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High efficiency liquid chromatography techniques coupled to mass spectrometry for the characterization of mate extracts

Paola Dugo[a], Francesco Cacciola[a], Paola Donato[a], Rosângela Assis Jacques[b], Elina Bastos Caramão[b], Luigi Mondello[a,c,*]

[a] Dipartimento Farmaco-chimico, Facoltà di Farmacia, Università di Messina, Viale Annunziata, 98168 Messina, Italy
[b] Departamento de Química, Universidade Federal do Pampa (UNIPAMPA), Carlos Barbosa s/n°,Bagé 96400-970RS, Brazil
[c] Campus-Biomedico, Via A. del Portillo 21, 00128 Roma, Italy

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ABSTRACT

There is growing interest related to rapid screening and full characterization of the constituents of plants with medicinal properties; among these, "Mate" or Yerba Maté is a tea-like beverage widely consumed in South America, obtained from the dried leaves of *Ilex paraguariensis*. The high content in polyphenols accounts for *in vitro* and *in vivo* antioxidant activity of the extracts obtained from this plant; on the other hand, the high complexity of the samples extracted, depending on the method employed, may preclude complete resolution by conventional HPLC techniques. For this purpose, a comprehensive two-dimensional liquid chromatography (LC × LC) system, comprised of an RP-Amide first dimension and a partially porous octadecylsilica column in the second dimension, has been compared with a one-dimensional system. The latter was operated using a partially porous octadecylsilica column, with diode array (DAD) and electrospray/ion trap-time of flight (ESI/IT-TOF) detection for the most complex extracts. The employment of the hybrid mass spectrometer allowed unequivocal identification of several compounds in the mate extracts. Using LC × LC–MS^3, it was possible to discriminate between congeners of chlorogenic acids, along with monoacyl- and diacylchlorogenic acid esters.

1. Introduction

"Mate" or "Yerba maté" is a tea-like beverage prepared by infusion of the dried and minced leaves of *Ilex paraguariensis*, a native South American tree, where it is widely consumed [1]. The high content of caffeine and theobromine accounts for its peculiar flavour, with a characteristic bitter taste, as well as for its mild stimulating effects [2]. Recent studies have demonstrated *in vitro* and *in vivo* antioxidant activities, and hepatoprotective, choleretic, diuretic, hypocholesterolemic, antirheumatic, antitrombotic, anti-inflammatory, antiobesity or antiageing properties of mate extracts [3]. Some of the mate pharmacological properties have been related to the high content of caffeoyl derivatives and other phenolics [4]. The most conventional way to determine the water soluble antioxidative compounds in plant materials is represented by solvent extraction and liquid chromatography [5–8]. Recently, Kivilompolo and Hyötyläinen have compared conventional extraction techniques such as refluxing with methanol, shaking with methanol or ethanol, and hydrodistillation, with dynamic sonication assisted with ethanol for the isolation of phenolic acids contained in six herbs of the *Lamiaceae* family [9]. To the best of our knowledge, only infusions and organic extracts of mate have been analyzed by liquid chromatography with diode array detection (LC-DAD) or electrospray mass spectrometry detection (LC-ESI-MS) in order to evaluate their polyphenolic profile [10–13]. Other studies, focused on the isolation of potentially active organic compounds, employed non-conventional extraction methods such as ultrasound, maceration and supercritical fluid extraction (SFE) prior to GC–MS analysis [14–16].

Although one-dimensional LC chromatography is widely applied to the analysis of herb extracts, the complexity of many extracts, sometimes exceeds the separation capacity of any single system. The degree of separation prior to MS detection is of the utmost importance to improve the reliability of the MS results, by decreasing the matrix interferences and/or co-elutions. In a comprehensive LC system, all fractions from the first column are continuously sampled and transferred, by a switching valve, to the second column for further separation. Thus, the whole sample is analysed in both dimensions. Typically, a comprehensive LC system combines two columns with different separation mechanisms in order to achieve a high-resolution separation and reliable identification [17–21]. The fraction injected onto the secondary column

* Corresponding author at: Dipartimento Farmaco-chimico, Facoltà di Farmacia, Università di Messina, Viale Annunziata, 98168 Messina, Italy.
Tel.: +39 090 6766536; fax: +39 090 358220.
E-mail address: lmondello@unime.it (L. Mondello).

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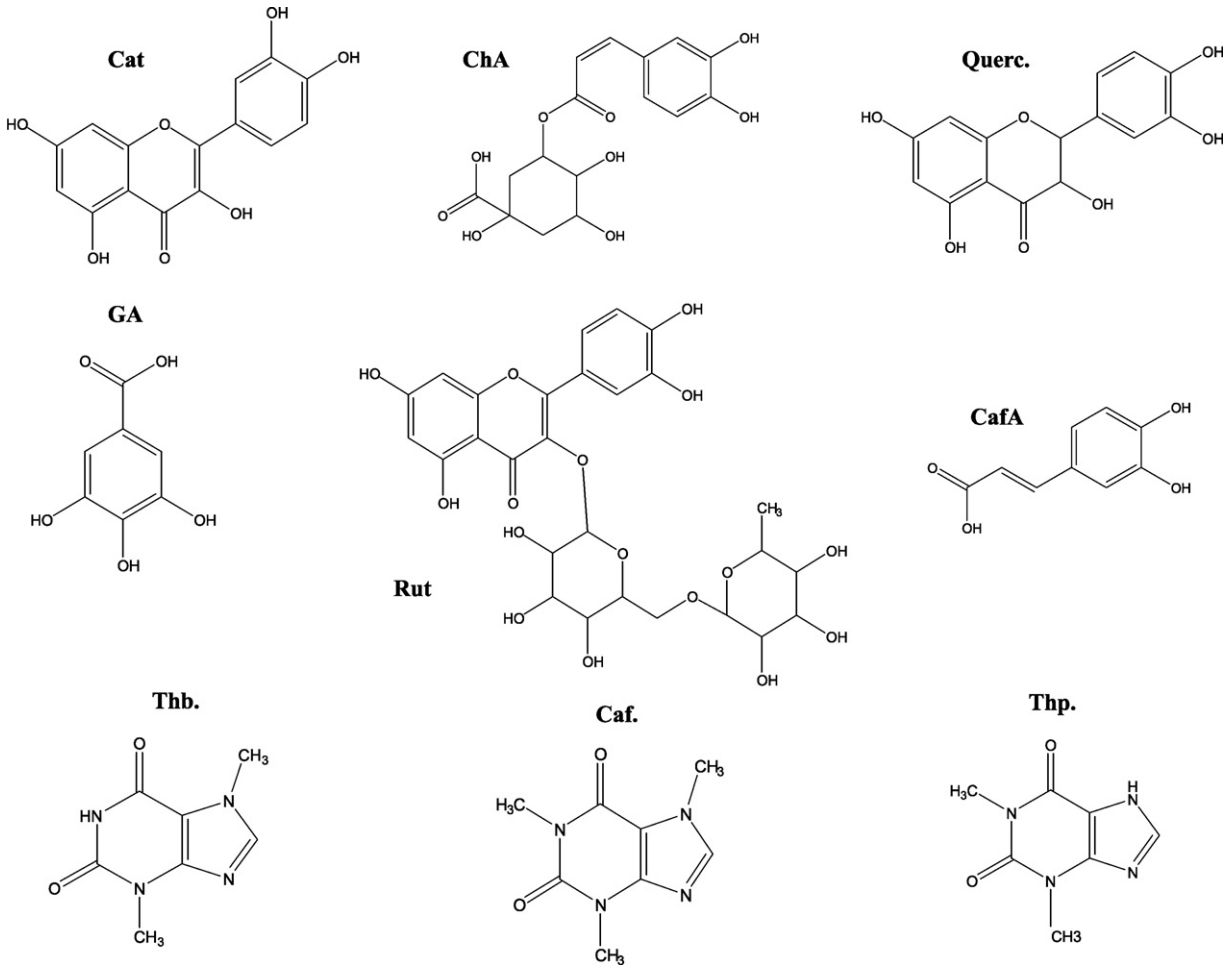

Fig. 1. Structures of the standard antioxidants tested (abbreviations as in Table 2).

should be completely analyzed before the following transfer occurs. The modulation time, which is dependent on the first dimension (D1) flow rate and second dimension (D2) analysis time, needs to be carefully tuned and maintained as brief as possible, because the separation achieved in the first dimension must not be lost [22,23]. For this reason, microbore columns in the first dimension and fast columns in the second dimension are commonly employed [21,24].

As second dimension, as well as for the one-dimensional separations, a partially porous column, based on a new particle technology (Fused-Core™), was used. The column particles consist of a 1.7 μm solid core with a 0.5 μm porous silica surrounding shell (2.7 μm). The major benefit of such particles is the small diffusion path compared to that of a totally porous particle, thus reducing the axial dispersion of the solutes, minimizing band broadening. The advantages offered by these columns have been demonstrated in several studies [25–29].

Comprehensive LC (RP × RP) systems have already been developed and applied for the determination of antioxidants in plants extracts [9,30]. In these two papers, combinations of cyano-C18 [9] and amino-C18 [30] columns were investigated for *Lamiaceae* and *Stevia Rebaudiana* extracts, respectively.

In the present contribution, we employed LC and LC × LC for the characterization of extracts of minced leaves of mate, comparing several extraction methods (infusion, ultrasonication, supercritical fluid extraction, maceration with methanol). The antioxidant profile of the extracted samples was revealed through DAD and MS detection.

2. Experimental

2.1. Materials and samples

The standards of caffeine, theobromine, theophylline, gallic acid, chlorogenic acid, catechin, caffeic acid, rutin and quercetin were purchased from Sigma–Aldrich (Milan, Italy) and Extrasynthese (Genay-France) and are illustrated in Fig. 1.

The Ascentis RP-Amide (250 × 1.0 mm; 5 μm) and the 2.7 μm Ascentis Express columns (150 × 4.6 mm and 30 × 4.6 mm) were obtained from Supelco (Bellefonte, PA).

ACN, MeOH, and water, HPLC grade were purchased from VWR International S.r.l. (Milan, Italy). ACN, water, formic acid, MS grade were obtained from Riedel-de Haën (Germany). All the mobile phases were adjusted to an appropriate pH value (pH 3) with formic acid in order to suppress the ionization of phenolic acids. Stock solutions of each standard, at a concentration of 1000 mg/L, were prepared by weighting the appropriate amount in aqueous methanol (50:50, v/v). The standard mixture was obtained by dissolving each standard solution in water in order to obtain an approximate concentration of 110 ppm.

The mate samples, purchased at a local supermarket in Brazil, were extracted using different procedures (infusion, maceration, ultrasonic and supercritical fluid extraction) and dissolved in aqueous methanol prior to HPLC analysis.

The mobile phases as well as the samples were filtered using a Millipore (Bedford, MA, USA) 0.45 μm filter and degassed by ultrasonication before use.

2.2. Instrumentation

One-dimensional LC analyses were carried out on a Shimadzu LC system (Shimadzu, Milan, Italy), consisting of an SCL-10A*vp* controller, two LC-10 AD*Vp* pumps, a DGU-14A degasser, a SIL-10A*vp* autosampler, a CTO-10A*vp* column oven and an SPD-10A*vp* photo diode array detector. For data acquisition, the LCsolution Version 1.21 SP1 software (Shimadzu, Milan, Italy) was used.

Comprehensive LC analyses were performed on a Shimadzu Prominence LC-20A (Shimadzu, Milan, Italy), which consists of a CBM-20A controller, two LC-20AD dual-plunger parallel-flow pumps (LC-1), an LC-20 AB solvent delivery module, equipped with two dual- plunger tandem-flow pumps (LC-2), a DGU-20 A5 degasser, an SPD-M20A photo diode array detector, a CTO-20 AC column oven, a Rheodyne two-position 6-port injection valve model 8125 equipped with a 5 μL loop (Rheodyne, Rohnert Park, CA). The two dimensions were connected by using an electronically controlled 2-position 10-port Supelpro switching valve (Supelco, Milan, Italy), placed inside the column oven and equipped with two 20 μL sample loops. Both dimensions and the switching valve were controlled by the LCMSsolution software (Version 3.50.346, Shimadzu). The LC × LC data were elaborated and visualized in two and three dimensions using the Chromsquare Version 1.0 software (Chromaleont, Messina, Italy). The HPLC systems were coupled to an ion trap-time of flight (IT-TOF) mass spectrometer (Shimadzu) through an atmospheric pressure electrospray ionization (ESI) source (Shimadzu).

2.3. Extraction procedures

2.3.1. Infusion

Mate infusions were prepared by boiling 7.5 g of dried minced leaves in 225 mL of water for 5 min. After this time, the samples were cooled down at room temperature, then filtered through filter paper (0.45 μm pore size) and evaporated to dryness.

2.3.2. Maceration

Maceration was performed with approximately 7.5 g of mate minced dried leaves in 225 mL of methanol. The mixture was left at room temperature for 10 days and occasionally stirred [14]. After this time, the extracts were filtered through filter paper and evaporated to dryness.

2.3.3. Ultrasound assisted extraction (UAE)

Dried and minced leaves (7.5 g) from mate were extracted with 225 mL of methanol in an ultrasonic bath (Fischer Scientific-FS 14H), thermostated at 75 ± 0.5 °C (potency: 90 W; frequency: 40 kHz). Temperature and extraction time (180 min) had been optimized for this technique in a previous study [14].

2.3.4. Supercritical fluid extraction

SFE was performed on a laboratory scale unit, as presented in previous works [15,16], consisting of a CO_2 reservoir, two thermostatic baths, a syringe pump (ISCO 260D), a 0.2 dm^3 jacketed extraction vessel, an absolute pressure transducer (Smar, LD301) equipped with a portable programmer (Smar, HT 201) with a precision of ±0.31 bar, a collector vessel with a glass tube and a cold trap. Amounts of *circa* 25 g of dried mate leaves were introduced into the extraction vessel. The CO_2 was pumped at a constant rate of 1 g min^{-1} into the bed, which was supported by two 300 mesh wire disks at both ends, and was kept in contact with the vegetable matrix for at least 1 h to allow system stabilization. Afterwards, the extract was collected opening the micrometering valve, and the CO_2 mass flow was accounted for the pump recordings. After that, the mass of the extracted oil was weighed, the glass tube was reconnected to the equipment and this procedure was performed in approximately 400 min. The experiments were accomplished at temperature of 40 °C, and pressure of 175 bar. A whole experimental run lasted in general 10 h, including all steps involved: sample weighing, temperature stabilization (baths, extractor), depressurization, etc.

2.4. LC conditions

One-dimensional analyses were carried out on an Ascentis Express C18 column (150 × 4.6 mm, 2.7 μm particles) in gradient elution with water (A) and ACN (B) (0 min 0% B; 20 min 5% B; 50 min 40% B) at a flow rate of 1 mL/min. The injection volume was of 10 μL and the temperature was set at 25 °C. The PDA detector covered a wavelength range from 215 to 420 nm (8 μL detector cell volume, cell temp. 40 °C, sampling rate 12.5 Hz, time constant 0.025 s) and the chromatograms obtained were extracted for visual inspection at 280 nm.

Comprehensive LC analyses were performed using an RP-Amide column (250 × 1.0 mm, 5 μm) in the first dimension and an Ascentis Express C18 (30 × 4.6 mm, 2.7 μm) in the second dimension. In order to reduce the flow rate in the first dimension, an Accurate flow splitter (LC Packings, Amsterdam, The Netherlands) was used between the LC-1 pump and the injector. In the first dimension, gradient elution (0 min 20% B; 60 min 70% B; 70 min 90% B) was used at a flow rate of 10 μL/min and at a temperature of 25 °C.

Second dimension analyses were carried out with gradient elution (0 min 0% B; 0.90 min 20% B; 1.20 min 30% B; 1.50 min 100% B; 1.69 min 100% B; 1.70 min 0% B) at a flow rate of 4 mL/min and a temperature of 45 °C. The total effluent deriving from the DAD was split at a ratio of 1:20 in order to reduce the flow rate to a consistent degree prior to MS detector. The 10-port valve was switched every 2 min.

Twenty μL of the extracts were injected at a temperature of 25 °C. DAD conditions were the same as employed for one-dimensional analyses.

2.5. MS conditions

Mass spectrometry data were acquired in the comprehensive LC–MS-IT-TOF instrument: 0.2 mL/min from the LC unit were directed to the mass spectrometer through an atmospheric pressure electrospray ionization source, which was operated in scan mode (both positive and negative ionization) for the accurate determination of parent ion *m/z*, and in MS^3 mode (negative ionization) to obtain fragment ion *m/z*. Resolution, sensitivity, and mass number calibration of the ion trap and the TOF analyzer were adjusted using a standard sample solution of trifluoroacetic acid (TFA, approx. 0.25 mL/L) and sodium hydrate (approx. 0.1 g/L). After the calibrant had flowed, cleaning operation of the tube and ESI probe was made by using acetonitrile (0.2 mL/min, 20 min). MS operating conditions were as follows: curve desolvation line (CDL) and heat block temperature, 200 °C; detector voltage, 1.57 kV; nebulising gas (N_2) flow, 1.5 mL/min; collision gas and cooling gas (argon), 43 and 102 kPa, respectively. Parent ion *m/z* was acquired in the 170–200 range (positive ionization mode) and in the 330–680 range (negative ionization mode), with an ion accumulation time of 30 ms (ion trap, repeat = 3). MS/MS fragment ions were acquired in the 50–400 *m/z* range under the following conditions: precursors ion isolation in the 330–680 *m/z* range (width 3 Da); ion accumulation time 50 ms; tolerance 0.05 *m/z*; repeat = 1; collision-induced dissociation (CID) energy 50%; execution trigger (BPC) intensity at 95% stop level. MS^3 fragment ions were acquired in the 50–200 *m/z* range under the following conditions: precursors ion isolation in the 50–400 *m/z* range (width 3 Da); ion accumulation time 70 ms; tolerance 0.05 *m/z*; repeat = 1; collision-induced dissociation (CID) energy 70%; execution trigger (BPC) intensity at 95% stop level.

LCMSsolution Version 3.50.346 software from Shimadzu was used for data acquisition and processing.

3. Results and discussion

The objective of this work was to fully characterize the polyphenolic and xanthinic content of mate leaves, extracted with different techniques. Separation was performed by using RPLC and RPLC × RPLC, prior to DAD and mass spectrometric detection.

3.1. Analysis of mate extracts by one-dimensional liquid chromatography

Fig. 2(A–D) illustrates the chromatograms (extracted at 280 nm) obtained on a partially porous C18 column (150 × 4.6 mm, 2.7 μm particles) for the mate extracts obtained through each technique.

Different gradients comprised of water and acetonitrile were tested in order to ensure the separation of most compounds with a satisfactory degree of resolution. In particular, the low elution strength of mobile phase for the first 20 min of analysis allowed peak focusing of the most polar phenolic acids. For the remaining 30 min, a linear gradient up to 40% of acetonitrile revealed to be suitable for the elution of all compounds. A visual inspection of chromatograms A and B reveals that the polyphenolic profile obtained is practically the same by employing the two extraction methods: a total of nine, baseline resolved compounds were identified, as will be later discussed (see Table 1 for peak assignment and compound characterization). Nevertheless, it is possible to note that nitrogen-containing compounds such as theobromine and caffeine (assigned as peaks 3 and 8, respectively) are better extracted with SFE (B), rather than maceration (A); on the other hand, the maceration extract is characterized by a higher content of chlorogenic acids, especially dicaffeoylquinic acids (peaks 18–20). A much higher number of components are extracted through infusion and UAE, probably due to mass transfer enhancement and facilitated contact between the extraction solvent and the vegetable cells. Since the improved effectiveness of the two latter extraction methods resulted in a much more complex chromatographic profile, we compared the performance of both LC and comprehensive LC methods for the two extracts, as will be discussed in the following section. The chromatograms obtained from the one-dimensional LC analysis of the extracts obtained by UAE and infusion (Fig. 2C and D) show a much higher number of extracted compounds: a total of twenty-six components were identified, that is three times more than those found in the mate samples extracted through maceration and SFE. The high polarity of the extraction solvent, *viz.*, methanol, certainly accounts for the different composition of the extracts obtained. The chromatographic profile obtained, under the same experimental conditions, from the LC analysis of the infusion and UAE extracts is practically identical, the only exception being the absence of peaks labelled as 1 and 2 in the UAE chromatogram, and identified as gentisic acid and 3,4-dihydroxy-phenylacetic acid (Table 1). Apart from the peak relative intensities, the main difference in the performance of these two latter extraction methods (infusion and UAE) can be observed in the extraction of compounds labelled as 11–14 and 22–26 in Fig. 2(C and D), which were not detected in the extracts obtained by maceration and SFE (Fig. 2A and B). The first group of compounds (11–14) are monoacylchlorogenic acids, while the later eluting congeners (22–26) are diacylchlorogenic acids, as will be discussed later on. The expansion windows, reported in Fig. 2C and D, show how these minor components are all well separated. It is noteworthy that all compounds are eluted within the 42 min analysis time with a satisfactory degree of resolution, except for compounds 7 and 8, which partially co-elute at around 28 min run time (chlorogenic acid and caffeine, respectively).

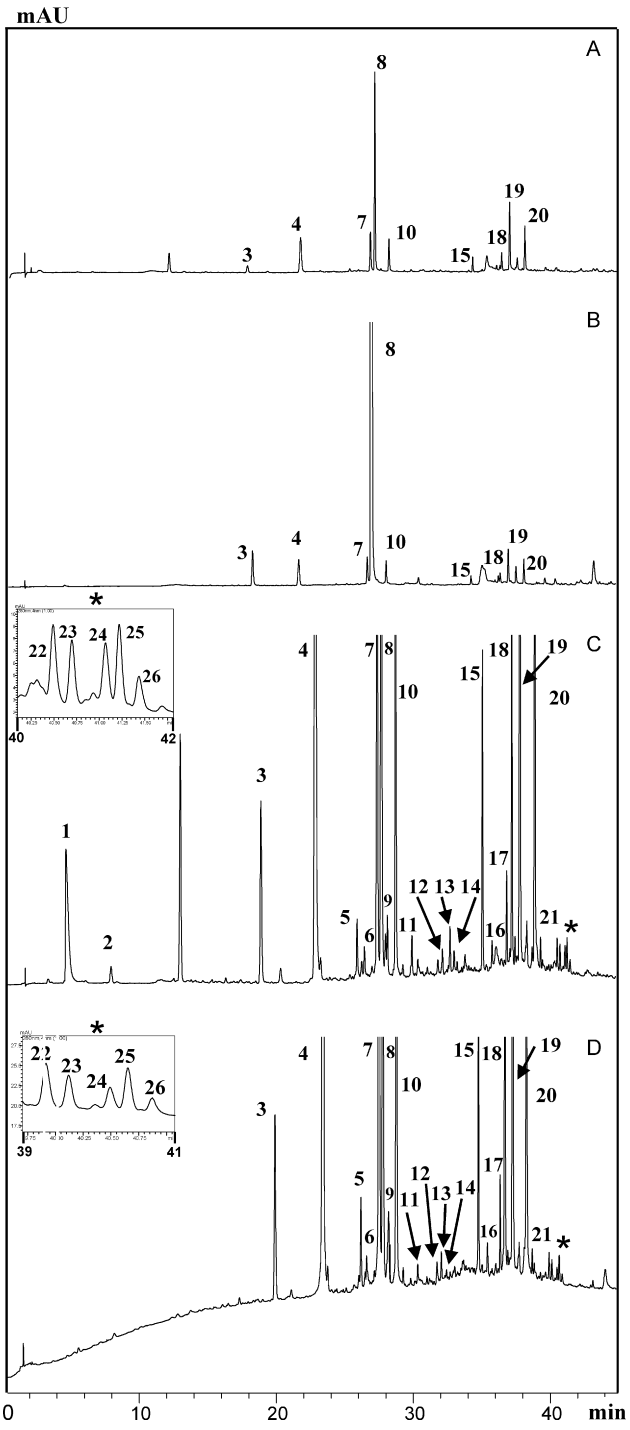

Fig. 2. LC chromatograms of mate extracts prepared by maceration (A), SFE (B), UAE (C) and infusion (D) obtained on an Ascentis Express C18 column (150 × 4.6 mm, 2.7 μm particles). Peak assignment as in Table 1. For experimental conditions, see the text.

3.2. Analysis of mate extracts by comprehensive two-dimensional liquid chromatography

Prior to the LC × LC separation of the real-world samples, a standard mixture of nine compounds, was analysed on the LC × LC system, comprised of an RP-Amide column (250 × 1.0 mm, 5 μm particles) as first dimension (D1) and a short partially porous C18 column (30 × 4.6 mm, 2.7 μm particles) in the second dimension (D2). The polar embedded RP phase provides enough separation selectivity, in combination with an octadecylsilica column, mak-

Table 1
HPLC-DAD and ESI-MS^3 (positive and negative ionization mode) fingerprint of green Yerba Matè (*Ilex paraguariensis*) extracts. The presence of any components in the different extracts is indicated by an asterisk.

Peak	Molecular formula	Selected ion	*m/z* calculated	*m/z* observed	Error (ppm)	MS^2 ions (*m/z*)		MS^3 ions (*m/z*)		UV–Vis (nm)	Compound	Inf.	UAE	Mac.	SFE
						Base peak	Fragments	Base peak	Fragments						
1	$C_7H_6O_4$	$[M-H]^-$	153.0188	153.0191	1.96					263	Gentisic acid		*		
2	$C_8H_8O_4$	$[M-H]^-$	167.0344	167.0346	1.19					277	3,4-dihydroxy-phenylacetic acid		*		
3	$C_7H_8N_4O_2$	$[M+H]^+$	181.0725	181.0726	0.55					272	Theobromine	*	*	*	*
4	$C_{16}H_{18}O_9$	$[M-H]^-$	353.0872	353.0860	3.39	191	179, 135	85	172, 127	325, 298 (sh)	Neo-chlorogenic acid (3-CQA)	*	*	*	*
5	$C_{18}H_{14}O_7$	$[M-H]^-$	341.0661	341.0649	3.14	281	179			324, 298 (sh)	Dicaffeic acid	*	*		
6	$C_{16}H_{18}O_8$	$[M-H]^-$	337.0923	337.0924	0.29	163				314	Coumaroylquinic acid (CQA)	*	*		
7	$C_{16}H_{18}O_9$	$[M-H]^-$	353.0872	353.0865	1.98	191	179, 161	85	172, 127	325, 298 (sh)	Chlorogenic acid (5-CQA)	*	*	*	*
8	$C_8H_{10}N_4O_2$	$[M+H]^+$	195.0882	195.0889	3.58					271	Caffeine	*	*	*	*
9	$C_{18}H_{14}O_7$	$[M-H]^-$	341.0661	341.0669	2.34	251	179			324, 298 (sh)	Dicaffeic acid	*	*		
10	$C_{16}H_{18}O_9$	$[M-H]^-$	353.0872	353.0870	0.56	173	179	93	111	325, 298 (sh)	Crypto-chlorogenic acid (4-CQA)	*	*	*	*
11	$C_{16}H_{18}O_9$	$[M-H]^-$	353.0872	353.0876	1.13	191	179, 173	123		325, 298 (sh)	Caffeoylquinic acid (1-CQA)	*	*		
12	$C_{17}H_{20}O_9$	$[M-H]^-$	367.1029	367.1036	1.90	193	191	134	152	324, 298 (sh)	Feruloylquinic acid (3-FQA)	*	*		
13	$C_{17}H_{20}O_9$	$[M-H]^-$	367.1029	367.1044	4.08	191	173	85		324, 298 (sh)	Feruloylquinic acid (5-FQA)	*	*		
14	$C_{17}H_{20}O_9$	$[M-H]^-$	367.1029	367.1032	0.82	173	191	93		324, 298 (sh)	Feruloylquinic acid (4-FQA)	*	*		
15	$C_{27}H_{30}O_{16}$	$[M-H]^-$	609.1456	609.1458	0.32	301				254, 354	Rutin	*	*	*	*
16	$C_{21}H_{20}O_{12}$	$[M-H]^-$	463.0876	463.0886	2.16	301				256, 344	Quercetin-3-O-glycoside	*	*		
17	$C_{21}H_{20}O_{11}$	$[M-H]^-$	447.0927	447.0923	0.89	285				266, 344	Kaempferol-3-O-glycoside	*	*		
18	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.1189	515.1201	2.32	353	335	173	179, 135, 93	327, 296 (sh)	Dicaffeoylquinic acid (3,4-di-CQA)	*	*	*	*
19	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.1189	515.1187	0.38	353		191	179, 173, 135, 85	327, 296 (sh)	Dicaffeoylquinic acid (3,5-di-CQA)	*	*	*	*
20	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.1189	515.1191	0.38	353		173	191, 179, 135	327, 296 (sh)	Dicaffeoylquinic acid (4,5-di-CQA)	*	*	*	*
21	$C_{25}H_{25}O_{12}$	$[M-H]^-$	529.1346	529.1343	0.57	353	367, 335	173		238, 327	Caffeoylferuloylquinic acid (3F, 4C-QA)	*	*		
22	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.1189	515.1179	1.94	353		179	191	327, 296 (sh)	Dicaffeoylquinic acid (1,5-di-CQA)	*	*		
23	$C_{27}H_{28}O_{13}$	$[M-H]^-$	559.1451	559.1446	0.89	397		179		238, 327	Caffeoylsinapylquinic acid (CSQA)	*	*		
24	$C_{25}H_{24}O_{12}$	$[M-H]^-$	529.1346	529.1321	4.72	367	353, 335	173	193	238, 327	Caffeoylferuloylquinic acid (3C,4F-QA)	*	*		
25	$C_{25}H_{24}O_{12}$	$[M-H]^-$	529.1346	529.1334	2.27	367	335	193	173	238, 327	Caffeoylferuloylquinic acid (3F, 5C-QA)	*	*		
26	$C_{25}H_{24}O_{12}$	$[M-H]^-$	529.1346	529.1341	0.94	367	393	173		238, 327	Caffeoylferuloylquinic acid (4C, 5F-QA)	*	*		

Table 2
List of standard antioxidants tested.

Abbreviations	Trivial name	Systematic name	Compound type	Manufacturer
Thp.	Theophylline	1H-Purine-2,6-dione, 3,7-dihydro-1,3-dimethyl	Xanthine derivative	Sigma–Aldrich
Thb.	Theobromine	1H-Purine-2,6-dione, 3,7-dihydro-3,7-dimethyl	Xanthine derivative	Sigma–Aldrich
GA	Gallic acid	3,4,5-Trihydroxybenzoic acid	Benzoic acid derivative	Sigma–Aldrich
Caf.	Caffeine	1H-Purine-2,6-dione, 3,7-dihydro-1,3,7-trimethyl	Xanthine derivative	Sigma–Aldrich
Querc.	Quercetin	3',4',5,7-tetrahydroxyflavon-3-ol	Cinnamic acid derivative	Sigma–Aldrich
ChA	Chlorogenic acid	3-[3-(3,4-Dihydroxyphenyl)-1-oxo-2-propenyloxy]-1,4,5-trihydroxycyclohexane carboxylic acid	Cinnamic acid derivative	Sigma–Aldrich
Cat	Catechin	(2S,3R)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-1-(2H)-benzopyran-3,5,7-triol	Flavan-3-ol derivative	Sigma–Aldrich
Rut	Rutin	3,3',4',5,7-Pentahydroxyflavone-3-rutinoside	Flavonol-glycoside derivative	Extrasynthese
CafA	Caffeic acid	(E)-3-(3,4-Dihydroxyphenyl)-2-propenoic acid	Cinnamic acid derivative	Sigma–Aldrich

ing it an interesting opportunity for RPLC × RPLC separations of complex samples, containing closely eluting components. As previously tested [21], the short reconditioning times of the 3 cm column enables the application of fast repetitive gradients in the second dimension and short modulation times (the two-position, 10-port valve was switched every 2 min) to ensure adequate peak sampling, thus not impairing D1 resolution. The standard compounds are listed in Table 2, and Fig. 3 illustrates the comprehensive LC chromatogram.

Different gradients, with mobile phases composed of water and acetonitrile, were tested in both dimensions, considering the analysis time, the degree of separation in the first dimension, as well as the possibility of peak focusing at the head of the second dimension column. Relative speeds and looping time for the switching valve were also carefully selected in order to ensure the adequate sampling of peaks eluting from D1, thus avoiding loss in resolution and wrap-around phenomena [23]. Furthermore, the low mobile phase flow rate through the first column enabled satisfactory peak focusing at the second column inlet, despite the high percentage of organic solvent in the transferred fractions. As it can be seen, all the target compounds, which can be considered as representatives of the samples tested, are separated with a good degree of orthogonality.

The comprehensive LC chromatogram, for the UAE mate extract, is shown in Fig. 4. Fig. 4A and B corresponds to expansions of two different 2D zones, extracted at a lower absorbance scale (the plot was extracted at 280 nm, which is suitable for the different classes of compounds under investigation). Despite these components are present in rather low amounts, their identification was possible due to the good degree of separation attained, and the high sensitivity of the mass detector.

As expected, the retention behaviour of the sample components on the polar embedded stationary phase was somewhat different from that observed on the octadecylsilica column, *e.g.* compounds 3–6 which were nicely separated in the one-dimensional analysis, all co-elute around 30 min retention time on the RP-Amide column used in D1, and are then resolved within 0.67 min on the D2 C18 column. On the other hand, the elution order was maintained for all the peaks detected. However, the employment of the 2D set-up allowed the resolution of a few critical pairs observed in the one-dimensional analysis: the most remarkable example is represented by peaks 7 and 8, identified as chlorogenic acid and caffeine, respectively, for which an inversion in the retention order was observed. In fact, chlorogenic acid is more strongly retained on the more polar RP-Amide stationary phase; as a result, the partial co-elution observed on the octadecylsilica column was resolved. Another example is represented by peaks 8 and 9, identified as caffeine and dicaffeic acid, respectively: in this case the different selectivity of the stationary phases was useful to achieve satisfactory resolution. Apart from these examples, there seems to be no particular advantage in the use of the comprehensive set-up, over the one-dimensional analysis. In fact, the small diffusion path of the shell-packed particles of the one-dimensional column reduces axial dispersion and minimizes peak broadening; in this way, the mass-transfer component of the van Deemter curve decreases, enabling higher resolving power analysis.

3.3. Comparison of performance results by LC and RPLC × RPLC

The separation performance of the LC and RPLC × RPLC systems was calculated in terms of peak capacity, considering the first and last peaks in the chromatograms according to Eq. (1) [31]:

$$P_c = 1 + \frac{t_g}{(1/n)\sum_1^n w} \quad (1)$$

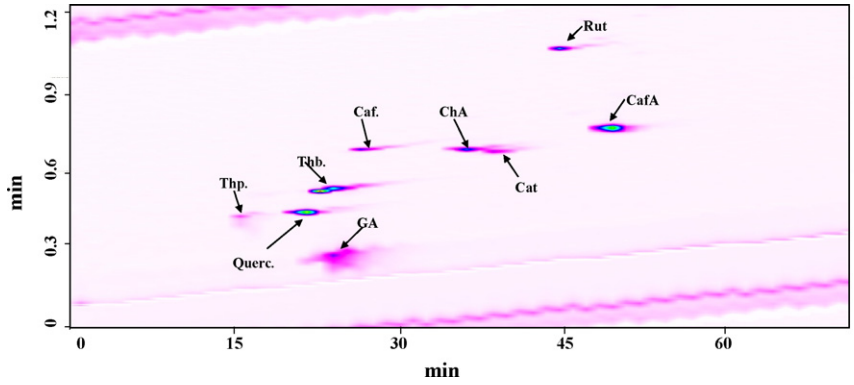

Fig. 3. 2D plot of the LC × LC analysis of a mixture of standard compounds obtained using an RP-Amide column (250 × 1.0 mm, 5 μm particles) in the first dimension and an Ascentis Express C18 column (30 × 4.6, 2.7 μm particles) in the second dimension. Peak assignment as in Table 2. For experimental conditions, see the text.

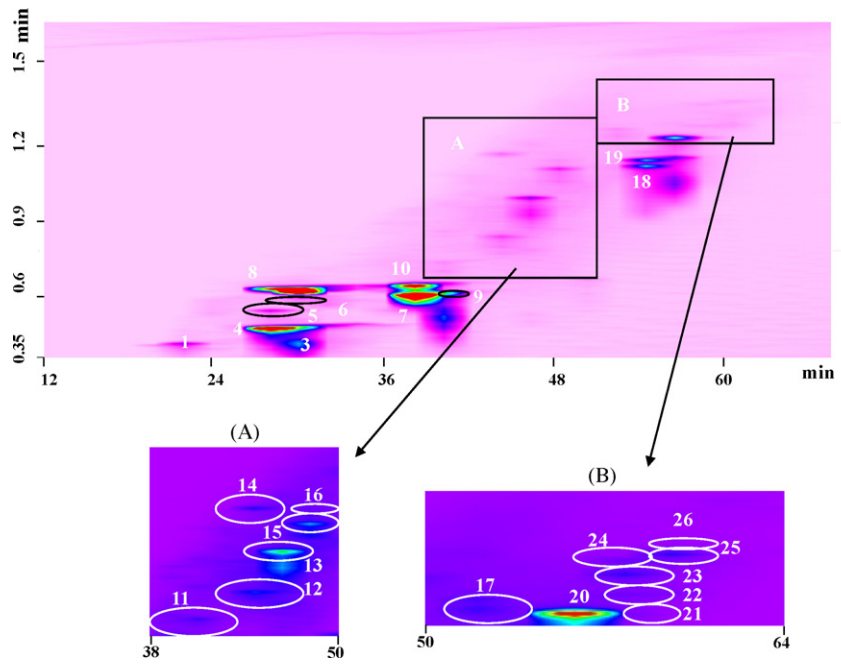

Fig. 4. 2D plot of the LC × LC analysis of the UAE extract of mate leaves obtained using an RP-Amide column (250 × 1.0 mm, 5 μm particles) in the first dimension and an Ascentis Express C18 column (30 × 4.6 mm, 2.7 μm particles) in the second dimension. Peak assignment as in Table 1. For experimental conditions, see the text.

in which t_g is the gradient run time, n is the number of peaks selected for the calculation, and w the average width of the n peaks. The calculated theoretical peak capacity values were of 195, for the one-dimensional, and 503 for the comprehensive LC set-up. Significantly, the latter value is only 2.5 times higher than that obtained on the single column, and results from the contribution of both dimensions. It is worth noting that the first dimension column, operated below optimum flow rate conditions for the aforementioned reasons, caused a decrease in D1 peak capacity compared to the second dimension column (D1: 10.69 *vs.* D2: 47.10). Furthermore, in the comprehensive LC application a double amount was injected, with respect to the one-dimensional analyses, because the former is usually affected by a sensitivity decrease due to dilution effects caused by the high second-dimension flow rates [32].

3.4. Identification of phenolics and other mate extract constituents

Identification of the mate constituents was carried out on the basis of their retention times on the octadecylsilica column, on diode array spectra, MS ions and MS/MS or MS^3 fragments (if detected), and on literature-derived information and/or on that of pure standards, when available. Chromatographic and spectral data were collected for a total of 26 compounds identified in the UAE extract obtained from mate leaves, which showed the most complete profile. Table 1 includes, in order of peak elution: molecular formula (as generated by the software), selected ion (for mass detection), calculated and observed mass-to-charge ratio and the relative experimental error, MS/MS and MS^3 fragment ion, UV absorption maxima (from DAD), proposed structure for the compounds detected, along with their presence in the different extracts. Molecular formulae were also calculated by the Composition Formula Predictor software (Shimadzu), setting a low tolerance (since the average error in mass accuracy was below 3 ppm), so that most of the identified compounds were in position 1 (hit) in the list of possible candidates. When necessary, as for the structural elucidation of isomers, fragment ions were also included in the search.

Identification of the compounds summarized in Table 1 will be discussed following the order of their retention times, except for peaks 1 and 2 (gentisic acid and 3,4-dihydroxy-phenylacetic acid), whose identification was carried out on the basis of previous considerations, and will be discussed at the end of this section. Peaks 3 and 8 (found in all the tested samples) had similar spectral features, both showing only one absorption maximum around 272 nm, and both giving only the protonated parent ion $[M+H]^+$. These two compounds in fact belong to the same chemical class, *i.e.* they are methylxanthines. The first, with a t_R of 19 min, was identified as theobromine, and detected as $[M+H]^+$ ion at m/z 181.0726; it was previously described in extracts obtained from sonication of mate leaves, analyzed by GC–MS [14]. The second, with a t_R of 28 min, was identified as caffeine, and detected as $[M+H]^+$ ion at m/z 195.0889. The only difference in their chemical structures consists in the methyl substitution at the N − 1 atom in caffeine (1,3,7-trimethylpurine-2,6-dione hydrate); on the other hand, the presence of the unsubstituted NH– in theobromine (3,7-dimethylpurine-2,6-dione) confers higher polarity and, as a consequence, decreases the degree of interaction with the C18 stationary phase. Caffeine was also detected in mate, through GC–MS analysis of extracts obtained from sonication and maceration of dried leaves [14]. Streit et al. also reported caffeine in infusion extracts characterized by LC–UV [10], while Bravo et al. detected this methylxanthine through LC–MS analysis of the acid–methanol/acetone extract of mate dried leaves [11].

Peaks 4, 7, 10, and 11 all showed the same $[M-H]^-$ parent ion at an average m/z value of 353, a secondary MS^2 ion at m/z 179 (caffeic acid) and identical UV spectra, typical of caffeic acid derivatives, with a maximum at 325 nm and a shoulder at 298 nm. They were detected in all the samples tested, and assigned as structural isomers of chlorogenic acid. Other authors already described these monoacylchlorogenic acids as the main constituents of samples obtained by infusion [10,12,13] or acid–methanol/acetone extraction of mate leaves [11]. Distinction between the four isomers, differing in their chemical structure by the position of the caffeoyl residue on the quinic acid ring, was performed considering their retention times and fragmentation pattern in negative ionization mode; structure elucidation was further supported by comparison with the literature [33]. Interestingly, three out of four compounds, *i.e.* peaks 4, 7, and 11, all gave m/z 191 (quinic acid) as MS^2 base peak,

while *m/z* 173 (dehydrated) was the most intense MS^2 fragment ion for peak 10, with *m/z* 191 as secondary peak. The MS^2 spectrum of compound 4 presented *m/z* 135 as secondary ion, a decarboxylation product of *m/z* 179 (caffeic acid), while a fragment at *m/z* 161 was detected for compound 7, as a result of loss of water from *m/z* 179; the product of dehydration of quinic acid was detected as a minor MS^2 fragment of compound 11 (*m/z* 173). Further fragmentation resulted in MS^3 ions at *m/z* 85 (from the MS^2 precursor at *m/z* 191) and at *m/z* 93 (from the MS^2 precursor at *m/z* 173); they both may result from fragmentation of the quinic acid moiety, because they were also detected in feruloylquinic acid isomers, as discussed later on. Minor MS^3 ions at *m/z* 172 and 127 may arise from deprotonation, and subsequent decarboxylation of *m/z* 173. On the basis of these data, as well as the retention order already reported [11], compounds 4, 7, 10, and 11 can be assigned as neo-chlorogenic acid (3-CQA), chlorogenic acid (5-CQA), crypto-chlorogenic acid (4-CQA) and caffeoylquinic acid (1-CQA), respectively.

Peaks 5 and 9 were identified as two isomers of dicaffeic acid, on the basis of their spectra and MS fragments. Dimers of caffeic acid have already been reported in the acid–methanol/acetone extract of mate dried leaves, characterized by LC–MS [11]; however, identification could not be achieved due to incomplete chromatographic resolution and lack of data on the fragmentation pattern. The UV spectra of peaks 5 and 9 showed a λ_{max} at 324 nm, which is characteristic of caffeic acid, and a shoulder at 298 nm, commonly detected in quinic acid derivatives. In terms of MS detection, both compounds generated $[M-H]^-$ ions at *m/z* 341, although their fragmentation patterns were different. In fact, the $[M-H]^-$ ion at *m/z* 341.0649 (peak 5) generated an ion at *m/z* 281 as MS^2 base peak, while peak 9, whose $[M-H]^-$ ion was detected at *m/z* 341.0669, gave an ion at *m/z* 251 as the most abundant fragment. The fragment at *m/z* 281 is likely to arise from the loss of an acetic acid (CH_3COO-) group from the parent ion, suggesting that at least one of the two cinnamoyl (–CH=CHCOOH) substitutes of the phenyl ring is not involved in the formation of the dimer structure. The fragment at *m/z* 251 corresponds to the loss of a 3,4-dihydroxyphenyl ring (hydrated ion), and, hence, it may hypothesized as follows: both dimers derive from the condensation of two caffeic acid molecules, and consequent dehydration. Caffeic acid is, in fact, detected as secondary MS^2 ion (*m/z* 179) from the fragmentation of both isomers. We propose, for compound 5, the existence of an ester-like bond between the carboxylic function of one caffeic acid molecule, and one of the two hydroxyl groups of the catechol ring of a second unit (most probably, the sterically favoured one in 4-*p*). On the other hand, compound 9 might consist of an anhydride, formed through condensation of two cinnamoyl moieties; such a hypothesis would be in accordance with the longer retention time (28 min) observed for this isomer, due to a stronger interaction with the octadecylsilica stationary phase, as a consequence of its lower polarity with respect to peak 5 (t_R less than 26 min). These two isomers were detected only in the samples obtained by infusion and UAE.

Peak 6 showed an UV maximum at 314 nm (similar to that of *p*-coumaric acid) and a $[M-H]^-$ parent ion at *m/z* 337.0924, with only a secondary MS^2 ion at *m/z* 163 (*p*-coumaric acid). This compound, identified only in the infusion and UAE samples, has already been reported [11] and characterized as coumaroylquinic acid.

Peaks 12–14, with t_R of approx. 32 min, were characterized by an $[M-H]^-$ ion at an average of *m/z* 367 and by UV spectra very similar to those of compounds 4, 7, 10 and 11, with a λ_{max} at 324 nm, which is characteristic of caffeic acid, and with a shoulder at 298 nm, characteristic of quinic acid derivatives; such a behaviour has already been described in the LC–MS analysis of acid–methanol/acetone extracts of mate leaves [11]. Comparing retention times reported in the literature [11], MS^2 and MS^3 fragments [33], the following peak assignment was performed: peak 12 (deprotonated parent ion at *m/z* 367.1036, MS^2 base peak at 193, MS^3 base peak at 134) was identified as 3-feruloylquinic acid (3-FQA); peak 13 (deprotonated parent ion at *m/z* 367.1044, MS^2 base peak at 191, MS^3 base peak at 85) was identified as 5-feruloylquinic acid (5-FQA); peak 14 (deprotonated parent ion at *m/z* 367.1032, MS^2 base peak at 173, MS^3 base peak at 93) was identified as 4-feruloylquinic acid (4-FQA).

Peaks 15–17 were identified as flavonoids; more precisely, the first two compounds result from glycosylation of the flavonol quercetin at different positions, resulting in quite dissimilar UV spectral features. Peak 15 shows UV maxima at 254 and 354 nm, is detected with an $[M-H]^-$ ion at *m/z* 609.1458, and further generates an MS^2 peak at *m/z* 301, corresponding to the aglycone quercetin. The latter compound was identified as rutin, as also reported in the literature [11–13]. Peak 16, characterized by UV maxima at 256 and 344 nm, presented an $[M-H]^-$ ion at *m/z* 463.0886, and further gave an ion at *m/z* 301 as MS^2 peak, as well. This compound, identified as quercetin-3-O-glycoside, has already been described as a mate constituent [11]. Peak 17, which presented UV maxima at 266 and 344 nm, and was detected only as an $[M-H]^-$ ion at *m/z* 447.0923, was identified as kaempferol-3-O-glycoside [11].

Peaks 18–20, and 22 were positively identified as dicaffeoylquinic acid congeners (di-CQA), with deprotonated parent ions $[M-H]^-$ at *m/z* 515, and common spectral characteristics of caffeic acid derivatives with λ_{max} at 327 nm, and a shoulder at 296 nm. Together with the corresponding monoacylderivatives, *i.e.* the caffeoylquinic acid isomers identified as peaks 4, 7, 10, and 11, they represent the major components of the mate extracts [11–13]. The MS^2 spectra, of the dicaffeoylquinic acid congeners, were dominated by a strong fragment ion at *m/z* 353, corresponding to the loss of one caffeoyl group [M-caffeoyl-H_2O]$^-$; a secondary ion at *m/z* 335, resulting from further dehydration, was detected for peak 18. Abundant MS^3 fragmentation occurred, generating base peaks at *m/z* 173 (dehydration product of quinic acid) for compounds 18 and 20, at *m/z* 191 (quinic acid) and at *m/z* 179 (caffeic acid) for compounds 19 and 22, respectively. Secondary MS^3 ions at *m/z* 135, 93 and 85 were also detected, as for the chlorogenic- and feruloylquinic acid congeners, already described. In agreement with data found in the literature [33], peak 18 was identified as 3,4-dicaffeoylquinic acid (3,4-di-FQA), peak 19 as 3,5-dicaffeoylquinic acid (3,5-di-FQA), peak 20 as 4,5-dicaffeoylquinic acid (4,5-di-FQA), and peak 22 as 1,5-dicaffeoylquinic acid (1,5-di-FQA).

In the last part of the chromatogram, five minor components elute at a retention time of 40 min, all showing UV spectra similar to caffeic or chlorogenic acids, *i.e.* with UV maximum at 327 nm and a shoulder at 238 nm. Among these, four compounds had identical MS spectra in the negative ion mode, with $[M-H]^-$ ions at *m/z* 529, namely peaks 21, 24, 25, and 26. However, further fragmentation followed distinctive patterns, allowing a tentative structural assignment, also on the basis of the existing literature [11,33].

For peak 24, the precursor ion at 529.1321 gave a strong MS^2 fragment at *m/z* 367, which originates from the loss of one dehydrated molecule of caffeic acid [M-caffeic acid-H_2O]$^-$, as observed before for feruloylquinic acids, indicating the loss of the caffeoyl residue prior to the feruloyl residue. Weaker fragments at *m/z* 353 and 335 were detected as well, resulting from the loss of the feruloyl residue, and further dehydration. Considering MS^3, the base peak at *m/z* 173 [quinic acid-H_2O]$^-$ is accompanied by a weaker ion at *m/z* 179 [caffeic acid-H^+]$^-$. Compound 24 was identified as 3C, 4F-caffeoylferuloylquinic acid (3C, 4F-QA). Peak 25 (*m/z* 529.1334) yielded the same fragment at *m/z* 367 as MS^2 base peak, and one secondary ion; however, the absence of the MS^3 base peak at *m/z* 173 reveals the lacking of a substituent at position 4. Assignment of this compound as 3F, 5C-caffeoylferuloylquinic acid (3F, 5C-QA) was supported by the observation of [ferulic acid-H^+]$^-$ as MS^3 base peak at *m/z* 193, a common feature with 3-FQA (assigned as peak 12).

In contrast, the MS^2 spectra of peaks 21 and 26 gave an *m/z* 353 ion as base peak, indicating the loss of their feruloyl residue prior to caffeoyl; according to the information previously reported, these two compounds could be assigned as (3F, 4C)-, (3C, 5F)-, or 4C, 5F-caffeoylferuloylquinic acids. However, the presence of an *m/z* 173 ion as base peak in their MS^3 spectra, clearly define these two compounds as 4-substituted diacylchlorogenic acids, namely 3F, 4C-caffeoylferuloylquinic and 4C, 5F-caffeoylferuloylquinic acid.

Peak 23 was detected as an $[M-H]^-$ ion at *m/z* 559.1146, with a fragment ion at *m/z* 397 as MS^2 base peak, and an MS^3 spectrum dominated by a strong ion at *m/z* 179. This compound was identified as caffeoylsinapylquinic acid (di-CQA) [11], losing coumaric acid as previously described in the LC–MS analysis of acid–methanol/acetone extracts of mate leaves.

Compounds 1 and 2 were found only in the extract obtained by UAE, and detected as $[M-H]^-$ ions at *m/z* 153.0191 and 167.0346 (no fragment ions). In terms of UV spectra, a single absorption maximum at 263 nm for compound 1 and 277 nm for compound 2 were observed. On the basis of their spectral characteristics, and retention behaviour, a tentative identification was made by spiking the extract sample with pure phenolic acid standards, considering those compounds that, due to their polarity, were poorly retained on the C18 stationary phase (in particular, those eluting before theobromine previously identified as peak 3). On the basis of these information, peaks 1 and 2 were identified as gentisic and 3,4-dihydroxy-phenylacetic acid, respectively. To the best of our knowledge, these two compounds have never been reported before, as mate constituents.

4. Conclusions

The chromatographic methods optimized in the present research allowed the complete resolution and full characterization of polyphenols and xanthines in mate extracts. A total of 26 compounds were detected and identified through LC-DAD-MS and LC × LC-DAD-MS analysis of the UAE extract obtained from the dried leaves. The majority of components were represented by hydroxycinnamoyl quinic acids (80%), while methylxanthines and flavonol glycosides accounted for the rest. Remarkably, the high efficiency of the shell-packed C18 stationary phase offered comparable resolving power to that of the comprehensive RPLC × RPLC system used in this study.

The employment of the hybrid (IT-TOF) mass spectrometer allowed the structural assignment of a series of chlorogenic acids, together with their monoacyl- and diacyl esters, as reported in Table 1. Identification was achieved on the basis of the complementary information obtained from LC-DAD and sequential $MS–MS^3$ data.

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