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ANALYSIS OF PLANT PHENOLICS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Methods are described for the simultaneous qualitative and quantitative determination of aglycone plant phenolic acids by high-performance liquid chromatography using gradient elution. Known phenolic acids were determined in extracts from a cell suspension culture of maize. The compounds are identified by their retention times in two different mobile phase gradients, and by their absorbance ratios in simultaneous detection at 254 and 280 nm. The compounds are quantitatively determined by area measurements; the peak area was proportional to the amount in the range investigated (0.2–2 μg) for all compounds.

INTRODUCTION

Phenolic compounds have received considerable attention owing to their multiple function in plants, *e.g.*, their role in indoleacetic acid metabolism[1] and in differentiation[2] and their function in host–pathogen interaction[3]. High-performance liquid chromatography (HPLC), with its high speed and sensitivity, offers a promising system in analysing plant substances such as phenolics.

Studies on the separation of plant phenolics by reversed-phase HPLC[4–11] were mostly concerned with separation, where the phenolics are identified with a single retention time. This is inadequate for identification in plant extracts. The purpose of this study was to develop a method for the simultaneous qualitative and quantitative determination of aglycone phenolic acids in the same operational procedure. Further, the method should allow the specific detection of phenolics of physiological significance. This paper presents a method for determination of known plant phenolic acids and a method for quantification using reversed-phase HPLC. Reproducibility has been examined using standard compounds and examples are given for determining phenolics from plant extracts of a cell suspension culture of maize.

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EXPERIMENTAL

Isolation of phenolics from plant cells

Plant material. Cell suspension cultures of *Zea mays* cell line Z4 were used. These cells, originally derived from protoplasts[12], were kindly supplied by I. Potrykus, Friedrich-Miescher Institute, Basle, Switzerland.

Extraction and purification of phenolics from the cells. Ten grams fresh weight of cells were extracted in 100 ml of boiling ethanol for 40 min with reflux. The cells were removed by filtration through a glass funnel and the solvent was concentrated by evaporation to about 3 ml. Subsequently, the extract was transferred into a 30-ml centrifuge tube with approximately 10 ml of water and centrifuged at 25,000 *g* for 10 min, to remove cell debris. The supernatant was removed and after concentrating it to 3–4 ml the extract was transferred into a Centriflo membrane filter (Amicon, Lexington, MA, U.S.A.) with water up to 5 ml. The extract was centrifuged with low speed through the membrane filter to remove high-molecular-weight compounds. The resulting clear extract was applied to an ion-exchange column, when analysing for free aglycone phenolic acids. When analysing for both free and bound aglycone phenolic acids the 5-ml extract was hydrolysed with 5 ml of 1 *N* hydrochloric acid at 100°C for 30 min and then extracted three times with diethyl ether. The ether was removed from the combined extracts by evaporation and the phenolic acids were resuspended in 5 ml of water. Finally, the extract was applied to an ion-exchange column.

Group separation. To obtain a group separation into carboxylic acid phenolics and non-carboxylic acid phenolics, the extracts were applied to an ion-exchange column as described by Nagels *et al.*[13].

ECTEOLA-cellulose (MN 2100 ECTEOLA, Macherey, Nagel & Co., Düren, G.F.R.) equilibrated with acetate counter ions was poured into the column (10.5 × 1.4 cm I.D.) as a slurry. Non-carboxylic acid phenolics were eluted with 10 m*M* ammonium acetate solution at a flow-rate of 1 ml/min. The carboxylic acid phenolics were washed from the column with 0.1 *N* hydrochloric acid at a flow-rate of 1 ml/min. The flow-rate was controlled using a Minipuls 2 peristalistic pump (Gilson, France). This pump has four channels allowing the simultaneous control of the flow of three columns, while the fourth channel was connected with the outlet from one of the columns, pumping the eluate through a Uvicord III 2089 UV absorptiometer (LKB, Stockholm, Sweden). The fraction as detected on the Uvicord was approximately 50 ml. The recovery of the isolation and purification procedure was measured using standard compounds.

The concentrated extracts were applied to the HPLC column for determination of phenolic acids.

Determination of phenolics by HPLC

Apparatus. All analytical separations were performed with a Spectra Physics SP 8000 liquid chromatograph connected with a Waters 440 absorbance detector, allowing simultaneous detection at 254 and 280 nm. A Rheodyne 7120 injection unit was used, the injection volume being 100 μl. The peak area was calculated by a Spectra Physics integrator 4000. The peak height was measured manually.

Temperature. The analysis was performed at 40°C. This temperature decreases

the retention times relative to room temperature permitting the later compounds to be eluted with sufficiently low retention times.

Column. A 250 × 4.6 mm I.D. column containing Nucleosil C_{18} with a particle size of 10 μm (Macherey, Nagel & Co.) was used. The column was packed with a Magnus P6000 HPLC slurry packing pump, using a Knauer packing reservoir and Knauer column.

Solvents. Rathburn (Rathburn Chemicals, Walkerburn, Great Britain) and Li-Chrosolv (Merck, Darmstadt, G.F.R.) HPLC-grade methanol, 40 m*M* formic acid and redistilled water were used. All solvents were filtered through a fluff-free filter and stored under a helium atmosphere.

Mobile phases. A flow-rate of 4 ml/min for gradient A (from 5% to 60% methanol in 40 m*M* formic acid in 25 min) and for gradient B (from 10% to 25% methanol in 10 min and then from 25% to 40% methanol in 40 m*M* formic acid from 10 to 20 min) was used. To ensure reproducibility and to prolonge the lifetime of the column, cleaning was performed regularly by flushing with both pure water and methanol to remove salts and organic impurities accumulated on the column. Even elution with the solvents alone causes the accumulation of impurities on the column; this has been demonstrated with the two different types of HPLC-grade methanol.

Standards. All standards were obtained from commercial sources. Gallic, protocatechuic, chlorogenic, caffeic, vanillic, syringic, *p*-coumaric, ferulic and *trans*-cinnamic acids, umbelliferone and scopoletin were obtained from Sigma (St. Louis, MO, U.S.A.), 4-hydroxybenzoic acid and benzoic acid from Merck-Schuchardt (Munich, G.F.R.) and sinapic acid from Ega-Chemie (Steinheim, G.F.R.). The chemical structures of the standard compounds are shown in Table I. The concentration of all compounds used in the analysis of reproducibility was $5 \cdot 10^{-5}$ *M*.

RESULTS AND DISCUSSION

Qualitative determination of phenolic compounds

Phenolic acids were determined using two retention times in two different gradients, A and B, and the absorbance ratio at 254 and 280 nm from simultaneous detection at the two wavelengths. Known phenolic acids were demonstrated in plant extracts of maize cells. The use of retention times and absorbance ratios in identification requires that they can be measured in a reproducible manner. Therefore, an analysis of reproducibility was performed.

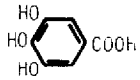
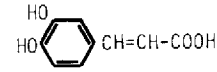
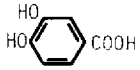
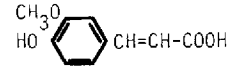
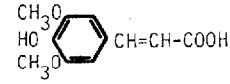
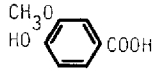
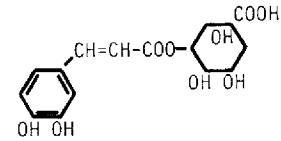
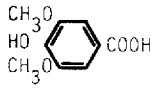
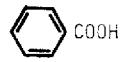
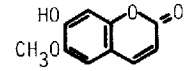
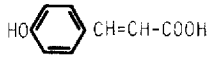
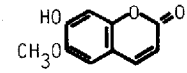
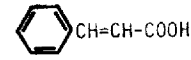
In order to use the absorbance ratio in the qualitative determination it is necessary that the compounds be well separated. From Tables II and III it is evident that the values of all but one of the capacity factors, k', are below 10 and also that separation factors, α, greater than 1 are obtained.

The retention times for the eleven phenolics used in the reproducibility study using gradients A and B are shown for a five-replicate analysis in Tables II and III. Relative standard deviations of the retention times in the range 0.4–1.0% were found, which fulfills the requirement of good reproducibility and is in accordance with other results[14,15].

Peak heights were measured in simultaneous detection at 254 and 280 nm. The absorbance ratios for each compound in a five-replicate analysis are shown in Tables

TABLE I

STRUCTURES OF PLANT PHENOLICS

Name	*Structure*	*Name*	*Structure*
Gallic acid		Caffeic acid	
Protocatechuic acid		Ferulic acid	
4-Hydroxybenzoic acid		Sinapic acid	
Vanillic acid		Chlorogenic acid	
Syringic acid			
Benzoic acid		Umbelliferone	
p-Coumaric acid		Scopoletin	
		Cinnamic acid	

II and III. It appears that this ratio is constant, and a relative standard deviation in the range 0.7–4.0% was found. This small variation renders the absorbance ratio well suited as a parameter in the determination of phenolic compounds.

The absorbance ratio and retention times in two gradients together are characteristic of individual phenolic compounds and allows their identification. To obtain these retention times requires two elutions, one in gradient A and one in gradient B. Including cleaning and equilibration of the column for the next injection, the total time required for identification by HPLC is 90 min. Compared with other methods, the present method is very fast and allows the simultaneous identification of several phenolics (Figs. 1 and 2); hence the method offers an excellent system for rapidly identifying plant phenolics.

Quantitative determination of phenolic compounds

For the quantitative analysis of phenolics, peak-area or peak-height measurements can be used. The reproducibility of peak-area measurements in a five-replicate analysis with standard compounds was found to be better than that for peak-height measurements. A relative standard deviation of peak areas for the available standards in the range 1.9–4.1% was found. Further, for all compounds the peak area was

TABLE II

REPRODUCIBILITY OF RETENTION TIMES AND ABSORBANCE RATIOS MEASURED IN GRADIENT A

S.D. = standard deviation; S.D. (%) = standard deviation as a percentage of the mean value. $k' = \frac{t_1 - t_0}{t_0}$; $\alpha = \frac{k'_1}{k'_2}$; t_0 = retention time of non-retained solvent.

Compound	*Retention time (sec)*			k'	α	*Absorbance ratio, 254/280 nm*		
	Mean	*S.D.*	*S.D.(%)*			*Mean*	*S.D.*	*S.D. (%)*
Gallic acid	161	1.4	0.9	1.0		1.77	0.02	0.8
					2.50			
Protocatechuic acid	290	2.3	0.9	2.6		5.23	0.04	0.8
					1.65			
4-Hydroxybenzoic acid	427	3.9	0.9	4.3		12.46	0.35	3.0
					1.38			
Vanillic acid	553	4.5	0.9	5.8		4.73	0.12	2.5
					1.13			
Chlorogenic acid	610	4.4	0.7	6.2		1.77	0.05	2.8
					1.15			
Syringic acid	661	5.4	0.8	7.1		1.21	0.03	2.1
					1.13			
Umbelliferone	725	5.2	0.7	8.0		1.12	0.09	3.8
					1.10			
Scopoletin	797	5.3	0.7	8.8		2.68	0.09	3.5
					1.07			
Ferulic acid	843	5.3	0.6	9.4		1.44	0.04	2.6
					1.05			
Sinapic acid	879	5.4	0.6	9.9		1.71	0.03	1.7
					1.38			
Cinnamic acid	1189	5.2	0.5	13.7		1.14	0.01	0.7

proportional to the amount of solute relevant to this study (less than 2 μg). Ferulic and *p*-coumaric acids showed more variation than the other compounds. Different batches of these compounds were tested and the same variation was observed. This variation is probably due to the presence of *cis*- and *trans*-isomers, which would be expected to cause broadening or splitting of the peaks and thereby also more variation in the measurement of the peak area.

Table IV shows the recovery of eleven phenolics extracted and purified according to the procedure used for the plant material. Gallic acid gives a poor recovery (only 24%) compared with the other phenolics analysed. For gallic acid as well as the other phenolics most of the losses take placing during the group fractionation. However, apart from gallic acid the recovery is high compared with that in a similar investigation[12].

Determination of plant phenolics by HPLC

The present method was developed in order to determine phenolic acids at concentrations of physiological interest in tissue culture, owing to their possible growth regulatory effects[2]. In investigations of possible growth effects of phenolics,

TABLE III

REPRODUCIBILITY OF RETENTION TIMES AND ABSORBANCE RATIOS MEASURED IN GRADIENT B

Abbreviations as in Table II.

Compound	*Retention time (sec)*			*k'*	*α*	*Absorbance ratio, 254/280 nm*		
	Mean	*S.D.*	*S.D. (%)*			*Mean*	*S.D.*	*S.D. (%)*
Gallic acid	119	1.0	0.8	0.5		1.72	0.03	1.5
					3.01			
Protocatechuic acid	203	2.0	1.0	1.5		5.34	0.05	0.9
					2.13			
4-Hydroxybenzoic acid	340	2.1	0.6	3.2		12.82	0.47	3.7
					1.49			
Vanillic acid	467	3.0	0.6	4.8		4.70	0.09	1.9
					1.12			
Chlorogenic acid	516	4.0	0.8	5.4		1.67	0.07	3.9
					1.12			
Syringic acid	573	3.5	0.6	6.1		1.26	0.05	4.0
					1.20			
Umbelliferone	676	3.5	0.5	7.4		1.13	0.04	3.6
					1.15			
Scopoletin	772	3.8	0.5	8.5		2.67	0.03	1.1
					1.09			
Ferulic acid	830	4.6	0.6	9.3		1.40	0.06	3.9
					1.08			
Sinapic acid	893	4.9	0.6	10.0		1.71	0.05	2.8
					1.42			
Cinnamic acid	1229	7.6	0.6	14.2		1.16	0.03	2.6

these compounds have been applied extogeneously to tissues[16,17]. From these investigations phenolic acids were shown to affect growth if present at high concentrations (above 10^{-5} *M*).

The chromatogram in Fig. 3 demonstrates the presence of phenolic acids, aglycone as well as glycone, in a hydrolysed and group fractionated extract of maize cells. From Table V it appears that the retention times and absorbance ratio for gallic, 4-hydroxybenzoic and vanillic acid standard compounds fits well with those observed for compounds 1, 3 and 4 in the chromatogram of the plant extract. Compound 6 is syringic acid, although the absorbance ratio differs slightly from that observed for the standard compound. It was not possible to identify compounds 2, a and b by comparison with the retention times and absorbance ratios of known phenolics. However, compound 2 could be protochatechuic acid, as the retention times fits with that of the standard, but it was not possible to calculate the absorbance ratio because of the small amount present. Using preparative HPLC the identities of compounds a and b are presently under investigation. The peak at the front of the chromatogram might be degradation products or charged compounds of some of the phenolics, especially gallic acid. This follows because in the recovery analysis the disappearance of gallic acid is followed by the occurrence of a peak at the front of the chromatogram, indicating that gallic acid is converted into charged compounds.

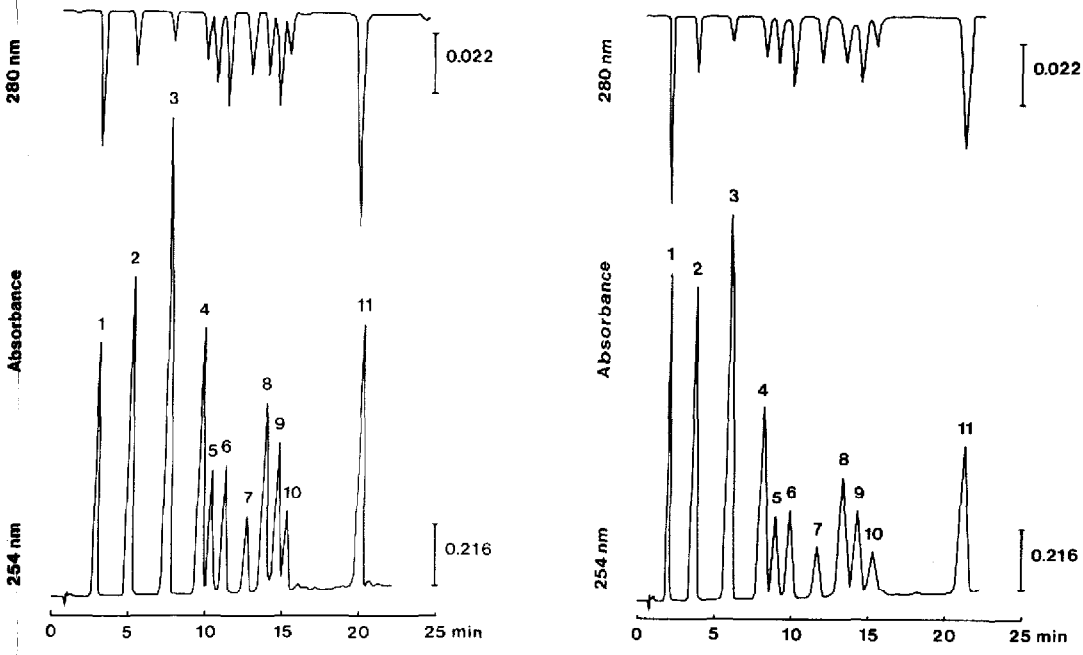


Fig. 1. Analysis of standard phenolic compounds on a Nucleosil C_{18} column. Gradient A; flow-rate, 4 ml/min; paper speed, 0.5 cm/min; full-scale absorbance, 2.0 at 254 nm and 0.2 at 280 nm; injection volume, 100 μl; amount of standards, 15 μl/ml of each compound. Peaks: 1 = gallic acid; 2 = protochatechuic acid; 3 = 4-hydroxybenzoic acid; 4 = vanillic acid; 5 = chlorogenic acid; 6 = syringic acid; 7 = umbelliferone; 8 = scopoletin; 9 = ferulic acid; 10 = sinapic acid; 11 = cinnamic acid.

Fig. 2. Analysis of standard phenolic compounds on a Nucleosil C_{18} column. Gradient B; same conditions and compounds as in Fig. 1.

TABLE IV

RECOVERY OF PHENOLICS USING THE EXTRACTION AND PURIFICATION METHODS DESCRIBED

Phenolic standard in a solution containing 15 μl/ml of each compound was used.

Compound	*Recovery (%)*	*Compound*	*Recovery (%)*
Gallic acid	24	Scopoletin	72
Protocatechuic acid	93	Umbelliferone	77
4-Hydroxybenzoic acid	95	Ferulic acid	68
Vanillic acid	96	Sinapic acid	86
Chlorogenic acid	64	Cinnamic acid	89
Syringic acid	77		

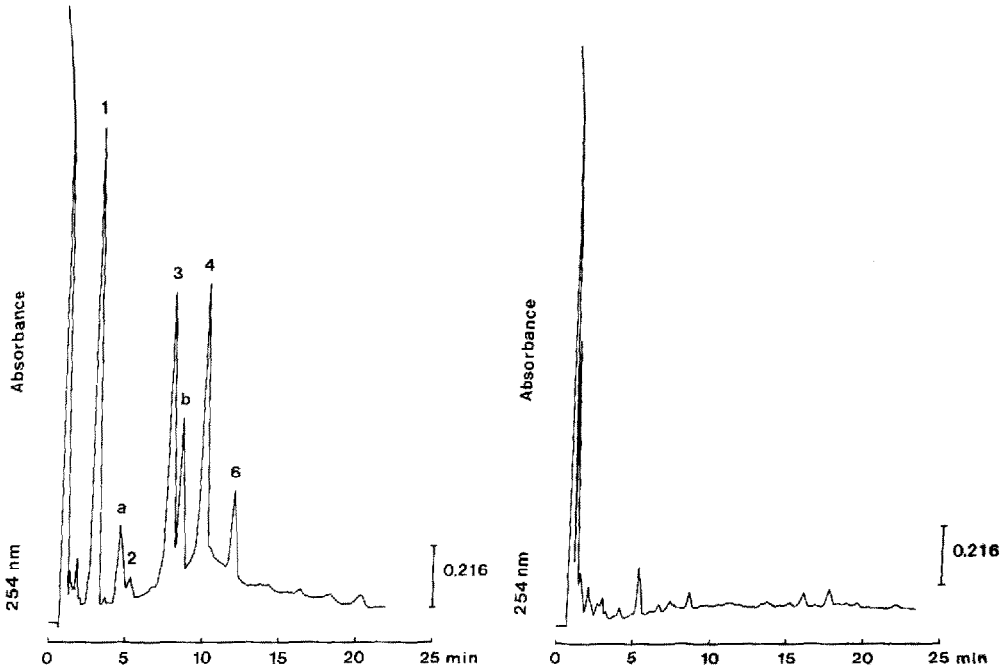


Fig. 3. Hydrolysed and group fractionated extract of maize cells, analysed on a Nucleosil C_{18} column. Gradient A; flow-rate, 4 ml/min; paper speed, 0.5 cm/min; full-scale absorbance, 2.0 at 254 nm; injection volume, 100 μl. Peaks: 1 = gallic acid; 2 = protochatechuic acid; 3 = 4-hydroxybenzoic acid; 4 = vanillic acid; 6 = syringic acid; a and b = unknown phenolic compounds.

Fig. 4. Non-hydrolysed group fractionated extract of maize cells, analysed on a Nucleosil C_{18} column. Conditions as in Fig. 3.

TABLE V

IDENTIFICATION OF PHENOLIC ACIDS IN EXTRACTS OF MAIZE CELLS

Phenolic acid	*Retention time (sec)*				*Absorbance ratio (254/280 nm),*	
	Gradient A		*Gradient B*		*Gradient A*	
	*Cells**	*St.***	*Cells**	*St.***	*Cells**	*St.***
Gallic acid	167	169	119	121	1.83	1.73
4-Hydroxybenzoic acid	443	440	331	329	12.65	12.43
Vanillic acid	563	555	451	447	4.71	4.60
Syringic acid	673	663	555	547	1.38	1.20

* Cells: extract of maize cells.
** St.: standard compounds.

Fig. 4 shows the chromatogram of a non-hydrolysed group fractionated extract of maize cells. It appears that aglycone phenolic acids were not present in these cells at concentrations higher than approximately 10^{-5} M; this result is in contrast to that for hydrolysed extracts of maize cells (Fig. 3). Indeed, free aglycone phenolic acids were not present at concentrations which in other systems are known to affect growth by external applications[16,17].

Owing to the excellent precision of the HPLC procedure used, the addition of internal standards is unnecessary for quantitative determination. This means that the quantitative determination of phenolic acids in plant extracts using peak-area measurements can be performed at the same time as the qualitative determination.

CONCLUSION

A method has been developed that allows the identification and quantification of phenolic acids in a very short time using HPLC. This much shorter analysis time compared with that of conventional methods allows many more samples to be processed with improved accuracy and can be very valuable in investigating phenolics of physiological interest. The method can be extended, with minor modifications, to the analysis of other phenolic compounds.

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