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Comparative investigation of UV, electrochemical and particle beam mass spectrometric detection for the high-performance liquid chromatographic determination of benzoic and cinnamic acids and of their corresponding phenolic acids

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Abstract

The capabilities of different detection techniques, UV, controlled-potential coulometry and particle-beam electron-impact mass spectrometry (PB-EI-MS) for the HPLC analysis of phenolic acids were studied; fifteen benzoic and cinnamic acid derivatives were considered. For the electrochemical detector (ED) a reversed-phase LC method was set up, whereas normal-phase partition chromatography, on a CN column, was used for UV and MS. Library-searchable EI mass spectra were obtained using the PB-MS technique with flow-injection analysis. UV detection was performed at 280 nm, whereas measurements with the LC–coulometric system were carried out using a porous graphite electrode. The detector responses were compared in terms of linearity, precision and limits of detection; for this purpose, the mass spectrometer was operated under selected-ion monitoring conditions. A linear dynamic range of at least 10^3 was found for the HPLC method with electrochemical detection, with detection limits ranging from 1 to 5 pg injected; the relative standard deviation (R.S.D.) was typically 0.6–3.0% at the 0.1 ng level ($n=4$). Using UV or PB-EI-MS detection, minimum amounts in the 5–50 and 2–5 ng ranges, respectively, could be detected. Calibration curves were linear from the limit of detection to at least 15 μg for most of the analytes detected by UV; the R.S.D. of the peak areas obtained in UV mode ranged from 1.2 to 3.1% at the 500 ng level ($n=4$). Non-linear behaviour over the entire amount range studied (from 10 ng to 10 μg) was observed using the LC–PB-MS technique, so that two different calibration fittings at low and high levels were calculated. Precision of the LC–PB-MS system was generally good (R.S.D. between 0.5 and 1.8% at the 100 ng level, $n=4$) except for caffeic acid (R.S.D. 7.5% at the 50 μg level, $n=4$).

Keywords: Detection, LC; Wine; Phenolic acids; Benzoic acid; Cinnamic acid

1. Introduction

Phenolic acids (various benzoic and cinnamic acid derivatives) play a primary role in defining the sensorial characteristics of wines and brandies, giving that "oak wood taste" typical of long aged products. Their presence and abundance is often related to the storage conditions and ageing process [1,2]; their concentration increase is probably due to extraction from wood as well as to the oxidation of aromatic compounds derived from lignine degradation [3].

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The interest in phenolic acids is also related to the lignin inhibition of nutrient uptake in animal feed [4]: in fact, the abundance of lignin (which oxidises to aryl aldehydes and acids) in materials such as cereal straw precludes its use in animal breeding [5]. Moreover, phenolic acids are known to have hormonal activity in plant growth and, more generally, to cover a multiplicity of functions in plant metabolism [6,7]. Phenolic acids derived from the biodegradation of organic matter [8] play an important role in the uptake of metals by plant roots [9].

In real samples, phenolic acids need to be determined at both low (μg/l) and high concentration levels. Most of the analytical methods proposed for the separation and determination of phenolic acids are based on HPLC techniques, with detection usually accomplished by either UV spectrophotometry or electrochemical methods (ED) [10–14].

Spectrophotometric detection has the drawback of relatively poor sensitivity; in fact, phenolic acids have generally rather low extinction coefficients and UV detection seems to have too low a sensitivity to perform direct quantitative analysis in matrices like wines or brandies: some authors used this detection mode only for identification purposes [1], whereas good quantitative analysis is performed only at high concentration levels [7].

For this class of compounds, ED, employed e.g. with voltammetric [14] or amperometric [15] methods and different cell designs, offers excellent sensitivity [16] and the capability of selective detection by varying the working potential; despite these characteristics, studies on the linear response range and sensitivity of these substances are lacking in the literature.

MS detection opens up a new perspective for the analysis of this class of compounds. Hyphenated HPLC–MS techniques have the great advantage of offering bidimensional resolution of complex mixtures, as would be required for phenolic acid analysis, and even allow compounds with overlapping chromatographic peaks to be distinguished. So far, the only published work in this field [17] concerns the use of the thermospray (TSP) interface for the analysis of cereal straw hydrolysates, which contain large amounts of phenolic compounds. Considering the analytical problem of the assay of phenolic acids at low levels in complex matrices, such as wines and aged alcoholic beverages, the present work explored the use of a particle beam (PB)-MS detector in a concentration range that was much lower than that reported in the above TSP work.

The separation method usually applied to these polar compounds is ion-pair or ion-suppression reversed-phase partitioning [1,10,16,18], with only a few authors using normal-phase partitioning chromatography [10]. Recently, a new chromatographic method suitable for the fast separation of a number of hydroxy derivatives of benzoic and cinnamic acids has been devised [19]; this method, applied with UV and MS detection in the present work, was specifically developed for the PB-MS system, which requires low water content and volatile mobile phases. For ED, a reversed-phase ion-suppression partitioning method has been developed and particular attention has been paid to the effects of pH variation and the amount of organic modifier. In this work the performances of these different detection systems in the determination of benzoic, cinnamic and veratric acids and of twelve phenolic acids derived from benzoic and cinnamic acids (listed in Table 1) were verified. The capabilities of each detection system were evaluated and compared in terms of linearity and limit of detection (LOD). At the same time, various chromatographic methods were developed to fit the different detection conditions. The different techniques were demonstrated for the determination of selected phenolic acids in an extract of a commercial brandy sample.

2. Experimental

2.1. Chemicals

All phenolic acid standards (97–99% purity) were purchased from Fluka (Buchs, Switzerland). Inorganic salts and sulphuric acid were supplied by Carlo Erba (Milan, Italy); hexane, diethyl ether, propan-2-ol and methanol (HPLC-grade) were purchased from Lab-Scan (Dublin, Ireland). Formic and acetic acids were obtained from Janssen Chimica (Geel, Belgium). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

Table 1
Phenolic acid ions monitored in SIM acquisition mode

Compound[a]	Ions monitored (m/z)[b]
Salicylic acid (2-hydroxybenzoic acid)	120,138
Benzoic acid	105,122
Cinnamic acid	103,148
Veratric acid (3,4-dimethoxybenzoic acid)	167,182
Gentisic acid (2,5-dihydroxybenzoic acid)	136,154
Vanillic acid (3-methoxy-4-hydroxybenzoic acid)	153,168
4-Hydroxybenzoic acid	121,138
o-Coumaric acid (2-hydroxycinnamic acid)	118,164,146[c]
p-Coumaric acid (4-hydroxycinnamic acid)	147,164
Ferulic acid (3-methoxy-4-hydroxycinnamic acid)	179,194
Protocatechuic acid (3,4-dihydroxybenzoic acid)	137,154
Syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid)	183,198
Caffeic acid (3,4-dihydroxycinnamic acid)	134,180
Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid)	209,224
Gallic acid (3,4,5-trihydroxybenzoic acid)	153,170

[a] Compounds listed on the basis of their elution order in normal-phase mode.
[b] Dwell time, 500 ms for each ion.
[c] Dwell time, 300 ms for each ion.

2.2. Standard and sample preparation

The phenolic acid standard solutions were prepared at the 10 g/l level in methanol and were gradually diluted to the working concentration levels required for the study of the linearity range and of the sensitivity of detection. Dilutions were performed in hexane–diethyl ether (80:20, v/v) for UV and MS detection modes and in water for the ED mode.

For the extraction of phenolic acids from a commercial brandy sample, the procedure described by Delgado et al. [20] was applied. A 50-ml volume of brandy was concentrated to approximately 15 ml under vacuum at 35°C. The concentrated sample was extracted with 5 ml of diethyl ether and the extraction procedure was repeated four times. The organic solvent was dried for 1 h with anhydrous sodium sulphate followed by evaporation under vacuum at 35°C. The residue was taken up with 500 μl of methanol–water (1:1, v/v). Prior to injection, the sample was filtered through a 0.45-μm membrane (Phenomenex, Torrance, CA, USA); in the case of electrochemical detection, the filtered sample was diluted 1:5 with methanol–water (1:1, v/v). Injected volumes were 5 μl for ED and 20 μl for UV and MS detection.

2.3. LC–UV

A Hewlett-Packard HP 1050 pump (Palo Alto, CA, USA), equipped with a Model 7125 injection valve with a 6-μl loop (Rheodyne, Cotati, CA, USA) was used. The injection volume was 6 μl. Separations were performed on a stainless steel column (200×4 mm I.D.) filled with 5 μm Nucleosil CN (Macherey-Nagel, Düren, Germany). The following quaternary system was used as the mobile-phase: eluent A, hexane–diethyl ether (80:20, v/v); eluent B, propan-2-ol–80% (v/v) aqueous formic acid (91:9, v/v); A–B volume ratio was 96:4. The overall flow-rate was held at 0.8 ml/min.

Spectrophotometric detection was performed using a variable-wavelength detector (Hewlett-Packard, HP 1050) at $\lambda=280$ nm. The output range of the detector (1 V) corresponded to 2 AUFS. Data were acquired on a Maxima system (Waters, Milford, MA, USA). For the determination of the linear dynamic range, the range of 10–5000 ng was explored. The instrumental precision was calculated by considering the repeatability of four measurements of chromatographic peak areas at the 500 ng level.

A linear regression fitting ($Y=i+mX$) was done on all the calibration data; when the intercept turned out

to be non-significant ($p>0.05$), a new regression was calculated according to $Y=mX$.

The calculations of the detection limits for the compounds studied were based on a signal-to-noise ratio of 3, throughout this work.

2.4. Electrochemistry and LC–ED

Voltammetric measurements were performed with a Metrohm (Herisau, Switzerland) Polarecord E506 polarographic/voltammetric control unit, coupled with a Metrohm E612 VA scanner. A three electrode system was used throughout, made of a Tacussel EDI 101 T glassy carbon electrode as the working electrode (WE), a platinum wire as the auxiliary electrode (AE) and an Ag–AgCl–KCl 3 *M* reference electrode (RE). A Linseis LY 1900 *x–y* recorder (Selb, Germany) was used. Hydrodynamic voltammograms were recorded with an ESA Coulochem 5100A coulometric detector (Bedford, MA, USA), coupled with a Perkin-Elmer model 250 HPLC pump system (Norwalk, CT, USA). All pH measurements in water–methanol mixed solutions [21] were performed with an Amel Model 335 pH meter (Milan, Italy).

The solutions for the cyclic voltammograms were prepared by diluting the standard solutions of the phenolic acids in a mixed solvent (acetate buffer pH 4 –methanol; 9:1, v/v) to 10^{-4} *M*; the acetate buffer was prepared by adjusting 0.036 *M* CH_3COONH_4 solution to pH 4 with glacial CH_3COOH. All samples were degassed with nitrogen for 10 min before the measurements were taken. The glassy carbon electrode was polished before each experiment with Al_2O_3 (1 μm), then dipped for 1 min in a weakly acidic solution (0.05 *M* H_2SO_4) and washed with deionized water. The voltammograms were recorded by scanning the potential between −200 and +1600 mV.

To correlate the results obtained from the cyclic voltammograms with the coulometric LC detector potential setting, the hydrodynamic voltammograms of caffeic and gentisic acids were obtained by performing flow injection analyses (20 μl injection volume) of the acid standards (6 mg/l in methanol) at different working electrode potentials in the range of −150 to +500 mV. The reference electrode of the detector is of unknown type, being covered by a patent.

Chromatographic separations were carried out using the Perkin-Elmer model 250 LC pump equipped with a Hewlett-Packard HP 1050 autosampler and a Spherisorb 5-ODS column (250×4.6 mm I.D., 5 μm). The mobile-phase was a mixture of methanol (0–13%), acetic acid (10–15%) (v/v) and aqueous ammonium acetate (0.005 *M*). The working flow-rate was 1.0 ml/min. Electrochemical detection was performed with the ESA Coulochem Detector mentioned above (1 V full-scale output). The system is made of two cells serially connected, both of which contain a porous graphite working electrode together with associated reference- and counter electrodes. The potential applied to the first cell (E1) was −150 mV, whereas the potential applied to the second cell (E2) varied from +200 to +900 mV, depending on the analyte. In order to avoid decreases in sensitivity, the detector cell was periodically flushed with a 0.05 *M* H_2SO_4 solution and repetitive potential steps (±1 V) were applied.

The standards injected ranged from 10 μg/l to 100 mg/l (injection volumes of 0.2–1 μl). Data acquisition was performed with a Turbochrom 4 PE Nelson data acquisition system (PE Nelson, Cupertino, CA, USA).

2.5. LC–MS

For the chromatographic separations with MS detection, a Hewlett-Packard HP 1090 liquid chromatograph equipped with an HP 1050 autosampler was used. The column and chromatographic conditions used for the separations were the same as for UV detection (Section 2.3). The flow-rate was 0.4 ml/min in flow injection analysis (FIA) mode and 0.8 ml/min using the chromatographic column.

A Hewlett-Packard Model HP 5989A quadrupole mass spectrometer and a particle beam LC–MS interface Model HP 59980A were used. The mass spectra were recorded under electron impact (EI) conditions, scanning from 90–250 u (1 scan/s). The source and quadrupole temperatures were 200 and 100°C, respectively. Linearity and detection limits were determined using selected-ion monitoring (SIM). Regarding the PB interface parameters, the temperature of the desolvation chamber (50°C), the

nebulizing gas inlet pressure (helium, 172 kPa) and the nebulizer capillary position were set according to the results of a preliminary optimization procedure, which was performed by injecting 100 ng of ferulic acid and by monitoring the m/z 136 and 154 ions (dwell times, 300 and 700 ms, respectively) in SIM mode.

SIM detection was performed by monitoring the ions listed in Table 1, using a dwell time of 500 ms per ion, except in the case of *o*-coumaric acid, for which a dwell time of 300 ms was chosen for each ion; in this case three ions were scanned, since, besides the base peak, two fragments having high and similar abundances were detected. Data were acquired using the HP MS 59940A Chem Station (HP-UX series). Mass spectra of the analytes were compared with Wiley library reference spectra. The linearity of the dependence of the detector responses on the quantities of phenolic acids injected was studied both in FIA mode (1–1000 ng) and using the chromatographic column (0.01–10 μg).

3. Results and discussion

3.1. LC–UV

Normal-phase partition chromatography of the phenolic acids using a CN bonded-silica column has already been discussed in previous work [19]. In this paper, the performance of the UV detector, in terms of linearity and detection limits, was studied in order to achieve data comparable with those obtained with the other detection systems considered. UV detection did not appear to be an adequate technique for the analysis of low concentrations of the phenolic acids because of the low absorptivity values of these compounds at the operative wavelength of 280 nm; these values are further lowered by the mobile phase used. In addition, it was not possible to use wavelengths corresponding to the maximum absorbance for the single components, due to the small differences in retention times.

Limits of detection of some selected compounds, determined with this detector at 280 nm, ranged from 5 ng (caffeic acid) to 50 ng (vanillic acid). In general, all of the cinnamic acid compounds gave a higher response than obtained for the corresponding benzoic acid derivatives that were analyzed. The linearity of the response was verified from the LOD to the saturation of the UV signal. As can be inferred from the results presented in Table 2, calibration curves were linear from the LOD to at least 15 μg for most of the compounds. The relative standard deviation (R.S.D.) of the peak areas ranged from 1.2% for ferulic acid to 3.1% for *p*-hydroxybenzoic acid (n=4; 500 ng level).

These results attest a satisfactory repeatability of the method, whereas the minimum detectable amounts confirm the poor sensitivity of spectrophotometric detection.

3.2. Electrochemistry and LC–ED

A preliminary part of this work was devoted to finding the optimum working electrode potential to be used with the coulometric detector. The redox activity of the phenolic acids was tested by cyclic voltammetry. The peak current potentials are reported in Table 3.

The electrochemical behaviour of phenolic acids is not completely understood. The compounds bearing

Table 2
Linearity between the amounts of selected phenolic acids and chromatographic peak areas using the LC–UV method. Calibration fitting: $Y=i+mX$

Compound	Range (ng)	$i\times10^5$	m	n	r	LOD[a]
4-Hydroxybenzoic acid	100–50000	–	245±3	30	0.996	10
p-Coumaric acid	10–5000	−4.21±1.93	3558±91	21	0.992	10
Ferulic acid	10–15000	–	1642±3	34	0.993	10
Vanillic acid	50–5000	−1.74±0.44	841±2	21	0.992	50
Protocatechuic acid	10–15000	−1.22±0.46	577±8	43	0.996	10
Caffeic acid	10–15000	13.85±3.16	4719±66	38	0.997	5

[a] Limit of detection, in ng

Table 3
Peak potentials for the oxidation and the reduction of selected phenolic acids on the glassy carbon electrode[a]

Compound	E^{b}
Gentisic acid	+510[c],+260[d]
Caffeic acid	+555[c],+285[d]
Gallic acid	+580[c]
Protocatechuic acid	+650[c],+335[d]
Sinapic acid	+670[c]
Syringic acid	+730[c]
Ferulic acid	+770[c]
Vanillic acid	+910[c]
p-Coumaric acid	+930[c]
o-Coumaric acid	+975[c]
Salicylic acid	+1145[c]
4-Hydroxybenzoic acid	+1170[c]
Veratric acid	+1450[c]

[a] All samples were 10^{-4} *M* solutions in the following medium: 0.036 *M* CH_3COONH_4–CH_3COOH buffer, pH 4–methanol (9:1, v/v).
[b] Millivolts *vs.* Ag/AgCl at 50 mV/s.
[c] Anodic peak.
[d] Cathodic peak.

two OH groups in the *ortho* or *para* position are expected to be easily oxidized to yield *o*- or *p*-quinones [14], as is found for enzymatic oxidation [22]. This is a reversible reaction, since, by cyclic voltammetry, a quasi-reversible behaviour was observed for gentisic, protocathecuic and caffeic acids.

Regarding the electrochemical oxidation mechanism of the monohydroxy or methoxy derivatives, the reactions are irreversible and the reaction products seem to affect the detector electrode sensitivity, forming a partially passivating film [16]. In fact, it was necessary to clean the electrode periodically by means of an acid solution by alternatively applying large positive (+1 V) and negative (−1 V) potentials.

From the hydrodynamic voltammograms of caffeic acid and gentisic acid (Fig. 1) it can be inferred that a diffusion limited current is reached at a voltage that is about 200 mV lower than the cyclic voltammetry peak potentials (Table 3); this is due mainly to the difference between the reference electrodes of the two measuring systems. Hence, the choice of the working potential for the detector was made taking into account different factors, i.e. the results of the cyclic voltammetry measurements, the oxidation potential of the least easily oxidisable compounds and the impossibility of working at $E2>1000$ mV

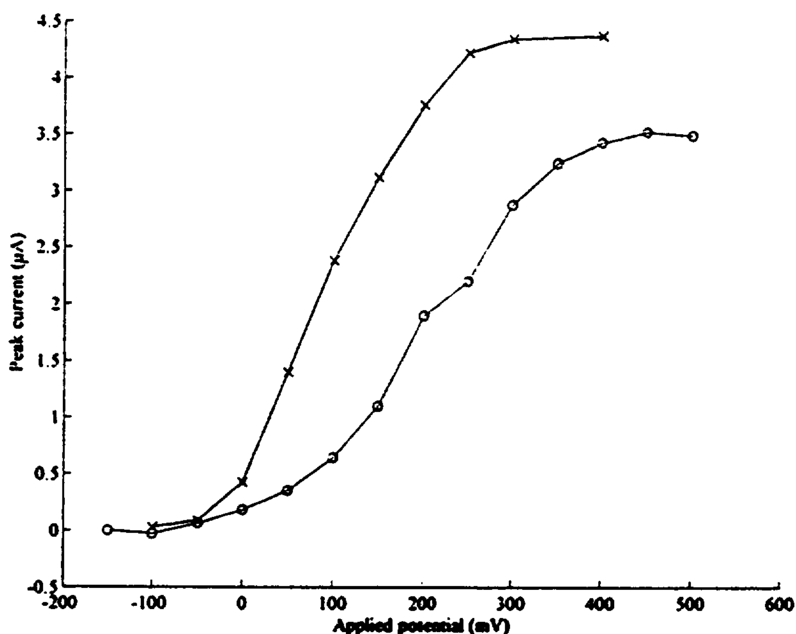

Fig. 1. Hydrodynamic voltammograms of caffeic acid (o) and gentisic acid (x). Mobile phase, 0.036 *M* CH_3COONH_4–CH_3COOH, pH 4–methanol (9:1, v/v); flow-rate, 1.0 ml/min. Amount injected: caffeic acid, 120 ng; gentisic acid, 100 ng.

due to high background currents. At $E2$=900 mV, all the compounds considered were detected, except for veratric acid, which is oxidized at +1200 mV; consequently, the latter compound was excluded from this ED work. Benzoic acid and cinnamic acid were not detected; in fact, in voltammetric experiments they did not show any anodic wave into the 0–1600 mV range.

The chromatographic separation of a mixture of twelve phenolic acids was studied with mobile phases containing variable ratios of methanol, acetic acid and 0.005 *M* aqueous ammonium acetate. As expected, the higher the percentage of acid, the sharper the peak became. In fact, a decrease of the pH allows more efficient suppression of the ionization process for the analytes to occur. In addition, low percentages of methanol reduce the elution times. Fig. 2 shows the effect of variations on the capacity factors obtained using mobile phases of (a) methanol–acetic acid–0.005 *M* ammonium acetate (13:10:77, v/v), (b) methanol–acetic acid–0.005 *M* ammonium acetate (8:15:77, v/v) and (c) acetic acid–0.005 *M* ammonium acetate (15:85, v/v), whereas the influence of the same eluents on the resolution is illustrated in Fig. 3. It is evident that even a low percentage of methanol, volumes of acetic acid being equal, is important for obtaining faster separations (Fig. 2). In addition, it is remarkable that mobile phase (b), which contains a higher percentage of acetic acid, not only gives the shortest analysis time, but also enhances the resolution with respect to mobile phase (a) (Fig. 3). The only exception is that of salicylic, *o*-coumaric and ferulic acids, which in any case were not well separated using eluent (a). On the other hand, the separation between these compounds can be obtained only by using the eluent made up of acetic acid and 0.005 *M* ammonium acetate, which results in longer retention times and broader and non-symmetrical peaks. For these reasons, the calibrations were performed using the methanol–acetic acid–ammonium acetate (8:15:77, v/v) mobile phase having a pH value of 2.3. The elution order of the analytes reflects the number of OH groups on the aromatic ring.

Operating at oxidation potential values lower than 900 mV, it was possible to detect some phenolic acids selectively. Injections of a standard mixture were performed at 700, 500, 400, 300 and 200 mV. In

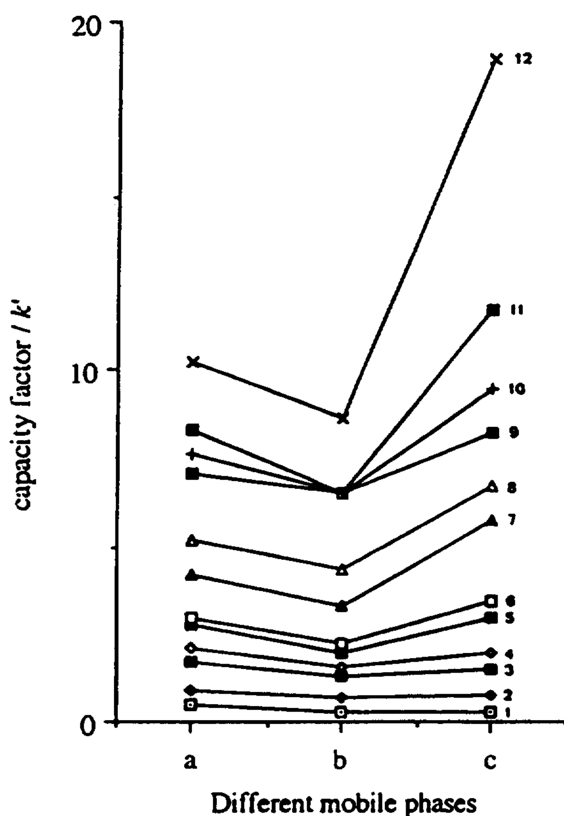

Fig. 2. Effect of the composition of the mobile phase on the capacity factors (k') of standard phenolic acids under RPLC–ED conditions. Eluent: methanol–acetic acid–0.005 *M* ammonium acetate (a) 13:10:77 (v/v), (b) 8:15:77 (v/v/v) and (c) 0:15:85 (v/v); flow-rate, 1.0 ml/min; $E2$=900 mV. Elution order: (1) Gallic acid, (2) protocatechuic acid, (3) gentisic acid, (4) *p*-hydroxybenzoic acid, (5) caffeic acid, (6) vanillic acid, (7) syringic acid, (8) *p*-coumaric acid, (9) salicylic acid, (10) *o*-coumaric acid, (11) ferulic acid and (12) sinapic acid.

Fig. 4, three chromatograms obtained at 900, 500 and 200 mV are shown. When $E2$ was set at 200 mV (Fig. 4c) only gallic acid, protocatechuic acid, gentisic acid, caffeic acid and sinapic acid were detectable, whereas at 500 mV (Fig. 4b) only protocatechuic acid and salicylic acid disappeared from the LC profile.

Detection limits and the linearity were checked and results are summarized in Table 4. For all compounds tested, a linear dynamic range of at least three orders of magnitude was explored. Except for 4-hydroxybenzoic acid, two different calibration fittings at low and high levels (more than 1 ng) were

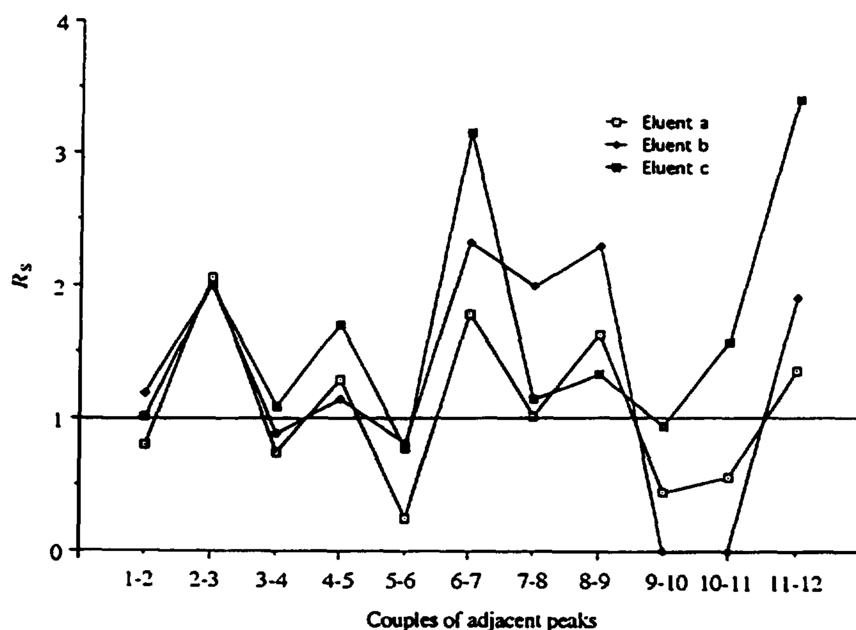

Fig. 3. Effect of the composition of the mobile phase on the resolution (R_s) of adjacent peaks of standard phenolic acids. $R_s=1$ for baseline separation. Conditions and compounds as in Fig. 2.

calculated, with a sharp decrease in sensitivity for the high levels, as indicated in Table 4. In all cases investigated, the response was linear with a correlation coefficient, r, of at least 0.999 ($n=10$). The best behaviour was observed for 4-hydroxybenzoic acid, which exhibited linearity in a dynamic range greater than four orders of magnitude. Using the chromatographic column, the minimum detectable amounts of the analytes were 1 pg, for ferulic and 4-hydroxybenzoic acids, and 5 pg, for vanillic and p-coumaric acids ($S/N=3$). Table 4 shows calibration graphs where the amount of analyte is referred to both in ng

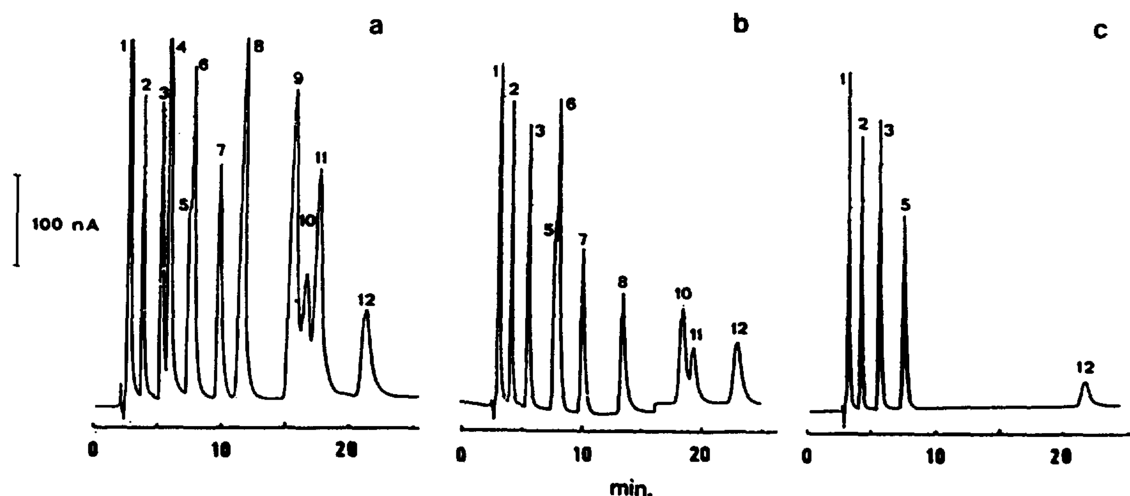

Fig. 4. Effect of the decrease of the oxidation potential on the ED of the standard solution of Fig. 2. Eluent: methanol–acetic acid–0.005 M ammonium acetate (13:10:73, v/v); flow-rate, 1.0 ml/min. (a) $E2$, 900 mV; (b) $E2$, 500 mV; (c) $E2$, 200 mV.

Table 4
Linearity between the amounts of selected phenolic acids and chromatographic peak areas using the LC–ED method. Calibration fitting: $Y=i+mX$

Compound	Range (ng)	$i\times10^4$	$m\times10^{6}$ [a]	$m'\times10^{7}$ [b]	n	r	LOD[c]
4-Hydroxybenzoic acid	0.005–100	–	5.35±0.04	73.83±0.52	30	0.999	0.001
p-Coumaric acid	0.01–0.5	8±3	10.2 ±0.10	167.28±0.16	15	0.999	0.005
	0.5–100	280±60	7.39±0.02	121.19±0.33	10	0.999	
Vanillic acid	0.01–0.5	–	5.51±0.08	92.57±1.34	11	0.999	0.005
	1–50	500±100	3.37±0.02	56.62±0.34	12	0.999	
Ferulic acid	0.05–1	5±2	4.62±0.03	89.63±0.58	10	0.999	0.001
	1–100	–	3.18±0.02	61.69±0.39	10	0.999	

[a] m, [peak area]/[ng of analyte].
[b] m', [peak area]/[pmoles of analyte].
[c] Limit of detection, in ng.

and in pmol; the slope values, m' (peak area/pmol), are roughly proportional to the charge number of the electrochemical oxidations at the chosen potential value.

Good repeatability of the method was found, relative standard deviations ranging from 0.6% for 4-hydroxybenzoic acid to 3.0% for *p*-coumaric acid at the 0.1 ng level (n=4).

3.3. LC–MS

In order to investigate the applicability of the particle beam HPLC–MS technique to the analysis of phenolic acids, preliminary studies were conducted under FIA conditions. It is well known that LC–PB-MS is not suitable for highly polar compounds, such as the di- and trihydroxy derivatives of the cinnamic and benzoic acids, for which the transfer through the PB interface is not efficient because of difficulties in the desolvation process of the acids. This behaviour was particularly evident under reversed-phase conditions, using aqueous–alcoholic mixtures as the eluent. High amounts of the examined compounds (about 5 μg) had to be injected to obtain poor quality mass spectra, which were not comparable to those of the mass spectra data bases.

In contrast, when operating under normal-phase conditions, it was verified that the use of non-polar or slightly polar organic eluents (like hexane–diethyl ether mixtures with organic modifiers) let the analytes cross the interface without deprotonation and allowed them to reach the ion source efficiently. Low amounts (5–10 ng) of phenolic acids were sufficient to obtain EI library-searchable mass spectra. On the other hand, the use of organic solvents limited the acquisition mass range, so that it was impossible to record below 90 u. In Table 5 the MS data for the compounds examined, with the exception of benzoic acid, are summarized; due to its high volatility, benzoic acid could hardly be detected at the low ng level, probably being pumped away together with the eluent in the PB interface. Structural assignment and relative abundance of the fragment ions are contained in Table 5. All of the PB-EI mass spectra were in agreement with the corresponding Wiley library reference spectra.

It should be noted that up to now, very few examples of the use of non-polar solvents in LC–PB-MS have been reported [23,24]. Winkler et al. [23] have described the normal-phase separation of retinol acetate isomers using an improved MAGIC (monodisperse aerosol generating interface for chromatography) LC–MS system; full scan EI mass spectra were obtained with 50 ng injections on-column. The advantages in the use of normal-phase chromatography with respect to reversed-phase separations have been highlighted by Zinkl et al. [24] in the LC–PB-MS analysis of pesticides; the normal-phase approach offered significant advantages resulting in highly resolved sharper peaks and greater sensitivity for the compounds of interest.

After the optimization of the PB interface parameters under FIA conditions, LC–MS experiments were performed with the CN column. Even though the best flow-rate for this interface is 0.3–0.4 ml/min, in normal-phase mode it is possible to operate at 0.8 ml/min, without any appreciable loss of sensitivity. This may be explained considering that, in the case

Table 5
Mass spectral data of the phenolic acids in FIA–PB-EI–MS mode[a]

Compound	$[M]^+$	$[M-H]^+$	$[M-OH]^+$	$[M-H_2O]^+$	$[M-CH_3]^+$	$[M-COOH]^+$	Others
Salicylic acid	138 (100)			120(100)			92(80)
Cinnamic acid	148(80)	147(100)	131(25)			103(49)	
Veratric acid	182(100)		165(7)		167(33)		
Gentisic acid	154(40)		137(11)	136(100)			108(31)
Vanillic acid	168(100)		151(17)		153(74)		125(21), 97(26)
4-Hydroxybenzoic acid	138(78)		121(100)			93(30)	
o-Coumaric acid	164(39)		147(11)	146(56)			118(100)
p-Coumaric acid	164(100)		147(41)			119(26)	
Ferulic acid	194(100)		177(11)		179(25)		133(19)
Protocatechuic acid	154(96)		137(100)	136(6)		109(29)	
Syringic acid	198(100)		181(7)		182(37)		127(18),168(3)
Caffeic acid	180(100)		163(26)	162(8)			134(45),136 (29)
Sinapic acid	224(100)		207(4)		209(18)		194(2)
Gallic acid	170(100)		153(81)	154(7)			136(19)

[a] Relative abundances enclosed in parenthesis for each ion.

of the use of non-polar or low-polar eluents, the desolvation process is less difficult due to the lower ΔH_{vap} for these solvents over the high-polar organic solvents or water, as already discussed by Voyksner et al. [25] regarding reversed-phase eluents.

A mixture of ten phenolic acid standards was injected, obtaining the LC–PB-MS chromatogram illustrated in Fig. 5. In general, the peak broadening caused by the PB interface was not particularly noticeable; only in the case of sinapic and gallic acids, the most polar compounds, was this broadening so wide that it was not possible to obtain a real chromatographic peak. However, for these analytes poor peak shape was observed even with UV detection.

Table 6 summarizes the EI data concerning the linearity and the detection limits obtained in FIA mode (a) and with the chromatographic column (b, c). Under FIA conditions, the linearity of MS responses (i.e. the intensity of the ion signals monitored in SIM mode) *versus* the amounts of phenolic acids was established over a dynamic range of 10^3 for most of the analytes. In contrast, non-linear behaviour at low levels was observed for the most polar derivatives, such as gentisic, protocatechuic, caffeic and gallic acids, for which efficiency transmission through the PB interface is particularly low on decreasing the concentration. This results in calibration plots with a linear range at the higher levels and a deviation from linearity at the lower levels, in agreement with the theoretical model proposed by Apffel and Perry [26]; for these acids, correlation coefficients, *r*, ranged from 0.976 to 0.995. When using the LC column, the experimental

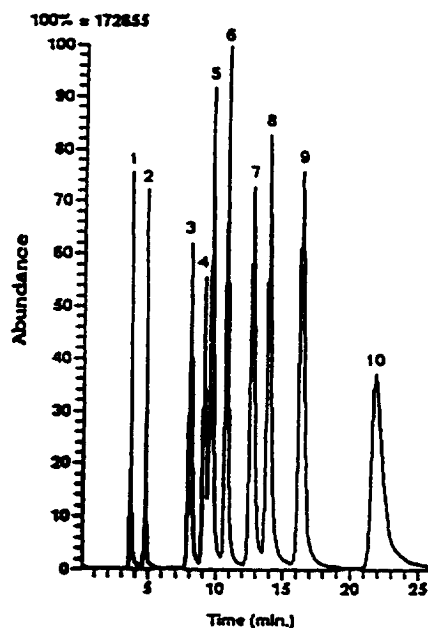

Fig. 5. LC–PB-MS total ion chromatogram of a mixture of ten phenolic acids obtained using a CN bonded-silica column (scan range 90–250 u). Elution order: (1) salicylic acid, (2) cinnamic acid, (3) veratric acid, (4) gentisic acid, (5) vanillic acid, (6) *p*-hydroxybenzoic acid, (7) *o*-coumaric acid, (8) ferulic acid, (9) syringic acid and (10) caffeic acid.

Table 6
Linearity between the amounts of phenolic acids and chromatographic peak areas using the PB-MS method. Calibration fitting: $Y=i+mX$

Compound	Range (ng)	$i\times10^5$	$m\times10^4$	n	r	LOD[a]
(a) FIA-mode						
Salicylic acid	20–10000	−1.40± 0.61	0.143±0.001	31	0.998	1.0
Benzoic acid	10–1000	–	0.406±0.001	21	1.000	1.0
Cinnamic acid	10–1000	−31.78± 7.39	16.79 ±0.17	21	0.999	1.0
Veratric acid	1–1000	−23.17± 4.74	10.52 ±0.12	26	0.998	0.1
Gentisic acid	5–1000	−62.34±14.50	12.79 ±0.34	22	0.993	0.3
Vanillic acid	1–1000	–	15.56 ±0.13	27	0.999	0.2
4-Hydroxybenzoic acid	1–1000	−38.77± 8.15	17.01 ±0.20	27	0.998	0.2
o-Coumaric acid	1–1000	−26.58±10.51	28.37 ±0.28	30	0.999	0.1
p-Coumaric acid	1–1000	–	17.99 ±0.23	29	0.998	0.1
Ferulic acid	1–1000	18.71± 4.11	13.24 ±0.12	39	0.998	0.1
Protocatechuic acid	1–1000	−42.32±12.79	17.50 ±0.34	28	0.995	0.2
Syringic acid	1–1000	–	14.05 ±0.10	27	0.999	0.1
Caffeic acid	5–1000	−13.45± 5.25	5.34 ±0.13	25	0.993	0.7
Sinapic acid	1–1000	−12.87± 4.46	12.75 ±0.11	25	0.999	0.5
Gallic acid	5–250	−1.70± 0.71	0.86 ±0.06	15	0.972	0.3
(b) LC-mode, low-amount range						
4-Hydroxybenzoic acid	10–500	−18± 4	7.0 ±0.2	12	0.997	2.0
p-Coumaric acid	10–500	−8± 2	3.10 ±0.07	12	0.997	5.0
Ferulic acid	10–500	−24± 7	6.7 ±0.3	12	0.991	3.0
Vanillic acid	10–500	−7± 2	2.27 ±0.07	12	0.995	3.0
Protocatechuic acid	10–500	−7± 2	2.18 ±0.06	12	0.995	5.0
(c) LC-mode, high-amount range						
4-Hydroxybenzoic acid	1–10	−40±20	11.2 ±0.3	12	0.997	
p-Coumaric acid	1–10	−16± 5	5.94 ±0.09	12	0.999	
Ferulic acid	1–10	–	11.3 ±0.4	12	0.993	
Vanillic acid	1–10	−30±10	10.5 ±0.2	12	0.999	
Protocatechuic acid	1–10	−77± 6	10.4 ±0.1	12	0.999	
Caffeic acid	5–50	−270±60	5.8 ±0.2	9	0.996	

[a] Limit of detection, in ng.

data were not fitted by a unique linear equation over the whole concentration range considered for all analytes: in fact, the data at the low level (10–500 ng for the compounds reported in Table 6b) gave rise to a sensitivity that was significantly lower than that obtained at high levels (1–10 μg except for caffeic acid, whose linear range was 5–50 μg). This could be explained by considering the combination of two factors; peak broadening due to the chromatographic column and analyte transfer through the PB interface, which becomes less efficient by decreasing the concentration. Recently, the non-linear behaviour of the LC–PB-MS system at low concentrations has been discussed by Creaser and Stygall [27] in a review dealing with the coupling of HPLC with MS through the PB interface.

The minimum detectable amount ranged from 0.1 to 1.0 ng in FIA, depending on the analyte (0.1–0.7 μl injected). When operating with the column, the detection limits for six of the compounds examined were higher than in FIA. This behaviour has been noticed in other cases [28,29], with a ten-fold decrease in sensitivity passing from the FIA mode to the use of an ordinary chromatographic column. In the case of caffeic acid, the last eluting compound under the LC conditions used, the loss in sensitivity was dramatic because of considerable peak broadening. Nevertheless, the LC–PB-EI-MS method can provide quantitation of phenolic acids in matrices like wines and other alcoholic beverages, the minimum detectable amounts being much lower than those found in the case of LC–TSP-MS [17]. In fact, under the presently reported conditions, detection limits were in the order of 1 μg and thus satisfactory

for the analysis of phenolics produced by the chemical degradation of plant material such as wheat straw.

Precision of the LC–PB-MS system was evaluated by performing four repetitive analyses using 100 ng of the compounds reported in Table 6(b), which gave a R.S.D. ranging from 0.50% for *p*-hydroxybenzoic acid to 1.8% for protocatechuic, ferulic and *p*-coumaric acids. For caffeic acid, which gave a large peak shape, repeatability was worse (R.S.D. of 7.5% for four 50 μg on-column injections).

3.4. Analysis of phenolic acids by LC–UV, LC–ED and LC–PB-MS techniques in a brandy sample

The different techniques were demonstrated for the analysis of phenolic acids present in a real sample such as an extract of commercial brandy. This type of matrix is known to be characterized by low concentrations (from ppb to ppm) of phenolic acids [20]. Analysis of this alcoholic beverage, performed using UV, ED and PB-MS detection modes, gave the results shown in Fig. 6. Coulometric detection provided advantages in comparison to either UV or MS detection; eight phenolic acids were detected and quantified by the electrochemical method in the low ng range, as reported in Table 7. The matrix effect did not represent a problem in the case of ED, except for gallic acid, for which the accuracy of the determination is scarce due to partial overlapping with other peaks (Fig. 6a). Selectivity in chromatographic detection could be further increased by varying operative oxidation potential values, as discussed above. In contrast, UV detection of the phenolics was not feasible because of the large interference from various peaks and, in particular,

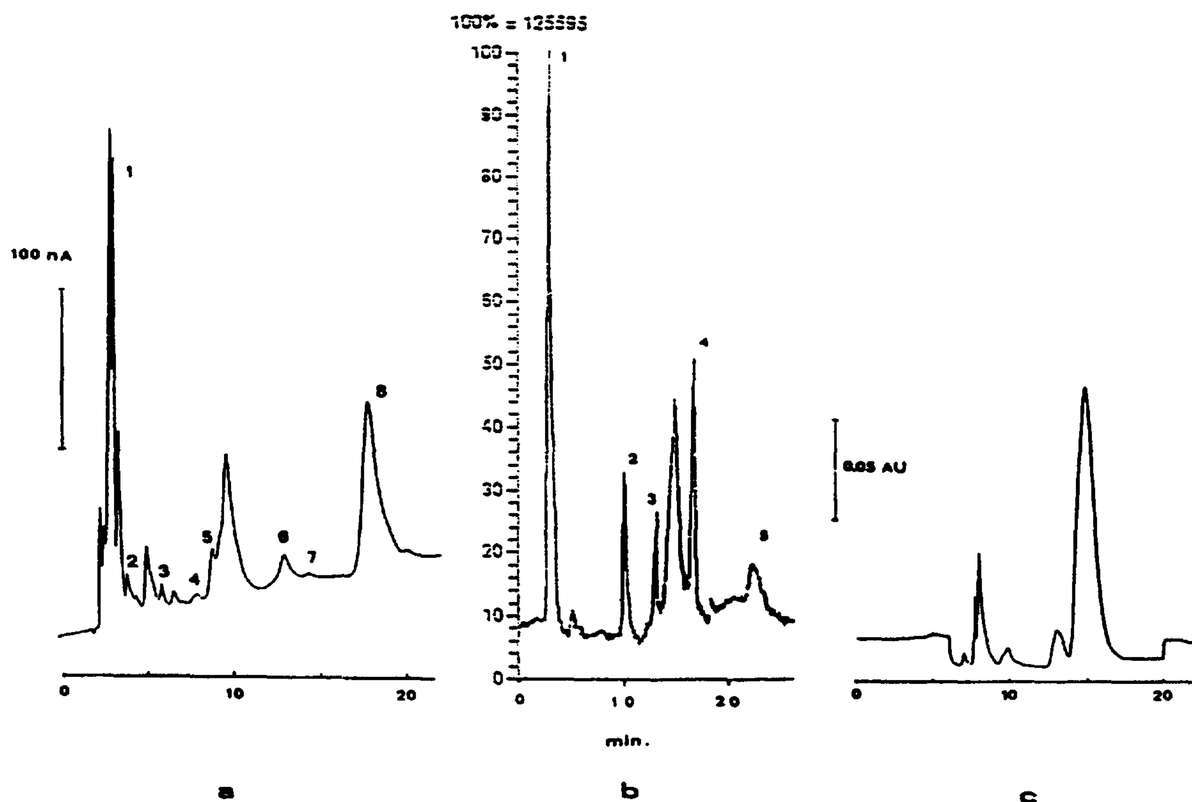

Fig. 6. Comparison of chromatograms of the brandy extract obtained with (a) ED, $E2=900$ mV, RP mode. Elution order: (1) gallic acid, (2) protocatechuic acid, (3) *p*-hydroxybenzoic acid, (4) caffeic acid, (5) vanillic acid, (6) syringic acid, (7) *p*-coumaric acid and (8) salicylic acid; (b) PB–EI-MS detection, SIM acquisition, NP mode; (c) UV detection, $\lambda=280$ nm, NP mode. Elution order: (1) salicylic acid, (2) vanillic acid, (3) protocatechuic acid, (4) syringic acid and (5) caffeic acid.

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