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Preliminary investigation into development of HPLC with UV and MS-electrospray detection for the analysis of tea catechins

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Abstract

There is an increasing interest in the biological and technological role of natural antioxidants present in green tea extracts. This is due to the inhibition of the oxidative process showed by tea catechins, which is higher than those of synthetic antioxidants (such as BHT) and other vegetal extracts (rosemary, oregano, grape seeds). In a first step of the work a rapid reversed phase HPLC method for the determination of catechins in green tea extracts, using a binary gradient system, was developed. Commercial green tea extracts were analyzed and the different catechins quantified. EGCG ((−)-epigallocatechin-3-gallate) and EGC ((−)-epigallocatechin) were proposed as index of the antioxidant quality of tea extracts. Subsequentely, the previous chromatographic method was applied on a HPLC–MS system in order to verify the accuracy of some HPLC-DAD results and compare the two detection modes, on such a polyphenolic mixture. The use of mass spectrometry detection in quantification of catechins ensured an higher specificity of the method and a constant qualitative control of the identity of chromatographic peaks thanks to the concurrent acquisition of more than one mass signal (as M + 1 and M + Na pseudomolecular peaks). © 2002 Published by Elsevier Science Ltd.

Keywords: RP-HPLC-DAD; HPLC/MS; Green tea; Catechins; Antioxidants

1. Introduction

The growing interest in the antioxidant properties of the polyphenolic compounds contained in vegetables and fruits derives from their strong activity and low toxicity compared with those of synthetic phenolic antioxidants, such as BHT (butylated hydroxytoluene) as reported by several authors (Marinova & Yanishlieva, 1997; Nakatani, 1996). These products could be helpful against human cancers, arteriosclerosis, ischaemia events and inflammatory diseases, which are partially caused by exposure to oxidative stress (Halliwell, 1996; Namiki, 1990).

In relation to the increasing use of vegetable extracts as food antioxidants, nutrition complements, or even as drugs it is necessary to define their quality, by identifying and quantifying their major components.

Green tea (*Camellia sinensis*) is an excellent source of polyphenol antioxidants, known as green tea catechins (GTCs). Green tea is a dried, unfermented product, in which GTCs are more preserved than in partially fermented (oolong tea), or fully fermented tea (black tea). In fact, fermentation process involves browning reactions which are catalyzed by polyphenol oxidase. Non-fermented green tea predominantly contains flavanols (such as catechins, flavandiols) and phenolic acids (such as gallic acid, cumaric acid or caffeic acid). During fermentation, the polyphenols are enzymatically oxidized and polymerized to theaflavins and thearubigins (Liebert, Licht, Böhm, & Bitsch, 1999) and a simultaneous increase of caffeine and sugars contents is also registered (Sanderson & Graham, 1973). Based on flavan-3-ols chemical structure, catechins which show three hydroxyl groups in the B ring (positions 3′, 4′, 5′) are called gallocatechins, while a gallic acid substitution in the position 3 of the C ring, is characteristic for catechin-gallate. The more represented catechins of green tea are seven: (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (+)-catechin, (C), (−)-epigallocatechin-3-gallate (EGCG), (−)epicatechin (EC), (−)-gallocatechingallate (GCG), (−)-epicatechingallate (ECG). The content of the GTC isomers can vary among different green teas, depending on the species, the climate, the cultural practices and, in the case of green tea extracts (GTEs), on the conditions and technology used for the extraction and storage.

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Many studies have been published on the antioxidant properties of catechins and their mechanism. Chen and Chan (1996) reported the following order of catechins antioxidant activity EGC > EGCG > EC > ECG as a result of canola oil oxidation tests. Therefore, the antioxidant ability of different GTEs and, as a consequence, their quality vary according to their GTCs qualitative and quantitative composition to the concentration and more or less drastic drying treatments, as pointed out by Gallina Toschi, Bordoni, Hrelia, Bendini, Lercker, and Biagi (2000).

In this work a rapid RP–HPLC method for the separation and the quantification of catechins, gallic acid and caffeine, was set. Detection was performed by diode array detector (DAD) at 270 nm and by mass spectrometry (MS), in a positive electrospray mode (API–ES+); adequate calibration of ES parameters was required to optimize the response (Lazou, De Geyter, De Reu, Zhao, & Sandra, 2000). In order to correctly identify each peak, the mass spectra of the GTCs were compared with those of the corresponding standards and the samples were spiked with the GTC standards when analyzed by HPLC–UV; this ensured a correct identification of the single GTC, avoiding errors generated by the similarity of the UV spectra of such compounds and the possibility of peak overlapping. Finally, a comparison between the quantitative determination of GTCs by UV and MS detections was also carried out.

2. Materials and methods

2.1. Samples

Green tea extracts (GTEs) were kindly donated by Di Minno D. & C. S.r.l. (Milan, Italy), Indena (Milan, Italy) or purchased from a local market.

GTE-1 was defined as an aqueous spray-dried extract, containing caffeine (identified by TLC). The extract was dissolved in a distilled water/formic acid solution (99.7/0.3 v/v), after sonication for 10 min at 30 °C, in order to induce faster solubilization.

GTE-2 had polyphenol content higher than 60% (w/w), an EGCG content higher than 40% (w/w) and a caffeine content lower than 0.1% (w/w) (HPLC determination).

GTE-3 had a polyphenol content equal to 75%, an EGCG content equal to 30%, a content of other catechins equal to 40% and a caffeine content lower than 8% (HPLC determination).

GTE-4 (from local market) was an aqueous extract of Chinese whole green tea leaves, (*Camellia thea Link*.) which was prepared in our laboratory. Green tea leaves (1.5 g) were exactly weighed (±0.0001), dried for 2 h at 80 °C and immersed in 100 ml of hot distilled water (100 °C) for 10 min. The tea infusion was passed through a cellulose filter (15 cm in diameter) (Superfiltro, Milan, Italy) and was taken to a final volume of 200 ml; 5 ml of this solution were then diluted to 25 ml with distilled water. Of this last solution 20 ml were injected into RP–HPLC. The product was defined by caffeine content of 1.4% (HPLC determination).

GTE-5 (from a local market) was an aqueous extract of minced green tea leaves, obtained in our laboratory using the same extraction procedure described for GTE-4.

BTE-1 (black tea extract) was an aqueous extract of minced black tea leaves, obtained in our laboratory following the same extraction procedure described for GTE-4 and GTE-5.

2.2. Standards

Gallic acid (GA, 98%), (−)-gallocatechin (GC, purity not specified), (−)-epigallocatechin (EGC, 98%), (+)-catechin (C, 98%), (−)-epigallocatechin-3-gallate (EGCG, 95%), (−)-epicatechin (EC, purity not specified), (−)-gallocatechingallate (GCG, 98%), (−)-epicatechingallate (ECG, 98%), caffeine (CAF, 99.9%), were purchased from Sigma Chemical Co. (St. Louis, MO, USA); BHT (butylated hydroxytoluene, 99%) was supplied by Carlo Erba (Milan, Italy); tyrosol (2-(4-hydroxyphenyl)ethanol, TYR, 97%) was supplied by Fluka (Neu-Ulm, Switzerland).

2.3. Solvents

HPLC-grade methanol and formic acid were from Carlo Erba (Milan, Italy), HPLC-grade acetonitrile was supplied by Prolabo (Paris, France). All the other chemicals and solvents were high-analytical grade ones. Double distilled water was prepared in our laboratory from deionized water.

3. HPLC–DAD analysis of tea extracts

The HPLC analyses were performed on a HP Series 1050 (Hewlett Packard, Wilmington, DE, USA), equipped with a quaternary pump delivery system, a Rheodyne injection valve (20 μl capacity, Cotati, CA, USA) and a HP diode-array UV–Vis detector Series 1050; integration and data elaboration were performed by the Chemstation software (HP). A Luna™ 5 μm C18, 25 cm×4.6 mm i.d. (Phenomenex, Torrance, CA, USA) column with Reodyne precolumn filter 7335 model, was used. All solvents were filtered with 0.45 μm Millipore filter disk and degassed by stripping of helium. A gradient elution was carried out using the following solvent systems: mobile phase A, double distilled water/methanol/formic acid (74.7/25/0.3; v/v/v); mobile phase B, acetonitrile/formic acid (99.7/0.3; v/v). The linear gra-

dient elution system was: 100% A for 8 min, to 100% B in 33 min, standing at 100% B for 5 min and returning to 100% A, after other 5 min. The flow rate was 1.0 ml min^{-1} and the quantification of the catechins was performed at 270 nm. Of each sample 20 μl were injected, after filtration through a 0.45 μm filter disk.

Identification of the catechins, gallic acid and caffeine was carried out by comparing the retention times and the UV absorbance of the unknown peaks to those of the standards. Calibration curves for GA ($R^2 = 0.999$), EGC ($R^2 = 0.996$), C ($R^2 = 0.999$), EGCG ($R^2 = 0.999$), EC ($R^2 = 0.999$), GCG ($R^2 = 0.998$), ECG ($R^2 = 0.997$) and caffeine ($R^2 = 0.999$) were done using standard solutions at five different concentrations between 0.5 and 0.01 mg ml^{-1}. All aqueous solutions of GTEs and the BTE-1 were prepared in duplicate and analyzed in triple for a total of six replicates per sample.

4. HPLC-MSD analysis of tea extracts

The HPLC-MS analysis were performed on a HP Series 1100 (Hewlett Packard, Wilmington, DE, USA) equipped with a binary pump delivery system, a degaser (model G1322A), an autosampler (automatic liquid sampler, ALS, model G1312A) and a HP-mass spectrometer detector (MSD, model G1946A); integration and data elaboration were performed by the Chemstation software (Hewlett Packard).

The same column and filter of the HPLC–DAD analysis were used. The same gradient elution and the same flow rate described above were carried out.

All the GTCs standards and GTE-2 were analyzed by HPLC–MS. Tyrosol was chosen as internal standard (IS). Mass spectra of catechins, gallic acid and tyrosol were recorded by positive ions detection mode using an Electrospray (API–ES) ionizing source with nitrogen as drying gas. The selected values for Spray Chamber parameters were as follows: capillary potential, 4000 V; gas temperature, 350 °C; drying gas flow, 13 l min^{-1}; nebulizer pressure, 60 psig.

Identification of compounds by HPLC–MS analysis was carried out by comparing retention times and mass spectra of the unknown peaks to those of the standards.

Characteristic ions for each catechin were studied at different voltages, applied between the mass capillary and the first skimmer (fragmentor values).

HPLC–MS detection of GTE-2 was performed by single ion monitoring (SIM) mode, setting the fragmentor value at 60 V, with two different ions for each compound (M + 1 and M + Na), except for tyrosol, which was quantified by a single ion (m/z 121, M-17). Quantification of GTE-2 catechins was performed by the extraction of each compound's ions from the original SIM-chromatogram. Calibration curves for GC ($R^2 = 0.999$), EGC ($R^2 = 0.999$), C ($R^2 = 0.999$), EGCG ($R^2 = 0.998$), EC ($R^2 = 0.9945$), GCG ($R^2 = 0.998$), ECG ($R^2 = 0.998$) and tyrosol ($R^2 = 0.999$), were done using standard solutions at four different concentrations, between 0.025 and 0.003 mg ml^{-1}. Aqueous solutions of GTE-2 were prepared in triplicate and analysed in double for a total of six replicates.

5. Results and discussion

A rapid RP–HPLC method for the separation of catechins, GA and caffeine was developed. To avoid interaction between the free hydroxyl groups of the polyphenols and the stationary phase, all the standard solutions were prepared in acidified distilled water (with formic acid); this is required also because catechins are more stable in acidic media as reported by Zhu, Zhang, Tsang, Huang, and Chen (1997).

The Luna™ column (Phenomenex, Torrance, CA, USA) was chosen because of its high stationary phase surface and a constant supports dimension that permitted a complete separation of catechins and caffeine in a short time (18 min) (Gallina Toschi et al., 2000). Catechin contents of GTEs and BTE-1, by HPLC–DAD method, are reported in Table 1.

The catechin contents of GTE-2 and GTE-3 were similar (697 and 757 mg/g, respectively) as indicated on the label. On the other hand, GTE-1 catechin content was very low (93 mg/g) indicating a lack of purification and concentration steps. In addition, the latter showed a GTCs composition quite different from the others samples (with an excess of GCG and a lower amount of EGCG) that could suggest a partial degradation, probably derived from different or more drastic conditions of drying. EGCG, which is considered an antioxidant activity marker by Miller, Castelluccio, Tijburg, and Rice-Evans (1996), is highly present in GTE-2 and

Table 1
Gallic acid, catechins and caffeine contents, by RP–HPLC, in GTEs and BTE-1 (mg/g of extract)[a]

	GTE-1	GTE-2	GTE-3	GTE-4	GTE-5	BTE-1
GA	10.8	7.8	5.4	1.7	2.0	7.7
EGC	12.9	101.8	39.9	16.5	19.0	7.9
C	8.0	9.2	52.9	n.d.	1.3	3.8
CAF	44.8	n.d.	60.4	18.7	24.6	35.0
EGCG	26.0	412.9	299.9	29.6	44.8	6.6
EC	5.2	43.6	56.8	3.9	5.0	4.9
GCG	30.8	11.6	160.6	2.4	6.1	1.7
ECG	10.1	118.4	147.5	10.2	14.0	9.2
Total catechins[b]	93.0	697.5	757.6	62.7	90.1	3.4

[a] UV detection at 270 nm. GA = gallic acid; GC = gallocatechin; EGC = epigallocatechingallate; C = catechin; EGCG = epigallocatechingallate; EC = epicatechin; GCG = gallocatechingallate; ECG = epicatechingallate. GTEs and BTE samples are described in Section 2.

[b] Total Catechins does not include GA.

GTE-3, but is 10–20 fold lower in GTE-1. This fact could explain the weak antioxidant activity showed by GTE-1 in the Oxidative Stability Istrument (OSI time value) (Gallina Toschi et al., 2000; Jebe, Matlock, & Sleeter, 1993). As reported by the manufacturers, no caffeine was found in GTE-2 indicating the higher purification of this extract as compared to GTE-1 and GTE-3. The low content of total catechins demonstrated by BTE-1 (34.4 mg/g) respect to GTE-4 (62.7 mg/g) and GTE-5 (90 mg/g) was supposed to be due to the polyphenol oxidation and/or polymerization promoted by the fermentation of tea leaves. The augmentation in caffeine content during the fermentation, already recorded by other authors (Liebert et al., 1999) could explain the high percentage of this compound in black tea (35 mg/g).

Table 2
Ions detected for qualitative and quantitative analysis of GTCs and tyrosol (IS)[a]

Catechin	Retention time (min)	M + 1	M + Na
GA	4.6	171	193
GC	4.6	307	329
EGC	7.5	307	329
C	8.5	191	313
EGCG	13.2	459	481
EC	14.5	291	313
GCG	15.2	459	481
ECG	16.6	443	465
Tyrosol (IS)	10.3	121[b]	–

[a] UV detection at 270 nm. GA = gallic acid; GC = gallocatechin; EGC = epigallocatechingallate; C = catechin; EGCG = epigallocatechingallate; EC = epicatechin; GCG = gallocatechingallate; ECG = epicatechingallate. GTEs and BTE samples are described in Section 2.
[b] M-17.

GTE-2 was chosen for further HPLC–MS analysis. Two different ionization sources, electrospray ionization (EI) and chemical ionization (CI), both at atmospheric pressure, were compared in analysis of catechins, but the sensitivity of detection was similar. Electrospray mode was chosen for quantification.

It was preferred to carry out a positive ions' detection because of its quite better signal-to-noise response, onto GTCs analysis. Tyrosol was found to be an ideal internal standard because of its phenolic structure. Furthermore, using the above gradient elution, tyrosol retention time (RT) was about 10 min, so it should permit a good resolution and separation from C (RT = 8.8) and EGCG (RT = 13.6 min).

In order to increase sensitivity in MS detection for quantitative analysis, ions M + 1 and M + 23 (identified as the adduct M + Na, present as impurity) for each GTC, were chosen as the most abundant and representative signals. Tyrosol (IS) was detected by means of its M-17 fragment (Table 2).

Voltage between mass capillary and the first skimmer, named fragmentor, was chosen to be 60 V; this value represented the best compromise among optimal fragmentor for all *M + 1 plus M + Na* ions detected in every GTC.

In Fig. 1 chromatograms of GTE-2 recorded by both UV–Vis detector ($\lambda = 270$ nm) and MSD, in a positive SIM mode are compared. Mass spectra of each peaks

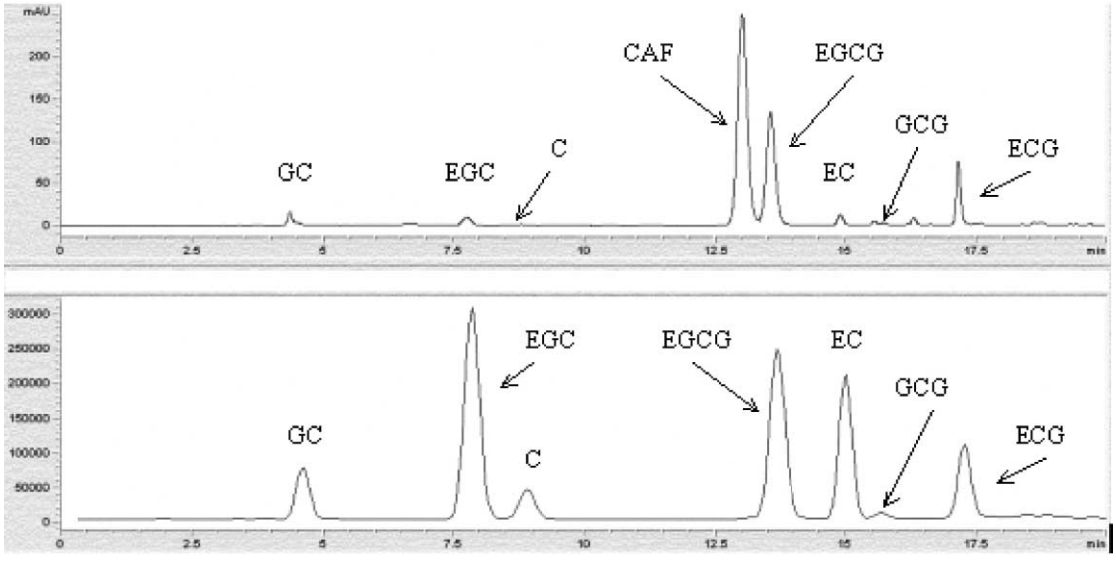

Fig. 1. (Upper trace) chromatogram of GTE-2, recoverd by UV–Vis detector, 270 nm; (lower trace) Chromatogram of GTE-2, recovered by MS detector. Column Luna™ 5 μ C 18, 25 cm×4.6 mm i.d.; Reodyne precolumn filter 7335 model. Method HPLC: mobile phase A (double distilled water/methanol/formic acid, 74.7/25/0.3, v/v/v) and mobile phase B (acetonitrile/formic acid, 99.7/0.3, v/v). The linear gradient eluition system was: 100% A for 8 min, to 100% B in 33 min, standing at 100% B for 5 min and returning to 100% A after 5 min. The flow rate was 1 ml min^{-1}; 20 μl of sample were injected, after filtration through a 0.45 μm filter disk. EGCG = epigallocatechingallate; EC = epicatechin; GCG = gallocatechingallate GC = gallocatechin; EGC = epigallocatechingallate; C = catechin; gallocatechingallate; ECG = epicatechingallate.

were compared with those of standard catechins and showed the true identity of the first eluted compound to be GC rather than GA. In order to establish the possibility of co-elution of the two compounds, each one was detected separately by an extract ions function on the original SIM chromatogram. The complete absence of GA (relatively to its detection limit, DL = 3 ng) was found.

Table 3
Quantitative determination (HPLC–MS) of main catechins in GTE-2 extract[a]

Catechin	mg/g±S.D.	Percent of total (P%)	Relative percent (%)
GC	35.6±1.0	3.6	3.9
EGC	140.7±5.1	14.1	15.4
C	13.7±0.7	1.4	1.5
EGCG	478.9±35.4	47.9	52.4
EC	91.4±3.2	9.1	10.0
GCG	7.3±0.4	0.7	0.8
ECG	146.2±3.8	14.6	16.0
Total	914.0±33.8	91.4	100.0

[a] UV detection at 270 nm. GA = gallic acid; GC = gallocatechin; EGC = epigallocatechingallate; C = catechin; EGCG = epigallocatechingallate; EC = epicatechin; GCG = gallocatechingallate; ECG = epicatechingallate. GTEs and BTE samples are described in Section 2.

In this viewpoint, choosing of a double ions detection for each analyte in Mass chromatograms, furnished a more precise control of their identity; relative abundance of M + 1 and M + Na signals for every detected catechins was controlled to be correspondent to that found in the complete mass spectrum of such molecules. In this way a concurrent qualitative control was achieved in quantitative analysis. Table 3 reports the quantification of GTCs in GTE-2, obtained by mass detection.

6. Conclusions

In this work, five samples of green tea (GTE) and one of black tea were analyzed and significant differences in their GTCs composition were found. Since the antioxidative activity of such extracts greatly depends on the total amount of catechins and their relative presence, it could be suggested to utilize one or two catechins as markers of the quality of GTE extract. The best choice for this scope are EGCG and ECG, because they are the most abundant catechins in GTE extracts and they also exhibit the highest antioxidant activity (Chen & Chan, 1996).

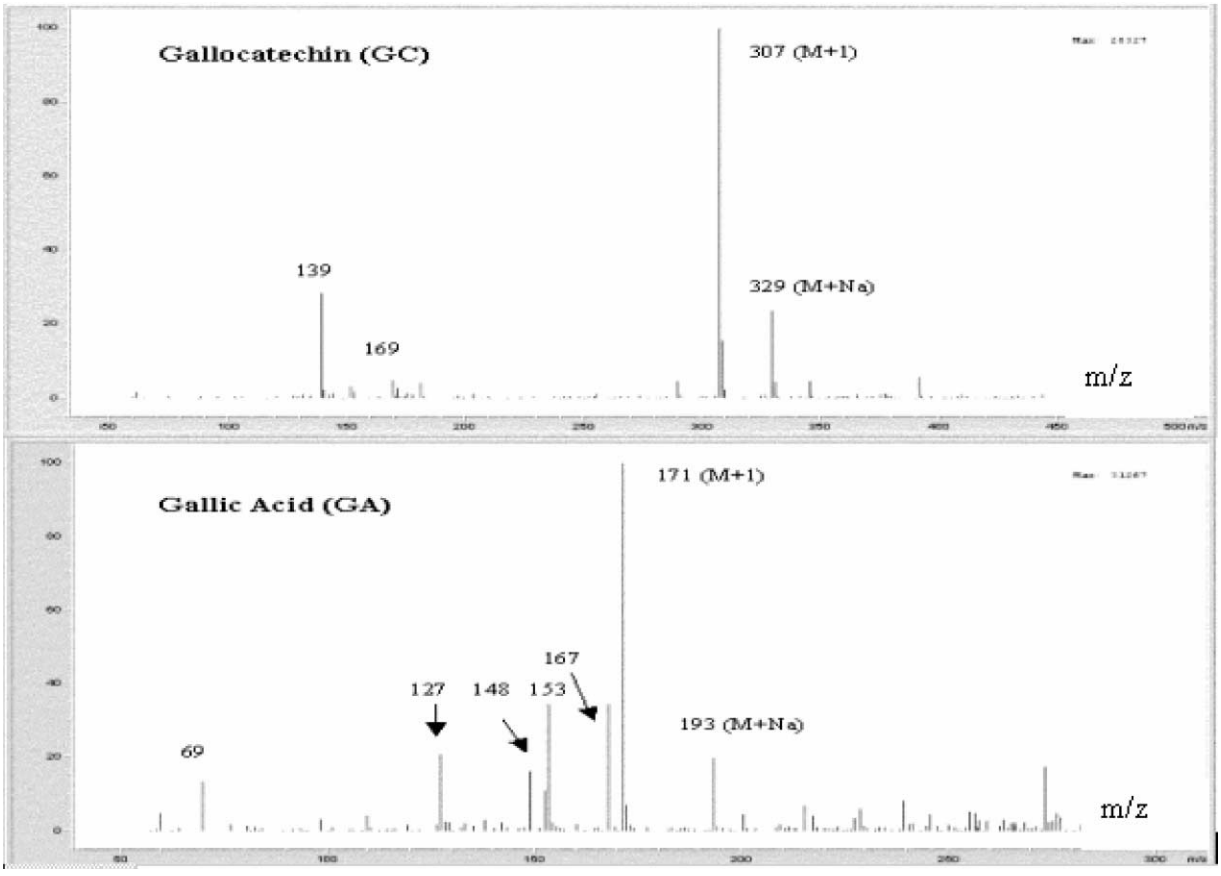

Fig. 2. Mass spectra of gallic acid and gallocatechin, recorded by ES+ at fragmentor set on 60 V spray chamber parameters: capillary potential, 4000 V; gas temperature, 350 °C; drying gas flow, 13 l min^{-1}; nebulizer pressure, 60 psig.

Table 4
Detection limits for GTCs, by DAD and MSD[a]

Catechin	Detection UV (ng)	Detection MS (ng)
GA	5	3
GC	–	12
EGC	60	11
C	60	11
EGCG	30	39
EC	20	9
GCG	10	33
ECG	9	35

[a] UV detection at 270 nm. GA = gallic acid; GC = gallocatechin; EGC = epigallocatechingallate; C = catechin; EGCG = epigallocatechingallate; EC = epicatechin; GCG = gallocatechingallate; ECG = epicatechingallate. GTEs and BTE samples are described in Section 2.

MS detection allowed to recognize the presence of gallocatechin (GC) in the catechin mixture, by comparing the typical fragmentation of each analyte; in fact, GC can be mistakenly identified as gallic acid (GA), because GC and GA co-elute under the HPLC conditions here employed. Fig. 2 shows the mass spectra of GA and GC recorded by ES+, with the fragmentor set at 60 V.

Detection limits of GTCs, evaluated by DAD and MSD, ranged between 3 and 60 ng (Table 4), exhibiting both detectors comparable sensitivity levels; these values resulted ten times better than those obtained elsewhere (Lee & Ong, 2000), otherwise on the same range of picomoles (Dalluge & Nelson, 2000). This indicates that, under those conditions, MS can achieve a similar performance to that of the UV system, even in presence of strong absorbing chromophores, such as polyphenols. However, mass spectrometer parameters could be adequately chosen in order to enhance sensibility towards a single catechin, whenever required. A double signal detection (as M + 1 and M + Na pseudomolecular peaks) clearly furnished a richer qualitative information but not the highest sensibility.

Above all, the main advantage of interfacing DAD and Mass detectors arises from the possibility of collecting a considerable amount of informations about each analyte. In particular, MS detection greatly enhances selectivity by means of the extract ions function, even if two or more analytes display a partial or a complete overlapping; in this case, it is required that the main ions should be distinct. Moreover, MS detection is very specific with respect to the type and shape of molecules, thus being able to provide important information on their fragmentation and to confirm their structure. The application of MS detection can be extremely useful for the study of more complex matrices, such as other vegetable extracts. On the other hand, optimization of MS analysis parameters can require much more significant effort.

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