" detectors such as LSD and RI give good responses, the use of many TAG standards is mandatory. In addition, the coelution can make difficult the identification of the peaks in a chromatogram.

Another problem to be faced by analytical techniques is the TAG structure determination. Generally, the detailed compositional analysis of natural oils and fats, which can be a complex mixture of TAGs, is difficult since the molecular species have very similar chemical and physical properties. In a TAG with a molecular weight of 850−900, the presence of even one double bond can make a strong difference as each double bond in a TAG structure reduces its retention time so much that its

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value becomes comparable to the retention time of a TAG lacking two carbons (*11*). Because the presence of TAG isomers is common in lipids, the use of two or three columns in series and the use of columns with silver ions bounded onto the stationary phase (*12*−*15*) are commonly used to achieve a complete separation of all TAG species.

The use of modern hyphenated techniques such as liquid chromatography coupled to mass spectroscopy (LC-MS) can also overcome the difficulties in identifying some TAGs (*16*, *17*), but the use of tandem mass spectroscopy (MS/MS) is mandatory to completely elucidate the TAG isomers. For instance, LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry) was used to distinguish between the isomer pairs capryl-lauryl-myristoyl-glycerol (CaLaM) and caprilyl-lauryl-palmitoyl-glycerol (CyLaP) in ouricuri (*Syagrus coronata*) oil (*18*). Fatty acid residues of TAGs present in ouricuri oil were identified by tandem mass spectra of the corresponding sodium adducts of molecular ions $[M + Na]^+$. Even when a complete separation of TAG is not achieved, tandem mass spectrometry can be a useful tool to identify the TAG species. For example, LC-MS/MS was successfully used to identify the TAG isomer pair POO and PLS, which coeluted in the Malaysian cocoa butter by analyzing the diacylglycerols fragmented ions (*19*).

The TAG adduct ions can be very informative; therefore, the use of different doping agents allows a complete structural characterization. Hsu and Turk (*20*) studied the TAG species obtained by electrospray ionization tandem mass spectroscopy via lithiated adducts with low energy collisional activated dissociation (CAD). The relative abundance of $[M + Li - RCOOH]^+$, $[M + Li - RCOOLi]^+$, and RCO^+ ions reflected the position of each fatty acid on the glycerol backbone. Cheng and Gross (*21*) studying the fragment ions of ammoniated adduct ions $[M + NH_4]^+$ produced by ESI and fragment ions of sodiated adduct ions $[M + Na]^+$ fast atom bombardment (FAB)-produced could completely elucidate the TAG structure by eight ions formed through the fragmented ions (FAB-produced $[M + Na]^+$). Hvattum (*22*) identified the position of the acyl chain through the fragments of $[M + NH_4]^+$ ions. The energetically less favorable neutral loss of the *sn*-2 fatty acid from $[M + NH_4]^+$ precursor ion allows determination of the exact position of each fatty acid on the glycerol backbone.

Given the great potential of LC-MS and LC-MS/MS in TAG analysis and the lack of information on the TAG composition of the commercially important coffee variety *C. canephora* P., we have determined the TAG composition of this species by liquid chromatography and positive ion electrospray tandem mass spectrometry (LC-ESI-MS/MS).

EXPERIMENTAL SECTION

Reagents and Solvents. All reagents and solvents were of analytical or chromatographic grade. Acetonitrile, 2-propanol, and hexane were purchased from Fisher (Fair Lawn, NJ) and were used without further purification. Formic acid was acquired from Aldrich Chemical (Milwaukee, WI).

Coffee Sample. The details of obtaining coffee sample have been previously described elsewhere (*23*).

Coffee Oil. The coffee oil was obtained after grinding the cherry coffee beans on a food processor and extracting them with hexane for 6 h in a Soxhlet extractor. The hexane layer was dried using anhydrous sodium sulfate and paper filtered, the solvent was removed using the rotatory evaporator under reduced pressure at 35 °C, and the crude oil was stored in a freezer. The crude oil was analyzed without the previous purification by dissolving in the mobile phase (10 mg/mL).

Fatty Acid Analysis by Gas Chromatography. Fatty acid methyl esters (FAMEs) were prepared according to Christie (*24*). A Shimadzu (model 17A) gas chromatograph fitted with a flame ionization detector autosampler (model AOC-20) and a workstation CBM-101 (Tokyo, Japan) were used with a 30 m × 0.25 mm (i.d.) fused silica column coated with the DB-1 stationary phase (Supelco, Bellefonte, PA) to identify the FAMEs. The column temperature was programmed from 65 °C (held for 3 min) to 260 °C at 4 °C min^{-1} and held at this temperature for 20 min; injector and detector temperatures were 260 °C.

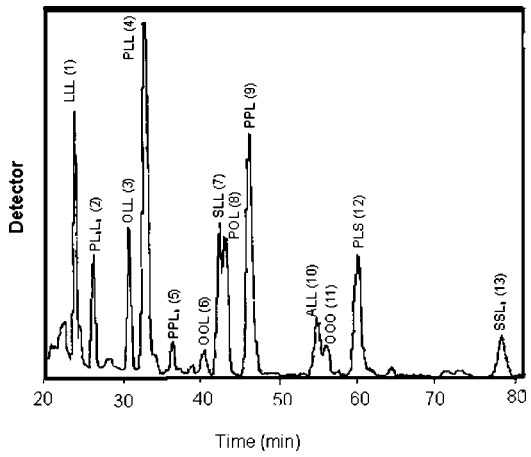

Figure 1. Typical HPLC trace obtained with a crude hexane extract of a cherry bean coffee sample with PDA detector. Peaks 1–13 correspond to TAGs LLL, PLnLn, OLL, PLL, PPLn, OOL, SLL, POL, PPL, ALL, OOO, PSL, and SSLn, respectively.

Separation of TAGs Using HPLC. The HPLC system used for the TAG separations was a Surveyor LC pump (San Jose, CA) and autosampler from Thermo-Finningan (San Jose, CA) combined with a photodiode array (PDA) detector (San Jose, CA) and an ion trap mass spectrometer detector (San Jose, CA) in series. The TAGs were separated with a Microsorb RP C-18 column (length = 15 cm, internal diameter = 4.6 mm, and particle size = 5 μm; Rainin Inc., Oakland, CA) and a Supelcosil RP C-18 (length = 25 cm, internal diameter = 4.6 mm, and particle size = 5 μm; Supelco Inc.) column in series. Samples (20 μL) were injected via an autosampler and a PDA detector and set to scan between 200 and 600 nm, and the effluent that was led to the PDA detector and to the mass spectrometer simultaneously was monitored. Acetonitrile/2-propanol/hexane (v/v/v, 57:38:5) (*18*) was the mobile phase for the isocratic TAG separation at a flow rate of 1 mL/min for 100 min.

Analysis of TAG by LC-ESI-MS/MS. The TAG standards and the coffee oil sample were separated with the LC system previously described. The columns were connected to a Thermo-Finningan LCQ Deca XP mass spectrometer equipped with an atmospheric pressure ion source to sample positive ions from the electrospray interface. Formic acid (0.5%) in acetonitrile/water (1:1) was added postcolumn at 5 μL/min to improve the ionization. The whole column effluent (1 mL/min) was directed to the mass spectrometer. Data acquisition and processing were performed using a Xcalibur NT 1.2 data system (Woburn, MA). The ion-source parameters were optimized with respect to the positive molecular ion of TAG, and the cone voltage was set to 50 eV. Nitrogen was used both as a sheath gas and as an auxiliary gas at a flow rate (instrument settings) of 60.0 and 20.0 (arbitrary units), respectively. The mass spectra, between 300 and 1200 amu, were obtained in normal mode with an ion scan rate of 5500 amu/s. Helium was used as a collision gas for collision-induced dissociation (CID), and the optimized relative collision energy (RCE) was set at 45% for MS^2. The instrument was tuned with tricaprin acquired from Sigma-Aldrich (St. Louis, MO) and triolein (Nu-Check-Prep. Inc., Elysian, MN). Calibration was done with a mixture of caffeine, MRFA (a short, four amino acid peptide), and Ultramark 1621 (perfluoroalkylphosphazine) solutions (Sigma-Aldrich). The percentages of each TAG separated by HPLC were based on the detector's area response.

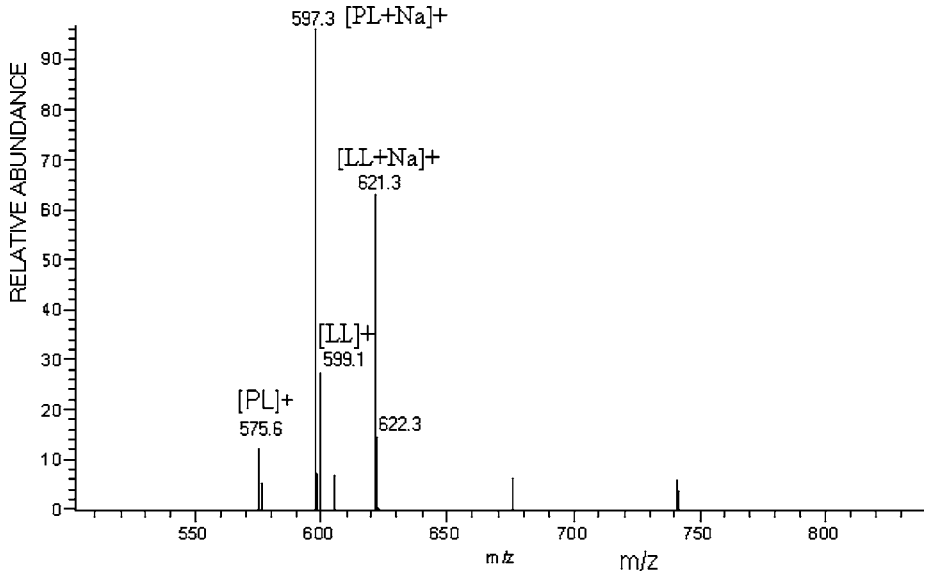

Figure 2. Tandem mass spectrum of sodium adduct ion $[M + 23]^+$ of TAG PLL showing the fragmented ions corresponding to the losses of neutral fatty acids and fatty acids sodium salts (L and P).

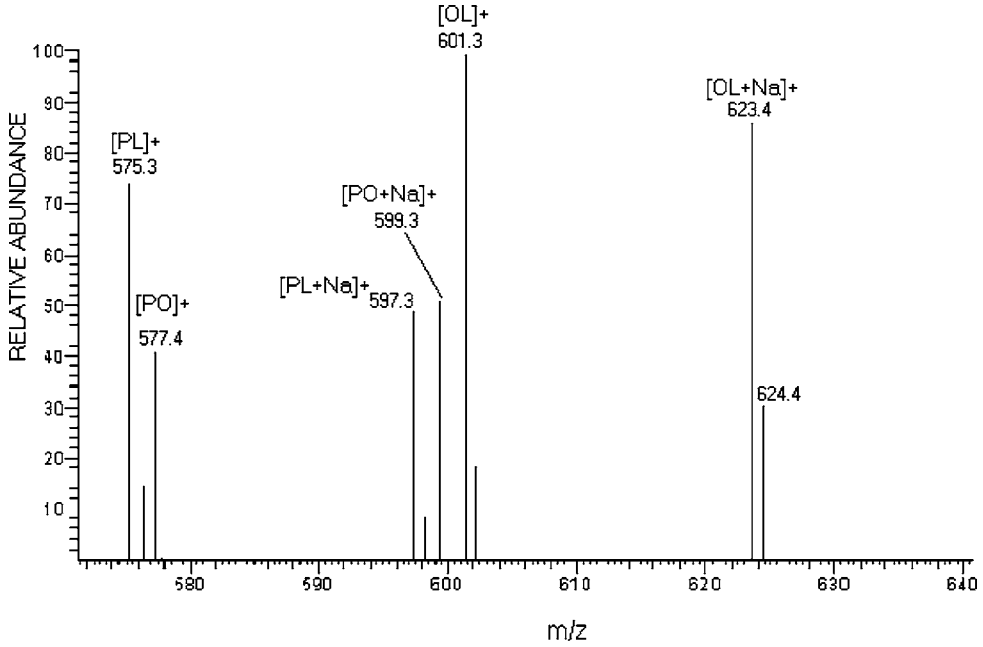

Figure 3. Tandem mass spectrum of sodium adduct ion $[M + 23]^+$ of TAG POL showing the fragmented ions corresponding to the losses of neutral fatty acids and the sodium adduct ions (L, O, and P).

RESULTS AND DISCUSSION

Fatty Acid Composition. Ten fatty acids, typical of coffee bean lipids, were identified on the *Coffea canephora* P. oil. Palmitic (16:0) and linoleic acid (cis 9,12-18:2) dominate by far (32.32 and 43.64%, respectively). The total lipids contain moderate and almost equal quantities of stearic (18:0) and oleic (cis 9-18:1) acids (10.33 and 11.50%, respectively), while myristic (14:0), palmitoleic (16:1), arahidic (20:0), and behenic (22:0) acids are present in less than 1% each. The sample also contains ca. 1% of linolenic acid (18:3).

Separation of TAG Using HPLC. To avoid the recurring coelution problem, two columns, therefore, a higher number of theoretical plates, were used in series to optimize the TAG separation. In addition, the flow rate was set to 1 mL/min and the solvent system acetonitrile/2-propanol/hexane (v/v/v, 57:38:5) was used, as it showed good results previously (*18*). In fact, a satisfactory separation was obtained (**Figure 1**), despite isocratic elution.

Analysis of TAG Using HPLC-MS/MS. HPLC-MS/MS revealed, for the majority of peaks that were evaluated, that the sodium adduct of molecular ion $[M + 23]^+$ was much more intense than protonated molecular ion $[M + 1]^+$, except for TAG PLnLn where the formation of $[M + 18]^+$ ion was observed. The $[M + 18]^+$, $[M + 23]^+$, $[M + 39]^+$, and $[M + 7]^+$, representing cationized ions bearing water, Na, K, Li, etc. have been described in the literature for lipid analysis (*25*−*29*).

The first peak (11.76% of total TAGs, **Figure 1**) was promptly identified as the TAG LLL based on the ion $[M + 23]^+$ with *m*/*z* at 901.8. The second peak (2.94% of total TAGs, **Figure 1**) was identified as the TAG PLnLn by tandem mass spectrometry. Formation of the corresponding $[M + 18]^+$ with *m*/*z* at 870 was observed. When tandem mass spectrometry was applied, two fragment ions were observed as follows: the base

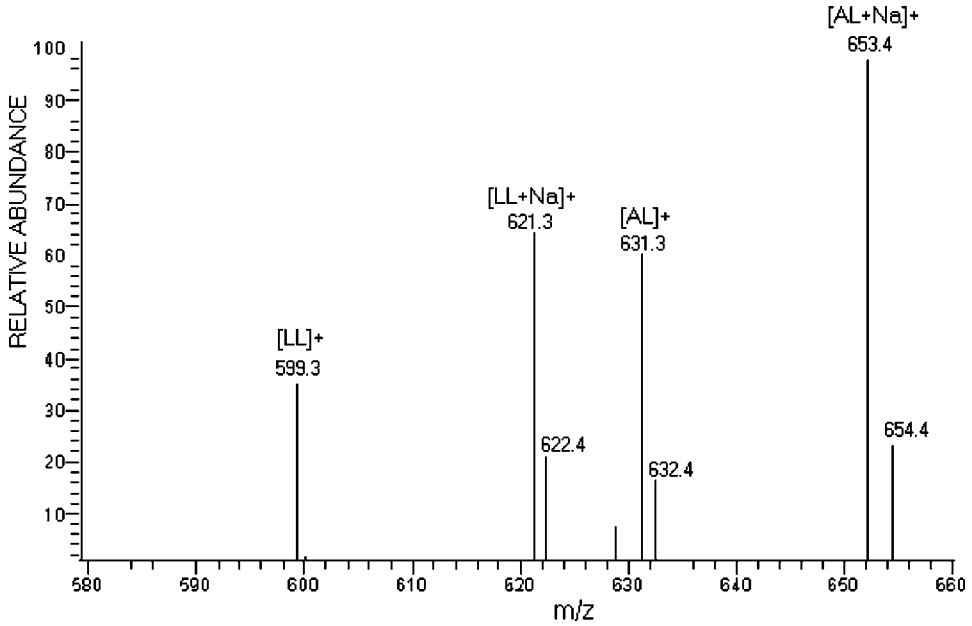

Figure 4. Tandem mass spectrum of sodium adduct ion $[M + 23]^+$ of TAG ALL showing the fragmented ions corresponding to the losses of neutral fatty acids and fatty acids sodium salts (A and L).

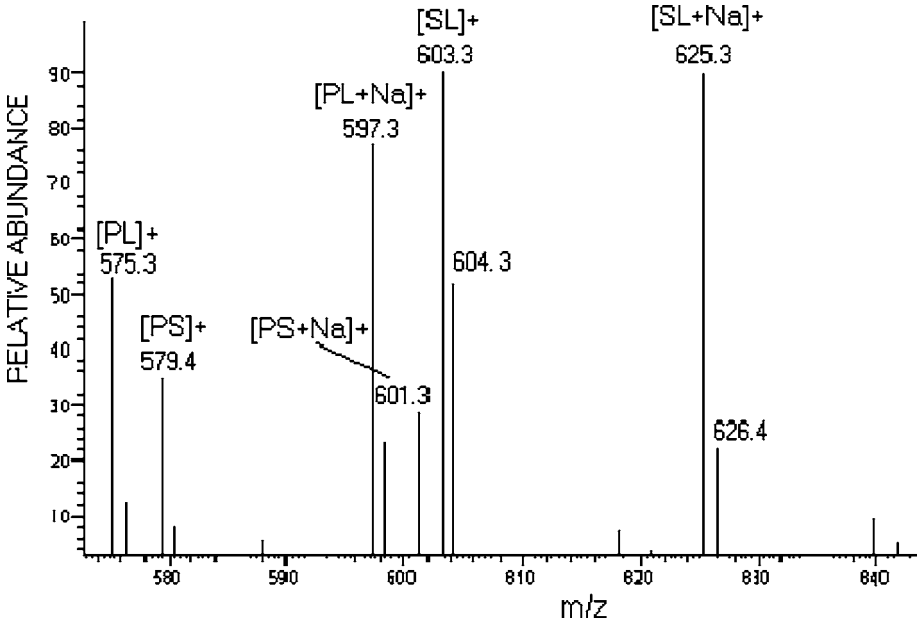

Figure 5. Tandem mass spectrum sodium adduct ion $[M + 23]^+$ of TAG PSL showing the fragmented ions corresponding to the losses of neutral fatty acids and fatty acids sodium salts (L, S, and P).

peak, formed by the neutral loss of palmitic acid (256.2 u) originating from the diacylglycerol adduct ion $[LnLn + 18]^+$ (*m*/*z* 613.3) and, upon neutral loss of linolenic acid fragment (278.2 u), the diacylglycerol adduct ion $[LnP + 18]^+$ (*m*/*z* 591.5), although it was formed with very low intensity (1.44%).

The third peak (7.77% of total TAGs, **Figure 1**) was identified as OLL due to the ion $[M + 23]^+$ (*m*/*z* 903.8). Peak four (25.90% of total TAGs, **Figure 1**), the most abundant TAG, was identified as PLL. The $[M + Na]^+$ of PLL (*m*/*z* 877.8) when analyzed by MS/MS presented two patterns of fragmentation produced by the neutral loss of fatty acid $[M + Na - RCOOH]^+$ and the neutral loss of sodium salt of fatty acid $[M + Na - RCOONa]^+$. The TAG PLL, when submitted to CID energy, produced four diacylglycerols fragmented ions. **Figure 2** shows the tandem mass spectrum of $[M + 23]^+$ ion. The fragmented ions with *m*/*z* at 621.3 and *m*/*z* 597.3 correspond to the sodium adduct of diacylglycerol ions $[LL + Na]^+$ and $[PL + Na]^+$ that were formed by the neutral loss $[M + Na - RCOOH]^+$ of palmitic (256.2 u) and linoleic acids (280.2 u), respectively. The other two less intense fragmented ions with *m*/*z* at 575.6 and 599.1 correspond to the diacylglycerol ions $[PL]^+$ and $[LL]^+$, respectively. The former was originated by the neutral loss of sodium salt of linoleic acid $[M + Na - RCOONa]^+$ and the latter by the neutral loss of sodium salt of palmitic acid, respectively.

The fifth peak (1.66% of the total TAGs, **Figure 1**) presented the ion $[M + 23]$ (*m*/*z* 851.9) and was identified as the TAG PPLn. Peaks six and seven (1.68 and 8.28%, respectively, of the total TAGs, **Figure 1**) both presented *m*/*z* at 905.8. The use of two columns was probably responsible for the separation of this isomers pair. To confirm which isomer eluted first, fragmentation by CID of these ions present in each peak was studied. The results indicated that the peak six corresponded to TAG OOL and peak seven to TAG SLL. Peak six presented a

base peak with *m*/*z* at 623.2, characteristic of a diacylglycerol sodium adduct $[OL + Na]^+$ originated by the neutral loss of oleic acid (282.2 u). A peak observed at *m*/*z* 625.2 corresponds to the ion $[OO + Na]^+$, formed by neutral loss of linoleic acid (280.2 u). The other ions present in the mass spectrum (*m*/*z* 601.4 and 603.4) were formed by the neutral losses of the sodium salt of oleic and linoleic acids, respectively. The tandem mass spectrum of the next peak, an SLL isomer, presented the fragmented ions with *m*/*z* at 621.5 (base peak) and 625.6 corresponding to the neutral losses of stearic acid (284.3 u) and linoleic acid (280.2 u), respectively.

Peak eight (8.76% of the total TAGs, **Figure 1**) presented *m*/*z* at 879.8 was identified as POL according to its $[M + Na]^+$ fragment. **Figure 3** shows the tandem mass spectrum of TAG POL. As expected, the presence of three different fatty acids in the TAG POL led to six fragmented ions corresponding to the neutral losses of the three distinct fatty acids and their respective sodium salts.

Peak nine (13.74% of the total TAGs, **Figure 1**) presented *m*/*z* at 853.7 and corresponds to the $[M + Na]^+$ fragment of TAG PPL. The MS^2 spectrum confirmed the presence of fatty acids palmitic and linoleic, as observed by the presence of dilinoleylsodium adduct ion $[LL + Na]^+$ (*m*/*z* 597.3) and dipalmitoylsodium adduct ion $[PP]^+$ (*m*/*z* 573.3), respectively.

Peak 10 (3.51% of the total TAGs, **Figure 1**) was identified as ALL (910.5 u) due to its sodium adduct peak $[M + Na]^+$, *m*/*z* 933.8 (**Figure 4**). By analysis of the corresponding tandem mass spectrum, it was possible to identify fragments corresponding to the losses of arachidic and linoleic acids and their respective sodium salts.

Peak eleven (2.33% of the total TAGs, **Figure 1**) was identified as OOO based on *m*/*z* at 907.8 $[M + Na]^+$. Peak twelve (8.73% of the total TAGs, **Figure 1**) was identified using tandem mass spectrum as TAG PSL. **Figure 5** shows the tandem mass spectroscopy of PSL $[M + Na]^+$ peak (*m*/*z* 881.7). Fragments of ions with *m*/*z* 625.3 (base peak) and 603.3 (second most stable ion) were formed by the neutral loss $[M + Na - RCOOH]^+$ of palmitic acid and its sodium salt $[M + Na - RCOONa]^+$, respectively. The other two ions corresponding to $[M + Na - RCOOH]^+$ and $[M + Na - RCOONa]^+$ fragments of linoleic acid were also observed (*m*/*z* 601.3 and 579.4, respectively). Fragments of ions corresponding to the neutral loss of stearic acid and its sodium salt were identified as ions with *m*/*z* at 597.3 $[M + Na - RCOOH]^+$ and *m*/*z* 575.3 $[M + Na - RCOONa]^+$, respectively. Finally, peak 13 (2.91% of the total TAGs, **Figure 1**) was identified as the TAG SSLn presented fragmentations of linolenic (278.2 u) and stearic acid (284.3 u), respectively.

The HPLC conditions used in the present work furnished a good separation allowing the identification of isomers OOL and SLL. Comparing the TAG composition here reported for *C. canephora* P. with data reported for *C. arabica* (*9*), it is possible to observe that TAGs LLL, OLL, PLL, OOL, SLL, POL, PPL, ALL, and PSL were present in both species. TAGs PPLn, OOO, and SSLn were only detected in *C. canephora* P. oil. POO, PSL, PPO, and PSO, detected in *C. arabica* oil, were not identified in *C. canephora* P. oil. SLLn, reported as a constituent of *C. arabica* oil, may have been incorrectly assigned in the literature (*9*). In general, the TAG qualitative composition of coffee varieties is similar but the relative percentage of the TAGs in distinct varieties can vary. In this case, LC-MS/MS was shown to be an efficient analytical technique for oil analysis especially for food lipids, where precise data are necessary in order to evaluate the functional properties.

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