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Evidence of noncovalent complexes in some natural extracts: Ceylon tea and mate extracts

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Abstract

Considering the high complexity of natural extracts, because of the presence of organic molecules of different chemical nature, the possibility of formation of noncovalent complexes should be taken into account. In a previous investigation, the formation of bimolecular complexes between caffeine and catechins in green tea extracts (GTE) has been experimentally proven by means of mass spectrometric and ^{1}H nuclear magnetic resonance experiments. The same approaches have been employed in the present study to evaluate the presence of bimolecular complexes in Ceylon tea and mate extracts. The obtained results show that in the case of Ceylon tea extracts, protonated theaflavin is detectable, together with theaflavin/caffein complexes, while caffeine/catechin complexes, already detected in green tea, are still present but at lower concentration. This aspect is evidenced by the comparison of precursor ion scans performed on protonated caffeine for the two extracts. The spectra obtained in these conditions for GTE and Ceylon tea show that the complexes of caffeine with epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG), highy abundant in the case of GTE (signal-to-chemical noise ratio in the range 50-100), are negligible (signal-to-chemical noise ratio in the range 2-3) in the case of Ceylon tea. Mate extracts show the formation of bimolecular complexes involving caffeine but not catechins, and chlorogenic acid becomes responsible for other complex formation. Under positive ion and negative ion conditions, accurate mass measurements allow the identification of malealdehyde, chlorogenic acid, caffeine, two isomers of dicaffeoylquinic acid, rutin, and kaempferol-3-*O*-rutinoside. These data indicate that the formation of complexes in natural extracts is a common behavior, and their presence must be considered in the description of natural extracts and, consequently, in their biological activity.

KEYWORDS

natural products, bimolecular complexes, precursor ion scans, accurate mass measurements, ^{1}H NMR, Ceylon tea, Mate

1 | INTRODUCTION

Can molecules be considered as isolated objects in space? Surely in a very rarefied space, where the density of matter is infinitesimal, the probability of noncovalent interaction between two molecules is very low but not anything. In fact, it has been proven that the formation of molecular complexes is a phenomenon observed in most of the universe,[1] and it represents an essential stage for the formation of complex molecular systems. At atmospheric pressure, because of the much higher frequency of interactions, the formation of numerous complexes takes place, above all, in the condensed phase (solution). These phenomena have been and are still considered the basis of "chemical evolution."[2] These aspects have been studied by a branch of modern physics, the theory of complexity,[3] emerging in the last decades in scientific research. It studies the so-called complex systems, renouncing linearity assumptions in dynamic systems and investigating their behavior more thoroughly.

The number and frequency of molecular interactions depend on the complexity of the system under study. In the case of natural substrates, consisting of hundreds (or thousands) of different molecular species in the condensed phase, the frequency of interactions will be very high. Thus, a natural substrate should not be considered as a set of isolated molecules but as an entity consisting of species generated from continuous processes of molecular interaction, in a situation of equilibrium dependent on thermodynamic conditions. We have, therefore, to observe a complex system,[3] consisting of the molecules present in the natural extract, which interact in a nonlinear way[4] with each other through the formation of noncovalent bonds.

In this frame, mass spectrometry can be a valid tool to achieve a quite accurate general view of natural extracts,[5] in particular by using different ionization techniques able to evidence classes of compound exhibiting different chemico-physical properties. As an example, electrospray ionization (ESI) operating in the positive ion mode allows to obtain a map of the basic species present in the extract (leading to $[M + H]^+$ ions), while in the negative ion mode previleges the detection of acid compounds (because of the formation of $[M - H]^-$ ions).

A hypothesis on the peculiar pharmacological behavior of biologically active natural compounds is based on the occurrence of molecular interactions following the rules of supramolecular chemistry.[6]

In particular, in the case of caffeine, an interesting review has been recently published[7] on the molecular recognition of caffeine in solution and solid state: A wide variety of receptors has been designed for this aim and found to exhibit a high affinity toward caffeine. The modern bioorganic chemistry concerns the design of synthetic molecules that mimic various aspects of enzyme chemistry and allows to understand their essential roles in biological systems. In this case, molecular recognition of caffeine with polyphenols and carboxylic acids has been described.[7-9]

Caffeine posseses a number of features, which optimize its effectiveness for complexation with polyphenolic substrates: The phenolic group is a good proton donor. It can be assumed that in complexation with caffeine, hydrogen bonding between the polyphenol (proton donor) and caffeine (proton acceptor) takes place, giving a specific contribution to the stability of the complex.[7]

In line with this view, some investigations were performed to establish the presence of caffeine/catechin complexes in green tea extracts (GTEs).[10] ^{1}H NMR spectroscopy was employed to compare profiles from GTEs with caffeine/catechin mixtures in different molar ratios, showing that peaks related to caffeine in GTEs are generally upfield shifted compared with those of free caffeine. Illustrative ^{1}H NMR spectra of caffeine/catechin mixtures (in molar ratio 1:0.6, mimicking the molar ratios present in GTE) compared with profiles of individual molecules and GTE extract show that specific signals in caffeine/gallate-type catechin mixtures undergo chemical shift changes comparable with those observed in GTE.[10]

In parallel, ESI-MS/MS experiments performed on GTEs, by means of precursor ion scan and neutral loss scan experiments, proved unequivocally the presence of caffeine/catechin complexes.[10] To be confident on the results obtained by ESI/MS and to exclude the nature of the detected complexes as artifacts generated by ESI conditions, further investigations were performed by an LC-MS method operating in high resolution conditions. The reconstructed ion chromatograms of the exact mass ions corresponding to caffeine/catechin species were obtained, showing the presence of complexes of caffeine with gallate-type catechins at different retention times. Furthermore, this last approach evidenced the presence of the same complex (ie, with the same m/z value) with different structures, exhibiting different retention times, in agreement with the findings of Ujihara and Hayashi.[11] Both MS and product ion MS/MS methods confirmed the nature of caffeine/catechin complexes of the detected ions, showing the collisionally induced formation of protonated caffeine from the molecular species of the complexes.

Considering these promising results, it was thought of interest to investigate on the presence of noncovalent molecular complexes in other natural extracts by the same experimental approaches. The results obtained for Ceylon tea and mate extracts are reported here.

2 | EXPERIMENTAL

2.1 | Samples

Ultrahigh purified H_2O was prepared by using a PurelabUltra water purification system (ELGA, UK). Absolute EtOH 99.8% and MeOH 99% were purchased from Merck-VWR. Absolute MeOH LC-MS grade and formic acid LC-MS grade were purchased from Extrasynthese (Genay Cedex France).

Green tea, Ceylon tea, and mate were extracted with EtOH 70%/H_2O 30% (v/v) (drug solvent ratio, 1:8) at 50°C for 8 hours. The extracts were concentrated under vacuum, and the concentrates were lyophilized for 72 hours. The resulting freeze-dried extracts were stored in the dark and under dry conditions until use (drug extract ratio, DER 8-4:1).

2.2 | Sample preparation for MS and MS/MS analysis

The solutions analyzed by MS and MS/MS were prepared as follows: Green tea, Ceylon tea, and mate powder extracts were dissolved in Milli-Q H_2O at a concentration of 1 mg/mL. The solutions were then filtered through a pore size filter of 0.22 μm and further diluted to obtain a 100-μg/mL working solutions in MeOH/H_2O (50:50 v/v).

Mass spectrometry measurements were performed by using an API 4000 triple quadrupole mass spectrometer (AB SCIEX, Massachusetts). The sample solutions were infused by the use of a programmable syringe pump (KD Scientific, Massachusetts), at a flow rate of 600 μL/h. ESI source parameters were as follows: source temperature, 300°C; curtain gas (nitrogen), 40 psi; and nebulizer gas (air) GS1 and GS2, 10 and 15 psi, respectively.

2.2.1 | MS analysis

Full scan spectra in the positive ion mode were recorded with ion spray voltage set at 4500 V, entrance potential at 10 V, and declustering potential at 20 V; for negative ion measurements, ion spray voltage was set at −4500 V, entrance potential at −10 V, and declustering potential at −20 V.

2.2.2 | MS/MS analysis (precursor ion and neutral loss scans)

For collisional experiments, CAD was set at 4 (arbitrary units); for positive measurements collision, cell exit potential (CXP) and collision energy (CE) were, respectively, 15 V and 30 eV, while for negative measurements, CXP and CE were −15 V and −30 eV respectively.

2.3 | Sample preparation for UHPLC ESI-QToF analysis

The ground sample of Ceylon tea (0.25 g) and mate were extracted with 25 mL of MeOH/H_2O 50:50 (v/v) under ultrasound at 35°C. After 30 minutes, the samples were centrifuged 10 minutes at 4000 rpm. The supernatant was collected in a 50-mL volumetric flask, and the pellet extracted under the same conditions. After centrifugation, the second extract was combined with the first, and the volume was adjusted to 50 mL, controlling the temperature at 20°C. The sample was filtered on a 0.20-μm cellulose acetate syringe filter; then 200 μL of the filtered solution was added to the internal standard sulfadimethoxine-d_6 (4 μL of a 0.005-mg/mL methanol solution), affording the corresponding analysis solution.

Experiments were performed under high resolution LC-MS conditions: LC-ESI MS all-ions analysis was carried out by an HPLC1290 Series (Agilent Technologies INC., Santa Clara, California) system equipped with a vacuum degasser and a quaternary pump. The effluent was analyzed by a QToF 6545 mass spectrometer equipped with an ESI interface Dual AJS ESI (Agilent Technologies Inc) operating in positive or in negative ion mode at 40 000 resolution. The chromatographic separation was performed on a Waters Cortecs UPLC C18, 1.6 μm, 2.1 × 100 mm and equipped with a VanGuard precolumn, 1.6 μm, 2.1 × 5 mm. The column temperature was 40°C, the flow rate was 0.3 mL/min, and the injector volume was 3 μL. The mass spectrometer parameters were set as follows: mass range, m/z 50 to 1700; entrance capillary voltage, 3500 V; fragmentor, 100 V; skimmer, 65 V, OCt 1 RF Vpp 750 V; nozzle voltage, 1000; gas flow, 11 L/min; gas temperature, 325°C. The ESI interface nebulizer gas pressure was set at 35 psi, and the collision energy at 0-20-30-40. The mobile phase consisted of MeOH/0.1% HCOOH (LCMS grade) (B) and ultrapure H_2O/0.1% HCOOH (LC-MS grade) (A) according to the elution gradient reported in Table 1.

2.4 | ^{1}H NMR

^{1}H NMR spectra were recorded on a Bruker AMX-300 instrument at 25°C using tetramethylsilane as internal reference. Standard solutions of tea extracts were prepared by dissolving 4 mg of the extract in 0.6 mL of D_2O. Standard solutions of individual caffeine, different catechins, and chlorogenic acid were prepared in D_2O at the concentrations of the analytes observed in the extract solutions.

3 | RESULTS AND DISCUSSION

3.1 | Ceylon tea

Tea has been considered since ancient times to exhibit medicinal properties.[12-15] Tea beverage contains xanthine derivatives such as caffeine, theophylline, theobromine, and the glutamide derivative theanine. These substances have well-known stimulant properties and have also been reported to have beneficial effects on memory and on the immune system. Tea also contains many nutritional components, such as vitamin E, vitamin C, fluoride, and potassium.

TABLE 1 Elution gradient employed for the UHPLC ESIQToF analysis: (A) ultrapure water/0.1% HCOOH and (B) methanol/0.1% HCOOH

Min	A%	B%
0.00	99	1
1.00	99	1
2.00	75	25
10.00	50	50
11.00	50	50
15.00	25	75
17.00	15	85
19.00	1	99
19.50	1	99
21.00	99	1
Stop time: 21 min		
Post time: 3 min		

The astringency of the beverage is due to phenolic constituents known as catechins, a group of compounds that are closely related to tannins. Tannins are acidic because of the phenolic hydroxyl groups present on the gallic acid moiety. They also act as antioxidants and form complexes or chelates with metals. Most of phenols in tea are catechins (see Figure 1), hydroxylated flavanols, and their gallic acid esters. During fermentation process, if employed, these catechins undergo phenolic oxidative coupling reactions that yield red-colored catechin dimers such as thearubigins.

Catechins are believed to have a range of beneficial health effects[13,15] such as neuroprotective activity and anti-inflammatory, antiulcer, antiviral, antibacterial, and antiparasitic effects. The most studied catechin in relation to health contributing potential is epigallocatechin 3-gallate (EGCG), which constitutes 50% to 75% of the total flavonoid content in green tea.

Ceylon tea is a popular type of black tea.[16] It is known for its bold flavor, but it can vary greatly in taste, depending on where it is grown. The process of manufacture, after leaf plucking, is based on a series of treatments, ie, withering, rolling (a mechanized process in which the leaf cells are ruptured to release enzymes and bring them in contact with air so that aeration can take place), aeration (sometimes known as "fermentation" or "oxidation"), and drying (the leaf is dried in a dessicator or "firing chamber" at 99°C to 104°C to prevent further chemical changes. This shrinks and darkens the leaf, resulting in the product known as black tea).[16]

All these treatments are absent in the case of green tea (GT) production. In this case, after the tea leaves are plucked, they are dried to prevent fermentation, so inhibiting any activity that causes oxidation. Tea leaves are stirred constantly for even drying. Withering is also used, which spreads the tea leaves on racks of bamboo or woven straw to dry in the sun or using warm air. Again, the leaves must be moved around to ensure uniform drying.

At this point, a question arises: Do all treatments required for the production of Ceylon tea impact on the composition, making the extracts different to those of green tea? In more detail, are catechin amounts decreased, and are the bimolecular caffeine/catechin complexes, detected in GTEs, still present?

The ^{1}H NMR spectra of GTE and Ceylon tea extracts are reported in Figure 2. This comparison shows that the content of catechins (mainly EGCG) originally present in GTEs[10] is drastically reduced in Ceylon tea, whereas caffeine content remains practically unaltered. Therefore, also bimolecular caffeine/catechin complexes are supposed to be reduced in Ceylon tea extracts. The aromatic proton signal of free gallic acid is significantly increased in Ceylon tea, further indicating degradation of catechins. The aromatic signal of gallic acid was assigned by comparison with the signal arising from a pure sample of gallic acid. In the present work, the drastic decrease of catechin contents in Ceylon tea extracts compared with green tea was verified. Because of the low amount of catechins in Ceylon tea, the chemical shift variations of the aromatic protons of EGCG or ECG, as we did in a previous work,[10] was not discussed.

In the previous investigation on GTE, it was found that ESI operating in negative ion mode is an effective tool to describe the acid components of the extract introduced by direct infusion,[10] leading in particular to a valid mapping of the polyphenolic species. Consequently, the same approach was employed in the study of Ceylon tea extracts. The ESI-(−) spectra of GTE and Ceylon tea extracts are shown in Figure 3. It is evident that the same species are present in the two extracts, but with major differences in their relative abundances, as emphasized by the data reported in Table 2. Deprotonated theaflavin (see Figure 1) (*m/z* 563) is detectable in the case of Ceylon tea (see Figure 3), while in GTE spectrum, theaflavin-caffein complex is detectable at *m/z* 757, in agreement with the model proposed by the Williamson's group[9]: The self-association (K_a = 2301 M^{-1}) of

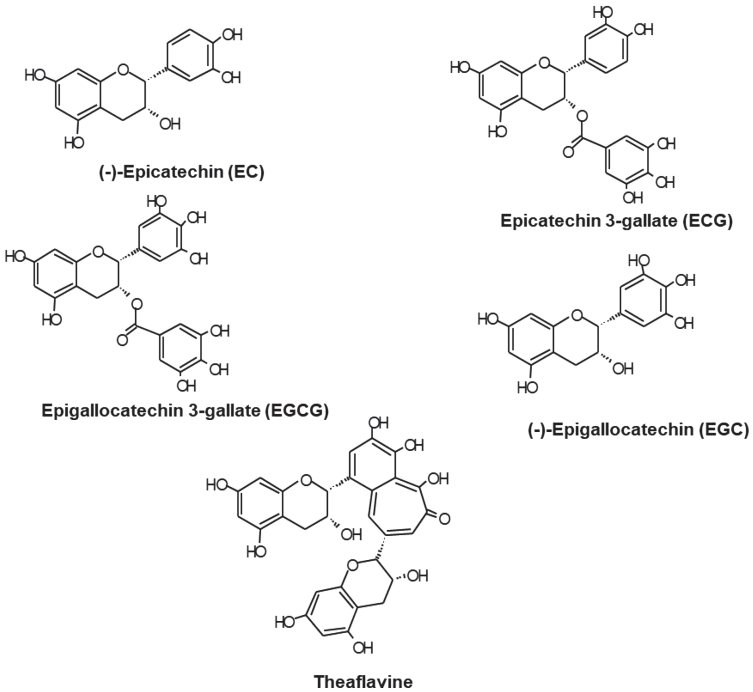


FIGURE 1 Stuctures of epicatechin, epicatechin-3-gallate, epigallocatechin-3-gallate, epigallocatechin, and theaflavine

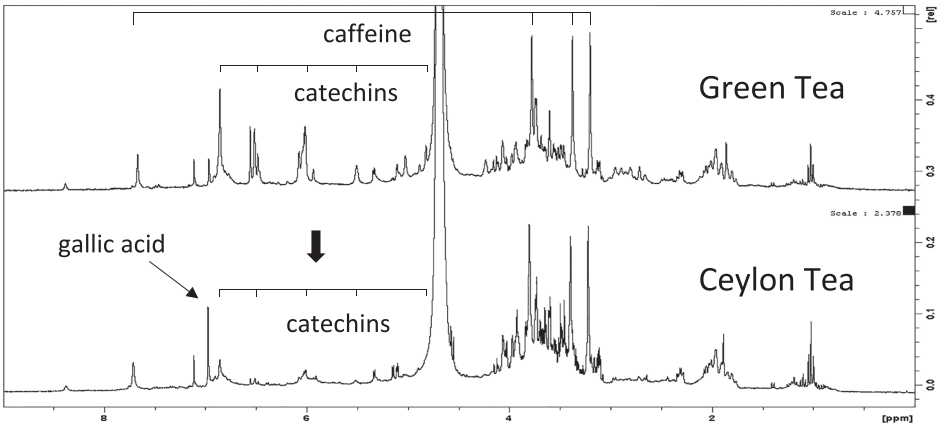


FIGURE 2 Comparison of ^{1}H NMR profiles of green tea extract (GTE; top trace) versus Ceylon tea extract (bottom trace). It shows that the content of catechins (mainly EGCG: epigallocatechingallate) present in GTEs is reduced in Ceylon tea, whereas caffeine content remains practically unaltered. The aromatic proton signal of free gallic acid significantly increases in Ceylon tea, further indicating degradation of catechins

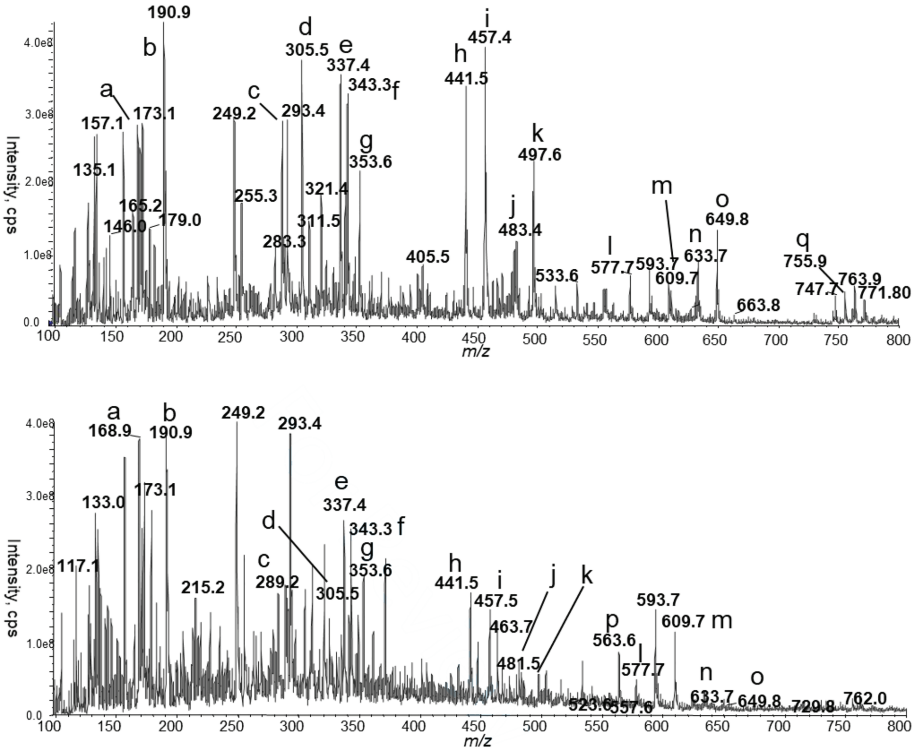


FIGURE 3 Comparison of ESI-(−) spectra of green tea extract (GTE) (top) versus Ceylon tea extract (bottom). The relative abundances of the most significant species are reported in Table 2. Lower levels of catechins in the Ceylon tea are observed, confirming the ^{1}H NMR data

theaflavin and caffeine was studied using NMR methods, showing that caffeine forms a stacked complex with theaflavin.

Lower levels of catechins are observed in Ceylon tea extracts, confirming ^{1}H NMR data. As a consequence, it is expected that the presence of the catechin/caffeine complexes, detected in the case of GTE, should be strongly reduced in the case of Ceylon tea extracts. This aspect is well evidenced by the comparison of precursor ion scans performed on protonated caffeine for the two extracts. The obtained spectra are reported in Figure 4: The complexes of caffeine with EGC, ECG, and EGCG, highy abundant in the case of GTE (signal-to-chemical noise ratio in the range 50-100), are negligible (signal-to-chemical noise ratio in the range 2-3) in the case of Ceylon tea.

An important point must be discussed: Are the detected complexes originally present in the GTE and Ceylon tea extracts, or they just represent species produced by the ESI conditions? In fact, ESI mass spectrometry can easily lead to the aggregation of ionic and neutral components present in the analyzed sample. However, to exclude this aspect, in the previous study on GTE,[10] both direct infusion and LC MS data were compared, showing that also in the latter conditions, the complexes were detectable at different retention times, so giving

TABLE 2 Relative abundances of the $[M - H]^-$ ions present in ESI-(−) spectra of green tea and Ceylon tea extracts

			Relative Abundance of $[M - H]^-$ ions	
Symbol	*m/z*	Compound	Green Tea	Ceylon Tea
a	169	Gallic Acid	70	90
b	191	Quinic Acid	100	100
c	289	EC	75	30
d	305	EGC	85	30
e	337	Cumaroyl quinic acid	80	65
f	343	Theogallin	80	62
g	353	Chlorogenic acid	58	45
h	441	ECG	78	30
i	457	EGCG	95	26
j	483	EC + CA	23	11
k	499	EGC + CA	50	8
l	577	Procyanidin	15	6
m	609	Rutin	12	29
n	635	ECG + CA	20	5
o	651	EGCG + CA	28	2
p	563	Theoflavine	2	15
q	757	Theoflavine + CA	5	1

Abbreviations: CA, caffeine; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocathechin; EGCG, epigallocatechin gallate.

evidence on the validity of the direct infusion approach and the related precursor ion scan data. Then it can be assumed that the same behavior is present in the case of Ceylon tea and that the data reported in Figure 4 can be considered valid.

These results can be justified by supposing that the treatments above described for the Ceylon tea production are partially lesive either for catechins and/or for the catechin/caffeine complexes. It must be stressed that catechins and their derivatives are thought to contribute to the beneficial effects ascribed to tea.[13] Tea catechins and polyphenols are effective scavengers of reactive oxygen species in vitro and may also function indirectly as antioxidants through their effects on transcription factors and enzyme activities. The fact that catechins are rapidly and extensively metabolized emphasizes the importance of demonstrating their antioxidant activity in vivo. In humans, modest transient increases in plasma antioxidant capacity have been demonstrated following the consumption of tea and green tea catechins. The effects of tea and green tea catechins on biomarkers of oxidative stress, especially oxidative DNA damage, appear very promising in animal models, but data on biomarkers of in vivo oxidative stress in humans are limited.

3.2 | Mate

The *Ilex paraguariensis* plant, called yerba mate plant[17,18] is grown and processed in South America, specifically in northern Argentina, Paraguay, Uruguay, and Southern Brazil. Seeds used to germinate new

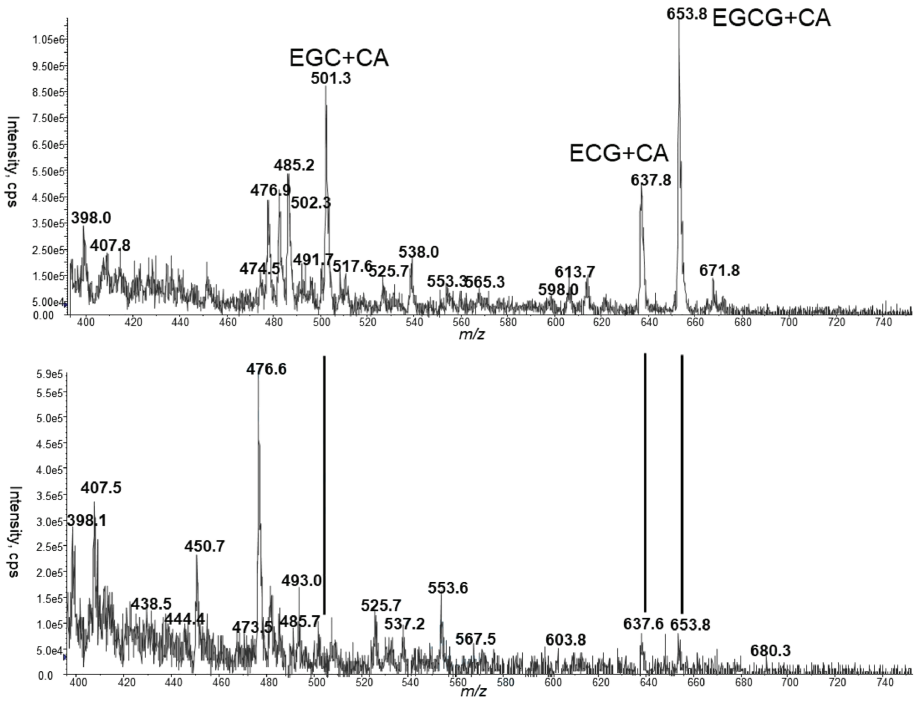


FIGURE 4 Precursor ion spectra for protonated caffeine obtained for GTE (top) and Ceylon tea (bottom) extracts. The complexes of caffeine (CA) with epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG), highy abundant in the case of GTE (signal-to-chemical noise ratio in the range 50-100), are in the case of Ceylon tea particularly scarce (signal-to-chemical noise ratio in the range 2-3)

plants are harvested from January to April only after they have turned dark purple. After harvesting, they are submerged in water to eliminate floating nonviable seeds and detritus like twigs and leaves. When yerba mate is harvested, the branches are often dried by a wood fire, imparting a smoky flavor.

The main bioactive compounds present in yerba mate extracts are reported in Table 3,[18-20] while the structures of the caffeoyl, *p*-coumaroyl, and feruloyl derivatives of quinic acid, representing the most abundant species present in Mate extracts, are illustrated in Figure 5. They are particularly rich in chlorogenic acids, a family of esters of *trans* cinnamic acids and quinic acid. These compounds are also major phenolic compounds in coffee, strawberries, apples, pineapples, blueberries, and sunflowers. Chlorogenic acids are metal and free-radical scavengers, able to interfere with glucose absorption and modulating gene expression of antioxidant enzymes.[18,19]

The ^{1}H NMR spectrum of a mate extract shows the presence of caffeine, chlorogenic acids, and monomeric sugars (sucrose and glucose). In the aromatic region, a significant amount of chlorogenic acids is detected, while the contribution of gallocatechins is negligible.

The same mass spectrometric methods used in the case of GTE,[10] ie, either LC-MS in high resolution conditions or precursor ion scans performed on protonated caffeine and deprotonated chlorogenic acid, have been employed for the detection of possible noncovalent complexes in mate extracts.

The LC-ESI-(+)MS chromatogram of a mate extract, obtained in high resolution conditions, is reported in Figure 6, while the chromatogram obtained in ESI-(−) conditions is reported in Figure 7. The related results are summarized in Tables 4 and 5. Under positive ion conditions, accurate mass measurements allow the identification of malealdehyde, chlorogenic acid, caffeine, two isomers of dicaffeoylquinic acid (species h and k in Table 4, reasonably originating from 3,5-DCQ and 4,5-DCQ that, as shown in Table 3, are the most abundant DCQ isomers), rutin, and kaempferol-3-*O*-rutinoside, while the elemental composition of other species obtained by accurate mass measurements do not lead to their structural identification.

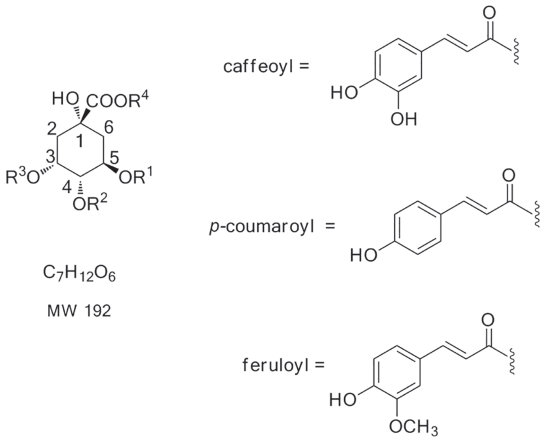
The comparison of the LC-MS data with those obtained by direct infusion of the extract in ESI-(+) conditions (fourth column of Table 4) shows that direct infusion leads to poor results: Only few of the species evidenced by LC-MS are detectable and only few, other protonated ions are present. For example, an abundant ion at *m/z* 287 detected in the direct infusion ESI-(+) spectrum only could be justified by protonated kaempferol, on the basis of product ion MS/MS experiments.

On the contrary of what obtained by ESI-(+), the data achieved by ESI-(−) with the two techniques agree well (Table 5), allowing the identification of many compounds (except neochlorogenic acid and kaempferol-3-*O*-rutinoside, reasonably undetectable for ion suppression effects) and giving a further evidence of the capability of ESI-(−) in the detection of acidic species also by direct infusion. The $[M - H]^-$ species of malic acid, quinic acid, neochlorogenic acid, chloro/cryptochlorogenic acid, the two isomers of dicaffeoylquinic acid (species f′ and h′ in Table 5), rutin, and kaempferol-3-*O*-rutinoside have been detected.

Considering the results achieved on the molecular recognition of caffeine[7] and the high abundance of chlorogenic acid in the mate

TABLE 3 Main bioactive compounds present in yerba mate extracts (DCQ = dicumaroylquinic acid)[18-20]

Chemical Compounds	Dry Weight Composition %
Caffeoyl derivatives	10.000
Chlorogenic acid	2.800
Caffeic acid	0.023
3,4-DCQ	0.855
3,5-DCQ	3.040
4,5-DCQ	2.890
Saponins	5-10
Xantines:	
Caffeine	1-2
Theobromine	0.3-0.9
Theophylline	traces
Rutin	0.060
Quercetin	0.0031
Kaempferol	0.0012



	MW
R^1 = caffeoyl, $R^2 = R^3 = R^4$ = H	354
R^1 = *p*-coumaroyl, $R^2 = R^3 = R^4$ = H	338
R^1 = feruloyl, $R^2 = R^3 = R^4$ = H	368
R^1 = caffeoyl, $R^2 = R^3$ = H, R^4 = Me	368
R^1 = H, $R^2 = R^3$ = caffeoyl, R^4 = H	516
$R^1 = R^3$ = caffeoyl, R^2 = H, R^4 = H	516
$R^1 = R^2$ = caffeoyl, R^3 = H, R^4 = H	516
R^1 = H, $R^2 = R^3$ = caffeoyl, R^4 = Me	530
$R^1 = R^3$ = caffeoyl, R^2 = H, R^4 = Me	530
$R^1 = R^2$ = caffeoyl, R^3 = H, R^4 = Me	530
$R^1 = R^2$ = caffeoyl, R^3 = H, R^4 = Et	544

FIGURE 5 Molecular structures and molecular weight of the main chlorogenic acids present in mate extracts

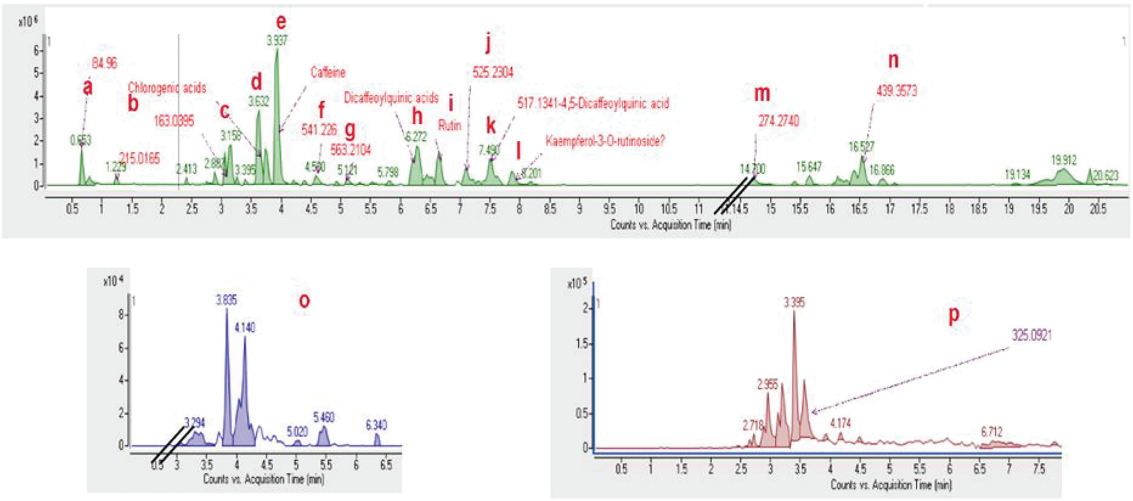


FIGURE 6 Top: LC-ESI-(+)MS chromatogram of mate extract, obtained in high resolution conditions. The related results are summarized in Table 4. In positive ion conditions, accurate mass measurements allow the identification of malealdehyde, chologenic acid, caffeine, two isomers of dicaffeoylquinic acid (species h and k in Table 4), rutin, and kaempferol-3-*O*-rutinoside while the elemental composition of other species do not lead to structural identification. Bottom: reconstructed ion chromatograms obtained in high resolution conditions of ions o and p ($C_{23}H_{21}N_4O_8$ and $C_{12}H_{13}N_4O_7$, respectively) corresponding to complexes of protonated caffeine with kaempferol and oxalacetic acid

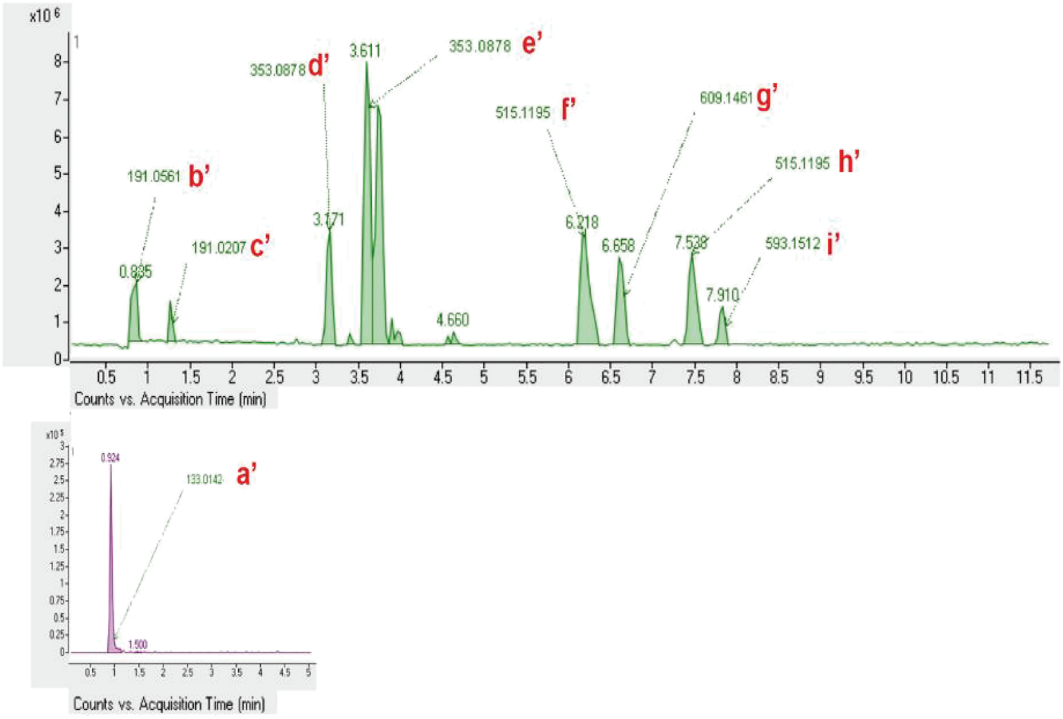


FIGURE 7 Top: LC-ESI-(-)MS chromatogram of mate extract, obtained in high resolution conditions. On the contrary of what obtained by ESI-(+), the data achieved by ESI-(−) (Table 5) are strongly analogous to those obtained by direct infusion of the same extract. The $[M - H]^-$ species of malic acid, quinic acid, neochlorogenic acid, chloro/cryptochlorogenic acid, the two isomers of dicaffeoylquinic acid (species f' and h' in Table 5), rutin, and kaempferol-3-*O*-rutinoside have been detected. Bottom: reconstructed ion chromatogram obtained in high resolution conditions of ions a' (*m/z* 133.0142) corresponding to deprotonated malic acid

extracts (see Figures 6 and 7), it was considered interesting to investigate the possible formation of noncovalent complexes with other molecular species present in the extract by precursor ion scans performed on protonated caffeine (*m/z* 195) and $[M - H]^-$ ion of chlorogenic acid (*m/z* 353). As shown in Table 6, complexes of protonated caffeine with malealdehyde, oxalacetic acid, kaempferol, and theanine have been detected. Quite surprisingly, no complexes with catechins were present. This result is in agreement with the data

TABLE 4 Data obtained in ESI-(+) by LC-MS in high resolution (second and third columns) and their comparison with those achieved by direct infusion of mate extract (fourth column: √ = detected, X = undetected)

LC-MS HR ESI-(+)	Accurate Mass	Compound or Elemental Formula	Direct Infusion
a	85.0289	Malealdehyde	√
b	215.0165	$C_7H_7O_2$	X
c	163.0395	$C_9H_6O_3$	X
d	355.1028	Chlorogenic acid	X
e	195.0878	Caffeine	√
f	541.2260	$C_{35}H_{31}O_7$	√
g	563.2104	$C_{35}H_{30}O_7Na$	X
h	517.1350	Dicaffeoylquinic acid	X
i	611.1608	Rutin	X
j	525.2304		√
k	517.1350	Dicaffeoylquinic acid	X
l	595.1662	Kaempferol-3-*O*-rutinoside	X
m	274.2740		√
n	439.3573		X
o	481.1316	$C_{23}H_{21}N_4O_8$	X
p	325.0921	$C_{12}H_{13}N_4O_7$	X
q	365.1197	$C_{25}H_{16}O_3$	√

TABLE 5 Data obtained in ESI-(−) by LC-MS in high resolution (second and third columns) and their comparison with those achieved by direct infusion of mate extract (fourth column:√ = detected, X = undetected)

LC-MS HR ESI-(−)	Accurate Mass	Compound or Elemental Formula	Direct Infusion
a′	133.0142	Malic acid	√
b′	191.0561	Quinic acid	√
c′	191.0207		√
d′	353.0878	Neochlorogenic acid	X
e′	353.0878	Chloro/Cryptochlorogenic acid	√
f′	515.1195	Dicaffeoylquinic acid	√
g′	609.1318	Rutin	√
h′	515.1195	Dicaffeoylquinic acid	√
i′	593.1512	Kaempferol-3-*O*-rutinoside	X

obtained by ^{1}H NMR, LC-ESI(+), and LC-ESI(−), which did not evidence the presence of catechins. Interestingly, the complexes of caffeine with kaempferol and oxalacetic acid were also detected under LC-MS conditions (Table 4, ions o and p, $C_{23}H_{21}N_4O_8$ and $C_{12}H_{13}N_4O_7$, respectively), as proven by the reconstructed ion chromatograms obtained in high resolution conditions (see lower part of Figure 7). These data put in evidence the presence of two isomers of the caffeine-kaempferol complex and four isomers of the caffeine-oxalacetic acid complex, detected at different retention times. This aspect could be justified by the presence of different coordination sites oriented versus the different nitrogen atoms and/or π systems. In the case of precursor ion scan of $[M - H]^-$ ion of chlorogenic acid, a quite simple spectrum is obtained, and the related peaks can be justified by the presence of complexes with methylfurane, *p*-cumaric acid, and a three-component complex among chlorogenic acid, *p*-cumaric acid, and caffeic acid. For the scarcely abundant ion detected at *m/z* 708, it was impossible to propose a reasonable structure.

TABLE 6 Precursor ion scans of protonated caffeine (*m/z* 195, top) and $[M - H]^-$ of chlorogenic acid (*m/z* 353, bottom) for a mate extract. First column: detected ionic species; second column: mass increase with respect the selected precursor ion; third column: possible molecular species involved in the complex formation

ESI-(+) Precursor Ion Scan of Ion at *m/z* 195 (Protonated Caffeine)		
247	+52	
279	+84	Malealdehyde
287	+92	
327	+132	Oxalacetic acid
477	+282	
481	+286	Kaempferol
370	+175	Theanine
ESI-(−) Precursor Ion Scan of Ion at *m/z* 353 ($[M - H]^-$ of Chlorogenic Acid)		
435	+82	Pyrane?
517	+164	4-Hydroxycinnamic acid (p-coumaric acid)
697	+ 164 + 180	+p-coumaric acid and +caffeic acid
708	+355	

The results show that a deep difference is present among tea and mate extracts. In particular, catechin amounts seem to be, in the latter case, strongly reduced, and the biological activity of mate should be solely related to chlorogenic acids contained at high level. Because of their various biological properties, such as antispasmodic, antioxidant, inhibition of the HIV-1 integrase, and inhibition of the mutagenicity of carcinogenic compounds, they are very important plant secondary metabolites. In the light of recently published results, they are also supposed to be helpful in fighting obesity and modify the activity of glucose-6-phosphatase involved in glucose metabolism. These suppositions, although unproven, were sufficient to fuel new research interest on CQAs properties.[18,19]

Total polyphenols and antioxidant activity of mate infusions have been compared with those of commonly consumed beverages like green and black teas, red, rosé and white wines, and orange juice.[20] Mate has a polyphenol content comparable with tea and orange juice, and when normalized for the polyphenol content of beverages, mate showed an antioxidant activity slightly higher than wines, orange juice, and black tea but lower than green tea.[20] Because of the presence of caffeoylquinic and dicaffeoylquinic acid isomers, mixed mono-, di-, and tri-esters of quinic acid, other hydroxycinnamates, and several quercetin and kaempferol glycosides, the consumption of mate infusions

would significantly contribute to the overall antioxidant intake, with biological effects potentially beneficial for human health (eg, for their hypocholesterolemic, antimutagenic, anti-inflammatory, and antiviral properties).

4 | CONCLUSIONS

The results obtained in the investigation on the presence of bimolecular, noncovalent complexes in Ceylon tea and mate extracts confirm the data previously achieved for green tea. Mass spectrometry is a valid tool with this respect leading to a quite accurate view of the components present in the extracts. In particular, LC-MS operating in high resolution conditions and MS/MS experiments performed by precursor ion scan allow to individuate and characterize the noncovalent complexes originating by the interactions between different components of the extract. The attention was focused on species originating protonated caffeine (whose molecular recognition on the basis of weak intermolecular forces has been illustrated by the wide variety of molecular receptors) and deprotonated chlorogenic acid (which is a major component of the extracts under study). This allowed to evidence clear differences between green tea and Ceylon tea extract but, over all, between tea extracts and mate samples. Summarizing the employed analytical approaches allow one to achieve valid information on the bimolecular complexes present in the extracts from a systemic point of view, which necessarily should reflect on their biological activity.

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