Ouricuri (*Syagrus coronata*) Triacylglycerol Analysis Using HPLC and Positive Ion Electrospray Tandem MS

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ABSTRACT: Among tropical oil sources in Brazil, the ouricuri (*Syagrus coronata*) tree is nearly ubiquitous. The exact TAG composition of oil from ouricuri fruits was elucidated with electrospray ionization/tandem MS. The oil contains the following FA: caprylic (Cy), capric (Ca), lauric (La), myristic (M), palmitic, stearic, oleic, and linoleic. An estimate of the relative TAG composition based on the peak areas using an ELSD indicated that the four primary TAG—CyLaLa, CaLaLa, CaLaM, and LaLaM—corresponded to 24.3, 19.8, 13.6, and 10.9%, respectively, of the total TAG content.

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The ouricuri plant (*Syagrus coronata)* is a tree commonly found among cerrado, or dry, bushy vegetation in many parts of Brazil. In some small communities, especially in the northeast of the country, the ouricuri fruit is exploited extensively as an oil source. The oil has been used for frying, cooking, and soap production.

Generally, the detailed compositional analysis of natural oils and fats, which can be a complex mixture of TAG, is a difficult task. The TAG can have different chiral configurations, and most of the TAG molecular species have very similar chemical and physical properties. A complete separation of all of the TAG species present in an oil sample is often a difficult task, demanding careful adjustment of the chromatographic conditions to obtain a good separation.

During the past decade several studies using equivalent carbon number (ECN) (1) were used to determine the composition of TAG (2), as well as the products from various enzymatic reactions involving TAG (3). However, some pairs of similar TAG, such as LLL and LnOL (where L = linoleic, Ln = linolenic, and O = oleic), contain the same number of double bonds and generally co-elute. Therefore, they cannot be differentiated with only this technique (4). Complementary analysis becomes necessary to identify the TAG composition unequivocally, especially when relatively unknown oils are the object of the investigation.

In the past, GC–MS analysis was an alternative, which included the isolation of each TAG peak, followed by oil hydrolysis, FA methylation, and analysis by GC–MS. This approach provides excellent compositional information, although it is very time consuming.

There have been important recent technical advances in chromatography as a result of the introduction of new columns, detectors, mass spectrometer systems, and ionization techniques that have improved the efficiency of separation and the investigators' ability to elucidate compound structures. For instance, in GC–MS the use of new high-temperature capillary columns that contain a phenyl methyl silicone stationary phase stable up to 370°C has been reported (5). New studies were reported based on this technique (6), although a cold on-column injection technique was used to reduce the substantial losses that can occur with hot injection due to thermal decomposition.

A variety of methods and ionization techniques are currently available to determine the exact TAG composition of an oil, such as FABMS (7), CI (8), desorption CI (DCI), tandem MS (9), LC–atmospheric pressure–CI–MS (LC-APCI-MS) (10–12), silver-ion LC/APCI-MS (13), electrospray ionization (ESI) MS (14), and electrospray tandem MS (15,16). Although the compatibility of APCI with a nonpolar chromatographic solvent system allows the analysis of an expanded range of molecules as compared to ESI, the identification of relatively saturated TAG may be difficult, because APCI does not yield any (or only small amounts) of the $[M + H]^+$ ion (13). However, the TAG fragmentation pattern obtained does allow identification by DAG fragments. Electrospray is an extremely soft means of forming gas-phase ions, avoiding fragmentation; therefore, molecular adduct ions such as $[M + 23]^+$ are obtained without interference from fragment ions (15).

To our knowledge there is no published report on the TAG and FA composition of ouricuri oil. Only one study of the triterpene contents of ouricuri wax was found in the literature (17). The objective of this research was to elucidate the exact TAG composition of ouricuri oil using ESI and tandem MS. The latter was used to complete the study on the FA composition of ouricuri oil, after TAG separation by HPLC.

EXPERIMENTAL PROCEDURES

Reagents and solvents. All reagents and solvents were of analytical or chromatographic grade. Acetonitrile, isopropanol, and hexane were purchased from Fisher (Fair Lawn, NJ) and were used without further purification. FAME were purchased from Supelco Inc. (Bellefonte, PA). Tricaprin was obtained

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from Sigma-Aldrich (St. Louis, MO) and triolein was obtained from Nu-Chek-Prep. Inc (Elysian, MN). The TAG standards were dissolved in the mobile phase at a concentration of 20 mg/mL. The three MS standards, Ultramark 1621 (perfluoroalkylphosphazine), caffeine, and MRFA (a short, four-amino-acid peptide), were purchased from Sigma-Aldrich.

Fat extraction. Ouricuri plants were acquired in the central market in Belo Horizonte, Minas Gerais, Brazil, and the plants were submitted to botanical classification at the Universidade Federal de Minas Gerais. Ouricuri (*S. coronata)* nuts were extracted as follows: After drying the nuts, the external husk was peeled manually and the nuts were crushed using a hydraulic press. A crushed nut sample, weighing 120 g, was blended with 1 L of hexane in a mixer and then held quiescently for 24 h. Next, the material was filtered, and the solvent/oil mixture was rotary-evaporated to remove the solvent. A second extraction of the nut residue was carried out, and the solvent was evaporated. The oil from both extractions was combined. The extracted nuts contained approximately 42% of ouricuri oil.

FA analysis by GC and GC–MS. The hydrolysis of the FA in the ouricuri oil samples was done according to the method of Christie (18). The samples were derivatized to form the methyl esters by adding 3 mL of BF_3/methanol (14% wt/vol) to the FFA mixture (30 mg) in a test tube fitted with a screw cap. The tube was placed in a boiling water bath for 10 min. The methyl esters formed were extracted with petroleum ether, dried, and dissolved in methanol for analysis. A Varian gas chromatography model 3400 CR6 with a FID was used to separate the FAME. The carrier gas was hydrogen. A 30 m × 0.25 mm i.d. SE-54 capillary column (Alltech, Deerfield, IL) was used. The injection volume was 2 µL, and the detector and injector temperatures were both 260°C. A split ratio of 1:100 was used, and the injection was made at a column temperature of 80°C. After a 1-min hold at 80°C, the column oven temperature was raised 10°C/min to 260°C. The FAME were identified by comparing retention times to pure standards purchased from Supelco. MS analysis of the FAME also was carried out with a Hewlett-Packard model 5970 mass spectrometer after separation of the FAME with a Hewlett-Packard model 5890 gas chromatograph (Palo Alto, CA). The carrier gas was helium. A 30 m × 0.25 mm i.d. SE-54 capillary column was used at the same temperature program described above. Mass spectral scans (45 to 350 m.u.) were recorded at 70 eV every 2 s, and the scans were started 2 min after injection.

HPLC analysis of TAG. Three different HPLC systems were used for the TAG separations. (i) A Shimadzu LC 10 AD pump combined with a Waters 410 refractive index detector (Milford, MA) (sensitivity setting = 32, scale factor = 20, positive polarity and temperature = 30°C) was used. The column was a Lichrospher 100 RP C-18 column (l = 25 cm, i.d. = 4.6 mm, particle size = 5 µm; Hewlett-Packard) with a pre-column. (ii) The second system consisted of a Surveyor LC pump and autosampler from Thermo-Finningan (San Jose, CA) combined with a photodiode array (PDA) detector and an ion trap mass spectrometer detector in series. (iii) The third system was a Waters 501 pump combined with a Sedex 55 ELSD (SEDERE, Alfortville, France). The PDA was set up to scan between 200 and 600 nm, and the ELSD operating conditions were 50°C and 2.0 bar. For chromatographic systems (ii) and (iii), a Microsorb RP C-18 column (l = 15 cm, i.d. = 4.6 mm, particle size = 5 µm; Rainin Inc., Oakland, CA) and a Supelcosil RP C-18 (l = 25 cm, i.d. = 4.6 mm, and particle size = 5 µm; Supelco Inc.) column were used in series. The same isocratic ternary mobile phase (acetonitrile/isopropanol/hexane, 57:38:5) (6) was used for all of the chromatographic systems at a flow rate of 1 mL/min.

HPLC-ESI/MS/MS. The TAG standards and the ouricuri oil samples were separated with the LC system described in (ii). The sample was prepared by dissolving the ouricuri oil in the mobile phase (acetonitrile/isopropanol/hexane). The concentration was about 10 mg/mL, and 20 µL was injected. 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. All of the column effluent (1 mL/min) was directed to the mass spectrometer. Data acquisition and processing were performed using an Xcalibur NT 1.2 data system (Thermo-Finnigan). The ion-source parameters were optimized with respect to the positive molecular ion of TAG, and the cone voltage was set to 50 eV. The capillary temperature was set to 350°C. The instrument was tuned on triolein in a positive mode. 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 *m/z* 300 and 1200, were obtained with an ion scan rate of 5500 amu/s. Helium was used as collision gas for collision-induced dissociation (CID), and the optimized relative collision energy was set to 45% for MS^2. The instrument was tuned with tricaprin and triolein. The calibration was done with a mixture of caffeine, MRFA, and Ultramark 1621 solutions.

RESULTS AND DISCUSSION

FA composition. The qualitative FA composition of ouricuri oil was achieved by GC after comparison of experimental and standard FAME retention times. Data obtained were in accordance with GC–MS results. In all spectra the $[M]^+$ ions of the FAME were observed with low intensity, the base peaks consisting of a fragment (*m/z* 74 amu) formed from a McLafferty rearrangement. The oil was composed primarily of lauric acid (*m/z* 214), followed by myristic (*m/z* 242), palmitic (*m/z* 270), oleic (*m/z* 296), stearic (*m/z* 298), and linoleic (*m/z* 294) acids. FAME of caprylic (*m/z* 158) and capric (*m/z* 186) acids were also identified. The FA profile was confirmed by the LC–MS TAG compositional analysis.

Optimization of the HPLC separation. Figure 1 contains two chromatograms of ouricuri oil. In Figure 1A, a single column was used (RP C-18, 5 µm, 250 × 4.6 mm) with a refractive index detector. In Figure 1B, two columns were used in series (C-18, 5 µm, 250 × 4.6 mm, and C-18, 5 µm, 150 × 4.6 mm) with an evaporative light-scattering detector (ELSD). As expected, better resolution was obtained with the dual-column

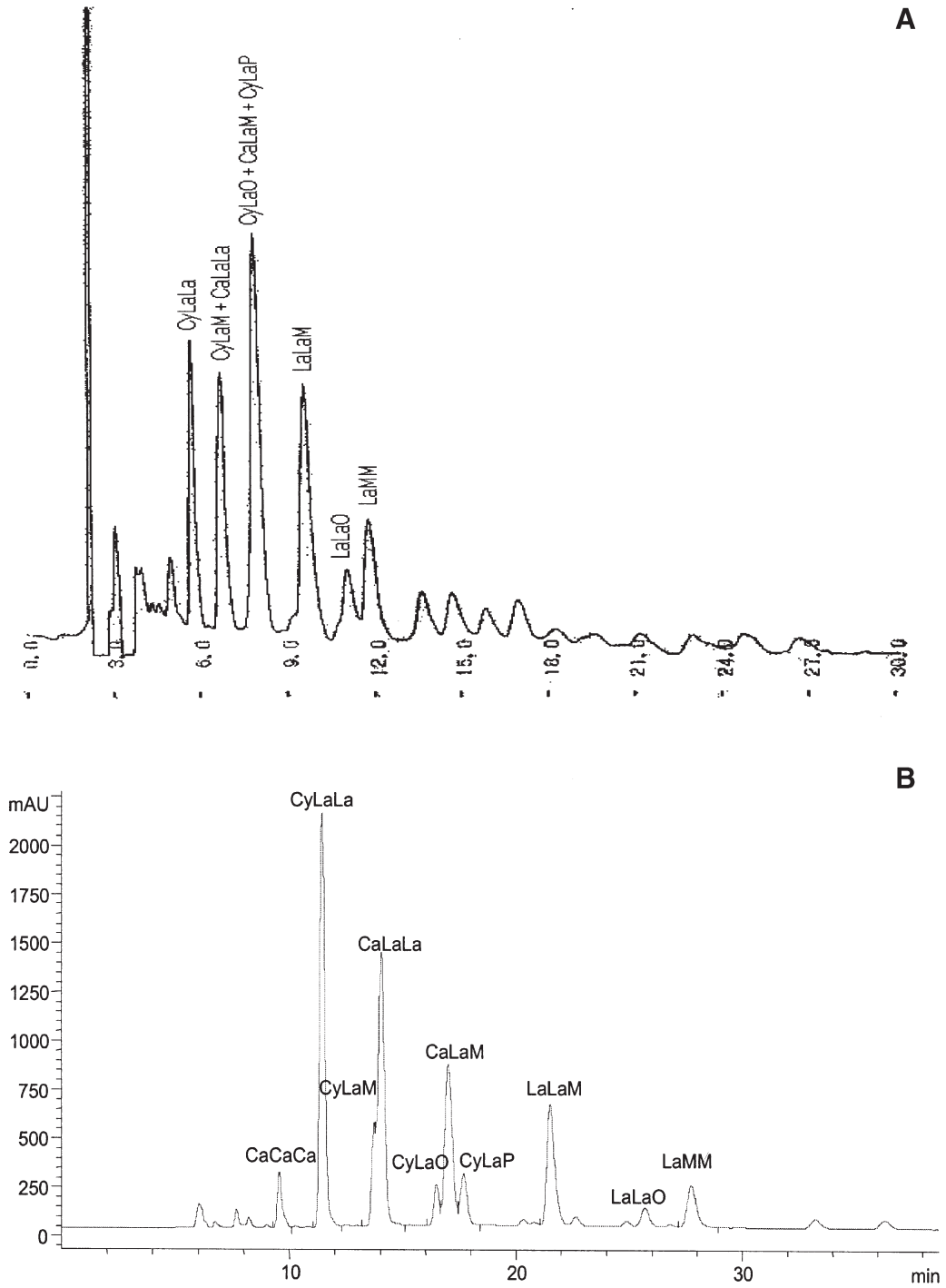

FIG. 1. Chromatograms of ouricuri oil using a single column (RP C-18 5 µm 250 × 4.6 mm) and a refractive index detector (A) and two columns (C-18 5 µm, 250 × 4.6 mm and C-18 5 µm, 150 × 4.6 mm) and an ELSD with a mobile phase of acetonitrile/isopropanol/hexane (57:38:5, by vol) (B). Ca, capryl; La, lauryl; Cy, caprilyl; M, myristoyl; O, oleic; P, palmitoyl; ELSD, evaporative light-scattering detector.

system. Some of the TAG that co-eluted as one peak (Fig. 1A) with the single column, such as capryl-lauryl-myristoyl-glycerol (CaLaM) and caprilyl-lauryl-palmitoyl-glycerol (CyLaP), were separated (Fig. 1B) and then identified by MS. Although a complete separation was not obtained even when two columns were used, additional components could be identified, such as the peak with a retention time (RT) of 14.04 min in Figure 1B, which appeared only as a small shoulder (peak at RT 7.34 min) in Figure 1A. Estimated TAG percentages based on the area response with the ELSD are shown in Table 1.

LC/ESI/MS analysis of TAG in ouricuri oil. The mass spectra of ouricuri oil showed sodium adducts of the molecular ions

TABLE 1
Composition of the Main TAG Present in Ouricuri Oil[a]

TAG	Composition (%)
Tricapryl-glycerol (CaCaCa)	3.12
Caprilyl-dilauryl-glycerol (CyLaLa)	25.76
Caprilyl-lauryl-myristoyl-glycerol (CyLaM)	4.54
Capryl-dilauryl-glycerol (CaLaLa)	21.03
Caprilyl-lauryl-oleoyl-glycerol (CyLaO)	2.76
Capryl-lauryl-myristoyl-glycerol (CaLaM)	14.47
Caprilyl-lauryl-palmitoyl-glycerol (CyLaP)	4.55
Dilauryl-myristoyl-glycerol (LaLaM)	11.53
Dilauryl-oleoyl-glycerol (LaLaO)	1.86
Lauryl-dimyristoyl-glycerol (LaMM)	4.53

[a]Amount of the relative TAG composition estimated based on the peak areas using ELSD.

$[M + Na]^+$ as base peaks. They were formed probably as a result of the presence of low levels of impurities in the sample. The major peaks represented $[CyLaLa + Na]^+$ (*m/z* 605.9), $[CyLaM + Na]^+$ (*m/z* 633.9), $[CaLaLa + Na]^+$ (*m/z* 633.8), $[CaLaM + Na]^+$ (*m/z* 661.8), and $[LaLaM + Na]^+$ (*m/z* 689.8). Table 2 lists the relevant TAG identified in the ouricuri oil sample with the major fragments detected by this technique, and Table 3 lists all of the TAG with their respective carbon numbers and the sodium adduct of the molecular ion. Figure 2 (A–D) contains the spectra of the sodium adduct molecular ions for CyLaLa, CaLaLa, CaLaM, and LaLaM. Although a quantitative analysis was difficult to obtain, an estimate of the relative TAG composition based on the peak areas from the ELSD indicated the TAG CyLaLa, CaLaLa, CaLaM, and LaLaM corresponded to 25.8, 21.0, 14.5, and 11.5%, respectively, of the total TAG composition. The results indicated that ouricuri oil has a moderately complex TAG composition, and optimization of the chromatographic conditions was valuable, particularly for the mass spectral analysis of TAG isomers. Tandem MS was used to distinguish between TAG pairs such as CaLaM and CyLaP, and CyLaM and CaLaLa.

The presence of a sodium adduct in the MS of lipids analysis is well documented (7,14,15). Some authors (15) observed that even when an acylglycerol is dissolved in a solvent with formic acid, the electrospray mass analysis produces only a weak ion current for the protonated molecule. In contrast to APCI, which produces ion fragments of MAG and DAG, ESI usually does not produce such ions due to the softness of this ionization technique.

LC/ESI/MS/MS analysis of TAG of ouricuri oil. In confirmation of the study of Duffin *et al.* (15), the MS/MS analysis of $[M + Na]^+$ yielded a limited CID mass spectrum showing DAG sodium adducts, whereas ions corresponding to either the FA or the MAG did not appear. Figure 3 contains the tandem mass spectrum for the TAG CyLaLa showing a fragmentation pattern that indicates loss of lauric (200.18 amu) or caprylic (144.12 amu) acid from the backbone of glycerol with formation of the corresponding CyLa-glycerol (*m/z* 405.2) and the DAG LaLa-glycerol sodium adducts (*m/z* 461.2), respectively. Other important peaks were observed at *m/z* 445.5 and 500.6. These peaks most likely represent clusters of acetonitrile adducts of *m/z* 405.2 and 461.2, probably due to the large amount of mobile phase that entered the ion trap. The ion trap can retain large amounts of neutral components from the mobile phase, and acetonitrile is particularly good at forming adducts with ions in a trap. The ions at *m/z* 439.2 and 383.2 arose from the neutral loss of the sodium salt of caprylic and lauric acids and correspond to LaLa-glycerol (*m/z* 439.2) and CyLa-glycerol (*m/z* 383.2) ions, respectively. The presence of the sodium adduct molecular ion and its fragmentation to the DAG ion provided the necessary information to differentiate among the TAG isomers.

TABLE 2
Ions Produced from TAG Present in Ouricuri Oil by Electrospray Ionization LC Tandem MS[a]

	$[M + Na]^+$ *(m/z)*	$[M + Na - RCOOH]^+$ *(m/z)*		$[M + Na - RCOONa]^+$ *(m/z)*
TAG			DAG	
CyLaLa	605.9	461.2	LaLa	439.2
		405.2	CyLa	383.2
CyLaM	633.9	489.3	LaM	467.3
		433.2	CyM	411.3
		405.3	CyLa	383.2
CaLaLa	633.8	461.1	LaLa	439.3
		433.3	CaLa	411.3
CaLaM	661.8	489.1	LaM	467.3
		461.3	CaM	439.3
		433.4	CaLa	411.3
CyLaP	661.8	517.2	LaP	495.3
		461.3	CyP	439.3
		405.2	CyLa	383.2

[a]For abbreviations see Table 1.

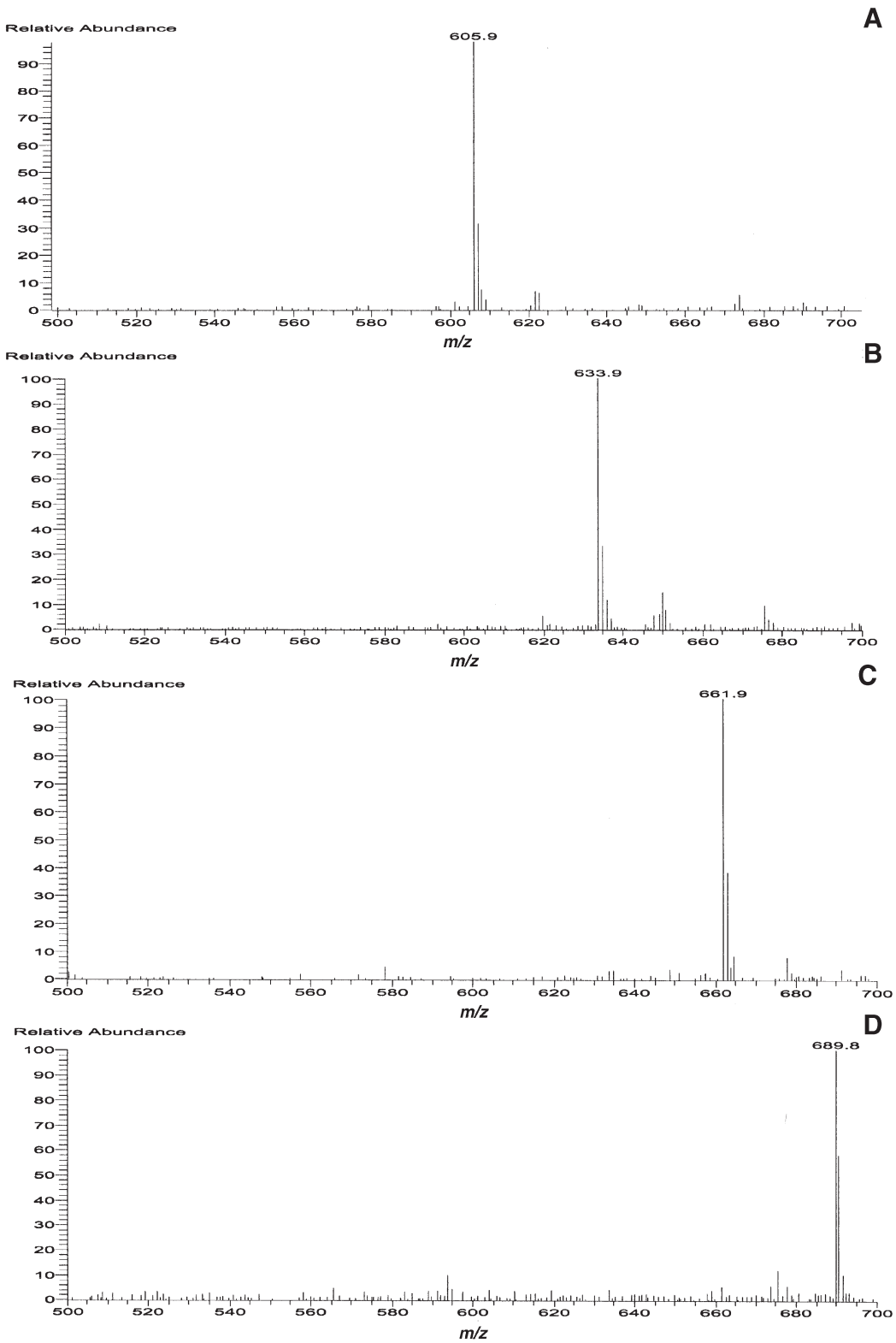

FIG. 2. Sodium adduct molecular ion $[M + Na]^+$ of the major TAG present in ouricuri oil. (A) $[CyLaLa]^+$ (*m/z* 605.9); (B) $[CaLaLa]^+$ (*m/z* 633.9); (C) $[CaLaM]^+$ (*m/z* 661.9); and (D) $[LaLaM]^+$ (*m/z* 689.8).

When two columns were used, three TAG that had previously co-eluted as one peak when a single column was used were well separated. Therefore, the isomers CaLaM and CyLaP were resolved by using tandem MS. Table 2 lists the sodium adduct and the DAG fragments present in the tandem mass spectra of TAG present in ouricuri oil. Sodium adduct molecular ions of CyLaP and CaLaM, both with *m/z* 661.8, produced different DAG fragmentation patterns upon tandem mass

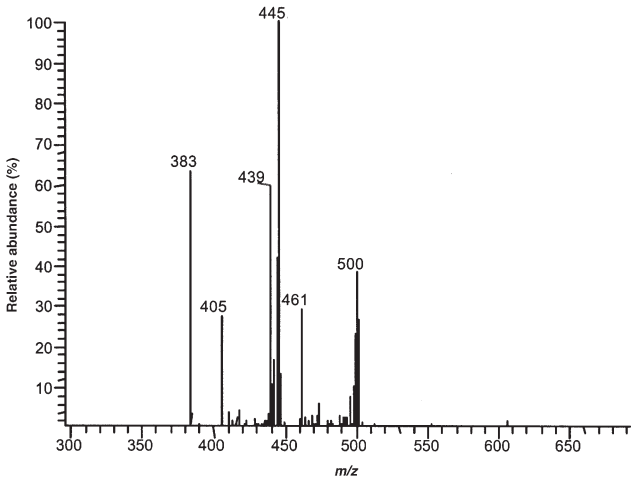

FIG. 3. Tandem mass spectrum of the TAG CyLaLa sodium adduct $[M + Na]^+$ (*m/z* 605) present in ouricuri oil. For abbreviations see Figure 1.

analysis. For CyLaP, three ions $[M + Na - RCOONa]^+$ were formed by the neutral loss of FA sodium salts and three others $[M + Na - RCOOH]^+$ were formed by the neutral loss of FA. The DAG sodium adduct ions LaP-glycerol (*m/z* 517.2), CyP-glycerol (*m/z* 461.3), and CyLa-glycerol (*m/z* 405.2) were formed by the neutral loss of caprylic (144.12 amu), lauric (200.18 amu), and palmitic (256.24 amu) acids, respectively. The ions at *m/z* 495.3, 439.3, and 383.2 are the corresponding DAG ions and result from the neutral loss of sodium salts of the respective FA. When CaLaM was analyzed by the same process, formation of fragments with *m/z* 489.1 (LaM-glycerol sodium adduct), 461.3 (CaM-glycerol sodium adduct), and 433.4 (CaLa-glycerol) was observed, corresponding to the neutral loss of capric (172.15 amu), lauric (200.18 amu), and myristic (228.21 amu) acids, respectively. The ions at *m/z* 467.3, 439.3, and 411.3 resulted from the neutral loss of the sodium salt of capric, lauric, and myristic acids, respectively, producing the corresponding DAG ion. Although the TAG isomers had formed the same sodium adduct molecular ion (*m/z* 661.8), the fragmentation patterns indicated distinctly different DAG ions, showing the differences in the chain length of the FA attached to the glycerol. Likewise, two other similar TAG (CyLaM and CaLaLa) found in the oil sample could be resolved. These two TAG had retention times that were close (RT 14.04 and 13.72 min), even when two columns were used (Table 3). A tandem mass spectrum indicated that for CyLaM, the DAG sodium adduct ions LaM-glycerol (*m/z* 489.3), CyM-glycerol (*m/z* 433.2), and CyLa-glycerol (*m/z* 405.3) were formed by the neutral loss of caprylic (144.12 amu), lauric (200.18 amu), and myristic (228.21 amu) acids, respectively. The ions observed at *m/z* 467.3, 411.3, and 383.2 resulted from the neutral loss of the FA of the respective sodium salts. When CaLaLa was analyzed by the same process, the ions at *m/z* 461.1 (LaLa-glycerol) and 433.3 (CaLa-glycerol) were found to correspond to the loss of capric (172.15 amu) and lauric (200.18 amu) acids, respectively. The ions at *m/z* 439.3 and 411.3 resulted from the neutral loss of the sodium salts of the FA.

Regarding the regiospecific distribution of FA, conflicting results, based on APCI and ESI, have been found in the literature. Laakso and Voutilainen (13) found that the abundance of ions formed by the loss of a fatty acyl residue from the *sn*-2 position was less than that formed by a loss of fatty acyl residue from the primary glycerol position when APCI MS was used. Duffin *et al.* (15) reported that the relative abundance of product ions resulting from the dissociation of FA chains from TAG was not significantly different, whereas Hvattum (16) found a smaller signal due to the neutral loss of the *sn*-2 FA residue. Both of the latter experiments (15,16) were conducted using ESI. In this preliminary study on ouricuri oil, the relative abundance of the DAG was not significantly different and, at this point, an absolute conclusion cannot be drawn concerning the regiospecific distribution of FA. The post-column addition of lithium acetate (19) can produce lithiated TAG that are useful for the analysis of the regiospecific distribution of FA, although this study has not been done with ouricuri TAG. This report confirms the importance of the optimization of chromatographic conditions and the use of MS/MS analysis for TAG identification, particularly if closely related TAG are present in the oil sample.

TABLE 3
Mass Spectral Data and Corresponding Retention Times of 10 Major TAG Present in Ouricuri Oil[a]

TAG	CN/DB	Retention time (min)	$[M + Na]^+$ (*m/z*)
CaCaCa	30:0	9.56	577.8
CyLaLa	32:0	11.43	605.9
CyLaM	34:0	13.72	633.9
CaLaLa	34:0	14.04	633.8
CyLaO	38:1	16.49	664.8
CaLaM	36:0	17.00	661.8
CyLaP	36:0	17.67	661.8
LaLaM	38:0	21.49	689.8
LaLaO	42:1	25.68	743.8
LaMM	40:0	27.73	717.9

[a]CN, carbon number; DB, double bond; for other abbreviations see Table 1.

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