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Various applications of liquid chromatography–mass spectrometry to the analysis of phenolic compounds

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Abstract

Different options of liquid chromatography–mass spectrometry were used to establish the most suitable ion source and conditions to analyse or detect some low-molecular mass phenols, flavan-3-ols, and apply such conditions to a complex sample (wine). Data presented in this work confirm the great utility of atmospheric pressure-ionisation electrospray mass spectrometry coupled to HPLC for analysis of phenolic compounds, under negative mode in the case of low-molecular mass phenols, and under both positive and negative modes in flavan-3-ol compounds. A fragmentor voltage of 60 V could be the most suitable for analysing the compounds under study.

Keywords: Flavan-3-ols; Phenolic compounds; Food analysis; Wine

1. Introduction

The study of phenolic compounds is of great interest due to their contribution to the organoleptic properties of fruits and beverages, such as colour, astringency, bitterness, flavour and browning [1–3].

Analyses of phenolic compounds is usually carried out using high-performance liquid chromatography (HPLC), which has been widely used coupled to diode-array detection (DAD) [4,5]. However, UV–Vis spectra of non-flavonoids and their derivatives are very similar, and often the possibility of unambiguous identification does not exist. This also occurs with flavan-3-ols and their derivatives.

In the last few years, mass spectrometry (MS) coupled to HPLC has improved the identification of these compounds that show similar UV–Vis spectra [6,7] as well as the identification of new compounds [8–10]. In addition, in contrast to UV–Vis detection, the compounds are allowed to coelute in HPLC–MS as long as they are different in molecular mass. Therefore, in some cases, the analysis time may be shortened.

The oldest MS techniques needed a vacuum pressure chamber to produce compound ionisation. Nowadays two different MS techniques are widely used and both are carried out at atmospheric pressure: atmospheric pressure chemical ionisation (APCI) and atmospheric pressure ionisation electrospray (API-ES). API-ES-MS coupled to HPLC has emerged as one of the most powerful techniques for analysis of biochemical compounds [11–13].

The aim of this work was to study the different options of liquid chromatography–mass spectrometry (LC–MS), establish the most suitable ion source and conditions for analysis of commercial phenolic stan-

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dards, and apply such conditions to a complex natural samples, like wines.

2. Experimental conditions

2.1. Phenolic standards

The commercial phenolic standards used in this study were: protocatechuic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzoic aldehyde, vanillic acid, vanillin, ferulic acid, *p*-coumaric acid, caffeic acid, gallic acid and (+)-catechin, provided by Sigma, and protocatechuic aldehyde, syringic aldehyde, esculetin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin-3-*O*-gallate, epigallocatechin-3-*O*-gallate provided by Extrasynthèse. Standards were dissolved in methanol–water (80:20). The solvents water and methanol were Milli-Q and HPLC (Lab-Scan) quality, respectively.

2.2. LC–MS analysis

Analyses were performed on a Hewlett-Packard HPLC system equipped with DAD and MS systems (HP 1100 LC–MS). The LC–MS system was controlled by Hewlett-Packard Chemstation software, which allowed full instrument control, simultaneous mass spectrometry and UV–Vis spectrum data acquisition and data analysis.

Method 1: fast elution of 5 flavan-3-ol standards. HP Hypersil ODS column (100×2.1 mm, 5 μm). Linear gradient from 5 to 30% of solvent B (acetonitrile) in 15 min, being solvent A, an aqueous solution of ammonium acetate (5 m*M*) and 4.5% formic acid. The flow rate was 0.4 ml/min; 5 μl of each sample were injected. Detection was carried out at a wavelength of 280 nm.

Method 2: separation of different phenolic compounds in natural samples. Spherisorb ODS2 (250× 4.6 mm, 3 μm). Solvent A was 4.5% of formic acid in water. Linear gradient from 0 to 50% of solvent B (solvent A–acetonitrile (90:10)) in 25 min; 50–80% in 35 min and 80% isocratic for 20 min. The flow-rate was 0.7 ml/min. Detection was carried out at 280 nm.

APCI and API-ES sources and positive and negative ionisation modes were used with different fragmentor voltages. Nitrogen was used as the nebulizing and drying gas.

API-ES conditions were: nitrogen pressure, 380 Pa; drying gas, 10 l/min at 350°C; ion spray voltage, 4000 V; and variable fragmentor voltage. Mass spectrometry data were acquired in the Scan mode (mass range, m/z 50–500 for low-molecular mass phenols and m/z 150–1000 for flavan-3-ol compounds). The SIM (selected ion monitoring) mode could be used when a search for some particular ions should be done.

3. Results and discussion

3.1. Low-molecular mass phenols

Spectra of phenolic compounds obtained with the APCI source are not presented since the results do not supply much information and sensitivity was very low. The same occurred to Robards et al. [12] in the determination of citrus flavonoids.

The results obtained in the flow injection analysis (FIA) mode in API-ES technique were better and proved to be more sensitive and with less background noise than APCI. Therefore, this mode was used to study the better fragmentor voltages in successive injections. Four different voltages were applied (60, 120, 180 and 210 V). Positive- and negative-ion modes were also assayed.

Positive-ion API-ES mass spectra do not clearly show the molecular ion $[M+H]^+$ no matter which fragmentor voltage used was, except for esculetin, whose molecular ion could be clearly observed. This could be due to the formation of oxonio cation. Thus, adducts and polymeric compounds were formed though the peaks obtained had a very low abundance.

Table 1 reports the main ions observed under the negative-ion mode when low-molecular-mass phenols, underwent fragmentor voltages of 60 and 120 V. Negative-ion API-ES-MS with a fragmentor voltage of 60 V produces mass spectra where the abundance of the molecular ions $[M-H]^-$ (deprotonated species) is high, and little fragmentation occurs. Increasing fragmentor voltage up to 120 V caused a reduction of the molecular ion signal and a new low-molecular-mass fragment ion was the base peak. These low-molecular-mass ions resulted from

Table 1
Ions of negative-ion API-ES mass spectra of low molecular mass phenols[a]

Compounds	M_r	Main ions observed (m/z)	
		Fragmentor 60 V	Fragmentor 120 V
Protocatechuic acid	154	153 (109)	109 (153)
Protocatechuic aldehyde	138	137	137 (108)
p-Hydroxybenzoic acid	138	137 (93)	93 (137)
p-Hydroxybenzoic aldehyde	122	121	121 (92)
Vanillic acid	168	167	108 (167, 123, 91)
Vanillin	152	151 (136)	136 (151)
Ferulic acid	194	193 (134, 149)	134 (193)
Syringic aldehyde	182	181 (166)	181 (166, 151)
p-Coumaric acid	164	147 (103)	147 (103)
Caffeic acid	180	179 (135)	135 (179)
Esculetin	178	177	177 (133)
Gallic acid	170	169 (125)	125 (169)

[a] M_r, molecular mass. Ions with lower abundance shown in parentheses.

the loss of a carboxylic $[M-45]^-$, hydroxyl $[M-17]^-$ or/and aldehyde $[M-30]^-$ group. It should be noted that acids and aldehydes, except for vanillin, present different fragmentation patterns. The molecular ion peak of esculetin and aldehydes remain intact even with a fragmentor voltage of 120 V, though some other characteristic fragment ions are obtained. On the contrary, vanillin and acids present the ion $[M-45]^-$ as the base peak instead of the molecular ion when high fragmentor voltages (120 V) are used. This behaviour should be related to the different lability of acids and aldehydes.

Mass spectra obtained by using higher fragmentor voltages (180 and 210 V) showed a lot of peaks with very low abundance, and other peaks which correspond to adducts and polymeric compounds.

Both fragmentor voltages, 60 and 120 V, could be used to analyse these compounds, and the derivatives that could be formed in a complex sample. However, a fragmentor voltage of 60 V could be more useful for analysing the compounds under study because the only prominent peak in the negative-ion API-ES mass spectrum is the molecular ion. Moreover, the abundance of these peaks is high, thus improving sensitivity.

3.2. Flavan-3-ols

Flavan-3-ol standard compounds were also studied in the FIA mode using the two ion sources (APCI and API-ES) and the two ionisation modes (positive and negative). As in the case of low molecular weight phenols, positive and negative-ion mass spectra obtained with APCI source did not show relevant results.

Table 2 reports the main ions observed under positive and negative-ion modes when flavan-3-ol compounds, underwent fragmentor voltages of 60

Table 2
Ions of positive- and negative-ion API-ES mass spectra of flavan-3-ol compounds[a]

Compounds	M_r	Main ions observed (m/z)		
		API+	API−	
		Fragm. 60 V	Fragm. 60 V	Fragm. 120 V
(+)-Catechin	290	291 (139)	289	289 (245)
(−)-Epicatechin	290	291 (139, 150)	289	289 (245)
(−)-Epigallocatechin	306	307 (139)	305	305
(−)-Epicatechin-3-*O*-gallate	442	443 (123, 273)	441	441 (289, 169)
Epigallocatechin-3-*O*-gallate	458	459 (139, 289)	457	169 (457)

[a] M_r, molecular mass. Ions with lower abundance shown in parentheses.

and 120 V. The results showed that when the fragmentor voltage was 60 V, only the molecular ions of the five compounds under study appear. With 120 V, some new fragments may be observed, though the molecular ion is still the most abundant one. The peak at m/z 169 obtained in epicatechin-3-*O*-gallate and epigallocatechin-3-*O*-gallate corresponds to the gallate ion. Epicatechin-3-*O*-gallate mass spectra showed another ion, m/z 289, corresponding to epicatechin. The peak at m/z 245 that is formed by fragmentation of catechin and epicatechin, may be the result of the loss of a $-CH_2-CHOH-$ group.

Adduct formation was evident in the negative-ion mass spectra at higher fragmentor voltages, which could be due to the high self-polymerisation capability of these compounds. These adducts were generally not observed with low fragmentor voltages. On the contrary, studies carried out by Robards et al. [12] in citrus flavanones did not show adduct formation with high fragmentor voltages. Such difference between flavan-3-ols, and citrus flavanones, could be due to the different heterocycle substituents that modify the molecule reactivity.

A fragmentor of 60 V was therefore chosen, and a mixture of the five compounds under study was analysed by negative-ion mode API-ES-MS coupled to HPLC–DAD. Fig. 1 shows the DAD chromatogram and selected mass chromatograms.

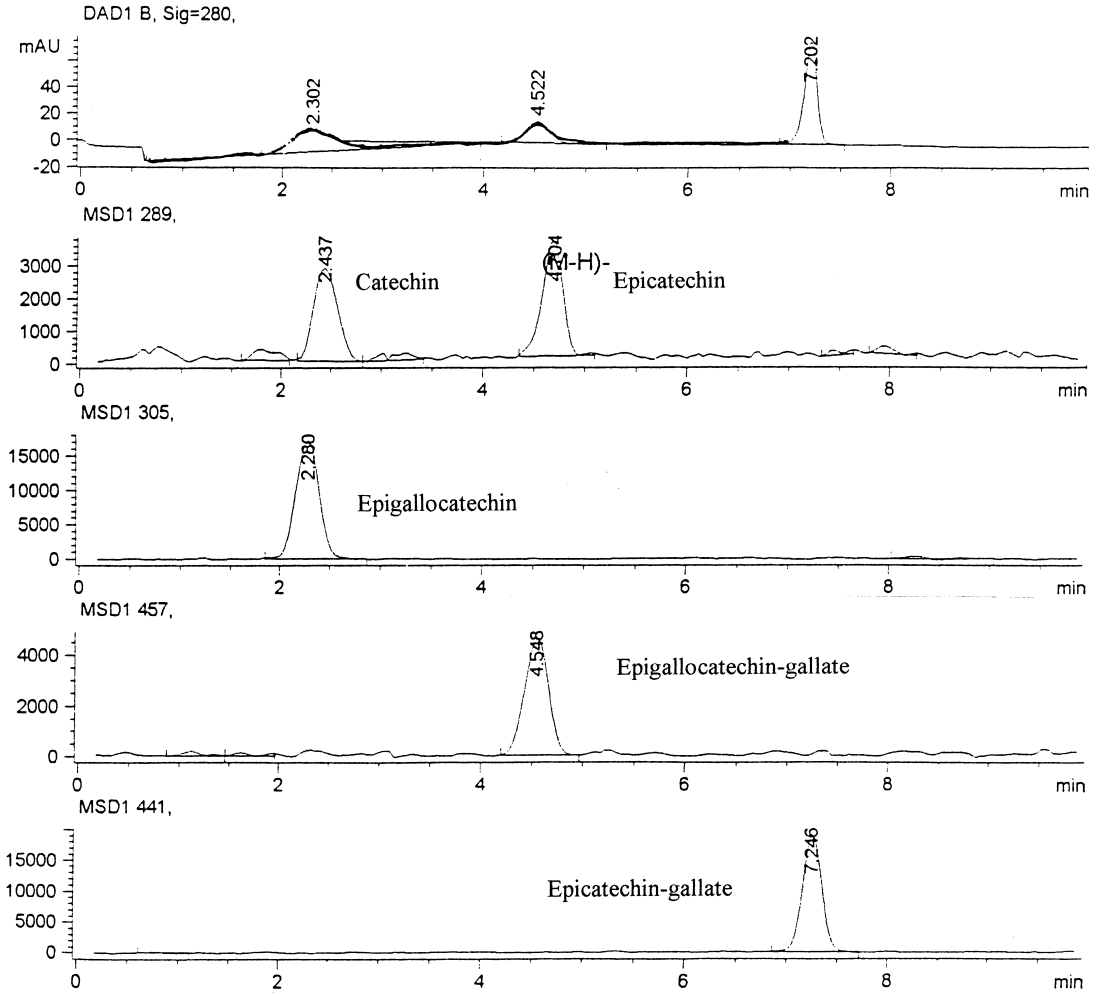

Fig. 1. DAD chromatogram and selected mass chromatograms of flavan-3-ols obtained by negative-ion mode API-ES-MS coupled to HPLC–DAD.

Comparison of mass chromatograms with DAD chromatogram, where only three peaks are clearly distinguishable, further demonstrates the utility of the LC–MS technique. Mass chromatograms at *m/z* 289 and *m/z* 305 establish the presence of catechin and epigallocatechin at the same retention time of 2.3 min. This also can be observed in mass chromatograms at *m/z* 289 and *m/z* 457, that correspond to epicatechin, and epigallocatechin-3-*O*-gallate, at the same retention time of 4.6 min.

These results could confirm the presence of other compounds whose chromatographic peaks overlap or coelute, by comparing the mass fragmentation patterns.

Most of the studies related to the detection of these type of compounds were carried out in negative-ion mode [6,7,14,15], since they are easily deprotonated [16,17]. However, Bailey et al. [8,18] obtained fast atom bombardment (FAB) mass spectra of proanthocyanidin polymers (flavan-3-ol units)

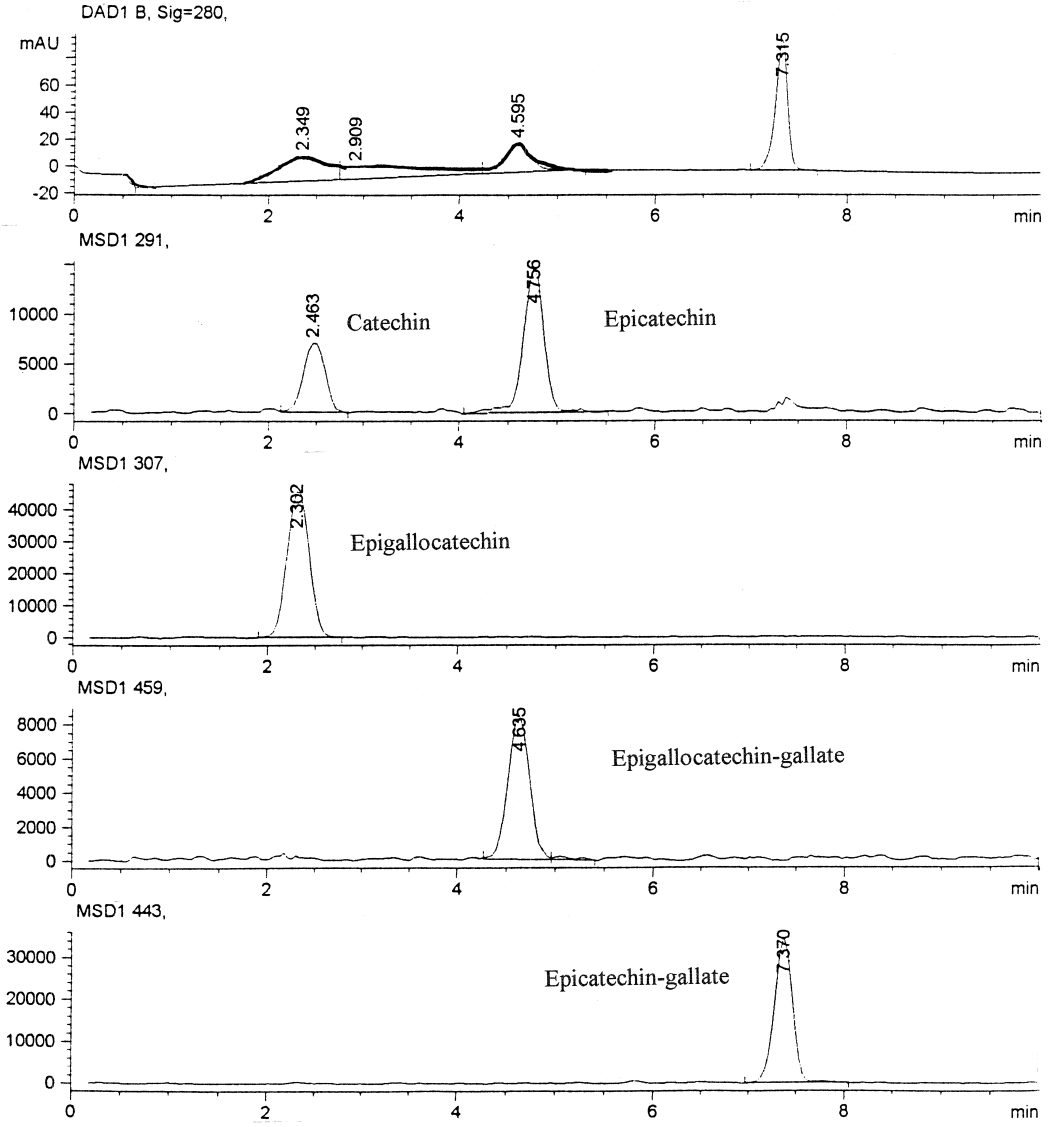

Fig. 2. DAD chromatogram and selected mass chromatograms of flavan-3-ols obtained by positive-ion mode API-ES-MS coupled to HPLC–DAD.

in the positive-ion mode. More recently Saucier et al. [19] and Cheynier et al. [20] have studied these compounds in wines using an LC–ESI-MS technique in the positive-ion mode to identify other compounds obtained by reaction of flavanols with acetaldehyde and other flavanols. Therefore, the study of the difference, especially in sensitivity, between the two ionisation modes has been subject of further study in this work. Since acid medium favours the formation of positive ions $[M+H]^+$, 4.5% of formic acid was added to the sample in order to improve the chromatographic results.

Table 2 shows the fragment ions obtained in positive-ion mode (API +) with a fragmentor voltage of 60 V. In this case, the base peak was also the molecular ion, although some other peaks may be obtained.

Mass spectral data shown in Fig. 2, have confirmed that it is possible to use the positive ionisation mode to identify flavan-3-ol molecules, with even better sensitivity than the negative ion mode. Because the natural samples are complex and usually acid and non-acid compounds could be present, the negative-ion mode was selected, because acid compounds are not detected in the positive mode.

After establishing and selecting MS conditions,

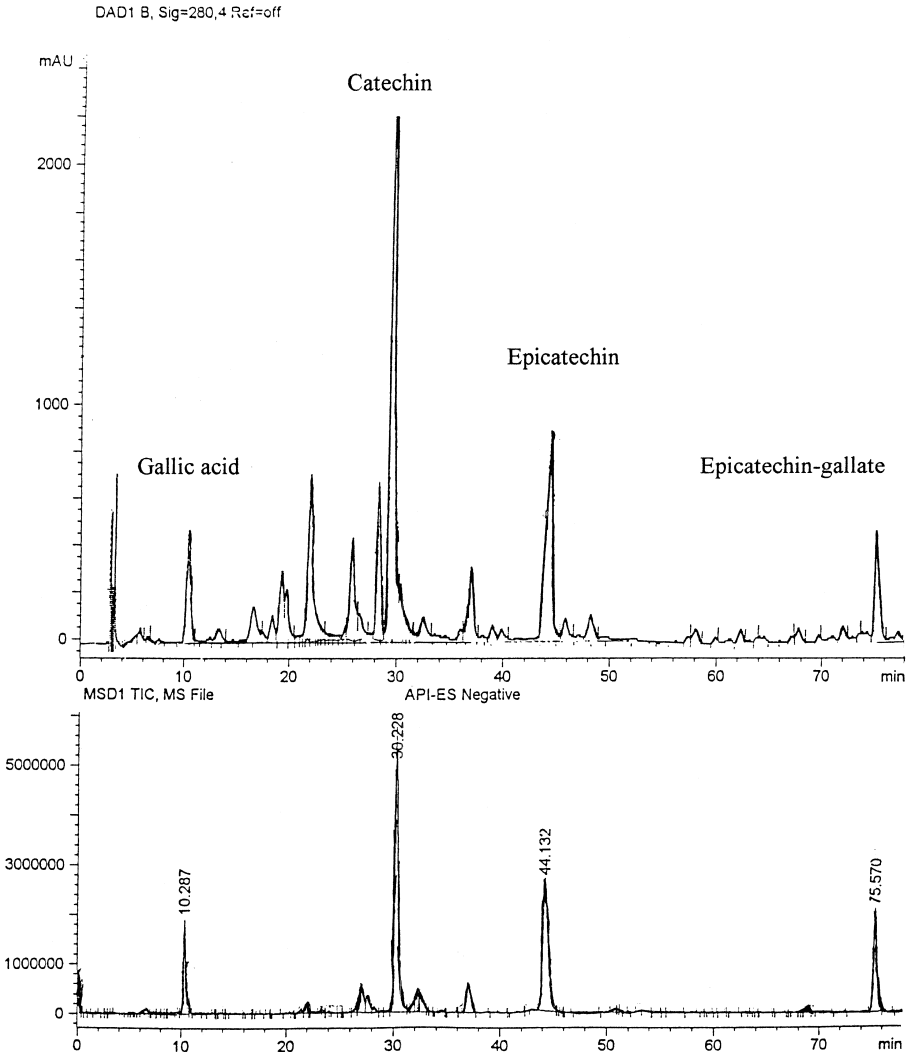

Fig. 3. DAD and TIC (total ion current) chromatogram of a wine sample using negative-ion API-ES detection.

HPLC method (method 2) was improved, and different natural samples were analysed. Fig. 3 shows the DAD trace and the total ion chromatogram (TIC) obtained for a wine sample. Catechin, epicatechin and epicatechin–gallate compounds were easily identified, in addition to gallic acid, which is the peak at 10.3 min.

Despite the obvious successes of HPLC [21,22], separations are not always feasible for resolution of a complex mixture containing many compounds. The data presented confirm the great utility of API-ES-MS coupled to HPLC for analysis of phenolic compounds, since coelution is not a problem in so far as they have different molecular masses. The major disadvantage of API-ES-MS is the operating cost.

Future works will involve further studies of the mass spectrometry of proanthocyanidins and polymers and their application to fruit and fruit derivatives.

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