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Use of porous graphitic carbon coupled with mass detection for the analysis of polar phenolic compounds by liquid chromatography

Jerome Vial^{b,*}, Marie-Claire Hennion^b, Amadeo Fernandez-Alba^a, Ana Agüera^a

^a*Departamento de Hidrogeología y Química Analítica, Universidad de Almería, La Cañada, 04120 Almería, Spain*

^b*Laboratoire “Environnement et Chimie Analytique”, associé au CNRS, Ecole Supérieure de Physique et Chimie Industrielles de la Ville de Paris, 10, rue Vauquelin, 75005 Paris, France*

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Abstract

Phenolic compounds present in olive mill wastewater (OMW) need to be quantified because of their pollution capacity toward the environment. In the present study, six representative phenolic compounds of OMW were chosen to develop a LC–MS method. The high polarity of the compounds caused problems when using traditional reversed-phase liquid chromatography (RPLC). Consequently, a method was developed on another kind of chromatographic phase: Porous Graphitic Carbon (PGC) involving the use of a tetrahydrofuran (THF) gradient. The influence of THF as mobile phase in LC–MS coupling, which is not common practice, was evaluated. In Atmospheric Pressure Chemical Ionisation (APCI) in the negative ion mode, the presence of THF in the mobile phase did not degrade the MS signal of the target compounds in the conditions studied. On the contrary, an improvement was even observed when the percentage of THF increased. The proposed PGC–LC–MS method was selective and linear for the six phenolic compounds analysed with limits of quantification lower than 5 ppm in all cases. The precision was satisfactory (pooled RSD around 6%). The analyses of OMW matrix spiked sample confirmed the good performance of the proposed method © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Water analysis; Mobile phase composition; Olive mill wastewater; Phenolic compounds; Porous graphitic carbon; Tetrahydrofuran

1. Introduction

Olive mill wastewater (OMW), produced in considerable amounts in Mediterranean countries, causes pollution of the environment. The presence of plant phenolic compounds in OMW can vary from trace level to a few hundred mg/l [1–4]. They can render wastewater treatment processes based on biological

degradation ineffective and can have a serious impact on receiving waters and soils, and their determination is of much interest for both environmental [5,6] and human care purposes [7]. Reversed phase liquid chromatography (RPLC) coupled with mass detection (MS) is one of the most adapted technique in the field of environmental analysis. However with very polar compounds like phenolic acids present in OMW, the low retention on classical RPLC columns, even when a high percentage of water is used in the mobile phase, often produces inadequate resolution. Another limitation encoun-

*Corresponding author.

E-mail addresses: jerome.vial@espci.fr (J. Vial), amadeo@ual.es (A. Fernandez-Alba).

tered with OMW samples is matrix effects, which is a consequence of the high organic carbon content of such wastewaters (>100 mg/l). To overcome this problem, high dilution ratios or sample handling is required. An elegant way to solve both the problem of poor retention and the need for a clean-up can be the use of Porous Graphitic Carbon (PGC) columns instead of alkyl bonded silica, taking advantage of its different retention behaviour [9]. To our knowledge there is no other study in the literature on the use of this phase for the LC–MS analysis of environmental samples. Six phenolic compounds likely to be present in OMW, five of them acids, were chosen as test compounds. Since retention on PGC is different from that on alkyl-bonded silica, a mobile phase containing tetrahydrofuran (THF) had to be used, and the compatibility and effectiveness of THF with Atmospheric Pressure Chemical Ionisation (APCI)-MS had to be evaluated. The characteristics of the method were evaluated and a short comparison with alkyl-bonded silica was carried out on a spiked OMW matrix.

2. Experimental

2.1. LC–MS System

The LC–MS system was a HP 1100 Series LC/MSD from Agilent (Palo Alto, CA, USA). The LC part was composed of a binary pump, an helium degasser module and an autosampler connected to the injection loop. The column effluent passed through a single wavelength ultraviolet (UV) detector before arriving in the MS interface. The APCI source was preferred to electrospray ionisation because of the signals obtained with APCI in the negative ion mode [8]. Parameters of the spray chamber were as follows: flow of drying gas, 8.5 l/min; nebulizer pressure, 50 psig; capillary voltage, 2500 V; corona current, 40 μ A; temperature of vaporizer, 450–500°C; fragmentation voltage, 60–70 V. These last two parameters had most influence on the ionic current. No exact value was given here since the optimum could a priori depend on the THF percentage and on the compound. For details, see Section 3. The HP Chemstation Revision — A.07.01 installed on a PC under Windows NT, drove the

system. The data acquisition for both UV and MS signals was also made on the Chemstation.

2.2. Compounds and solutions

The structure of the six target compounds and their main characteristics are shown in Table 1. Pure phenolic compounds were purchased from Aldrich (Milwaukee, USA). The solvents used were gradient grade and were provided by Merck (Darmstadt, Germany). For each compound, a stock solution at 400 ppm was prepared by dissolving 10 mg of product in 25 ml of pure methanol. To ensure a complete dissolution, a 10-min ultrasonication was applied. Samples used for LC analyses were prepared by dilution of the stock solutions with water. All the stock solutions were stored in amber flasks at 4°C and in these conditions no degradation was observed over at least 2 weeks.

2.3. LC method

A 100×4.6 mm I.D. 5 μ m PGC Hypercarb column from Hypersil (Shandon, UK) was used. The binary gradient of Table 2 used two solutions, A and B. A was methanol–acetonitrile–0.2 M formic acid (40/40/20 v:v:v); B was pure THF. Acidic conditions were used so that all the compounds are in their non-ionized form (pK_a values >4). All solvents were HPLC grade. The flow-rate was 1 ml/min, UV detection wavelength was 265 nm and injection volume was 10 μ l. In these conditions, as illustrated on Fig. 1, a baseline separation was observed for the six phenolic compounds. Neither the observed back-pressure, around 80 bar, nor the pH caused undue column ageing.

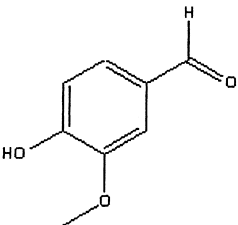
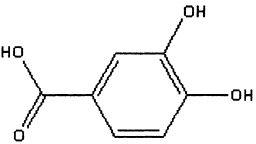
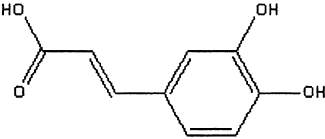
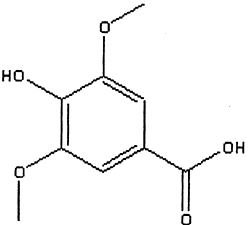
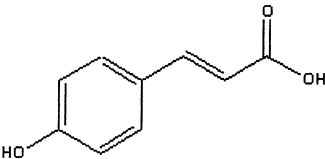
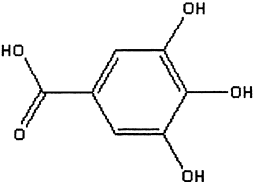
3. Results and discussion

3.1. Mass detection conditions

3.1.1. APCI fragmentation

For each of the six target compounds, two ions were selected by varying the fragmentation voltage. The higher the fragmentation voltage, the more fragmentation occurred. Values between 55 and 85 V were optimal for all compounds. The influence of

Table 1
Characteristic structures of the phenolic compounds analyzed

Name	Structure	p <i>K</i> _a
Vanillin		7.7
Protocatechuic acid		4.3; 8.7; 12.5
Caffeic acid		4.62; 9.07
Syringic acid		4.2; 9.1
<i>p</i> -Coumaric acid		4.64
Gallic acid		4.2; 8.6; 11.4; 12.8

temperature was less marked but not negligible and optimal values were between 400 and 500°C. The most intense signal (abundance of 100%), obtained

for the pseudo molecular ion $[M-H]^-$ in the experimental conditions was used for quantitative analysis whereas the second most intense (always \geq

Table 2
Mobile phase gradient used on the PGC column

Time (min)	% A	% B
0	100	0
15	100	0
35	50	50
40	0	100
44	0	100
45	100	0
60	100	0

20%) and its relative abundance in respect to the former, was used to confirm the identity of the compound. The ions chosen for each compound and

their relative abundances are shown in Table 3. Generally the main fragment ion corresponded to the loss of the carboxylic acid group.

3.1.2. Effect of THF

An important question that had to be answered concerned the percentage of THF that was possible to use in the mobile phase without degrading the capacity of ion formation. Effectively, formation and evolution of the charged microdroplets at the APCI interface can be affected by the different physico-chemical properties of the solvents used. Very little data were available about this problem so the experimental study was divided into two parts: First

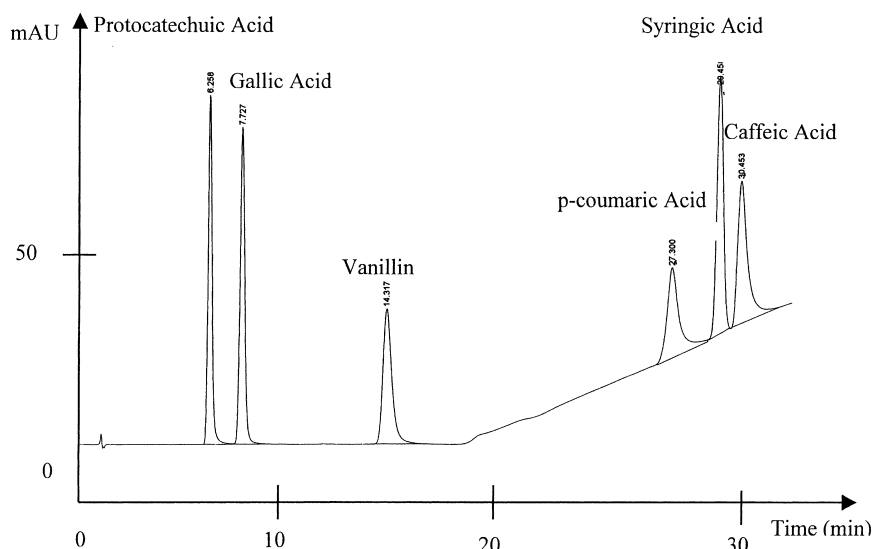


Fig. 1. LC–UV chromatogram of a mixture of the six phenolic compounds (20 ppm each) on the PGC column. Column: length, 10 cm; I.D., 0.46 cm; ambient temperature. Stationary phase: Hypercarb Porous Graphitic Carbon (Hypersil), 5 μ m. Mobile phase: acetonitrile–methanol–acidified water ([formic acid]=0.2 mol/l) 40/40/20 v:v:v and THF gradient; flow-rate, 1 ml/min; pressure drop, 80 bars; injected volume, 10 μ l; UV detection, 265 nm.

Table 3
Characteristic ions of selected phenols on APCI-MS

Name	[M-H] ⁻	Fragment ion	Relative abundance (%)
Vanillin	151	136 [M-CH ₄] ⁻	87
Protocatechuic acid	153	109 [M-COOH] ⁻	36
Caffeic acid	179	135 [M-COOH] ⁻	41
Syringic acid	197	182 [M-CH ₄] ⁻	56
p-Coumaric acid	163	119 [M-COOH] ⁻	81
Gallic acid	169	125 [M-COOH] ⁻	20

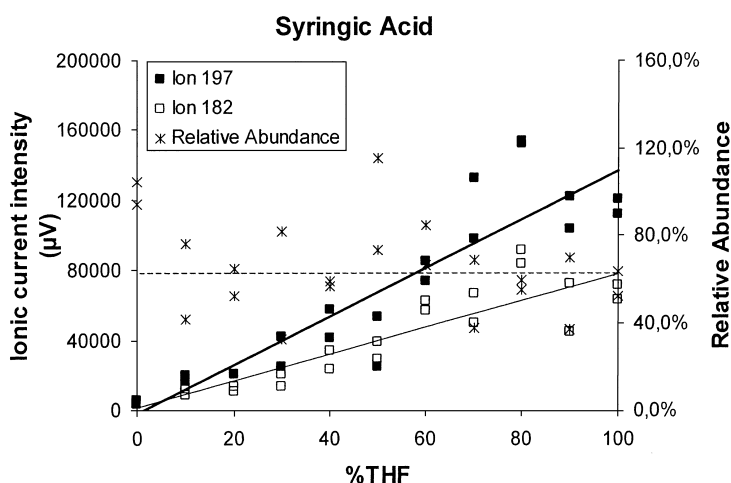


Fig. 2. Influence of THF on the ionization of syringic acid.

it was established whether the choice of the THF percentage affected the optimum vaporizer temperature and fragmentation voltage. A comprehensive study was carried out in Flow Injection Analysis (FIA) by varying the mobile phase composition in percentages between 0 and 50% for THF in respect to the other component that was A solution. For each THF percentage, temperature and voltage were varied, respectively, in the ranges 400 and 500°C and 55 and 85 V. Optimal conditions, i.e. providing best signal-to-noise, were found to be close to 70 V and 450°C. These optimal conditions were a compromise, since small differences were observed between the behaviour of the six compounds. Nevertheless, the results showed that optimal temperature and voltage were affected in a very minor way by the percentage of THF.

This ruggedness enabled to study continuously the effect of THF on ionization keeping constant the vaporizer temperature and the fragmentation voltage. The value chosen corresponded roughly to the

average value for all the THF percentages, i.e. 70 V for the fragmentation voltage and 475°C for the vaporizer temperature. Experiments were carried out for a THF percentage between 0 and 100% (in steps of 10%), the other component of the mobile phase was A solution. Experiments were carried out in duplicate. For each compound, the intensity of the ionic current corresponding to the two characteristic ions was recorded. Interpretation of the results was first based on a plot of the raw data as illustrated in Fig. 2 for syringic acid. The intensity of the noise increased little with the amount of THF. Then a model of linear variation was considered. Thanks to linear regression the parameters of the lines for all the ions and relative abundances were calculated. A Student's test on the slope was applied [11] to determine if the observed variations were significant. Results are shown in Table 4. If, as usual, the α level chosen was 5%, a slope could be considered significant when the probability to obtain a value greater than the observed t -value is less than 5% [i.e.

Table 4

Variations of the $[M-H]^-$ ionic current and of the relative abundance (ra) of the fragment ion as a function of the THF amount

Compound	Protocatechuic acid		Vanillin		Caffeic acid		Syringic acid		<i>p</i> -Coumaric acid		Gallic acid	
	$[M-H]^-$	ra	$[M-H]^-$	ra	$[M-H]^-$	ra	$[M-H]^-$	ra	$[M-H]^-$	ra	$[M-H]^-$	ra
Slope	219	0.002	1020	-0.004	477	-0.001	1388	-0.002	557	0.002	476	-0.0003
t -Value	2.48	1.46	7.60	-1.49	2.89	-2.59	9.58	-1.45	4.08	1.60	3.22	-0.39
Prob> t	0.02	0.16	<0.01	0.15	0.01	0.02	<0.01	0.16	<0.01	0.13	<0.01	0.70

($\text{prob} > |t| < 0.05$). The slopes were significantly different from zero for all the $[\text{M}-\text{H}]^-$ ions, which meant that THF percentage affected the ionization. But they were all positive, so the effect corresponded to an increase in the ionization with THF percentage and was rather an advantage. For all the compounds except caffeic acid, no significant variation of the relative abundance of the fragment ion was noticed. For caffeic acid, even if the slope was significantly different from zero, the value itself was of the same order of magnitude as for the other compounds. It meant that this different conclusion could be regarded as a statistical artefact due to a smaller dispersion for this particular set of data. On the whole, THF did not seem to affect significantly relative abundances, which was an advantage in terms of method ruggedness, especially when using a gradient. In conclusion, it has been demonstrated that, in APCI in the negative ion mode, the presence of THF in the mobile phase was not a drawback; on the contrary, it improved ionization of our phenolic compounds.

3.2. Rapid method validation

The use of THF also enabled to obtain repeatable analyses of the compounds with the PGC column. Without THF some acidic compounds were not completely eluted from the column which caused

degradation of the chromatogram injection after injection. This solvent seemed to be necessary for column conditioning. The elution order was quite different on C_{18} silica and on PGC, which confirmed the differences in retention mechanisms and the presence of interaction other than hydrophobic on PGC for polar compounds [9,10]. To assert that the PGC-LC-MS method had performances compatible with those required for routine analyses, a rapid validation of this method (without ruggedness study or round robin tests) was carried out.

3.2.1. Selectivity

For the six compounds, the minimum resolution was observed between the peaks of the syringic and caffeic acid and the value was never less than 1.5, which is acceptable even only with UV detection. Nevertheless, in the field of environmental analysis, the MS detection was generally preferred because of its inherent high selectivity, especially in single ion monitoring mode (SIM). If only the six phenolic compounds were considered, as shown on Fig. 3, the MS-SIM detection could even be considered specific.

3.2.2. Precision

The precision of the method was evaluated at a concentration level of 20 ppm by making two sets of repeated analyses on different days. The first set

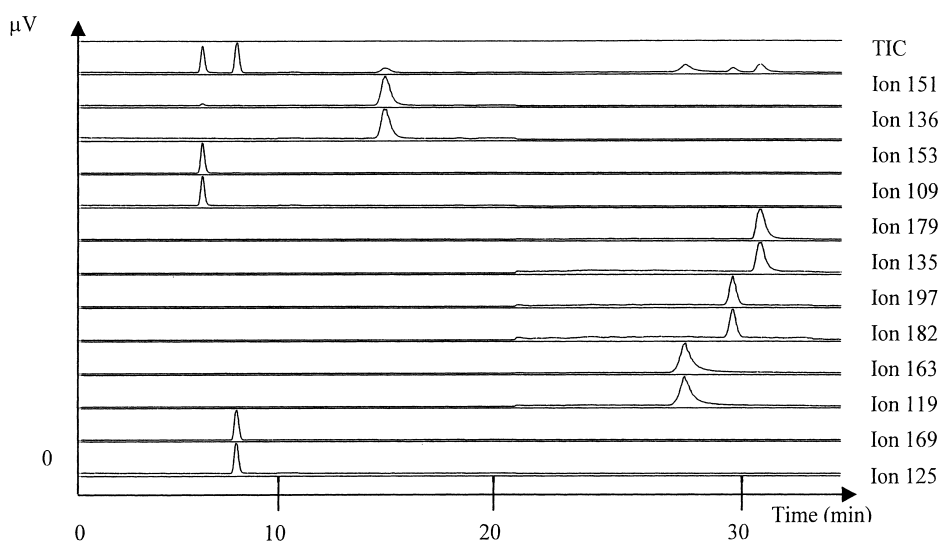


Fig. 3. LC-MS chromatograms of the six phenolic compounds obtained in SIM mode. TIC, total ionic current.

included 11 experiments and the second one 15. The responses measured on each chromatogram were: the retention time of each peak, the corresponding area of the $[M-H]^-$ ion and the relative abundance for the secondary ion. *F*-tests showed that dispersions observed within the two sets of data were not significantly different which enabled pooling the data to obtain more reliable estimates. So the final results, expressed as relative standard deviations (RSDs), were calculated from 26 experimental results. Very little dispersion was observed on the retention times, RSDs ranged from 0.3 to 1% which could allow an easy identification of the compounds. RSDs of $[M-H]^-$ ion areas ranged from 3.2 to 8.9% depending on the compound, with a pooled RSD around 6%. Performances of the method are sufficient for environmental analysis. Relative abundances were also little dispersed with a pooled RSD of 2%, which ensured a reliable product identification.

3.2.3. Analytical range

In the case where the method is applied to OMW effluents, dilutions applied would be adapted so that, in final analytical samples, concentrations are never above 20 ppm. Consequently it was not necessary to

examine the response of the system beyond this value, which was taken as the upper limit of our working range. A Eurachem-like, more reliable approach [12], was chosen in the present study to determine the limits of detection (LOD) and of quantification (LOQ) of the MS detection. Briefly, the principle consisted of determining the RSD of the analytical response, here the area, for different levels of concentration. Then, a modelling of the variation of the RSD was carried out and the LOQ was defined as the concentration value corresponding to a RSD equal to 10%. The LOD was defined as one-third of the limit of quantification. For each of the six compounds, concentrations ranging from 0.1 to 8 ppm were chosen. At each level the RSDs of the peak areas for the $[M-H]^-$ ions were estimated thanks to six repeated injections. Fig. 4 illustrates the methodology applied to *p*-coumaric acid. The numerical value of the LOQ found for this compound was 1.3 ppm, rounded off to 2 ppm. The results of LOQ determination for the six compounds are gathered in Table 5.

3.2.4. Linearity

The linearity of the response curves was evaluated

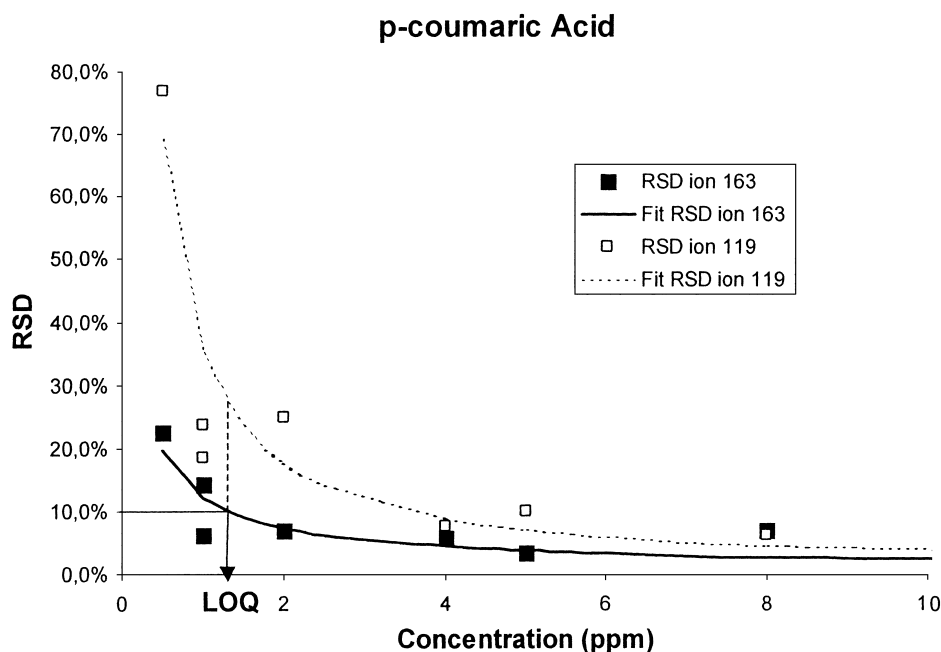


Fig. 4. LOQ determination for *p*-coumaric acid.

Table 5
LOQ for the six phenolic compounds

Compound	LOQ (ppm)
Vanillin	2
Protocatechuic acid	<0.5
Caffeic acid	5
Syringic acid	3
<i>p</i> -Coumaric acid	2
Gallic acid	2

using the same set of data as those used for the LOQ. For each compound area, values for the $[M-H]^-$ ion, ranging from the limit of quantification to 8 ppm,

were taken into account. Linearity of the MS detection did not set any problem. Nevertheless for all the compounds, except vanillin and syringic acid, the statistical test of zero intercept was rejected, which meant that in routine analysis it was recommended to use two levels calibration, or the standard additions method [13].

3.2.5. Accuracy by comparison with OMW matrix

Accuracy of the PGC-LC-MS method was evaluated by comparing the response obtained on a standard mixture of the six phenolic compounds each at 5 ppm with a OMW matrix, free from the six

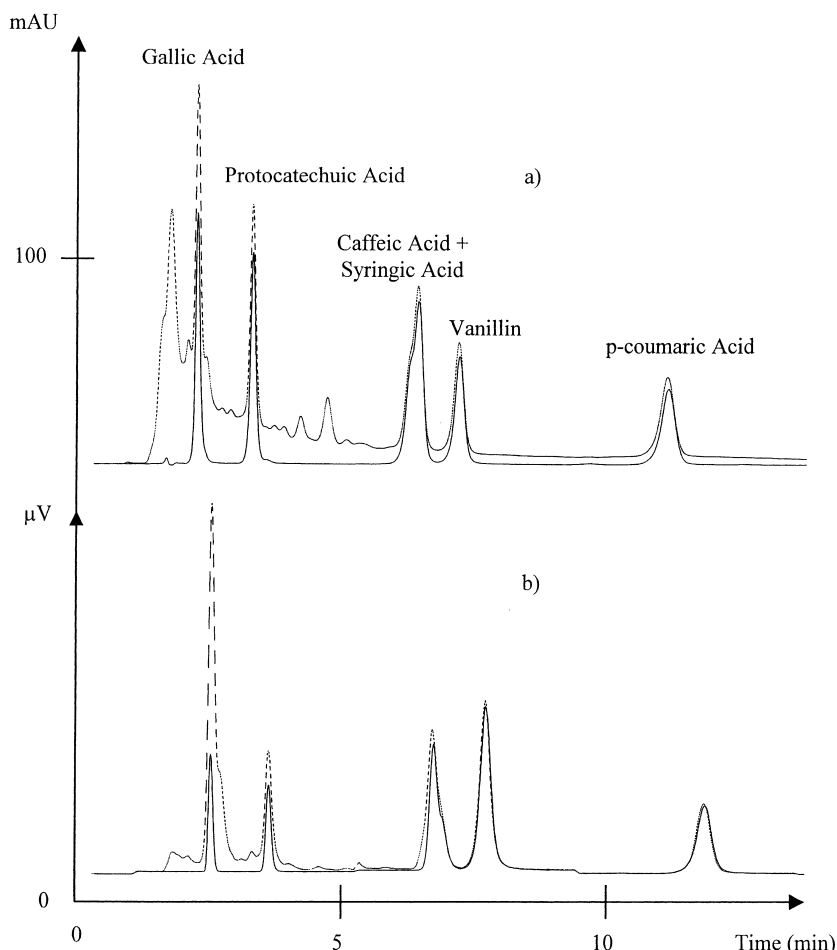


Fig. 5. Matrix effect on C_{18} column. (a) UV detection at 265 nm; (b) TIC in SIM mode (12 selected ions). Dotted line, OMW matrix spiked at 5 ppm; plain line, standard at 5 ppm. Column: length, 15 cm; I.D., 0.30 cm; ambient temperature. Stationary phase: C_{18} ODS1-NE, 5 μ m (Cluzeau). Mobile phase: acetonitrile–methanol–acidified water ([formic acid]=0.075 mol/l) 3/30/67 v:v:v; flow-rate, 0.5 ml/min; pressure drop, 140 bars; injected volume, 10 μ l.

compounds of interest, diluted 20 times in methanol, filtered and spiked at 5 ppm with the six phenolic compounds. A blank analysis of the OMW matrix carried out in SIM mode only gave a horizontal baseline. Both standards and spiked samples were injected six times. On the MS chromatograms obtained in SIM mode no interfering peaks were detected. The pooled RSD on the $[M-H]^-$ ions area was 3.0% for the standard and 2.5% for the spiked sample. The OMW matrix did not increase the dispersion of the results. Comparison of mean areas of standard solution and spiked OMW matrix did not show any significant difference except for caffeic acid, the area of which was between two and three times higher in spiked OMW matrix. In routine analyses, special care must be taken with this compound that should preferably be quantified by the standard addition method. Nevertheless, the overall accuracy of the PGC-LC-MS method could be considered satisfactory.

For the sake of completeness, a similar experiment was tried with a non-encapped C_{18} column. As illustrated in Fig. 5, differences between chromatograms of standard and spiked OMW were marked, with the presence of many interfering peaks and a baseline drift (both in UV and MS) until a retention time of about 7 min. This behaviour dissuaded us from further work with this column.

4. Conclusions

It has been shown that PGC, with its high retention toward polar compounds, is a proper stationary phase to determine phenolic compounds present in OMW. The use of a THF gradient is fully

compatible with APCI-MS in the negative ion mode. The PGC-LC-MS method was validated which ensured its performance level was compatible with the objective of the method. The precision characteristics were not as good as those of alkyl bonded silica LC-MS methods. On the other hand, due to the absence of matrix effects, the accuracy was better. Furthermore, the PGC-LC-MS method reduced sample handling such as clean-up. In the future, other phenolic compounds should be tested to enlarge the range of application of PGC-LC-MS.

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