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Analysis of *Camellia sinensis* green and black teas via ultra high performance liquid chromatography assisted by liquid–liquid partition and two-dimensional liquid chromatography (size exclusion × reversed phase)

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ABSTRACT

Green and black teas (*Camellia sinensis*) contain compounds ranging from simple phenolics to complex glycosides, many of which have well-recognized health benefits. Here, we describe two methodologies aiming to achieve a comprehensive analysis of hydro-alcoholic extracts of *C. sinensis*. In the first step, the extracts were partitioned in water, *n*-butanol, ethyl acetate and chloroform to separate the compounds according to their polarity, yielding less complex samples to be analyzed by ultra high performance liquid chromatography coupled with mass spectrometry (UHPLC–MS). Additionally, a comprehensive two dimensional liquid chromatography (2D-LC) technique, employing size exclusion chromatography (SEC) × reversed phase (BEH-C18) was developed. The following compounds were identified on the basis of retention time, UV-spectra and MS fragmentation patterns: catechins, theaflavins and their gallate derivatives; kaempferol, quercetin and myricetin mono-, di-, tri- and tetraglycosides; esters of quinic acid and gallic or hydroxycinnamic acids; purine alkaloids, such as caffeine and theobromine and many lipids. Additionally, there were many novel compounds that were previously undescribed, such as saponin isomers and gallic acid esters of four glycosides of myricetin, quercetin and kaempferol.

1. Introduction

Plant extracts are complex mixtures containing a great variety of compounds in different amounts. Conventional chromatographic techniques, such as high performance liquid chromatography (HPLC), used to analyze plant extracts are challenging. However, in recent years, a comprehensive two-dimensional liquid chromatography (2D-LC) has been exploited as a powerful analytical technique to study complex matrices. 2D-LC has a much greater resolving power compared to a single-dimensional LC. In the heart-cutting LC × LC system, only selected fractions from the first column are applied to the second dimension, whereas in a comprehensive LC × LC system, all the fractions over the first dimension are continuously sampled and transferred to the second dimension [1–3].

Comprehensive two-dimensional systems have been employed in the analysis of several natural compounds, such as polymers, organic acids and aromatics [4–8]. Typically, an LC × LC system combines two columns with different separation mechanisms in order to achieve better separation, identification and quantification. Good resolution of compounds prior to MS detection improves the reliability of the MS results by decreasing matrix suppression. In any LC × LC combination, the speed of the second dimension determines the overall analytical time. This speed should be as fast as possible, with good resolution. To achieve a fast speed, short columns packed with small particles or monolithic columns are utilized [2,3]. Thus, a combination of HPLC with ultra performance liquid chromatography (UHPLC) offers a good configuration for a comprehensive 2D system. For example, the separation power of ultra high performance liquid chromatography and comprehensive two-dimensional liquid chromatography of phenolic compounds in beverages were analyzed, and comprehensive 2D-LC proved to give clearly higher peak capacities compared with 1D HPLC and UHPLC analyses. Although the use of HPLC monolithic columns in the second dimension provided good results, employing UHPLC columns with gradient system was satisfactory in the analysis of selected phenolic compounds [9].

In another study, a high speed second dimension comprehensive LC × LC system allowed for the separation of components contained in *Stevia rebaudiana* extracts. Specifically, 10 glycosides were separated and identified from these extracts [10]. Also, in a recent study an ultra rapid chromatography was used in the second dimension

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for a comprehensive LC × LC method, which has been successfully used in the separation of complex degraded samples of alprazolam tablets. The combination of SB-CN and a C18 stationary phase provided enough orthogonality to build a 2D system. This UHPLC system has proven to be a promising tool for 2D separations, mainly in the second dimension where shorter analysis times are critical [11].

Historically, teas from *Camellia sinensis* have provoked much scientific interest concerning their biological effects and chemical composition [12–14]. The difference between the components in many tea preparations depends mainly on processing and growth/harvest conditions. Thus, teas are generally classified by the manufacturing process into the following four categories: unfermented (white and green), fermented (black), semi-fermented (oolong) and post-fermented (pu-erh tea, dark tea and related products) [15,16]. Even today, the chemical composition of *C. sinensis* has been explored, and novel compounds are continuously discovered [16].

There are numerous studies about the composition of *C. sinensis* demonstrating that teas contain purine alkaloids (xanthines), phenolic compounds (catechins, *O*-glycosylated flavonols, *C*-glycosylated flavones, proanthocyanidins and phenolic acids and their derivatives), terpenoids, fatty acids, essential oils and amino acids [17–23]. Oolong and black teas also contain the oxidation products of catechins, theaflavins and polymeric thearubigins [22,23].

Considering the great number of compounds, green and black *C. sinensis* teas provide a good model for the development of novel analytical approaches. Herein, we present the application of a comprehensive 2D-LC with real phytochemical samples, using an HPLC × UHPLC system that employs size exclusion chromatography (SEC) as a first dimension followed by reversed phase (BEH-C18) as a second.

2. Experimental

2.1. Chemicals

The following HPLC-grade solvents were purchased from Tedia: methanol, formic acid, chloroform, ethyl-acetate, *n*-butanol and acetonitrile. MilliQ (Millipore) water was used for deionized water. Standards of catechin, flavone, chlorogengic acid, caffeic acid, apigenin, theobromine, rutin, caffeine, myricetin, kaempferol and gallic acid were obtained from Sigma. All solvents and samples were filtered through a 0.22 μm membrane prior to chromatography.

2.2. Samples, extraction and fractionation

The samples (green and black teas) were purchased in a local market (Curitiba, State of Paraná, Brazil) as commercially processed leaves. Samples (15 g of each) were extracted by refluxing in 300 ml of a hydro-alcoholic solution (ethanol, 70%) for 1 h (3 times). The extracts were combined to give the samples G70 (green tea) and B70 (black tea), which were evaporated under reduced pressure, lyophilized and stored in a freezer at −20 °C.

A portion of each sample (400 mg) was subjected to liquid/liquid partitioning as follows: the samples were dissolved in H_2O (10 ml), $CHCl_3$ (10 ml) was then added and the mixture was vigorously stirred. The organic layer was removed to yield fractions G70C and B70C. Some insoluble matter was retained in the aqueous phase, to which ethyl acetate (10 ml) was added and stirred. Similarly, the organic layer was removed to yield fractions G70EAc and B70EAc. Subsequently, *n*-BuOH (10 ml) was added to the aqueous phase, stirred and the layers separated to give fractions G70Aq, G70B, B70Aq and B70B. The fractions were dried and stored in freezer.

2.3. Instrumentation

2.3.1. Single-dimensional UHPLC analysis

Ultra high performance LC consisted in a Waters ACQUITY UPLC™ System (Waters, Milford, MA) equipped with a binary solvent pump, column oven, auto-sampler, a photodiode array detector (PDA) and evaporative light scattering (ELSD) was used. An Acquity BEH-C18™ column, with 50 mm × 2.1 mm i.d. and 1.7 μm particle size (Waters), was employed. The separation occurred at 60 °C (column temperature) using a gradient of 0.1% formic acid (solvent A) and MeOH (solvent B). The following gradient was used for separation at flow rate of 0.4 ml min^{-1}: a linear increase of solvent B from 0 to 80% over 12 min, 80% to 100% B over the next 2 min (14 min) and held at 100% B for an additional minute (15 min). After returning to the initial conditions (0% B at 16 min), the system was re-equilibrated for an additional 2 min.

The fractions from the liquid/liquid partition were prepared in MeOH–H_2O (1:1, v/v) at 1 mg ml^{-1}. The injection volume was 10 μl and detection was provided by ELSD, PDA (200–400 nm) and by mass spectrometry (*m*/*z* 100–1600).

2.3.2. Off-line 2D-LC method SEC × LC

The first dimension was carried out using an HPLC LC10A (Shimadzu) equipped with a SEC-column Ultrahydrogel-120, with 300 mm × 7.8 mm and 5×10^3 Da size exclusion (Waters). The separation was developed in a gradient mode using H_2O (solvent A) and acetonitrile (solvent B), at a flow rate of 1 ml min^{-1} at 60 °C. The linear increase of solvent B was from 0 to 50% over 40 min, held until 50 min and then B returned to the initial condition at 60 min. Subsequently, the column was reconditioned with solvent A for 10 min. The crude samples, each at 50 mg ml^{-1}, were prepared in MeOH–H_2O (1:1, v/v) and the injection volume was 200 μl. Aliquots of 1 ml were collected over the entire running time, dried under N_2 stream using a Reacti-Vap evaporator (Thermo Scientific) and then transferred to UHPLC vials with 0.2 ml of MeOH–H_2O (1:1, v/v).

The second dimension was carried out using a UHPLC system equipped with a reversed phase (RP) BEH-C18 column as described above. The solvents were the same [formic acid 0.1% (A) and MeOH (B)], but the separation conditions were different. The flow rate was 1 ml min^{-1}, the column temperature was 80 °C and the gradient used was the following: 0% B held for 0.2 min, then 40% B at 4 min and 100% B at 6 min and held for an additional minute (7 min). The gradient returned to the initial condition (0% B in 8 min) and the column was reconditioned for an additional 2 min. The data from the PDA and ELSD detection, in the second dimension, were collected as ASCII files and processed by Sigma-Plot 12.

2.3.3. ESI-MS conditions

Positive and negative ESI-MS were carried out at atmospheric pressure ionization (API) with a triple quadrupole Quattro LC spectrometer (Waters), using N_2 as the nebulizer and desolvation gas. The samples were split post-column and injected into the ESI source at a constant flow rate of 100 μl min^{-1}. The energy parameters for negative and positive detection were the following, respectively: cone 40 V and capillary 2.4 kV; cone 60 V and capillary 2.8 kV with LiCl (5 mM) being added to the solvents. The samples were analyzed in a total ion current (TIC) mode (*m*/*z* 100–1600) and then fragmented using collision induced dissociation (CID) with argon as the collision gas, at energies ranging from 30 to 60 eV.

3. Results and discussion

3.1. Single-dimension UHPLC analysis

C. sinensis can be considered as a model for the development of analytical procedures for phytochemical mixtures. Highly complex

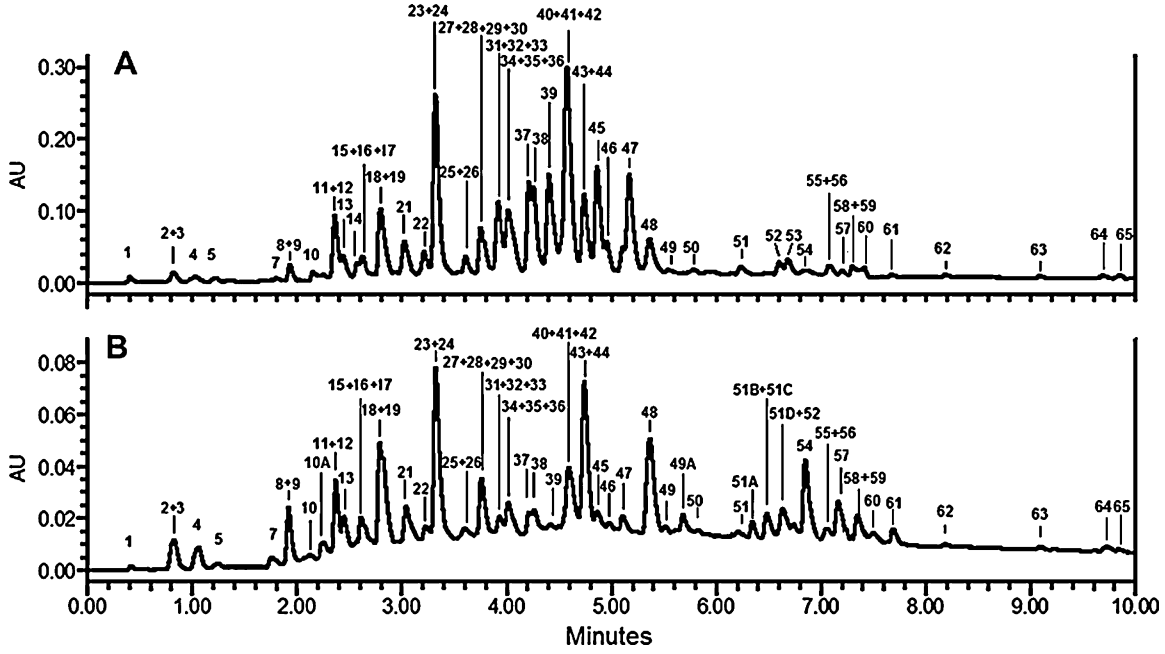

Fig. 1. UHPLC analyses of hydro-alcoholic extracts from green (A) and black teas (B) at PDA 325 nm.

extracts were obtained from green and black tea leaves by refluxing in 70% aq. ethanol. As expected, there was a greater variation in the polarity of the compounds, ranging from carbohydrates to lipids. The total UHPLC run was completed in less than 16 min (Fig. 1A and B), although some peak overlapping occurred. As a result of using a liquid/liquid partition prior to UHPLC analysis, the matrix complexity was reduced, allowing the identification of many constituents. Peak identification was based on retention time (t_R), UV-spectra, and MS fragmentation patterns by comparison with published data [17,22–26]. Compounds are shown in Table 1.

3.1.1. Analysis of green tea fractions

The aqueous phase (G70aq) obtained from the partition of crude hydro-alcoholic extract (G70) retained mainly carbohydrates (di- and trisaccharides), which were not resolved on the C18 column. The following acidic compounds were identified according to previous reports [24,27] (Fig. 2A, Table 1): quinic (peak 1) and its esters, 3- and 5-*p*-coumaroylquinic acid (peaks 13 and 23), 3-and 5-galloylquinic acid (peaks 2 and 4) and 3-,4- and 5-caffeoylquinic acid (peaks 7, 8, 19).

The butanolic fraction (G70B) contained the most flavonoid glycosides. In *C. sinensis*, these compounds are structurally diverse, containing kaempferol, quercetin, myricetin and apigenin as aglycones, that are glycosylated with arabinose, rhamnose, galactose and glucose or with oligosaccharides, which can contain esters of acetic or cinnamic acids [22,23,28–30]. These compounds were observed at λ 325 nm and confirmed by tandem-MS [22,24,31]

With the exception of those glycosides acylated by *p*-coumaric acid, the following compounds are flavonoid glycosides that eluted rapidly (within 3.5–5.5 min): flavonol monoglycosides (peaks 28, 30, 33, 34, 40, 42, 48, 49 50, 51 and 52), diglycosides (peaks 36, 44 and 62), triglycosides (peaks 29, 32, 39, 41, 45, 47, 54, 57, 59, 61 and 63), tetraglycosides (peaks 53, 55, 56 and 60) and some flavone diglycosides (peaks 35 and 37) (Fig. 2B, Table 1). Peak overlapping did occur, making their analysis difficult. Because the *p*-coumaroyl moiety is a non-polar site in the glycoside, the retention times of these flavonoid glycosides were greater than non-acylated glycosides, with t_Rs close to 7 min (Fig. 2B) (peaks 49, 52–57 and 59–63).

Another glycoside class, saponins, was present in fraction G70B. However, these compounds were poorly detected by PDA analysis. Thus, detection was provided by ELSD, and their acidic nature enabled good detection by ESI-MS (Fig. 2C). The saponins were the last compounds in G70B to be eluted from the BEH-C18 column, with t_R values ranging between 10 and 12 min (peaks 66–85, Table 1). The few investigations that have been carried out on saponins provided approximately 10 different structures found in green tea, including isomers containing angeloyl, tigloyl or (*E*)- or (*Z*)-cinnamoyl groups [26]. This substantial variability can cause peak overlapping, making these analyses challenging. Despite these difficulties, at least 20 different saponins were found (Fig. 2, C1–C6). These compounds were similar to previously reported saponins. Specifically, these saponins are novel isomers with different positions of acyl groups.

Four flavonol glycosides retained in the fraction G70B were identified for the first time. These glycosides are composed of myricetin, quercetin and kaempferol linked to a hexose (i.e., galactose or glucose) with a gallate ester (Fig. 2D). Other plants have been reported to contain similar glycosides, such as quercetin 3-*O*-glucosyl-6″-*O*-gallate from *Tellima grandiflora* and *Polygonum hydropiper* L. [32,33]. Because structural chemistry is outside the scope of the present work, we did not isolate these compounds for complete structural characterization. However, their MS spectra contained the following ions: peak 25 (Fig. 2D1) at *m*/*z* 631 $(M-H)^-$ with fragments at *m*/*z* 479 (M−galloyl), 317 (myricetin) and 169 (gallate); peak 38 (Fig. 2D2) with *m*/*z* 615 $(M-H)^-$ and fragments at *m*/*z* 463 (M−galloyl), 301 (quercetin) and 169 (gallate); and peaks 43 and 46 (Fig. 2D3) were similar at *m*/*z* 599 $(M-H)^-$ with fragments at *m*/*z* 447 (M−galloyl), 285 (kaempferol) and 169 (gallate). The structural difference between peaks 43 and 46 can possibly be due to the type of monosaccharide (i.e., glucose or galactose) or the position of linkages.

Flavan-3-ols (catechins) are the major flavonoids previously reported in green tea and are usually detected as gallate ester derivatives. These compounds were concentrated in the ethyl acetate fraction (G70EAc). The main flavan-3-ols found were epigallocatechin gallate (t_R 2.80 min, *m*/*z* 457 $[M-H]^-$) and epicatechin gallate (t_R 3.75 min) (Fig. 2E). Other components, such

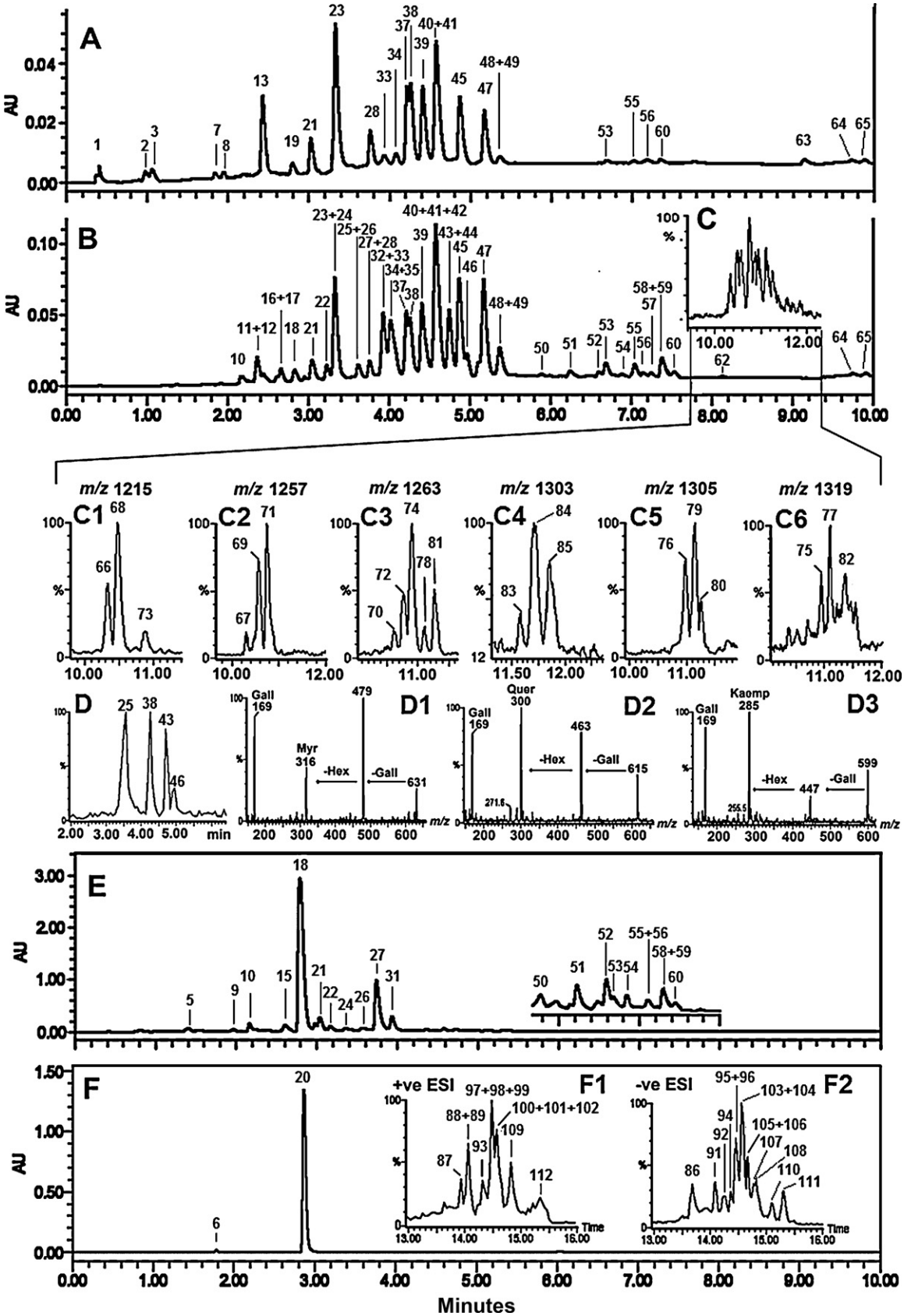

Fig. 2. UHPLC chromatograms of the following fractions of hydro-alcoholic extract from green tea: (A) aqueous fraction at 325 nm and (B) butanolic fraction at 325 nm. (C) Negative ESI-MS of butanolic fraction and extracted ions from saponins (C1–C5). (D) UHPLC–MS of extracted glycosides and their corresponding CID-MS spectra (D1–D3). UHPLC chromatograms of the ethyl acetate fraction (E) at 275 nm and the chloroform fraction (F) at 275 nm. (F1 and F2) represent positive and negative ESI-MS of lipids, respectively. Gall: gallate; Hex: hexose; Kaemp: kaempferol; Myr: myricetin; Quer: querctin.

Table 1
Identification of the compounds from green and black teas on single-dimension UPLC–MS.

Peak	R_t	[M−H]$^-$ (m/z)	Structure	Reference	Peak	R_t	[M−H]$^-$ (m/z)	Structure	Reference
1	0.46	191	Quinic acid	[19]	32	3.87	787	Myr-3-*O*-Hex-Rut	[19]
2	0.82	343	3-Galloylquinic acid	[19]	33	3.90	479	Myr-3-*O*-Gal	[19]
3	0.82	331	Galloyl-Glc	[21]	34	4.02	479	Myr-3-*O*-Glc	[19]
4	1.09	343	5-Galloylquinic acid	[19]	35	4.03	431	Api-*C*-Glc	[20]
5	1.39	305	(+)-Gallocatechin	[21]	36	4.06	625	Myr-3-*O*-Rut	[19]
6[a]	1.78	181	Theobromine	[19]	37	4.24	563	Api-6-*C*-Ara-8-*C*-Glc	[20]
7	1.82	353	3-Caffeoylquinic acid	[19,24]	38	4.25	615	Quer-Galloyl-Hex	–
8	1.91	353	5-Caffeoylquinic acid	[19,24]	39	4.41	771	Quer-3-*O*-Gal-Rut	[19]
9	1.97	761	Gallocatechin-(4–8)epigallocatechin-3′-*O*-gallate	[22]	40	4.58	431	Kaemp-Rha	[19]
10	2.16	305	(−)-Gallocatechin	[19]	41	4.6	771	Quer-3-*O*-Glc-Rut	[19]
10A	2.31	913	Theasinensis A	[22]	42	4.62	463	Quer-3-*O*-Gal	[19]
11	2.33	633	Strictinin	[21]	43	4.72	599	Kaemp-Galloyl-Hex	–
12	2.34	577	Procyanidin dimer	[21]	44	4.74	609	Quer-3-*O*-Rha-Gal	[19]
13	2.45	337	3-*p*-Coumaroylquinic acid	[19,24]	45	4.88	755	Kaemp-3-*O*-Gal-Rut	[19]
14	2.52	483	Digalloyl-Glc	[19]	46	4.96	599	Kaemp-Galloyl-Hex	–
15	2.62	745	Gallocatechin catechingallate	[19]	47	5.18	755	Kaemp-3-*O*-Glc-Rut	[19]
16	2.63	290	(+)-Catechin	[19]	48	5.40	433	Quer-pentoside	[19]
17	2.64	577	Procyanidin dimer	[21]	49	5.42	593	Kaemp-3-*O*-pCA-Glc	[19]
18	2.80	457	(−)-Gallocatechin gallate	[19]	49A	5.72	551	Epitheaflagalline-3-gallate	[20]
19	2.82	353	4-Caffeoylquinic acid	[19,24]	50	5.84	417	Kaemp-Pent	[19]
20[a]	2.95	195	Caffeine	[19]	51	6.26	463	Quer-3-*O*-Glc	[19]
21	3.05	289	(−)-Catechin	[19]	51A	6.36	563	Theflavin	[19]
22	3.22	457	(+)-Gallocatechin Gallate	[19]	51B	6.46	715	Theflavin-3-gallate	[19]
23	3.34	337	5-*p*-coumaroylquinic acid	[19,24]	51C	6.49	715	Theflavin-3′-gallate	[19]
24	3.37	609	Gallocatechin dimer	[21]	51D	6.61	867	Teaflavin-3-3′-digalate	[19]
25	3.56	631	Myr-Galloyl-Hex	–	52	6.62	593	Kaemp 3-*O*-6″-pCA-Glc	[19]
26	3.58	635	Trigalloyl-Glc	[19]	53	6.72	1049	Quer-3-*O*-Glc-Rha-(pCA-Ara)-Hex	[19]
27	3.75	441	(+)(-)-Catechin gallate	[19]	54	6.83	901	Quer-3-*O*-pCA-di Rha-Hex	[19]
28	3.76	447	Kaemp-3-*O*-Gal	[19]	55	7.05	1049	Quer-3-*O*-Glc-Rha-(pCA-Ara)- Hex	[19]
29	3.78	787	Myr-3-*O*-Hex-Rut	[19]	56	7.10	1033	Kaemp-3-*O*-pCA-Ara-Glc-Rha-Gal	[19]
30	3.80	447	Kaemp-3-*O*-Glc	[19]	57	7.18	885	Kaemp-3-*O*-pCA-di Rha-Hex	[19]
31	3.86	609	(−)-Gallocatechin-3.3′-di-*O*-gallate	[20]	58	7.33	1175	Unknown	–
59	7.33	901	Quer-3-*O*-pCA-di Rha-Hex	[19]	86	13.69	555	SQMG (C16)	[35]
60	7.36	1033	Kaemp-3-*O*-pCA-Ara-Glc-Rha-Gal	[19]	87[a]	13.93	813	MGDG (C20:2/C18:2)	–
61	7.68	885	Kaemp-3-*O*-pCA-di Rha-Hex	[19]	88[a]	14.06	797	PC (C18:3/C19)	–
62	8.15	739	Kaemp-3-*O*-pCA-Glc-Rha	[19]	89[a]	14.06	813	MGDG (C20:1/C18:3)	–
63	9.12	781	Kaemp-3-*O*-acetyl-di Rha-Hex	[19]	90	14.08	865	Unknown lipid	–
64	9.71	1170	Unknown	–	91	14.09	825	Unknown lipid	–
65	9.85	1060	Unknown	–	92	14.26	831	PI (C18:3/C16)	–
66	10.34	1215	Floratheasaponin A isomer	[23]	93[a]	14.32	944	DGDG (C18:3/C18:3)	–
67	10.36	1257	Foliatheasaponin I isomer	[23]	94	14.39	809	PI (C16/C16)	–
68	10.50	1215	Floratheasaponin A isomer	[23]	95	14.45	749	PG (C18/C16)	–
69	10.57	1257	Foliatheasaponin I isomer	[23]	96	14.46	837	SQDG (C18:3/C18:3)	[35]
70	10.61	1263	Isotheasaponin B1/B2 isomer	[23]	97[a]	14.48	782	PC (C18:2/C18:2)	–
71	10.75	1257	Foliatheasaponin I isomer	[23]	98[a]	14.48	783	MGDG (C18:3/C18:3)	–
72	10.80	1263	Isotheasaponin B1/B2 isomer	[23]	99[a]	14.50	781	MGDG (C18:3/C18:3)	–
73	10.90	1215	Floratheasaponin A isomer	[23]	100[a]	14.56	922	DGDG (C18:3/C16)	–
74	10.91	1263	Isotheasaponin B1/B2 isomer	[23]	101[a]	14.57	787	PC (C18:2/C18)	–
75	10.95	1319	Assamsaponin J isomer	[23]	102[a]	14.57	789	PC (C18:1/C18)	–
76	10.98	1305	Foliatheasaponin IV isomer	[23]	103	14.56	815	SQDG (C18:3/C16)	[35]
77	11.10	1319	Assamsaponin J isomer	[23]	104	14.56	817	SQDG (C18:2/C16)	[35]
78	11.10	1263	Isotheasaponin B1/B2 isomer	[23]	105	14.66	819	SQDG(C18:1/C16)	[35]
79	11.14	1305	Foliatheasaponin IV isomer	[23]	106	14.66	793	SQDG (C16/C16)	[35]
80	11.24	1305	Foliatheasaponin IV isomer	[23]	107	14.77	793	SQDG (C14/C18)	[35]
81	11.26	1263	Isotheasaponin B1/B2 isomer	[23]	108	14.81	820	Unknown lipid	–
82	11.37	1319	Assamsaponin J isomer	[23]	109[a]	14.83	767	Unknown lipid	–
83	11.58	1303	Isotheasaponin B3 isomer	[23]	110	15.10	627	Unknown lipid	–
84	11.71	1303	Isotheasaponin B3 isomer	[23]	111	15.30	528	Unknown lipid	–
85	11.86	1303	Isotheasaponin B3 isomer	[23]	112[a]	15.33	891	Unknown lipid	–

Kaemp: kaempferol; Myr: myricetin; Quer: quercetin; pCA: *p*-coumaroyl; Ara: arabinose; Gal: galactose; Glc: glucose; Hex: hexoside; Pent: pentoside; Rha: rhamnose; Rut: rutinoside. DGDG: digalactosyldiacylglycerol; MGDG: monogalactosyldiacylglycerol; PC: phosphatidylcholine; PG: phosphatidylglycerol; PI: phosphatidylinositol; SQDG: sulfoquinovosyldiacylglycerol; SQMG: sulfoquinovosylmonoacylglycerol.

[a] Identified in positive ESI-MS.

as gallocatechin, epigallocatechin, catechin, epicatechin and gallocatechin gallate (peaks 5, 10, 16, 21 and 22) were also found, but in smaller amounts, which is consistent with previous reports [17,24,34,35]. In addition to these compounds, condensed tannins (mainly dimers) were also found, which were identified on the basis of tandem-MS (Table 1) by comparison with previously reported data [36].

The chloroform fraction (G70C) from green tea was the last to be examined. At a first glance of the PDA detection (Fig. 2F), the chromatogram exhibited two peaks at t_R values of 1.78 and 2.95 min, which were identified as theobromine and caffeine, respectively (peaks 6 and 20), the most common purine alkaloids found in *C. sinensis* [17,22,24,37]. However, any lipid could be concentrated in this fraction. Therefore, the fraction was examined using

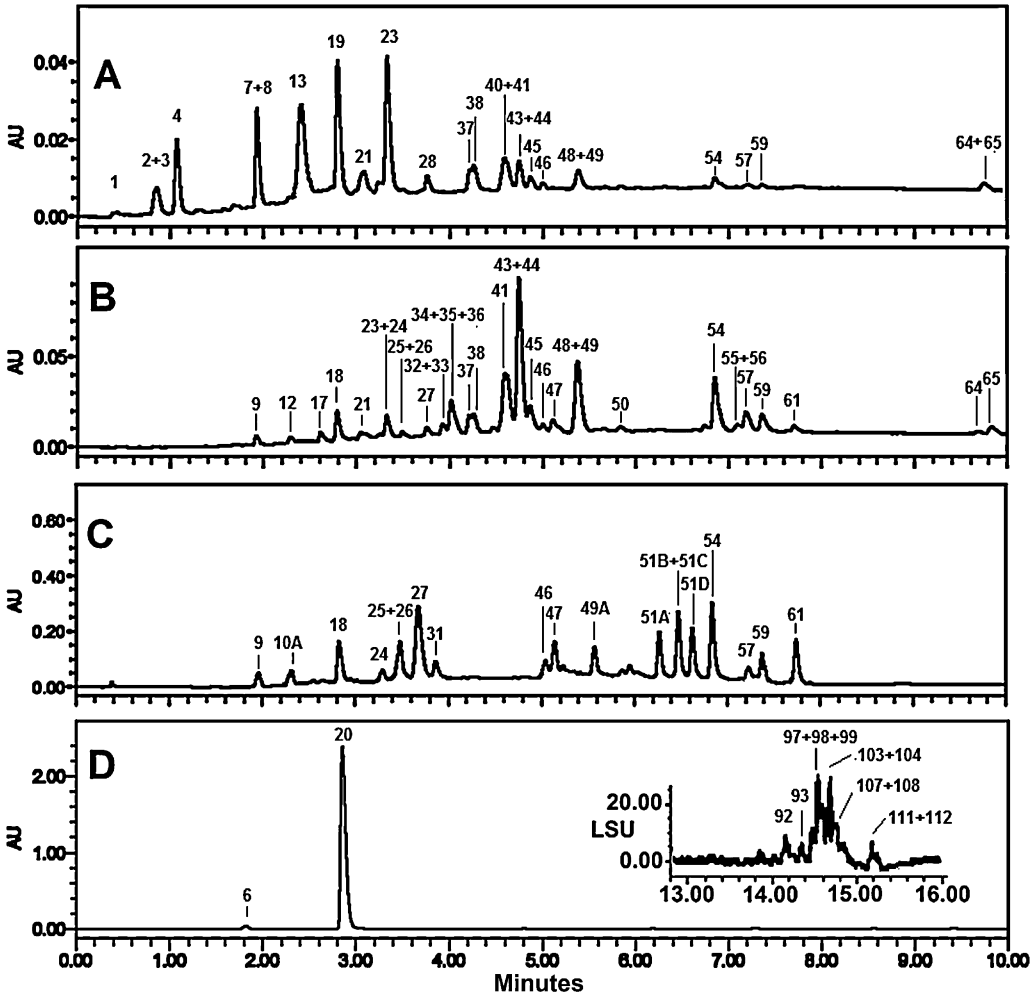

Fig. 3. UHPLC chromatograms of the following fractions of black tea hydro-alcoholic extract: (A) aqueous phase at 325 nm, (B) butanolic phase at 325 nm, (C) ethyl acetate phase at 275 nm, and (D) chloroform phase at 275 nm and lipids at ELSD (inserted).

positive and negative ESI-MS and ELSD detection, revealing a series of phospholipids and glycolipids (peaks 86–112) (Fig. 2F1 and F2). Using positive ion detection, the main lipids identified were phosphatidylcholine (PC, peaks 88, 97, 101 and 102) and the glycolipids monogalactosyldiacylglycerol (MGDG, peaks 87, 89, 98 and 99) and digalactosyldiacylglycerol (DGDG, peaks 93 and 100), which appeared as Li^+ adducts (Table 1). Using negative ion detection, a main glycolipid appeared at *m*/*z* 815 and was further confirmed via tandem-MS as a sulfoquinovosyldiacylglycerol (SQDG, peak 103) esterified by palmitic and linolenic acids. Other SQDG lipids were identified with different fatty acid combinations (peaks 96, 104–107) [38]. Phosphatidylglycerol (PG, peak 95) and phosphatidylinositol (PI, peaks 92 and 94) were the major phospholipids found using the negative UHPLC–MS detection. All the lipids detected contained many fatty acid combinations, mainly with palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids [38].

3.1.2. Analysis of black tea fractions

Similar to green tea, the fractionation of black tea provided good separation of compounds based on their polarity. In the aqueous fraction (B70Aq), the main compounds identified were quinic acid (peak 1) and their esters, such as 3- and 5-galloylquinic acid (peaks 2 and 4, respectively), 3- and 5-*p*-coumaroylquinic acid (peaks 13 and 23) (Fig. 3A) and 3-, 4- and 5-caffeoylquinic acid (peaks 7, 8 and 19).

Flavonoid glycosides concentrated in the B70B fraction were smaller components than those found in green tea, appearing as peaks 39, 41, 45 and 47. However, flavonoid glycosides containing esters with a *p*-coumaroyl moiety were more evident in black tea when compared with green tea (peaks 54, 57, 59, 61 and 62, Fig. 3B). These flavonoid glycosides are mainly composed of quercetin or kaempferol attached to different oligosaccharides from those found in glycosides lacking a *p*-coumaroyl group. These compounds were identified as quercetin-3-*O*-*p*-coumaroyldirhamnosylhexoside (peaks 54 and 59), kaempferol-3-*O*-*p*-coumaroyl dirhamnosylhexoside (peaks 57 and 61), kaempferol-3-*O*-*p*-coumaroylglucosylrhamnoside (peak 62) and kaempferol-3-*O*-acetyl-dirhamnosylhexoside (peak 63), which have all been previously reported [22,24]. Furthermore, in contrast to green tea (G70B), this fraction did not contain saponins.

A considerable decrease in flavan-3-ol content occurs in black tea as a result of enzyme activation (i.e., polyphenoloxidase) rather than catechin condensation products, such as theaflavins and thearubigins [39–42]. Here, the following theaflavins were highly concentrated in the ethyl acetate fraction (B70EAc), appearing on the RP-UHPLC between 6 and 7 min (Fig. 3C): theasinensis A, epitheaflagalline-3-gallate, theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate and theaflavin-3-3′-digallate (peaks 10A, 49A, 51A, 51B, 51C and 51D). However, although previously reported in the black tea, the thearubugins were not detected.

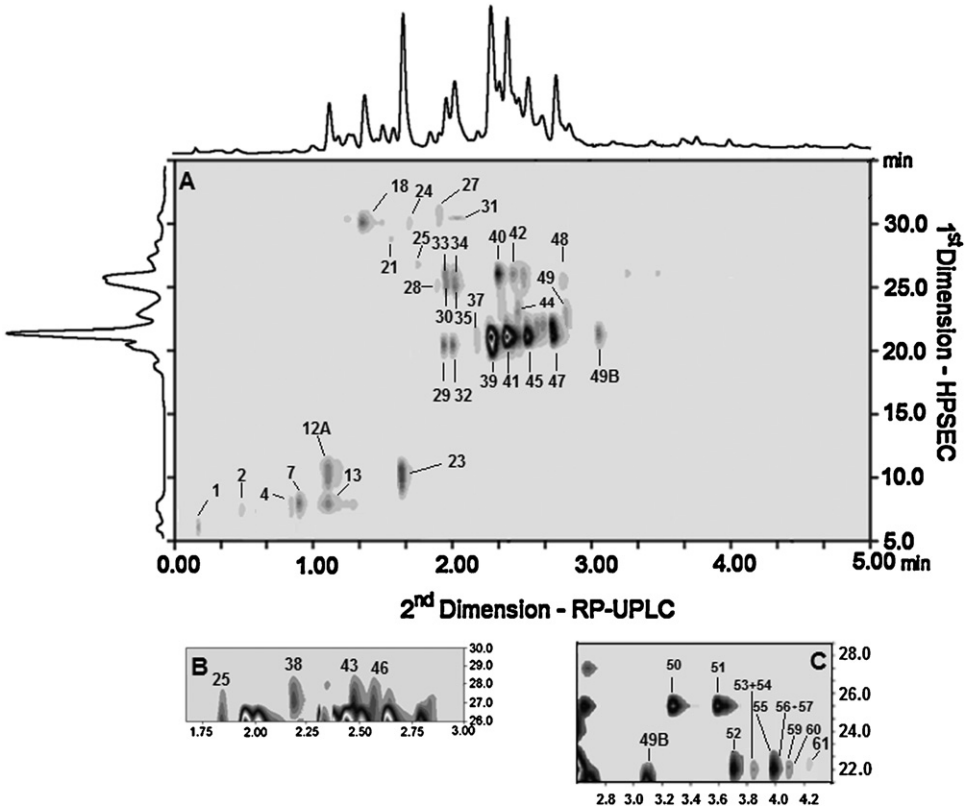

Fig. 4. (A) 2D-chromatogram (SEC × LC) of green tea hydro-alcoholic extract obtained at 325 nm showing mainly the phenolic compounds. (B) Region of peaks 25, 38, 43 and 46, stated as novel glycosides; and (C) the region of minor components.

The chloroform fraction (B70C) concentrated caffeine and theobromine, which appeared at 1.78 and 2.95 min (peaks 6 and 20) on the UHPLC-PDA (Fig. 3D). Examination by ELSD and MS detection revealed lipid contents in smaller amounts than those found in the green tea fraction G70C (Fig. 3D).

Some peaks co-eluted with each other, impairing their analysis (i.e., peaks 2 and 3; peaks 15, 16 and 17; peaks 18 and 19; peaks 27, 28, 29 and 30, peaks 40, 41 and 42 and many others). Peak overlapping associated with MS ion suppression caused by the matrix components is a common problem in phytochemical samples, resulting in serious problems when performing quantitative analyses. In an attempt to overcome these challenges, comprehensive two-dimensional liquid chromatography was applied.

3.2. Comprehensive two-dimensional LC method

Two-dimensional liquid chromatography is considered to be comprehensive when the entire effluent from the first separation, and not just a selected region, is subjected to further separation in a second chromatographic system [5,6,10]. The chromatogram can be plotted as a contour graph to display the components of the entire sample. This graph can be used to create a map or a fingerprint that covers different experimental conditions, such as different sample processing methodologies, which occurs in the preparation of green and black teas.

Considering the complexity of the matrix, we developed an offline 2D system (SEC × LC), combining HPLC × UHPLC. This

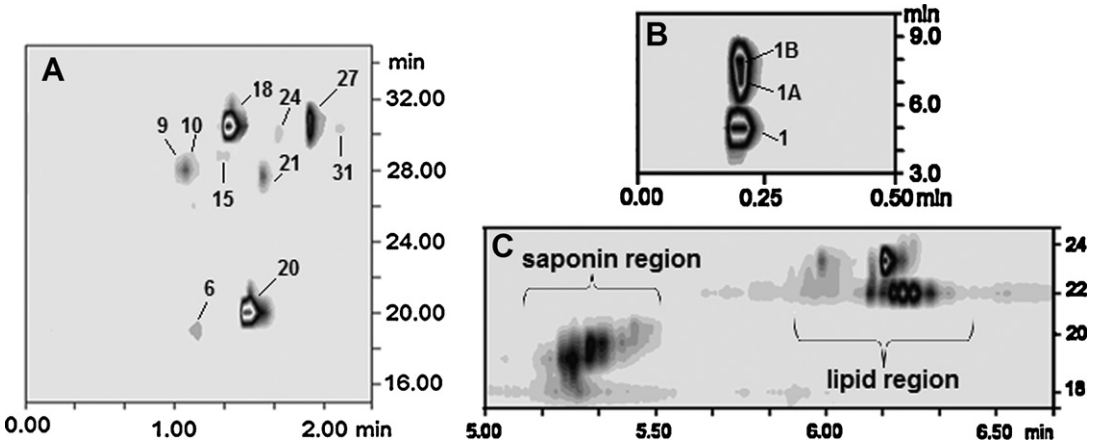

Fig. 5. (A) 2D-chromatogram (SEC × LC) of green tea hydro-alcoholic extract at 275 nm, showing mainly flanvan-3-ols and xanthines; and partial 2D-chromatograms obtained with ELSD of: (B) quinic acid and oligosaccharides and (C) saponins and lipids.

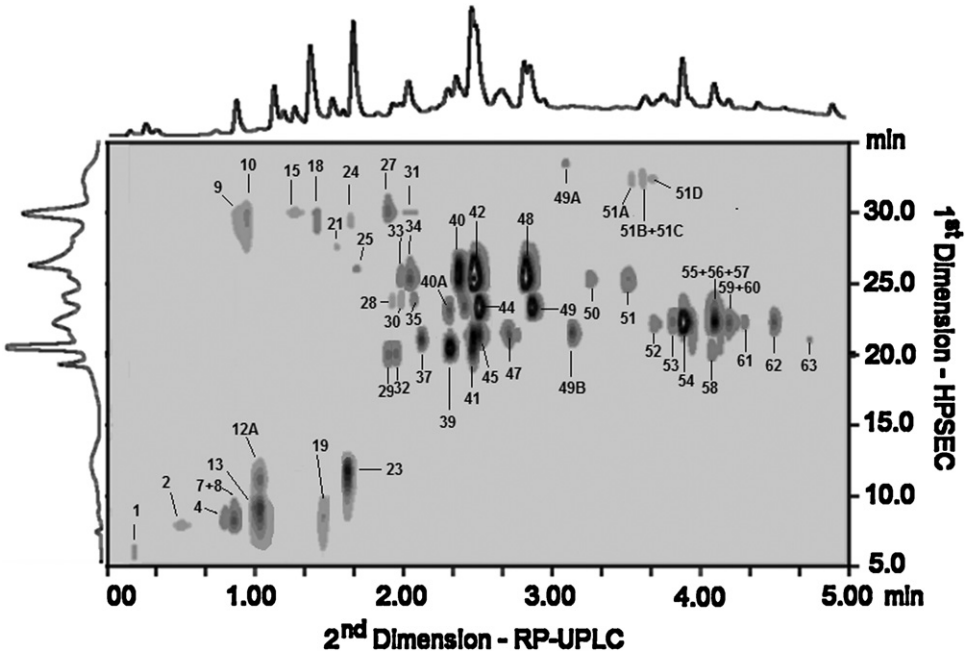

Fig. 6. 2D SEC × LC plot of black tea hydro-alcoholic extract obtained at 325 nm.

unusual application of SEC column (Ultrahydrogel 120 – Waters) was driven by our previous experience, in which many compounds from a plant extract (*Maytenus ilicifolia*), namely flavoinoids and their glycosides were well separated by SEC column [43].

3.2.1. Analysis of C. sinensis extracts

The higher peak capability and, consequently, the ability to resolve a more complex matrix, is the major benefit of the two-dimensional LC employed in the analysis of *C. sinensis* components. Thus, compounds that were not distinguished in a single-dimension could be separated and identified by 2D-LC analysis, making the results more reliable. Also, the chromatograms plotted in 2D-graphs allow for correlating the position of each compound as a function of two retention times, minimizing matrix interferences and misinterpretations.

Despite the many different types of compounds in crude extracts of green and black teas, as well as their low abundance, SEC analyses provided good results. As expected, the compounds were eluted mainly in the order of decreasing molecular weight, which allowed compounds that co-eluted in the single-dimensional analysis (UHPLC) to be distinguished and well resolved in two-dimensional analysis (i.e., peaks 18 and 19; 27, 28, 29 and 30; and peaks 40, 41 and 42). Flavonoids and their corresponding glycosides had notable interactions with the SEC column matrix (Figs. 4A–C and 5A). This was first reported for flavonol glycosides from *M. ilicifolia* in which the SEC column from Waters was capable of distinguishing between many compounds, including isomers [43].

However, carbohydrates such as di- and trisaccharides observed as lithiated ions at *m*/*z* 349 and 511, respectively, were eluted during the first 10 min (Fig. 5B). Regardless of their molecular mass, many other compounds had longer t_R values. The basis for SEC separation is not clear. Compound polarizability and, consequently, their solubility in the separation solvent must be considered as an important factor to describe the SEC separation, considering it was developed using a water/acetonitrile gradient. Nevertheless, mass spectrometry detection showed that lipids (i.e., SQDG at *m*/*z* 815, the main lipid detected in negative ion mode) were eluted with t_R values ranging close to 22 min (Fig. 5C). It is also worth noting that flavonol triglycosides with deprotonated ions at *m*/*z* 755 and 771 eluted close to the lipids. Additionally, many saponins, varying in molecular weight (1216–1320 Da) eluted close to lipids and flavonol triglycosides in the first dimension (Figs. 4A and 5C, Supplementary Table 2). Thus, since the polarities of flavonol glycosides, saponins and lipids are quite distinct, the solubility in the eluent was not the main factor leading to SEC separation. Notably, the major components from *C. sinensis*, the flavan-3-ols, eluted close to 30 min.

The main products of *C. sinensis* oxidation (the theaflavins from black tea), as detected by SEC analyses, eluted after 30 min and were well separated by molecular weight: theaflavin 3,3′-digallate (t_R 31.28 min, *m*/*z* 867), theaflavin 3- and 3′-gallate (t_R 32.68 min, *m*/*z* 715) and theaflavin (t_R 33.18 min, *m*/*z* 563). On the other hand, chlorogenic acids, found mainly in the black tea, had lower t_R values on the SEC column even with a molecular mass of 354 Da they were eluted at 8.5 and 8.86 min (Fig. 6). The acidic characteristics of these compounds could be the main reason for the low interaction with the SEC matrix as observed with free quinic acid, which had a t_R value of 5.55 min, and other ester derivatives of quinic acids.

The second dimension, based on comprehensive 2D-LC, was performed on a UHPLC with gradient 2 (described in the Experimental Section). This method was faster than using a RP gradient 1 because of the faster flow rate of 1 ml min^{-1} used now, allowed by the lower complexity of each fraction from the first dimension. The use of such a flow rate in a sub-2 μm particle size column can generate high backpressures, hindering analyses. In order to overcome this, recent methodologies have been employed using high temperatures, resulting in reduced viscosity and, consequently, a reduction in the backpressure [11,44]. Thus, we opted to use a temperature of 80 °C, which was within the column limit and gave rise to a backpressure of 11,000 psi (~750 bar), also within the column limit. In this system, degradation of the stationary phase was not observed, considering that no changes in the peak resolution and separation power were observed after this analysis.

Although the main compounds (typically the phenolics) eluted before 3 min, in order to have comprehensive analyses of phytochemicals, the second dimensional analysis was extended to 8 min to incorporate saponins and lipids, which impair the online comprehensive 2D-LC analyses.

The chromatograms of hydro-alcoholic extracts from *C. sinensis* green and black teas were plotted in 2D graphs. As a result, the difference due to the oxidation compounds present in black tea was evident (Figs. 4A and 5). As described above, the main change in the composition of black tea was the emergence of condensed theaflavins. However, other changes were the disappearance of saponins and a drastic decrease in lipid content as well as some flavonol glycosides. Thus, similar to 2D gel electrophoresis, 2D chromatographic graphs could be used to draw a comprehensive metabolic map.

4. Conclusion

The well-studied *C. sinensis* plant was chosen as a model for a 1D-LC and comprehensive 2D-LC development. Using liquid–liquid partitioning in single-dimensional analyses (1D), several classes of compounds were identified and, although outside of the scope of the present study, compounds not previously reported in *C. sinensis* were found. These compounds were saponin isomers and four flavonol glycosides containing gallic acid esters.

Regarding two-dimensional (2D) analyses, the first dimension was developed with an unusual SEC system, which proved to have interactions with the sample other than simple permeation based on molecular masses. These interactions were shown by the proximity of how many different compounds eluted, notably saponins (MW 1216–1320 Da), lipids (MW 749–944 Da) and caffeine (MW 194 Da). Each class of compounds, mainly phenolic classes, had elution profiles based on their molecular weight, allowing the separation of, for example, many flavonol glycosides with differing glycosyl moieties.

The combination of HPLC (SEC) and UHPLC (RP) provided the orthogonality required for a 2D system. As much as possible, UHPLC provided a fast analysis for complex matrices, making it efficient for second dimension analyses, considering that a reduced time in the second dimension has a key role in comprehensive 2D-LC. Smaller analysis times already had been achieved using UHPLC system in the second dimension. However, the present work intended to show the separation of compounds of very different chemical nature, yielding an analysis time greater than in previously reported [9–11]. Nevertheless, we believe that this is the first study covering the entire complexity of a plant extract, from sugars to lipids, including all the known products of secondary metabolism of *C. sinensis.*

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.11.038.

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