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GENERAL SCHEME OF ANALYSIS OF PHENOLIC COMPOUNDS IN PLANT EXTRACTS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A general procedure of analysis of phenolic compounds by reversed-phase high-performance liquid chromatography with ternary elution gradients has been designed. The method was applied to the separation and estimation of phenolic acids, phenolic aldehydes and coumarins from standard mixtures or from plant extracts. Examples of its application to the variations in phenolic acids during grape juice storage, to studies of the monomeric composition of poplar lignins and to estimation of the coumarin contents of sweet clover are given.

INTRODUCTION

Phenolic compounds are widely distributed in plants and are frequently present in products of plant origin of economic importance.

Paper chromatography has been extensively used for qualitative research in phenolics, ever since its introduction in the 1940's by Bate-Smith[20]. In contrast, the quantitative analysis of these compounds has been little developed owing to the great diversity of their chemical structures and the absence of a general procedure.

Low-pressure liquid chromatography[1] gave excellent separations of phenolic compounds but was not adapted to systematic studies owing to the long analysis times. The separation of some phenolics has recently been achieved by gas–liquid chromatography. However, the low volatility of polyphenols and the poor selectivity of the detectors render this method inappropriate for plant extracts (for details see the review by Van Sumere *et al.*[2]).

High-performance liquid chromatography (HPLC), first developed for phenolic compounds by Hostettmann and Jacot-Guillarmod[3] and Nagel and co-workers[4,5], and more recently by Murphy and Stutte[6]. Hardin and Stutte[7] and Hartley and Buchan[8], seems more promising. We present here a general method of analysis of polyphenols by reversed-phase HPLC, and show, by direct applications, that this method can easily be used to solve problems concerning the analysis of the polyphenol contents of plants.

EXPERIMENTAL

Apparatus

A Spectra-Physics SP 8000 liquid chromatograph was used. A 250 × 4.6 mm stainless-steel column, packed with 10-μm LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R., was used for the chromatographic separations. The SP 8000 includes a ternary gradient generator, an automatic injector with a 10-μl sample loop and a peak integrator. A microprocessor corrects for the variations due to the changes in viscosity of the gradient mixtures and allows constant flow conditions, giving highly reproducible retention times (better than 2%).

Optical density was monitored with a variable-wavelength detector (Gilson Holochrom) equipped with a 10-μl flow cell.

Solvents

Only methanol, acetic acid and water were used. The two organic solvents were freshly distilled and filtered through a sintered glass filter (G 5). De-ionized water was filtered through a 0.45-μm Millipore filter and then degassed by boiling. All the solvents were stored under a helium atmosphere.

Isolation of phenolic compounds from plant materials

Phenolic acids. Phenolic acids were isolated from grape concentrate obtained from *Vitis vinifera* var. "Carignan noir" (I.N.R.A. Station of Pech Rouge, Gruissan, France). After harvesting, the grapes were crushed and 1 g/l of gaseous SO_2 was added. The juice was then decanted, and concentrated in a tubular evaporator. One aliquot was kept frozen and another stored for 6 months at 10 C.

Before use, 20 ml of each concentrate were first diluted with water to the initial concentration of the juice. Then 2 % metaphosphoric acid, 20 % ammonium sulphate and 20 % ethanol were added and the phenolics extracted five times with 50 ml ethyl acetate. The combined extracts were taken to dryness. The residue was dissolved in water, adjusted to pH 4 and tannins were precipitated with 1 % gelatine solution in 10 % NaCl. The esters were then hydrolysed with 4 M NaOH for 4 h under nitrogen[9].

After adjustment of the pH to 2, phenolic acids were extracted by diethyl ether. The ether was then evaporated to dryness and the residue taken up in water and subsequently purified by extraction with diethyl ether, first at pH 8.5 (this extract was discarded) and then at pH 2. After evaporation of ether, the phenolic acids were dissolved in 1 ml methanol–1 % HCl and centrifuged for 15 min at 10,000 g to eliminate undissolved salts.

Phenolic aldehydes. Phenolic aldehydes were obtained after nitrobenzene oxidation of lignins isolated from sclerenchyma and xylem tissues of stems of *Populus nigra* var. *Italica*[10,11]. The aldehyde fraction was purified by successive extraction

with diethyl ether, first at pH 11 to eliminate nitrobenzene, and then at pH 8.5. This second extract was evaporated to dryness, and the residue dissolved in 0.5 ml methanol and centrifuged for 15 min at 10,000 g.

Coumarins. The coumarins were extracted from the leaflets of two genotypes of *Melilotus alba:* the CuCu BB reported as coumarin rich and the cucu bb known as coumarin poor[12-14]. One gram of leaflets was ground in 20 ml of 80˜ ethanol with an Ultra-Turrax and the phenolic material was extracted with 500 ml of the same solvent, as described by Alibert *et al.*[15]. The solvent was evaporated, the dry residue dissolved in 10 ml of a 0.1 *M* acetate buffer, pH 5, and the glycosides hydrolysed for 4 h at 37˚C by 0.3% emulsin (Nutritional Biochemicals)[16].

After acidification to pH 2, the coumarins were extracted by diethyl ether, the ethereal phase was evaporated and the residue dissolved in 0.5 ml methanol. This solution was centrifuged, as previously described for the other fractions, before HPLC analysis.

RESULTS

A HPLC separation of standard mixtures of phenolic compounds

During these experiments we tried to develop as simple as possible a method of analysis, allowing the separation of a wide variety of polyphenol classes without important modifications of the analytical system. After multiple preliminary essays we chose only one stationary phase: LiChrosorb RP-18 and methanol–acetic acid–water as solvent. Owing to the rapidity of the analysis, we preferred to separate the same sample twice under different conditions of elution and detection so as to obtain a complete separation, rather than to use a complicated elution mixture.

TABLE I

MOBILE PHASES FOR SEPARATION OF PHENOLIC ACIDS

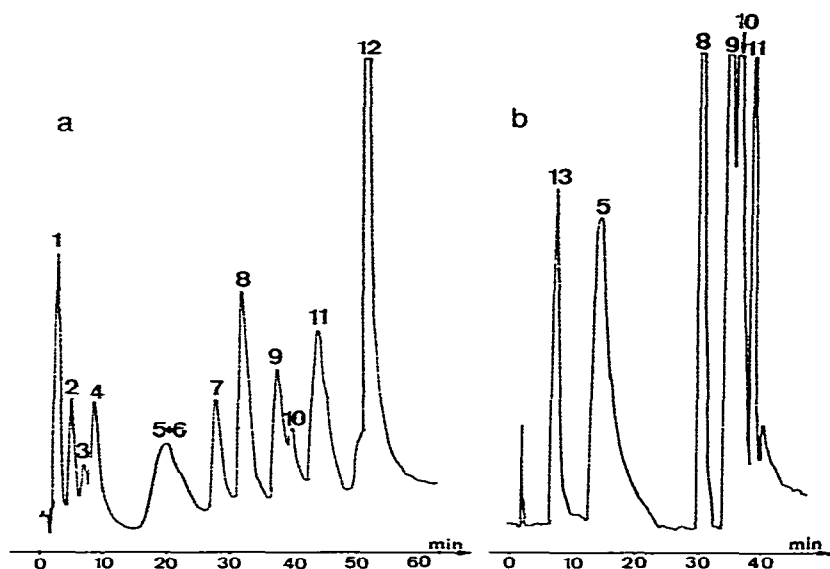| Mobile phase	Flow-rate (ml min)	Elution (min)	Time (min)	Methanol (%)	Acetic acid (%)	Water (%)
A	1	65	0	5	5	90
			2	5	1	94
			15	5	1	94
			16	5	5	90
			40	20	5	75
			41	65	5	30
			55	65	5	30
			56	100	0	0
			65	100	0	0
B	1	45	0	5	5	90
			2	5	1	94
			15	5	1	94
			16	5	5	90
			31	25	5	70
			32	100	0	0
			45	100	0	0

Fig. 1. Separation of phenolic acids: a, by mobile phase A with detection at 280 nm; b, by mobile phase B with detection at 335 nm. Numbers on the chromatograms correspond to the acids given in Table II.

Quantitative results are obtained directly by the data system of the SP 8000 liquid chromatograph. Different calibration methods are available; we used the external standard method where, in the "Cal Mode", the data system memorizes the values from successive standardization runs and corrects automatically the values of the analytical runs.

TABLE II

RETENTION TIMES (IN 0.1 min) OF PHENOLIC ACIDS

No.	Phenolic acid	Mobile phase A, 280 nm detection	Mobile phase B, 335 nm detection
1	Gallic	29	*
2	Protocatechuic	52	*
3	Salicylic	78	*
4	p-Hydroxybenzoic	85	*
5	Caffeic	193	144
6	Vanillic	193	*
7	Syringic	278	*
8	p-Coumaric	309	296
9	Ferulic	362	340
10	Sinapic	386	355
11	o-Coumaric	423	377
12	Cinnamic	482	*
13	Gentisic	*	76

* Not detected.

TABLE III

MOBILE PHASE FOR SEPARATION OF PHENOLIC ALDEHYDES

Flow-rate: 1 ml/min. Elution time: 25 min.

Time (min)	Methanol (%)	Acetic acid (%)	Water (%)
0	10	2	88
5	10	2	88
10	20	2	78
20	80	2	18
25	80	2	18

After 1 year of routine use, for amounts of 0.5–50 nmoles, the reproducibility of standards was better than 5%.

Phenolic acids. Phenolic acids are the most common phenolic compounds present in plants, and always as mixtures of benzoic and cinnamic acids.

Two mobile phase compositions were used to separate these substances (Table I). Mobile phase A, with detection at 280 nm, allows the quantitative analysis of most of the phenolic acids (Fig. 1a). However, gentisic acid does not absorb at this wavelength and caffeic and vanillic acids are poorly separated. Mobile phase B, with detection at 335 nm, allows quantitative analysis of these three acids (Fig. 1b)*.

Table II gives the retention times of the phenolic acids.

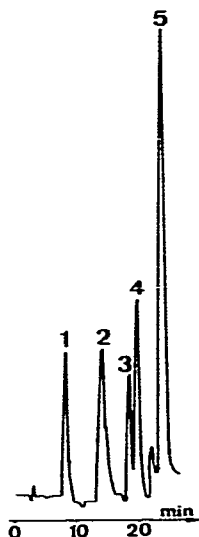*Phenolic aldehydes.* Phenolic aldehydes, the main flavour fraction of various

Fig. 2. Separation of phenolic aldehydes. Numbers on the chromatogram correspond to the aldehydes given in Table IV.

* The concentration of vanillic acid is given by $C_v = \left(A_{280} - \frac{K_c}{K'_c} \cdot A_{335} \right) \cdot \frac{1}{K_v}$

where A_{280} and A_{335} are the peak areas at 280 nm and 335 nm, respectively. K_c and K'_c are the response constants of the detector for caffeic acid at 280 and 335 nm respectively and K_v that of vanillic acid at 280 nm.

TABLE IV

RETENTION TIMES (IN 0.1 min) OF PHENOLIC ALDEHYDES

No.	Aldehyde	t_R
1	3,4-Dihydroxybenzaldehyde	78
2	4-Hydroxybenzaldehyde	133
3	Vanillin	176
4	Syringaldehyde	188
5	Benzaldehyde	225

TABLE V

MOBILE PHASES FOR SEPARATION OF COUMARINS

Mobile phase	Flow-rate (ml/min)	Elution time (min)	Time (min)	Methanol (%)	Acetic acid (%)	Water (%)
C	1	40	0	10	2	88
			30	50	2	48
			40	50	2	48
D	1	40	0	5	1	94
			12	20	1	79
			20	20	1	79
			30	80	1	19
			40	80	1	19

beverages, can easily be separated using the mobile phase given in Table III. Here the compounds are monitored at 290 nm (Fig. 2). The retention times of each compound are given in Table IV.

Coumarins. Coumarins constitute a large family of phenolic compounds particularly well represented in *Leguminosae*[17]. Most of them are separated on the RP-18 column using the solvent gradients reported in Table V. The coumarins were detected at 350 nm (gradient C) or 275 nm (gradient D). Table VI gives the retention times and the responses of the detector at these two wavelengths, while Fig. 3 shows the types of separation obtained under these conditions.

TABLE VI

RETENTION TIMES (IN 0.1 min) OF COUMARINS

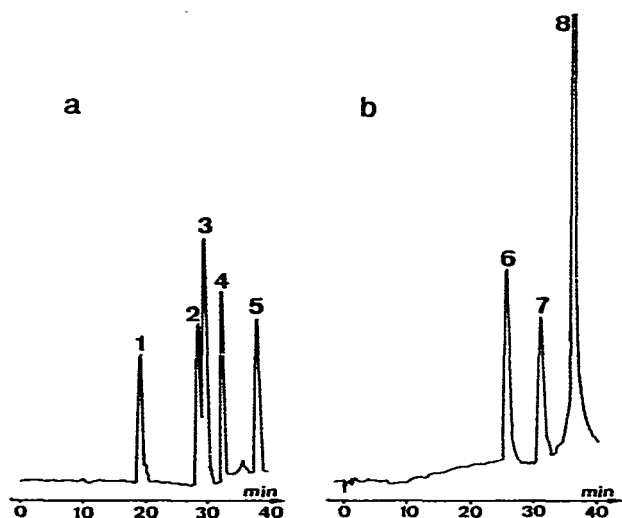| No.	Coumarin	Mobile phase C, 350 nm detection	Mobile phase D, 275 nm detection
1	Aesculetin	203	
2	Umbelieferin	277	
3	Scopoletin	304	Not
4	5,7-Dihydroxy-6-methyl-8-ethylcoumarin	340	separated
5	4-Methyl-7,8-diethylcoumarin	395	
6	Coumarin	Not detected	267
7	4-Hydroxycoumarin	Not detected	317
8	4-Methylcoumarin	Not detected	368

Fig. 3. Separation of coumarins: a, by mobile phase C with detection at 350 nm: b. by mobile phase D with detection at 275 nm. Numbers on the chromatograms correspond to the coumarins given in Table VI.

Application to the analysis of complex plant extracts

During our work on plant polyphenols we used this methodology to study the content or the variations of the phenolics. We present here some examples showing that the method can readily be adapted for various types of research.

Grape concentrate analysis. One of the main problems of the long-term storage of grape juice concentrate is the browning of the juices.

TABLE VII

RECOVERY OF PHENOLIC ACIDS USING THE EXTRACTION AND PURIFICATION DE-SCRIBED

In experiment 1, 10 nmoles of each acid were added to the sample; in experiment 2, 1 nmole of each acid was added.

Acids	Percentage recovery		Mean
	Experiment 1	Experiment 2	
Gallic	61	58	60
Protocatechuic	59	60	59
Salicylic	65	68	67
Gentisic	96	90	93
Caffeic	61	55	58
Vanillic	60	57	59
p-Hydroxybenzoic	64	58	62
Syringic	59	54	57
p-Coumaric	60	55	57
Ferulic	47	49	48
Sinapic	75	70	73
o-Coumaric	42	34	38
Cinnamic	83	80	81

TABLE VIII

AMOUNTS OF PHENOLIC ACIDS IN A GRAPE JUICE CONCENTRATE AFTER STORAGE FOR 6 MONTHS

Phenolic acid	Amount (µmol/l)	
	At −20°C	At 10°C
Gallic	12	12
Protocatechuic	37	36
Salicylic	36	23
p-Hydroxybenzoic	14	8.7
Caffeic	15	0
Vanillic	0.2	0
Syringic	48	25
p-Coumaric	37	29

Using our extraction and purification, the yield of phenolic acids was monitored by addition of standard mixtures to the concentrate after dilution with water. Table VII gives the percentage recovery of the different acids for two experiments and the mean values retained for automatic analysis. Table VIII shows that some phenolic acids (mainly caffeic, salicylic, p-hydroxybenzoic and vanillic) undergo degradation during a 6-month storage period while others (gallic, protocatechuic) seem to remain unchanged.

Studies on the monomer content of lignins. Next to cellulose, lignins are quantitatively the most important polymers in plants. The monomer content of lignin varies with the systematic classification of the examined plants[18], the age of the stems[19] or the nature of the lignified tissues[10]. The present technique has been applied to studies on lignins. As in the case of phenolic acids, the percentage recovery of the phenolic aldehydes was determined after addition of standard mixtures before nitrobenzene oxidation of a xylem sample of poplar stem. Table IX shows that the yields range from 70 to 84% for the three major monomeric units of lignins (*i.e.*, vanillin, syringaldehyde and 4-hydroxybenzaldehyde), but 3,4-dihydroxybenzaldehyde is destroyed by the degradation process of the lignins. Mean correction factors are given in the table.

TABLE IX

RECOVERY OF PHENOLIC BENZALDEHYDE USING THE NITROBENZENE DEGRADATION OF LIGNINS DESCRIBED

In experiment 1, 10 nmoles of each aldehyde were added to the sample; in experiment 2, 1 nmole of each aldehyde was added.

Aldehyde	Percentage recovery		Mean
	Experiment 1	Experiment 2	
3,4-Dihydroxybenzaldehyde	6.9	10	8
4-Hydroxybenzaldehyde	84	85	84
Vanillin	67	68	68
Syringaldehyde	67	72	70

TABLE X

MONOMER CONTENTS OF THE LIGNINS OF XYLEM AND SCLERENCHYMA OF ONE POPLAR STEM

Aldehyde	Amount ($\mu mol/g$)	
	Xylem	Sclerenchyma
p-Hydroxybenzaldehyde	12	0
Vanillin (V)	78	50
Syringaldehyde (S)	42	30
Ratio. S/V	0.54	0.60

Table X gives data on the monomer content of sclerenchyma and xylem of one poplar stem. We can show that these two types of tissue are different: sclerenchyma contains three monomeric units (guaiacyl, syringyl and p-hydroxybenzoyl corresponding, after degradation by alkaline nitrobenzene, to coniferylic, sinapylic and coumarylic alcohols), while xylem has only two constituents (guaiacyl and syringyl). Furthermore, the relative proportions of the guaiacyl (vanillin) and syringyl (syringaldehyde) units differ from one tissue to another.

This rapid HPLC technique seems useful for routine analysis in such fields.

Coumarin analysis. Research was originally conducted on *Melilotus* with the aim of developing strains of sweet clover deficient in coumarins; these compounds are normally found in relatively high concentrations and render the plant unpalatable to herbivores. No attempt was made in this case to determine the quantitative yield of the coumarins using the proposed extraction and purification since the main object of the work was essentially a comparison between coumarin contents of two strains of sweet clover.

Compared to the common CuCu BB genotype, the cucu bb genotype contains much lower amounts of scopoletin, coumarin and 4-hydroxycoumarin, as shown in Table XI.

Comparative studies on coumarin biosynthesis, transport and storage could be performed using this technique.

TABLE XI

COUMARIN CONTENTS OF TWO STRAINS OF *MELILOTUS ALBA*

Coumarin	Amount ($\mu mol,g$)	
	Genotype (CuCu BB)	Genotype (cucu bb)
Scopoletin	121	17
Coumarin	32	0
4-Hydroxycoumarin	664	30

CONCLUSIONS

We have developed a general technique of separation and quantitative analysis of plant phenolic compounds by reversed-phase HPLC. It is shown, through examples. that this rapid and sensitive method is easily adapted to numerous fields of application such as phytochemistry. food control and fundamental research.

We believe that this method is not restricted to the three types of phenolic compound described here. but can easily be extended. with minor adjustments. to the analysis of other phenolic compounds such as esters. flavonoids or anthocyanins.

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