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An investigation in the use of HPLC with UV and MS-electrospray detection for the quantification of tea catechins

M. Pelillo, M. Bonoli, B. Biguzzi, A. Bendini *, T. Gallina Toschi, G. Lercker

Dipartimento di Scienze degli Alimenti, Università di Bologna, Via Ravennate 1020, CAP 47023 Cesena, Italy

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Abstract

A rapid RP-HPLC method was applied to the analysis of a green tea extract (GTE). The use of a C-18 column with an internal diameter (ID) of 3.0 mm was compared with a similar one of 4.6 mm ID. Catechins quantification was performed both by ultra violet diode array detection (UV-DAD) and atmospheric pressure electrospray ionization-mass spectroscopy (API-ES-MS). Some statistical data were pointed out. High precision degree on the migration times was obtained: percent relative standard deviation lower than 0.7% was achieved by both the two detection systems. Advantages of mass detection were found to be the higher specificity and sensitivity of the signal, counterbalanced by stability of the UV-DAD signal over a significantly longer period. In fact, even if similar precision results on the quantification of green tea catechins between UV and MS detections have been found, the MS detection system was less accurate and provided less stable detector response. Finally, performance of narrower HPLC columns was evaluated in terms of detection limits: the 3.0 mm ID-column LODs of catechins were one order of magnitude lower than those of the 4.6 mm ID-column.

Keywords: Catechins; Diode array detector (DAD); Electrospray (ESI); Green tea; HPLC; Mass spectrometry detector (MSD)

1. Introduction

The growing interest on composition of phytoextracts can be explained by the biological activity of some of their constituents (Lindberg & Bertelsen, 1995; Namiki, 1990; Shahidi, 1997). Separation of natural compounds such as green tea catechins is generally carried out by HPLC using analytical columns with an internal diameter (ID) of 4.6 mm or higher (Dalluge, Nelson, Brown, & Sander, 1998; Gallina Toschi, Bordoni, Hrelia, Bendini, Lercker, & Biagi, 2000; Lee & Ong, 2000; Oszmianski & Sapis, 1989). Ultra violet diode array detection (UV-DAD) detector is a suitable detector for quantification this class of molecules and allows high sensitivity level for polyunsaturated species. However, UV detection does not discriminate different compounds having similar chromophore groups. More detailed structural information can be collected when a mass spectrometer is coupled with a UV-DAD. MS is a powerful tool for qualitative analysis to identify and confirm molecular structures of unknown compounds, and it is particularly useful for quantitative analysis, owing to its high sensitivity and selectivity (Lazou, De Geyter, De Reu, Zhao, & Sandra, 2000). Although both positive and negative detection mode provide a strong efficiency in signal to noise ratio for the analysis of this class of compounds, positive polarity is often preferred because provides more qualitative information on the compound structure and molecular weight than the negative mode (Dalluge & Nelson, 2000; Miketova et al., 1998; Pelillo, Biguzzi, Bendini, Gallina Toschi, Vanzini, & Lercker, 2002).

Electrospray LC/MS with an atmospheric pressure ionisation source (API-ES) needs an efficient liquid nebulization at the interface point, between the outlet of the chromatographic column and the mass spectrometer. HPLC methods can be applied to LC/MS as long as the number of spray chamber parameters optimized (applied voltages and temperatures, drying gas flow and pressure). In order to allow the whole desolvation of

* Corresponding author. Tel.: +39-547-636121; fax: +39-547-382348.
E-mail address: abendini@foodsci.unibo.it (A. Bendini).

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analytes, it has been suggested to perform the conventional HPLC separation using narrower columns. However, this is not a general rule for mass spectrometry detection; indeed, high amounts of solvent molecules are required in order to guarantee a reliable sample ionization when an atmospheric pressure chemical ionization (APCI) mode is used (Raffaelli, 1999), avoiding to decrease the flow-rate below 0.5 ml min^{-1}. Instead, a lower solvent flow rate is suitable for an API-ES system because it is a concentration-sensitive mode (Raffaelli & Bruins, 1991).

This work highlights how coupling options adopted between liquid chromatography and mass spectrometry can be strongly influencing the detection potentiality of this analytical system. Moreover, the possibility to use mass spectrometer, rather than UV detection, as quantitative detector to quantify catechins in green tea extracts, beyond its qualitative function, are also reported and discussed.

2. Materials and methods

2.1. Samples

Green tea extracts (GTE) were kindly donated by Indena (Milan, Italy). The polyphenol content was nominally higher than 60% (w/w). The EGCG content was higher than 40% (w/w) and caffeine was lower than 0.1% (w/w) (HPLC determination).

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%), were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Reagents

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

2.3. HPLC UV-DAD analysis

HPLC analyses were performed on a HP Series 1100 (Hewlett Packard, Wilmington, DE, USA), equipped with a binary pump delivery system, a degasser (model G1322A), an autosampler (Automatic Liquid Sampler, ALS, model G1312A), a HP diode-array UV-VIS detector (DAD, model G1315A) and a HP-Mass Spectrometer Detector (MSD, model G1946A); integration and data elaboration were performed by the Chemstation software (Hewlett Packard). Two Luna^{TM} 5 μm C-18 25 cm-long columns (Phenomenex, Torrance, CA, USA), having 4.6 and 3.0 mm as ID, respectively, with Rheodyne precolumn filter having the same stationary phase, were used. All solvents were filtered with 0.45 μm Millipore nylon filter disk. Gradient elution was carried out using the following solvent systems: mobile phase A, 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 gradient elution system was: $t = 0$ min, 100% A; $t = 8$ min, 100% A; $t = 33$ min, 100% B; $t = 38$ min, 100% B. The post-run time was 5 min. The flow rates were 1 and 0.5 ml min^{-1} for the 4.6 and 3.0 mm columns, respectively. Identification of compounds was carried out by comparing retention times and UV and mass spectra of the unknown peaks to those of the standards. The quantification of catechins by UV-DAD was performed at 270 nm. 1 μl of samples was injected, after filtration through a nylon 0.45 μm filter disk.

Calibration curves of standard catechins were arranged in the 10–50 ng range.

2.4. HPLC API-ES-MSD analysis

Mass spectra of catechins were recorded in the positive ionization mode using an electrospray (API-ES) ionizing source with nitrogen as drying gas. Spray chamber parameters were: capillary potential, 4000 V; gas temperature, 350 °C; drying gas flow, 9 l min^{-1}; nebulizer pressure 50 psig.

Quantitative analysis by MSD was carried out in the SIM (*Selected Ion Monitoring*) mode. The pseudomolecular ion ($[M+H]^+$) for each catechin was chosen as the most abundant and representative signals. Using the extracting ion chromatogram (EIC) tool from the total ion current allows recognizing partially or totally overlapped chromatographic peaks, indicating the great selectivity of mass detection.

The optimal ionization voltage applied between the mass capillary and the first skimmer (*fragmentor*) was evaluated for all molecules by FIA (*flow injection analysis*). Mass spectra of standard catechins were recorded in the 40–120 V range in order to determine the highest sensitivity for each molecule. A *fragmentor* value of 60 V was chosen as the best compromise among all ions detected and applied to the tea extracts.

2.5. Statistical analysis

Repeatability of the method (r) was calculated on the basis of the inter and intra-standard deviations (SD) on the basis of the total amount of catechins obtained by the two detectors, using the following formula:

$$r = t\,\mathrm{SD}\sqrt{2},$$

where t is the Student's value at $\alpha = 0.05$, and SD is the standard deviation. The calculated repeatability was compared with the difference ($|\Delta|_{TOT}$) between the total amount of catechins at the first quantification time and after a week. Repeatability can be assumed acceptable when $|\Delta|_{TOT} \leqslant r$.

3. Results and discussion

An HPLC method for the analysis of catechins was adapted from a previous study with a 4.6 mm ID column (Gallina Toschi et al., 2000; Pelillo et al., 2002) to a narrower one (ID = 3 mm). The 3 mm column required lower eluent flows than the 4.6 mm column to obtain a comparable separation, maintaining a relatively low pressure in the HPLC system (less than 210 bar).

An advantage of using a narrower column for HPLC-MS analysis is the saving of solvents, nebulizing and drying gas for mass spray chamber (both N_2, in our case). The demand of nitrogen is a real critical point considering that to dry a 1 ml min^{-1} flow of an aqueous solvents (from a 4.6 mm ID column) 13 l min^{-1} of drying gas and a nebulizer pressure of 60 psi are required, while 0.5 ml min^{-1} of the same phase (from a 3.0 mm ID column) needs 9 l min^{-1} of nitrogen or less and 50 psi of nebulizer gas to obtain a complete evaporation.

Retention times for all catechins are shown in Table 1. Using a 3.0 mm ID column with a flow of 0.5 ml min^{-1}, the last catechin (ECG) eluted at 17.36 min with a total analysis time comparable to that observed for the 4.6 mm ID column at 1 ml min^{-1} flow (Fig. 1). A delay of about 0.25 min between UV and MS detection is observed because they are configured in series. In terms of percent relative standard deviation (% RSD), variability of retention times calculated on five replications is very similar for both detectors and clearly less than 1%, confirming the great stability of the HPLC system.

The recovery amounts of each catechin obtained by both detectors, substantially, confirmed data obtained elsewhere for the same GTE sample (Biguzzi, Pelillo, Bendini, Gallina Toschi, Bonoli, & Lercker, 2001; Gallina Toschi et al., 2000). Table 2 shows that the single and the total amount of catechins reported as inter-repeatability (from five independent GTE solutions injected consecutively) and intra-repeatability (from the same solution injected five consecutive times) obtained by UV-DAD and MSD were similar. Moreover, the similarity between inter and intra – % RSD demonstrated that instrumental error was of the same order than the operator's one resulting by weighing and dilutions.

Table 1
Retention times (RT) and percent relative standard deviation (% RSD) of GTE catechins using the Luna^{TM} C-18 (5 μm) 250 × 3.0 mm column

Analyte	$RT_{UV\text{-}DAD}$	% $RSD_{UV\text{-}DAD}$	RT_{MSD}	% RSD_{MSD}
GC	3.99	0.35	4.24	0.42
EGC	6.87	0.62	7.06	0.61
C	7.87	0.45	8.06	0.55
EGCG	12.37	0.69	12.59	0.67
EC	14.82	0.38	15.07	0.38
GCG	15.95	0.28	16.20	0.27
ECG	17.36	0.21	17.56	0.21

The gradient is described in the text. The values are expressed as average of five injections ($n = 5$).

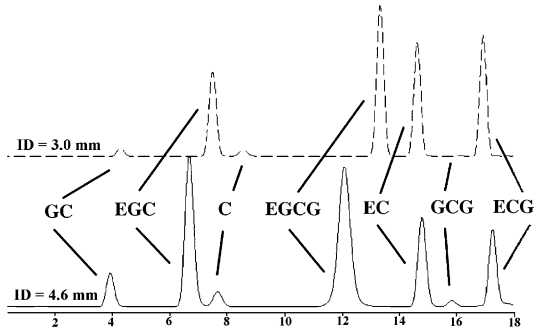

Fig. 1. SIM-MSD chromatograms of green tea catechins obtained by the C-18 Luna^{TM} 4.6 (lower trace) and 3.0 mm (upper trace) columns. The reported SIM-MSD signals were recorded selecting the $[M+H]^+$ ions of the analyzed catechins. Abbreviations: GC: gallocatechin; EGC: epigallocatechingallate; C: catechin; EGCG: epigallocatechingallate; EC: epicatechin; GCG: gallocatechingallate; ECG: epicatechingallate.

Calculated amounts of GC were significantly different between UV and MS detections, indicating a possible interference of a UV detectable molecule coeluting with the gallocatechin. Indeed, as reported in a previous work (Pelillo et al., 2002), gallocatechin and gallic acid might coelute. As shown in Fig. 2, when mass spectrum of the peak corresponding to the gallocatechin peak was recorded at the beginning (Fig. 2(a)), in the middle (Fig. 2(b)), and at the end of the peak (Fig. 2(c)), different ions were found. The three mass spectra showed characteristic ions of gallocatechin (m/z 307 is the $[M+H]^+$ ion, while m/z 329 is the $[M+Na]^+$ ion), but the mass spectrum found at the beginning of the peak showed the $[M+H]^+$ (m/z 171) and the $[M+H\text{-}H_2O]^+$ (m/z 153) ions of the gallic acid, as the mass spectrum of the standard gallic acid (Fig. 2(d)). This behavior confirmed that the UV detection of the gallocatechin peak could be affected by the coelution of the gallic acid, while monitoring the single ion of the gallocatechin in the MS detection this effect could be avoided. However, quantifications of other compounds by the two detectors were highly in agreement.

When quantification of the compounds was tested after one week, using previous calibration curves, the better robustness of UV detection respect to that of MS detection was verified (Table 2). UV-DAD quantification appeared to be only slightly less precise after one

Table 2
UV-DAD and MSD quantification (at the $t = 0$ and after 1 week) of GTE catechins (mg/100 mg extract) for the inter and intra-repeatability study

Catechin	First quantification				After 1 week quantification			
	Inter-repeatability		Intra-repeatability		Inter-repeatability		Intra-repeatability	
	Amount	% RSD	Amount	% RSD	Amount	% RSD	Amount	% RSD
$GC_{UV\text{-}DAD}$	4.40	2.95	4.23	3.58	4.58	2.10	4.55	2.50
GC_{MSD}	2.19	2.62	2.03	1.74	2.55	3.51	2.34	3.42
$EGC_{UV\text{-}DAD}$	11.92	1.38	11.82	1.27	12.13	2.17	12.07	0.96
EGC_{MSD}	11.53	1.95	10.88	1.65	13.32	3.99	12.40	3.42
$C_{UV\text{-}DAD}$	0.80	7.06	0.83	2.59	0.86	3.53	0.89	8.70
C_{MSD}	0.89	1.70	0.87	2.85	1.03	2.98	0.96	3.86
$EGCG_{UV\text{-}DAD}$	39.07	0.77	38.78	1.02	40.29	1.67	40.30	1.24
$EGCG_{MSD}$	36.13	2.81	33.71	1.60	43.71	4.06	39.64	5.54
$EC_{UV\text{-}DAD}$	5.57	2.22	5.55	2.57	5.91	4.11	6.21	5.37
EC_{MSD}	5.38	1.06	4.92	5.83	6.29	2.98	5.59	8.46
$GCG_{UV\text{-}DAD}$	0.72	3.94	0.69	4.35	0.79	6.60	0.85	4.56
GCG_{MSD}	0.77	7.01	0.65	6.52	1.01	5.19	0.85	16.95
$ECG_{UV\text{-}DAD}$	10.04	0.87	10.10	1.56	10.31	1.47	10.55	2.80
ECG_{MSD}	9.69	2.79	8.84	3.84	11.58	2.98	10.15	8.54
$TOT_{UV\text{-}DAD}$	72.52	0.88	71.98	0.77	74.88	1.72	75.40	0.96
TOT_{MSD}	66.58	2.46	61.91	2.08	79.51	3.75	71.93	5.69

The values are expressed as average of five injections ($n = 5$).

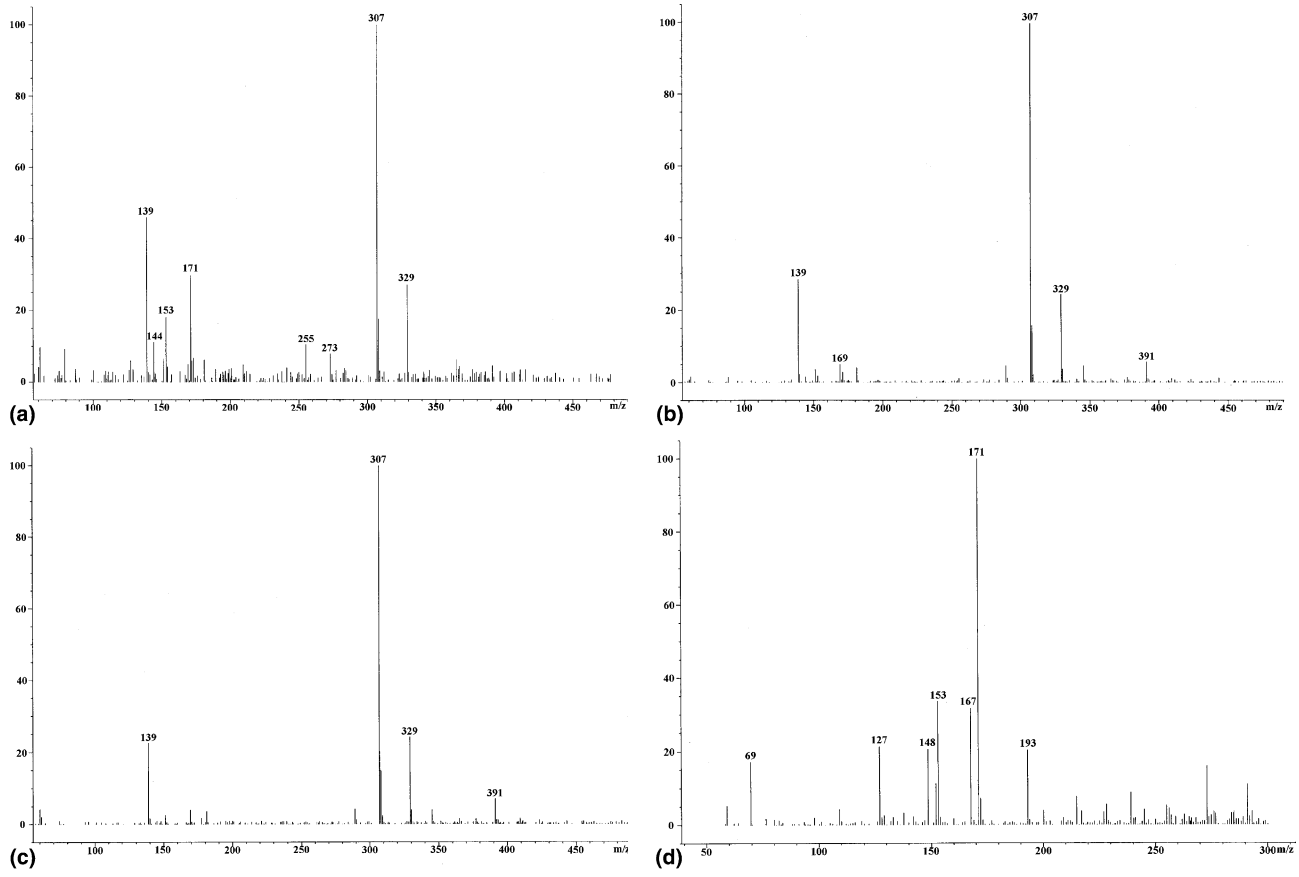

Fig. 2. Mass spectra of the peak corresponding to the gallocatechin, and mass spectrum of the standard gallic acid. (a) mass spectrum recorded at the beginning of the gallocatechin peak; (b) mass spectrum recorded in the middle of the gallocatechin peak; (c) mass spectrum recorded at the end of the gallocatechin peak; (d) mass spectrum of gallic acid.

week, whereas MSD gave a sensible shift in precision and an evident lack in accuracy. In particular, we could highlight that the total amount of catechins in the second MS quantification (after one week) was considerably different from the previous one, suggesting that regular calibration is needed for MSD analysis.

The dramatic decrease in precision for MS intra-repetitions after one week can be solely explained by assuming a less stable detector response under these analytical conditions.

The evaluation of method repeatability showed that the UV-DAD inter-repeatability was acceptable while the intra-repeatability was only slightly out of the range of acceptability (Table 3). The low value of standard deviation suggests that a modification in the sample concentration could be occurred in that case. On the other hand, statistics on MSD repeatability, demonstrated that the delay time from the first to the second quantification (1 week) did not allow to work under constant conditions for mass detection. A weak point of mass detection seems to be the state of the spray chamber that should be frequently cleaned when complex samples such as GTE are injected or long sequences are performed, in order to guarantee constant sensitivity.

Limits of detections (LOD) of catechins with both detectors were calculated as the amount corresponding to three times the noise recorded in chromatograms (S/N = 3). Resulting data (Table 4) show the higher sensitivity of MS detection, but this difference did not reach one order of magnitude. Performance of MSD in terms of sensitivity could be improved by reducing dead volumes between the two detectors.

One general recommendation (suitable for any kind of column) in order to improve mass separation efficiency and to give a better signal to noise ratio for mass spectra chromatograms is to bypass UV-DAD for final quantification and connect the column directly to the mass spectrometer.

In spite of more critical dead volumes, the use of a narrower column gives lower detection limits due to the increase in theoretical plate numbers. This is in agreement with the Van Deemter law where, since both the particle size and the lengths of the columns are identical, the separation efficiency is governed by the mass transfer in the mobile phase (depending on the ID of columns and, consequently, on the selected flow-rate) as reported by Skoog and Leary (1971). Calculated catechins' LODs for a 3.0 mm ID column separation, were lower than those relative to the same analysis with a 4.6 mm ID column (Pelillo et al., 2002): the resulting improvement was higher than one order of magnitude (more than 10 times lower for UV detection and almost 50 times lower for MS detection).

Table 3
Repeatability test (r) on the total amount of GTE catechins obtained by the inter and intra-studies[a]

	Inter-repeatability		Intra-repeatability	
	r	$\lvert\Delta\rvert_{TOT}$	r	$\lvert\Delta\rvert_{TOT}$
UV-DAD	2.65	2.36	2.31	3.42
MSD	4.27	12.93	3.13	10.02

$\lvert\Delta\rvert_{TOT}$ is the difference between the average values of total amounts of catechins for inter and intra-repetitions made: repeatability is preserved if $\lvert\Delta\rvert_{TOT} = r$.

[a] Evaluation of the 1 week-repeatability for UV-DAD and MSD on total catechins amount (mg/100 mg extract).

Table 4
Limits of detection (LODs) for catechins obtained by UV-DAD and MSD analysis[a]

Analyte	$LOD_{UV\text{-}DAD}$	LOD_{MSD}
GC	1.66 ± 0.07	0.33 ± 0.01
EGC	3.85 ± 0.03	0.37 ± 0.01
C	2.24 ± 0.09	0.60 ± 0.01
EGCG	1.92 ± 0.04	0.71 ± 0.01
EC	3.21 ± 0.11	0.67 ± 0.01
GCG	0.84 ± 0.04	0.50 ± 0.01
ECG	0.40 ± 0.02	0.41 ± 0.01

Results are expressed in nanograms.

[a] Limits of detection of catechins (average ± SD).

4. Conclusions

Quantitative HPLC-MSD analysis of catechins using a 3.0 mm ID C-18 column needs less than 20 min. Results are comparable to the relative 4.6 mm ID method but solvent flow-rate was reduced to a half, and 30% of the nebulizing gas used for the sample ionization in the spray chamber was also saved.

Sensitivity and efficiency of HPLC/API-ES-MS quantification method could significantly increased by using narrower HPLC columns and could be furthermore improved by reducing dead volumes. MS detection was found to be more sensitive than UV-DAD, and both detectors showed comparable precisions for quantification of catechins. Since repeatability conditions were maintained for a longer time in the UV-DAD quantification than in the MSD one, it would be recommendable that mass quantification underwent regular calibration, in order to compensate fall of robustness.

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