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Journal of Chromatography A, 730 (1996) 31–37

JOURNAL OF CHROMATOGRAPHY A

High-performance liquid chromatographic–electrospray mass spectrometric analysis of phenolic acids and aldehydes

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Abstract

The present work describes the development of an HPLC–electrospray mass spectrometric method for the analysis of phenolic acids and aldehydes. These compounds are important for the quality of foods and feeds, such as dietary fiber supplements, wine and lignicellulose by-products. Good separation was obtained with a phenyl column (3 μm particle size, 150 mm×3.9 mm I.D.), using MeOH–H_2O (30:70, v/v) as the mobile phase with 0.01% CH_3COOH and 0.2 m*M* tetraethyl ammonium iodide as the ion pairing agent, at a flow-rate of 0.3 ml/min. This system permits post column splitting of the eluate for analysis by electrospray mass spectrometry with a flow-rate of 11 μl/min. This new method is extremely sensitive and less than 6 pg/inj of the studied phenols can be identified and quantified. This method was applied to standard compounds as well as to components of high-fiber dietary supplements (primarily wheat bran), cornmeal, and oat bran.

Keywords: Food analysis; Phenolic compounds; Acids; Aldehydes

1. Introduction

The recent introduction of an electrospray interface (ESI) to high-performance liquid chromatography–mass spectrometry (HPLC–MS) equipment represents an important technological improvement [1–3]. Compounds over a wide range of molecular masses (up to 60 000) can be detected and identified on the basis of multiply charged molecular ions $[M+/-nH]^{n+/-}$, which are generated by mild ES ionization. The HPLC–ES-MS was originally designed for use with large molecules, such as proteins [4–8], but many recently published papers have demonstrated its excellent analytic capability for small organic polar compounds as well [9]. In a previous study we reported the use of this technique for bile acid (BA) analysis, using either a conventional or micro HPLC system [10]; with both systems the detection limit was on the order of 15 pg/inj, much lower than those achieved with other HPLC detectors, or when using a thermospray interface [11]. The ES interface is compatible with conventional solvent mixtures used for normal or reversed-phase HPLC, up to 80% of which can be

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0021-9673/96/$15.00
SSDI 0021-9673(95)01166-8

water, but only volatile buffers or counter-ions may be utilized. With this system, the flow-rate at the interface must be kept at <20 μl/min, which can be easily done by post-column splitting or by using a semimicro or micro HPLC apparatus [12].

In another paper, we reported the chromatographic analysis of low-molecular-mass plant phenolic compounds utilizing a thermospray interface [13]. While protonated molecules $[M+H]^+$ were formed and several compounds identified, this method showed poor sensitivity for these compounds, especially for gallic and ferulic acids.

In the present work we report an improvement in the analysis of plant phenolic compounds by applying HPLC–ES-MS: phenolic compounds are separated in a Nova-Pak phenyl column by ion pairing with tetraethylammonium iodide; the phenolic compounds are identified in the negative ion mode, in which only their deprotonated molecules $[M-H]^-$ are generated. This method was applied to the analysis of phenolic compounds extracted from some natural alimentary fiber sources including cornmeal, wheat bran dietary supplements, glucomannan and others, after alkaline hydrolysis of the original products. Phenolic compounds [14–17] are the building blocks of lignin, one of the main cell wall structural polymers; they also act as bridges, cross-linking lignin and structural polysaccharides such as hemicellulose and cellulose. This polymeric network is part of the so-called dietary fiber that affects the texture, esthetic acceptability and nutritional value of vegetable foodstuffs [18]; these fibers have been found to be important for many aspects of human health, including defense against disease [19]. Consequently, investigations aimed at determining the phenolic composition of vegetable fibers could have interesting practical applications. In particular, the determination of the low-molecular-mass phenolic acids and aldehydes after methanol extraction and mild alkaline hydrolysis allows us to study the role of the lignin:cellulose ratio and the structure of particular vegetable fiber components in the interaction of these components with other substances such as BA, cholesterol or other molecules. Many of these fiber sources are used as additives to foods, because of their ability to 'trap' intestinal components, or they are used as such in the treatment of constipation because of their ability to increase the bulk of the intestinal contents. Among the various intestinal components, BA are of particular importance since strong binding to these high-fiber substances can induce BA malabsorption with a consequent increase in their synthesis from cholesterol and, ultimately, reduction of the cholesterol pool.

2. Experimental

2.1. Chemicals

All chemicals and solvents used were of analytical grade. HPLC-grade methanol and acetic acid were purchased from Farmitalia, Carlo Erba (Milan, Italy); tetraethylammonium iodide (Et_4NI) from Aldrich (Aldrich Chemical Co. Ltd, Dorset, UK); and the phenol standards gallic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillic acid, vanillin, syringic acid, *p*-coumaric acid, syringaldehyde, ferulic acid and *p*-hydroxyphenylacetic acid from Sigma (St. Louis, MO, USA).

2.2. Instrumentation

Phenols and aldehydes were analyzed using an HPLC–MS system equipped with an electrospray ionization source (VG TRIO 2000, Fisons Instruments, Altrincham, UK). The system consists of a 600E Multisolvent Waters pump (Waters, Milford, MA, USA), connected to an autosampler (Waters 717). The eluate passes through a Waters Nova-Pak phenyl column (3 μm particle size, 150 mm × 3.9 mm I.D.; Millipore Corporation, Waters Chromatography, Milford, MA, USA), and a part is split off and introduced into the ES-MS system.

2.3. Analytical conditions

Electrospray MS conditions

The phenolic compounds were analyzed in the negative ion mode. Performance of the ES interface was optimized using purified and filtered air instead of nitrogen because the oxygen in air, by acting as an electron scavenger, prevents undesirable charging.

The ES was further optimized by direct loop

injection of the phenolic compounds. The lower detection limit (signal to noise ratio 3) was obtained under following conditions: probe voltage was 3.12 kV; counter electrode, 0.49 V; cone voltage, 56 V; and the source temperature was 65°C. The optimum flow-rate of the mobile phase was 11 μl/min after post-column splitting.

HPLC separation

Standard solutions of phenolic acids and aldehydes were obtained by dissolving the pure compounds in methanol to a final concentration of about 1 ng/μl.

In order to separate the mixture of standards and extracts in the column, we used a mobile phase consisting of MeOH–H_2O (30:70, v/v) with 0.01% CH_3COOH and 0.2 m*M* Et_4NI, with a pH of 5.7±0.1. This was performed under isocratic conditions, with a flow-rate of 0.3 ml/min. The column was thermostated at 10±0.1°C. Since the ES interface requires a flow-rate of ≤10–20 μl/min, it is necessary to split off a part of the eluate; a fixed, constant fraction is diverted in order to produce an optimal entry flow-rate into the ES source of 11 μl/min. As a result of this splitting, the absolute quantity of sample introduced into the spectrometer is only 3% of the injected amount.

Analysis of phenols in fiber

A quantitative and qualitative analysis of the phenols and aldehyde components of fiber from the following sources were performed using the HPLC–ES-MS system: cornmeal (for reference), and several high-fiber dietary supplements kindly provided by Giuliani S.p.A., Milan, Italy: Crusca Albios (primarily wheat bran), Propol KW, Glucomannan S, Pectin HM Slow, powdered oat bran, Amophol LG, psyllium husk and Rheolex.

Extraction of the phenolic compounds

Sequential extractions were performed on 100 mg samples each of high fiber dietary supplement (primarily wheat bran) and cornmeal: the first extraction was performed using 10 ml thick-glass tubes with 5 ml of methanol, and overnight shaking (inversion), at room temperature; the second, with 5 ml of 0.1 *M* sodium hydroxide at 110°C for 10 min. The two extracts consisted of free phenolics and alkali-labile lignin, respectively. The slurry was centrifuged at 3000 rpm, the supernatant decanted, and the residue washed with water (5 ml) after each extraction. The extracts and washing water of sodium hydroxide hydrolyzate were acidified with 37% HCl (20 μl) and diluted to 25 ml with water. The alkali-labile and free phenols solutions were filtered (0.22 μm nylon filter) and then injected into the HPLC–ES-MS system.

3. Results and discussion

3.1. ES optimization

Under the optimized ES source conditions, all the phenolic compounds tested generated intact deprotonated molecules $[M-H]^-$. The mass spectra of ferulic acid and vanillin are reported in Fig. 1(a) and Fig. 1(b), respectively. The peak intensity was similar for all tested standards, both acids and aldehydes.

The peak intensity is affected by the solvent used, and maximum values were observed only with methanol. Since the solvent must be compatible with the HPLC system (see below), the peak intensity was also determined with MeOH–H_2O (30:70, v/v) solvent: by increasing the amount of water to 70%, the peak intensity is reduced by 10% with respect to pure methanol. Under these solvent conditions, no adducts or other ions are generated. The *m/z* values of $[M-H]^-$ selected for HPLC analysis are listed in Table 1.

The flow-rate of the solvent entering the ES interface is also critical, and this must be kept as low as possible: the optimal rate is 11 μl/min, which is obtained by post-column splitting of the desired proportion of eluted standard from a conventional HPLC column having a flow-rate of 300 μl/min. Alternatively, a semimicro HPLC assembly using an Acurate system and micro column can also be adapted for this purpose.

3.2. HPLC separation

Previous studies have dealt with the separation of acidic phenols using a C_{18} column, but an unequivocal identification of possibly coeluting compounds is still a problem [20].

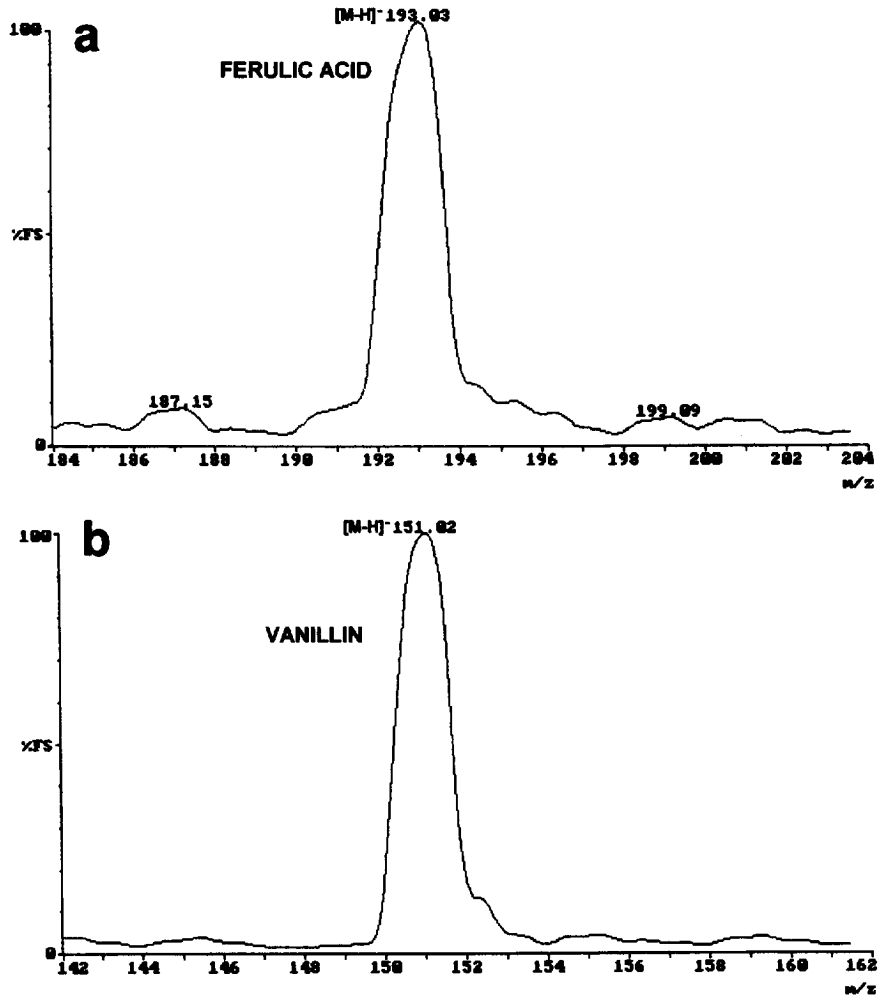

Fig. 1. Negative ion $[M-H]^-$ spectra of (a) ferulic acid and (b) vanillin.

Using the ES-MS system with selected ion monitoring, complete HPLC separation is not a crucial issue unless isomers are to be identified.

The best chromatographic separation of the 10 phenolic standards was obtained using a Nova-Pak phenylic column (3 μm particle size, 150 mm × 3.9 mm I.D.), at a flow-rate of 0.3 ml/min, with a MeOH–H_2O (30:70, v/v) mobile phase at pH= 5.67±0.1 to which Et_4NI 0.2 m*M* is added as an ion pairing agent. A typical chromatogram obtained with this selected ion monitoring method is shown in Fig. 2. Adequate separation of the 10 phenol standards was observed within 30 min. A crucial point for HPLC optimization with this technique is the use of Et_4NI, at a concentration of 0.2 m*M*, which results in more sharp symmetrical peaks; moreover, Et_4NI does not influence peak intensity or the analytical performance of the method. Correct identification and accurate quantification were accomplished despite incomplete resolution of some of the standards. The obtained retention times are reported in Table 1. The peak areas correlated well with the amount injected, and the slope of the regression line was similar for all phenolic compounds (Table 1).

Under the reported conditions, the detection limit of this method ranged from 1–6 pg/inj for all 10 standards, including the aldehydes, much lower than those previously achieved with conventional HPLC

Table 1
Molecular ions, retention time, detection limit and parameters of the calibration graphs of the HPLC–ES-MS analysis of phenolic acids and aldehydes

Phenolic acids and aldehydes	$[M-H]^-$	Retention time (min)	Detection limit (pg/inj)	a	b	s_a	s_b	r
Gallic acid	169.1	6.80	1.9	3.60	$4.05 \cdot 10^4$	0.41	$1.21 \cdot 10^3$	1.00
p-Hydroxybenzoic acid	137.1	9.27	1.1	5.44	$11.7 \cdot 10^4$	0.64	$15.4 \cdot 10^3$	0.99
p-Hydroxyphenylacetic acid	151.2	10.80	1.7	1.75	$33.6 \cdot 10^4$	0.52	$13.6 \cdot 10^3$	1.00
Vanillic acid	167.1	10.53	4.1	1.39	$8.14 \cdot 10^4$	0.17	$1.91 \cdot 10^3$	0.99
Syringic acid	197.2	11.66	3.2	1.22	$6.83 \cdot 10^4$	0.15	$1.21 \cdot 10^3$	0.99
p-Hydroxybenzaldehyde	121.1	13.01	1.0	3.97	$27.1 \cdot 10^4$	0.46	$1.78 \cdot 10^3$	1.00
Vanillin	151.1	16.16	2.0	1.33	$9.16 \cdot 10^4$	0.17	$4.52 \cdot 10^3$	1.00
p-Coumaric acid	163.2	16.20	3.3	4.45	$17.1 \cdot 10^4$	0.47	$1.32 \cdot 10^3$	1.00
Syringaldehyde	181.2	19.00	1.9	3.15	$8.46 \cdot 10^4$	0.42	$1.33 \cdot 10^3$	0.99
Ferulic acid	193.2	18.95	6.2	2.94	$10.6 \cdot 10^4$	0.40	$13.4 \cdot 10^3$	1.00

Equation of graphs: $A=a+bx$ where A=peak area; x=pmol injected; a=intercept; b=slope; s_a=standard deviation of intercept; s_b=standard deviation of slope; r=correlation coefficient.

methods or HPLC–MS with thermospray ionization (Table 1). Since the actual amount of each phenol entering the ES-MS detector is 3% of the quantity injected after eluate splitting, the detection limit is ~300 fg. The temperature of the HPLC column is important for resolution, which is also reflected by

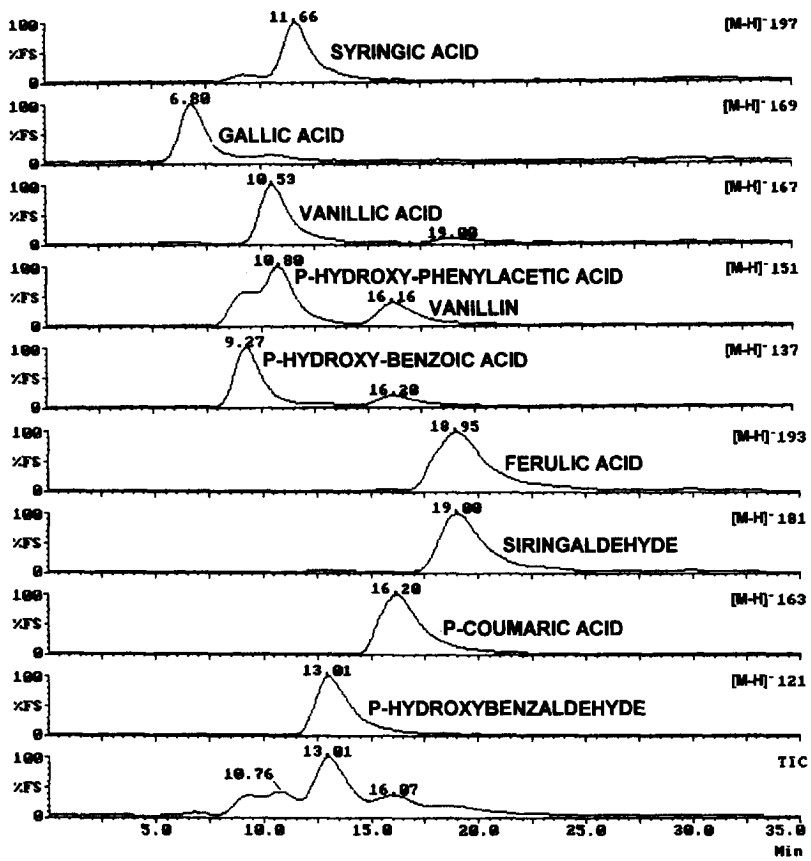

Fig. 2. Typical chromatogram obtained by HPLC–ES-MS analysis of the 10 phenolic standards.

Table 2
Percentage of alkali-labile lignin components present in the dietary fibers analyzed with the developed HPLC–ES-MS method

Fiber	Gallic acid	*p*-OH-benzoic acid	*p*-OH-benzaldehyde	Vanillin	*p*-OH-coumaric acid	Ferulic acid
Cornmeal	7.23%	–	9.36%	–	66.79%	16.63%
Crusca albios®	–	–	–	–	–	100%
Pectin	100%	–	–	–	–	–
Powdered oat bran	–	traces	4.55%	traces	62.62%	32.82%

the peak quality: the best analytical performance is achieved at 10–15°C, the range most compatible with the corresponding increase in the analysis time and column pressure. Carefully controlled column temperature (±0.2°C) is therefore necessary for obtaining highly reproducible retention time values. Day-to-day variation in the retention time over a 1-month period was less than 1% for each standard analyzed (Table 1).

The data obtained for alkali-labile lignin present in

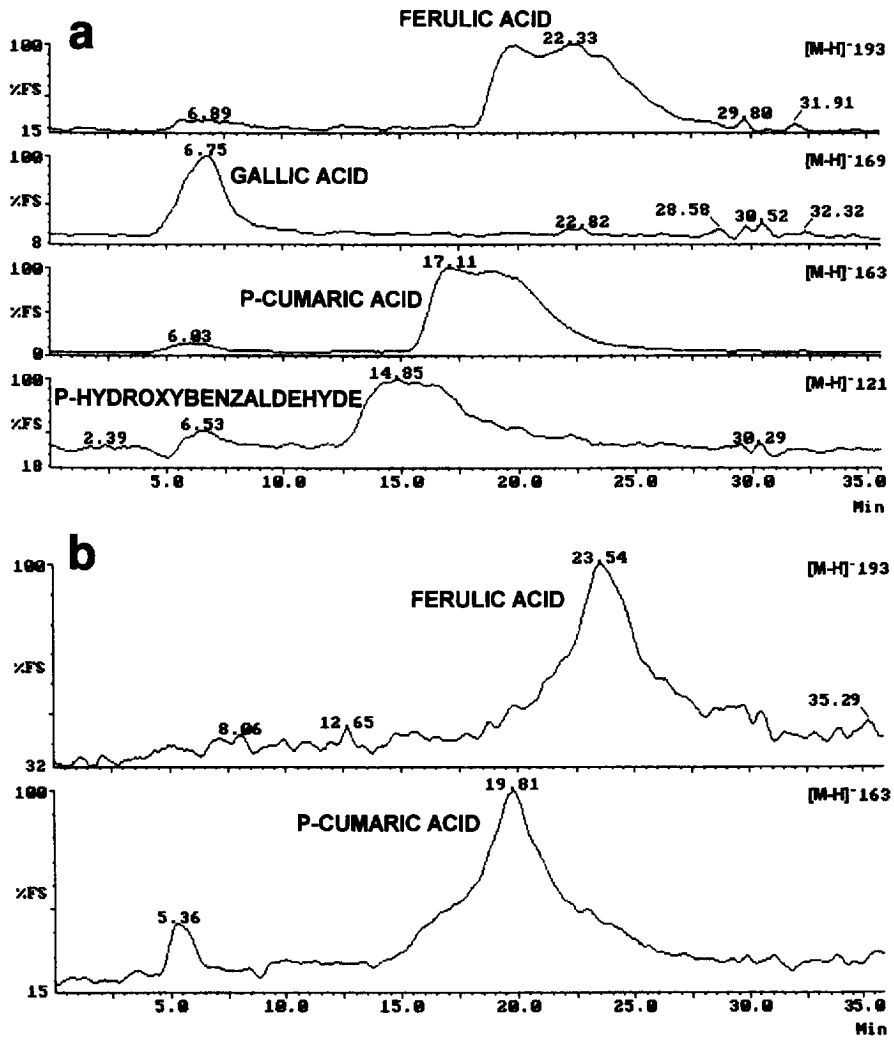

Fig. 3. Typical chromatograms of (a) cornmeal and (b) oat bran.

the dietary fibers are summarized in Table 2, and typical chromatograms of cornmeal (reference) and oat bran are shown in Fig. 3a and Fig. 3b. The most abundant phenolic compounds present in this fraction from, oat bran and cornmeal extracts, are ferulic acid and *p*-coumaric acid and, to a lesser extent, *p*-hydroxybenzaldehyde, gallic acid, *p*-hydroxybenzoic acid and vanillin. Cornmeal is the only substance studied which showed the presence of phenolic molecules (*p*-coumaric acid and *p*-hydroxybenzaldehyde) in the free phenolic fraction.

4. Conclusions

Negative ion electrospray MS provides a potent analytical tool for identification of plant phenolics in plant extracts. The developed HPLC–ES-MS method is extremely sensitive, the detection limit being 1 to 2 orders of magnitude lower than other developed methods, including positive ion thermospray-MS. The HPLC–ES-MS method is reproducible, simple and rapid, and is based on selected ion monitoring of deprotonated molecules $[M-H]^-$, which are formed by mild electrospray ionization. Complex mixtures of phenolic compounds can be evaluated without HPLC separation, unless positional isomers are present.

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