Phytochemistry, 1980, Vol. 19, pp. 651–657. Pergamon Press Ltd. Printed in Northern Ireland

FREE AND BOUND PHENOLIC ACIDS OF LUCERNE (*MEDICAGO SATIVA* CV EUROPE)

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(*Revised received* 30 *July* 1979)

Key Word Index—*Medicago sativa*; Leguminosae; phenolic acids; proteins; spectrophotometry; fluorimetry.

Abstract—The distribution of free and covalently-bound phenolic acids was studied in various fractions obtained from fresh lucerne shoots. *p*-Hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids were present, both free and bound, in all the fractions. Salicylic and sinapic acids occurred only in a bound, alkali-labile state and were found almost entirely in the 'aqueous phase' fraction after treatment of methanol–chloroform–water extract according to Bligh and Dyer. Many other common phenolics were absent. Amounts of the phenolic acids much larger than those extracted by methanol–chloroform–water were extracted subsequently by phenol–acetic acid–water and passed into the 'diffusate' fraction on dialysis of this extract against 70% acetic acid. Small, though significant, quantities of phenolic acids remained with the bulk protein in the 'bag contents' fraction. The extent to which the phenolic acids in these last two fractions are held to protein by covalent bonds or by secondary-valence attractions is discussed, particularly in relation to the isolation of *N*-feruloylglycylphenylalanine after partial hydrolysis. Suggestions are made for improving analytical procedures.‡

INTRODUCTION

Monties and Rambourg [1] have recently reviewed the sparse literature relating to the occurrence of phenolics in the important crop plant, *Medicago sativa*, with the general conclusion that variations between different cultivars may be substantial. However, of the common phenolic acids and derivatives, none other than chlorogenic acid has so far received mention (cf. also [2]). Although upper limits have been set to the extent of its occurrence in some cultivars, there are no conclusive published identifications of it.

In general in plants, phenolic acids occur both free and in covalently linked forms such as esters and amides [3]. When the free and conjugated forms have been extracted, purified and identified, they present no special problems. However, when one is dealing with a little-studied plant material, two complications need to be kept in mind: (1) whether the extracting medium is capable of removing quantitatively and without destruction the desired substances from the residual material, which may retain them by secondary-valence forces; (2) whether the hydrolysis procedures used are capable of liberating intact the phenolic acids from those compounds, known and unknown, to which they are bound by primary-valence links. In our present study, both these uncertainties have troubled us. The results which we now report have therefore general methodological interest within this field.

As to the free phenolic acids isolated, the amounts found may be low on account of imperfect extraction or damage or they may be high on account of hydrolysis of conjugated forms during the extraction. As to the phenolic acids believed to be in covalently-conjugated forms, we have not in general identified the parent substances chemically; what we have shown is that some of the hydrolysis procedures give rise to partial destruction of the substituted cinnamic acids, so that our values for covalently-conjugated cinnamics should be regarded as minimal.

One particular purpose of this work was to obtain the bulk leaf protein as free as possible from low-molecular contaminants, in order to study the covalently-linked phenolic acids, which are described in an accompanying paper [4]. In conclusion, we have correlated our results with those of Monties and Rambourg [1] who studied the same lucerne cultivar. However, they were analysing, on the whole, for different phenolics and by different methods. Moreover, they used different techniques from ours both for isolating the bulk protein and for attempted removal from it of associated phenolics.

RESULTS

Table 1 shows the dry-matter and nitrogen balances for the initial fractions derived from the lucerne shoots. Tables 2–5 give analyses for free and bound phenolic

‡In a Supplementary Publication, flowsheets are given for the detailed extractions and fractionations of the phenolic acids: SUP 90042 (13 pp.) deposited at the National Lending Library, Boston Spa, Yorkshire, LS23 7BQ, England, from whom copies can be obtained.

Table 1. Dry matter (DM) and nitrogen (N) balances for initial fractions from lucerne shoots (see Experimental)

Fraction	DM of fraction (as % of total DM)	N of fraction (as % of total N)	N (% of DM of fraction)
Aqueous phase	30.2	7.7	1.35
Chloroform phase	14.4	3.5	1.27
PAW extract	46.3	84.1	9.56
Bag contents	29.3	76.5	13.70
Diffusate	17.0	7.6	2.40
Ethanol wash	2.3	1.0	2.25
Residue	6.8	3.7	2.88
Whole shoots	(100)	(100)	5.26

Table 2. Free and bound phenolic acids of 'aqueous phase' fraction from lucerne shoots

Phenolic acids	Free phenolic acids (W1)*	Alkali-labile bound phenolic acids (W2)†	Acid-labile bound phenolic acids (W3)‡	Acid-labile bound phenolic acids (W4)‡	Acid-labile bound phenolic acids (W5)‡	Acid-labile bound phenolic acids (W6)‡	Total acid-labile bound phenolic acids (W3 + W4 + W5 + W6)
Salicylic acid	nd	225.1	58.0	nd	nd	nd	58.0
p-Hydroxybenzoic acid	3.2	129.0	54.9	28.0	6.4	2.9	92.3
Vanillic acid	0.6	19.7	13.6	3.0	nd	0.2	16.8
cis-*p*-Coumaric acid	4.6	147.8	2.0	1.8	3.2	0.7	7.6
trans-*p*-Coumaric acid	9.0	419.8	2.1	2.8	1.4	0.7	7.1
Total *p*-coumaric acids	13.6	567.6	4.1	4.6	4.6	1.4	14.7
cis-Ferulic acid	1.7	166.5	0.6	nd	0.6	0.2	1.4
trans-Ferulic acid	5.2	856.0	1.3	1.8	0.8	0.3	4.2
Total ferulic acids	6.9	1022.5	1.9	1.8	1.4	0.5	5.6
cis + *trans*-Sinapic acids	nd	302.5	nd	nd	nd	nd	nd

The results are expressed in μg/g dry wt lucerne shoots; for preparation of ether extracts W1–W6, see Supplementary Publication.

*Figures corrected for losses occurring during extraction, GLC, etc. (tested with labelled compounds).

†Figures corrected for losses occurring during extraction, alkali hydrolysis, G.C, etc. (tested with labelled compounds).

‡W3, W4, W5 acid hydrolysis with trifluoroacetic acid; W6, acid hydrolysis with HCl. The figures have only been corrected for losses during extraction and GLC. Important losses occur during acid hydrolysis (impossible to correct [4]).

nd = Not detected.

Table 3. Free and bound phenolic acids of 'chloroform phase' fraction from lucerne shoots

Phenolic acids	Free phenolic acids (C1)*	Alkali-labile bound phenolic acids (C2)†	Alkali-labile bound phenolic acids (C3)†
Salicylic acid	nd	nd	nd
p-hydroxybenzoic acid	0.8	0.5	nd
Vanillic acid	0.3	0.6	nd
cis-*p*-Coumaric acid	nd	0.4	3.2
trans-*p*-Coumaric acid	1.4	1.8	5.4
Total *p*-coumaric acids	1.4	2.2	8.6
cis-Ferulic acid	0.5	1.1	2.8
trans-Ferulic acid	1.2	3.2	8.0
Total ferulic acids	1.7	4.3	10.8
cis- + *trans*-Sinapic acids	nd	0.6	nd

The results are expressed in μg/g dry weight lucerne shoots; for preparation of ether extracts C1–C3, see Supplementary Publication.

*Figures corrected for losses occurring during extraction, GLC, etc. (tested with labelled compounds).

†Figures corrected for losses occurring during extraction, alkali hydrolysis, GLC etc.

nd = Not detected.

Table 4. Free and bound phenolic acids of 'diffusate' fraction from lucerne shoots

Phenolic acids	Free phenolic acids (D1)*	Alkali-labile bound phenolic acids (D2)†	Acid-labile bound phenolic acids (D3)‡	Acid-labile bound phenolic acids (D4)‡	Acid-labile bound phenolic acids (D5)‡	Acid-labile bound phenolic acids (D6)‡	Total acid-labile bound phenolic acids (D3+D4+D5+D6)
Salicylic acid	nd	nd	nd	nd	nd	nd	nd
p-Hydroxybenzoic acid	9.3	22.5	158.9	59.5	10.9	6.4	235.6
Vanillic acid	nd	142.1	28.8	3.7	0.6	0.4	33.5
cis-*p*-Coumaric acid	nd	343.6	nd	nd	nd	nd	nd
trans-*p*-Coumaric acid	5.1	865.7	14.2	4.1	nd	0.7	19.0
Total *p*-coumaric acids	5.1	1209.3	14.2	4.1	nd	0.7	19.0
cis-Ferulic acid	2.0	356.7	3.9	nd	nd	nd	3.9
trans-Ferulic acid	6.7	1370.3	20.4	3.5	1.1	0.2	25.2
Total ferulic acids	8.7	1727.0	24.3	3.5	1.1	0.2	29.1
cis- + *trans*- Sinapic acids	nd	nd	nd	nd	nd	nd	nd

The results are expressed in μg/g dry weight lucerne shoots; for preparation of ether extracts D1–D6, see Supplementary Publication.

*Figures corrected for losses during extraction, GLC, etc. (tested with labelled compounds).

†Figures corrected for losses during extraction, alkali hydrolysis, GLC, etc. (tested with labelled compounds).

‡D3, D4, D5, acid hydrolysis with trifluoroacetic acid; D6, hydrolysis with HCl. The figures have only been corrected for losses during extraction and GLC. Important losses occur during acid hydrolysis (impossible to correct [4]).

nd = Not detected.

Table 5. Free and bound phenolic acids of 'bag contents' fraction from lucerne shoots

Phenolic acids	Free phenolic acids (P1)*	Alkali-labile bound phenolic acids (P2)†	Acid-labile bound phenolic acids (P3)‡	Acid-alible bound phenolic acids (P4)‡	Total acid-labile bound phenolic acids (P3+P4)
Salicylic acid	nd	nd	nd	nd	nd
p-Hydroxybenzoic acid	1.7	9.0	12.6	1.5	14.1
Vanillic acid	0.6	4.9	3.9	nd	3.9
cis-*p*-Coumaric acid	nd	nd	nd	nd	nd
trans-*p*-Coumaric acid	2.0	12.2	nd	nd	nd
Total *p*-coumaric acids	2.0	12.2	nd	nd	nd
cis-Ferulic acid	nd	5.5	nd	nd	nd
trans-Ferulic acid	0.4	17.7	nd	nd	nd
Total ferulic acids	0.4	23.2	nd	nd	nd
cis- + *trans*-Sinapic acids	nd	nd	nd	nd	nd

The results are expressed in μg/g dry weight lucerne shoots; for preparation of ether extracts P1–P6, see Supplementary Publication.

*Figures corrected for losses during extraction, GLC, etc. (tested with labelled compounds).

†Figures corrected for losses during extraction, alkali hydrolysis, etc. (tested with labelled compounds).

‡P3, P4, acid hydrolysis with trifluoroacetic acid. The figures have only been corrected from losses during extraction and GLC. Important losses occur during acid hydrolysis (impossible to correct [4]).

nd = Not detected; note that P5 (CF_3COOH hydrolysis) and P6 (HCl hydrolysis) did not contain any detectable amounts of phenolic acids.

acids of 'aqueous phase', 'chloroform phase', 'diffusate' and 'bag contents' fractions respectively (see Experimental). All these results are expressed in terms of the dry matter of the original lucerne shoots subjected to fractionation.

Table 6 shows R_f values found for the various phenolic acids in a number of TLC systems (see Experimental). Table 7 shows wavelengths of their exitation and fluorescence maxima, used in obtaining the quantitative results reported in Tables 2–5 (see Experimental). It should be noted that salicylic and sinapic acids were found only in the 'aqueous phase' and 'chloroform phase' fractions, and there only in a bound state. Many other common phenolic compounds, which would have been manifested by the GLC runs, were apparently absent (cf. [5]).

DISCUSSION

Free phenolic acids

These were found in all the fractions. In 'aqueous phase' and 'chloroform phase' they were presumably genuinely free, since the methanol–chloroform–water (MCW) extraction [6] did not modify leaf pH. Thus, being largely in an ionized state, the phenolic acids distributed themselves mainly into the aqueous phase. In the other fractions, it is impossible to say whether they were retained by sorption of the unextracted residue or liberated by partial hydrolysis of covalently-combined forms which, in all fractions, were more abundant than the free phenolic acids. The substituted cinnamic acids were found in both *cis* and

Table 6. Values (× 100) of phenolic acids in four solvent systems

Phenolic acid	toluene–HCO_2Et–HCO_2H (5:4:1) (steamed)†	toluene–HOAc (9:1) (steamed)‡	C_6H_6–HOAc (19:1) (steamed)‡	2% HOAc* (unsteamed)
Salicylic acid	63	40	36	91
p-Hydroxybenzoic acid	46	14	14	80
Vanillic acid	51	31	36	84
cis-*p*-Coumaric acid	51	19	19	—
trans-*p*-Coumaric acid	51	19	19	48
cis-Ferulic acid	54	32	39	70
trans-Ferulic acid	54	32	39	37
cis-Sinapic acid	49	10	31	64
trans-Sinapic acid	49	10	31	30

**cis-* and *trans*-phenolic acids are separated in 2% HOAc.

Steaming: Sometimes the internal water phase of the plates was increased by keeping them, after spotting but before irrigation, in an atmosphere saturated with water for 15 min (†) [8] or by keeping the plates in water vapour escaping from a tea kettle. The procedure should be repeated twice, but plates should not be completely wet (‡) [20, 21].

trans forms, but the *trans* forms in every case predominated and it is reasonable to suppose that, if light had been excluded during the fractionations and analyses, only the *trans* forms would have been present (Van Sumere, C. F., unpublished work; [7]).

Covalently-bound phenolic acids

These were also found in all fractions, and in considerably greater amounts than the free acids. Since the greater part of them could be liberated by alkali treatment, it is reasonable to suppose that they occur in ester form, although some hydrolysis of amide linkage could also have occurred (Vande Casteele, K. and Van Sumere, C. F., unpublished work). The amounts liberated by the later acid hydrolyses were smaller and were presumably not from ester but from amide or some other type of linkage. It is to be noted that the amounts of substituted cinnamics liberated are diminished, owing to decomposition under acid conditions [8, 9]. Such decomposition is much less with the benzoic acids.

We have not, in general, attempted to identify the chemical compounds from which the phenolic acids were liberated. More bound phenolic acid was present in the 'diffusate' fraction than in the 'aqueous phase'. It is possible that several of the compounds were partially retained on the insoluble residue during the MCW extraction and released into the 'diffusate' at the dialysis step. On the other hand, salicylic and sinapic acid derivatives were found almost entirely in the 'aqueous phase'. These might have included sinapine and perhaps other choline esters held in the aqueous phase by their quaternary ammonium groups ([10–14]; Fenwick, R. G., personal communication). Moreover, the bound *p*-hydroxybenzoic acid was predominantly alkali-labile in 'aqueous phase' and predominantly acid-labile in 'diffusate'. These observations seem to prove that at least some of the compounds were cleanly partitioned into the different fractions.

Only small amounts of bound phenolic acids were found in 'chloroform phase', which indicates that the leaf lipids are largely free from this class of compound.

If present, chlorogenic acid would have been in 'aqueous phase' [2], but none was found, in agreement with Monties and Rambourg [1]. Nor was any caffeic acid, a possible decomposition product, detected [5].

In an accompanying paper [4], Van Sumere and colleagues, after partial hydrolysis of a somewhat differently prepared protein-rich lucerne fraction, identified *N*-feruloylglycylphenylalanine. It is not clear whether the parent protein or peptide would have been in the 'bag contents' or 'diffusate' fraction in the present scheme.

Hydrolysis procedures

The alkaline and acid hydrolysis procedures used were shown by control experiments to liberate the benzoic acid derivatives in good yields. However, poor yields of substituted cinnamic acids seem inevitable if acid hydrolysis is used, and it might be preferable to substitute repetitive alkaline hydrolyses. Even these, however, would probably not break phenolic glycoside bonds. For liberating substituted cinnamic acids from such compounds, treatment with a glycosidase would

Table 7. Excitation and fluorescence wavelength maxima of phenolic acids

Phenolic acids	Excitation maximum (nm)	Fluorescence maximum (nm)
Vanillic acid	304	354
cis- and *trans*-*p*-coumaric acids	344	444
cis- and *trans*-ferulic acids	358	464
cis- and *trans*-sinapic acids	364	478

be useful [8]. Once the phenolic acids had been obtained in the free state, analytical recovery was good (always within the range 70–100%), as judged by initially adding isotopically labelled compounds. Correction factors have been based on these recoveries and incorporated into the tables (see Experimental).

Chromatographic procedures

For quantitative determination of the simple phenolic acids, it was particularly important to follow the GLC step with hydrolysis of the trimethylsilyl (TMSi) derivatives and subsequent TLC, because the GLC cuts, as collected, often still contained several extraneous compounds.

Initial fractionation procedures

The use of phenol–acetic acid–water (PAW) as an extraction agent and the prolonged dialysis against 70% aqueous acetic acid subjected the 'diffusate' and 'bag contents' fractions to acidic conditions. These may well have caused hydrolytic cleavage of benzoic and (especially) cinnamic residues, with isomerization and destruction of the latter [8, 9]. A more rapid technique for obtaining a high-molecular fraction from a PAW extract by gel-permeation chromatography on Sephadex has been described [2], but even this operation took *ca* 2 hr; it might be hastened by using a shallow bed of Sephadex on a Buchner funnel. There is obvious scope for devising new non-acidic chaotropic solvent mixtures other than PAW for promoting the extraction of phenolics from plant materials with minimal damage to primary-valence bonds, and for avoiding subsequent chemical modification of any liberated phenolics.

The approach of Monties and Rambourg [1] to breaking hydrogen bonds between phenolics and protein by acetylating phenolic and other functional groups in both interacting categories, has notable merit, but it is a pity that they used such an acidic reagent mixture as acetyl bromide in acetic acid. Acetic anhydride–pyridine mixtures might well prove more useful.

For preparation of protein free from phenolics bound by secondary valencies, which was a subsidiary objective of the present work, it might be more convenient to use sorption of the phenolics on to such materials as polystyrene [15, 16]. It would be valuable to compare the behaviour of a variety of plant-protein preparations by subjecting them to various sequential operations of these kinds. The present state of the art of separating plant proteins from phenolics bound by secondary valencies is manifestly unsatisfactory [16].

Association of phenolic compounds with lucerne proteins

The lucerne bulk-protein preparation obtained by gel-permeation chromatography by Davies *et al.* [2] had a lower absorption at 350 nm than any of the protein-rich fractions studied in the present paper, namely, $E^{1\%}_{1\,cm} = 3.3$ in 0.1 M NaOH. Were this entirely due to ferulic and *p*-coumaric acids, that fraction would have contained approximately 0.4–1.2% w/w of these [7]. In the present work, we did not recover more than 0.013% w/w of such acids from the corresponding 'bag contents' fraction (Tables 1 and 5). As we cannot estimate our losses under the conditions which we used for preparation and hydrolysis, it could be that much more of these residues were present. On the other hand, much of the absorption at 350 nm could perhaps be due to coumestrol, as suggested by Monties and Rambourg [1]. These authors make no mention of the occurrence of phenolic acids in their preparations, apart from commenting on the absence of caffeic acid and its derivatives.

Nutritional implications

In general, we are in agreement with Monties and Rambourg [1] in not having found any significant quantities of *o*-diphenols in our respective fractions. It therefore seems unlikely that attack by quinonoids on nutritionally important amino acid residues (e.g. lysine residues in tobacco leaf protien [2]) is important in impairing the nutritive quality of lucerne leaf-protein concentrates. Reaction with the considerable quantities of linolenic acid residues present or thermal cross-linking, during ill-controlled drying and storage, seem more likely explanations for such impairments [17].

EXPERIMENTAL

Plant material. This was the same batch of lucerne shoots (*Medicago sativa* L. cv Europe) as previously studied [2].

Initial fractionation of lucerne shoots. This followed a previous procedure [18] to give 'aqueous phase', 'chloroform phase', 'PAW extract', 'ethanol wash' and 'residue', with an additional step to subdivide 'PAW extract' into 'diffusate' and 'bag contents' [2].

Analyses on initial fractions. These fractions were worked up for free and bound substituted benzoic and substituted cinnamic acids according to the detailed schemes shown in the Supplementary Publication. In brief, the following procedures were employed.

The aqueous phase (1750 ml) was concd to 1 l., acidified with 2 M HCl and extracted with Et_2O (1 l. × 6) (this Et_2O extract (fraction W1) contains the free-substituted benzoic and cinnamic acids). After concn and addition of EtOH to 10 ml, aliquots of the concentrate (see Supplementary Publication, p. 4) were treated with H_2O and then evapd to *ca* 1 ml. This soln was subsequently treated with 3 ml 6% (w/v) aq. Na_2CO_3 and then again extracted with Et_2O. The Et_2O layer was discarded and the carbonate layer was acidified with 6M HCl until pH 3.5. Thereafter the phenolic acids were again extracted with several portions of 5 ml Et_2O. The Et_2O extracts were combined (max vol. 25 ml) and the phenolic acids present were converted into TMSi derivatives. These derivatives were then separated by GLC, collected and further quantitatively analysed by TLC in combination with fluorimetry of spectrophotometry (see [5], [8] and the Supplementary Publication). After removal of the free phenolic acids the remaining aq. layer was successively hydrolysed (see below) with 2 M NaOH (the hydrolysate resulted, after neutralization (pH. 3.5) and Et_2O extraction, in fraction W2), trifluoroacetic acid (1M) (3 times repeated; resulting respectively in fractions W3, W4, W5) and HCl (2 M) (fraction W6). After each hydrolysis the liberated alkali-labile bound or acid-labile bound phenolic acids were extracted and analysed as described for the free phenolic acids.

The chloroform phase (980 ml) was concd to a small vol. Thereafter 150 ml H_2O and 200 ml 6% (w/v) aq. Na_2CO_3 were added. The alkaline soln was then twice extracted with

250 ml *n*-BuOH and 250 ml Et_2O. The organic layers were combined and treated with 40 ml 6% (w/v) aq. Na_2CO_3. The two carbonate layers were pooled, acidified with 6 M HCl until pH 3.5 and then extracted with Et_2O (×6). This Et_2O extract was as before analysed for free phenolic acids (fraction C1). The remaining aq. phase was hydrolysed with 2 M NaOH as described below and the liberated phenolic acids were analysed as indicated above (fraction C2). The *n*-BuOH–Et_2O layer was concd to a small vol. After addition of H_2O (total vol. 230 ml) and 8 M NaOH until 2 M, alkaline hydrolysis was performed as further described. The hydrolysate was subsequently twice extracted with *n*-BuOH and Et_2O and the organic layer, containing chlorophyll, was again treated with 40 ml 2 M NaOH. The two alkaline layers were combined, acidified with 6 M HCl until pH 3.5 and extracted with Et_2O (400 ml×6). The Et_2O extract (fraction C3) containing the liberated alkali-labile bound phenolic acids was analysed as before (see free phenolic acids). The acid aq. layer was discarded.

The diffusate fraction, diluted with H_2O to 200 ml, was extracted ×6 with 150 ml Et_2O. Both the Et_2O layer and the aq. phase were kept for further analysis. The Et_2O layer was concd, H_2O and solid Na_2CO_3 as well as 6% (w/v) aq. Na_2CO_3 were added until pH 6.6. After Et_2O extraction of the latter solution an Et_2O layer and an aq. layer No. 1 were obtained. The Et_2O layer was concd and 30 ml H_2O and enough 6% Na_2CO_3 to reach pH 6.6 were added This soln was then again extracted with Et_2O (150 ml×6). The Et_2O layer was discarded and both aq. layers Nos. 1 and 2 were combined, acidified with 6 M HCl (pH 3.5), and extracted with Et_2O (150 ml×6). The Et_2O extracts were combined, conc to 10 ml and analysed for free phenolic acids (fraction D1) as described above. The aq. layer, which was obtained after the first Et_2O extraction of the diffusate, was treated with 8 M NaOH until 2 M. After alkaline hydrolysis and acidification with 6 M HCl until pH 3.5 an Et_2O extract (fraction D2) was obtained which contained the alkali-labile bound phenolic acids of the diffusate. The acid aq. layer was further treated with trifluoroacetic acid until 1 M and hydrolysed. After neutralization until pH 3.5 and Et_2O extraction, fraction D3 was obtained. The same procedure was twice more repeated giving rise to fractions D4 and D5. The three last fractions contained the acid-labile bound phenolic compounds which were stable in acid medium. Subsequently also a hydrolysis with 2 M hydrolchloric acid was performed giving rise finally to fraction D6.

The bag contents (protein fraction; 850 ml) were first acidified with 6 M HCl until pH 3.5 and free phenolic acids present were extracted with Et_2O and analysed as described above (fraction P1). The aq. layer was then again (see diffusate) successively hydrolysed with 2 M NaOH and the hydrolysate resulted in fraction P2, which contained the alkali-labile bound phenolic acids. After acidification the remaining aq. layer was twice hydrolysed with trifluoroacetic acid (1 M) resulting in fractions P3 and P4. These fractions contained the acid-stable phenolic acids.

Radioactively-labelled phenolic acids were used to check the extraction schemes and chromatographic recoveries. For the free benzoic acids, the overall correction factor applied was 1.43 and for the free cinnamic acids 1.37. For the alkali-labile bound phenolic acids the overall correction factor was 1.35 and for the acid-labile bound phenolic acids 1.28. However, this last figure refers only to the substituted benzoic acids; destruction of the substituted cinnamic acids was too great to permit the use of any meaningful correction factor and the figures recorded are minimal (see Discussion).

Nitrogen. This was determined by a micro-Kjeldahl procedure [19].

Hydrolysis. (a) *Alkaline hydrolysis.* The extracts to be hydrolysed were treated with 8 M NaOH until 2 M. Thereafter hydrolysis was performed, under reflux and N_2, for 2 hr. Subsequently, the hydrolysate was acidified with 6 M HCl to pH 3.5 and the liberated phenolic acids were extracted with Et_2O (1 vol. ×6). (b) *Acid hydrolysis.* This hydrolysis was performed with trifluoroacetic acid, the concentrated acid being added to the extracts until 1 M. After refluxing for 3 hr under N_2, the acid medium was neutralized with conc Na_2CO_3 to pH 3.5 and the liberated phenolic acids were extracted with Et_2O (1 vol. ×6). In a second and comparative experiment hydrolysis was performed during 30 min with 2 M HCl. It should further be noted that acids such as *p*-hydroxybenzoic acid are quite stable in boiling acid but that hydroxycinnamic acids are rapidly destroyed in the same medium [8, 9].

Gas-chromatographic separation of the phenolic compounds present in the ether extracts. (a) *Preparation of the volatile trimethylsilyl (TMSi) derivatives of the phenolic compounds.* An aliquot of the above Et_2O extracts was first concd *in vacuo*. After addition of 1 ml H_2O and 2–3 ml 6% w/v Na_2CO_3 (pH 9) and 3–4 ml Et_2O, the carbonate fraction containing the salts of the phenolic acids, was separated from the Et_2O layer (discarded). After acidification of the alkaline aq. layer with 6 M HCl to pH 3.5, the phenolic acids were again repeatedly extracted with roughly equal volumes of Et_2O (6 times) and the Et_2O fractions were pooled (acid aq. layer discarded). The final Et_2O layer was then dried, concd *in vacuo* and quantitatively transferred into a tared bottle. After concn in N_2 and drying over P_2O_5 and NaOH, the dry wt of the extracted material was determined and the TMSi derivatives were prepared in sealed vials [5]. (b) *Gas chromatography.* After cooling the vials, 7–40 μl portions (amounts injected depending on the amount of dry wt and number of phenolics present in the Et_2O extracts) of the reaction mixture were injected in a Hewlett-Packard 5730A instrument. This instrument was equipped with Pyrex glass columns (3.0 m×2 mm ID) and a thermal-conductivity detector (temp. 350°). The glass columns were packed with Chromosorb W AW/DMCS (80–100 mesh) coated with 1.5% SE-30 plus 1.5% SE-52 (Varian Aerograph). The temp. of the injection port was 300°. The carrier gas was He, with flow rate 30 ml/min. The column temps. were programmed from 100 to 300°, with temp increases of 4° min. The collector temp. was further 250° and the recorder (0–1.0 mV) chart speed 1 cm/min, with the attenuator in position 1. Before the analysis of plant extract was performed, the separation of a known standard mixture of *ca* 30 different phenolic acids and related compounds was recorded. The same chart as well as the same start position were subsequently used for the recording of the peaks originating from the plant extracts. This procedure, which allowed the direct comparison of the retention times of the known and unknown, proved to be very helpful in the preliminary GLC identification of unknown compounds. The same procedure allowed also to make the proper selection for test substances which, after hydrolysis of the collected TMSi derivatives, had to be used during the TLC analysis. (c) *Collection and hydrolysis of the separated TMSi derivatives.* These were, as far as possible, collected as a single peak and then hydrolysed [5]. When the concn of a phenolic acid proved to be low in an extract, up to 10 collections of the same peak were made in the same micro-collector glass tube.

Quantitative TLC and determination of the phenolic

acids. The collected and hydrolysed TMSi fractions were further analysed by TLC on mixed layers consisting of Si gel G (Merck, Darmstadt, W. Germany) and cellulose MN (Macherey & Nagel, Düren, W. Germany) [8, 20, 21]. For this purpose, a series of adequate solvent systems (see Table 6) was employed.

When known amounts of reference substances were applied next to the unknowns, quantitative results could be obtained by combining TLC with fluorimetry (this was the case for vanillic, *p*-coumaric, ferulic and sinapic acids—see Table 7) or spectrophotometry (salicylic and *p*-hydroxybenzoic acids). The λ_{max} values of these last compounds in 0.1 M NaOH were respectively 296 and 280 nm. In all cases the unknown and known spots were removed from the plate and eluted with 4 ml 0.1 M NaOH. Final analysis was then performed with one of the above-mentioned spectroscopic methods [8].

Supplementary Publication Scheme—Information which supplements this article has been deposited with the National Lending Library, Boston Spa, Yorkshire LS23 7BQ, England. This supplementary information is available as microfiche or as enlargements from the Library's photocopying services.

Acknowledgements—The authors in Belgium thank the European Economic Commission and the Instituut tot Aanmoediging van bet Wetenschappelijk Onderzoek in Nijverheid en Landbouw (I.W.O.N.L.) for financial support. We all thank Drs B. Monties, J. C. Rambourg and W. D. Loomis for useful discussions and for showing us manuscripts before publication. We also thank Dr R. G. Fenwick for giving us several useful references.

REFERENCES

1. Monties, B. and Rambourg, J. C. (1978) *Ann. Technol. Agric.* **27**, 629.
2. Davies, A. M. C., Newby, V. K. and Synge, R. L. M. (1978) *J. Sci. Food Agric.* **29**, 33.
3. Herrmann, K. (1978) *Fortschr. Chem. Org. Naturstoffe*, **35**, 73.
4. Van Sumere, C. F., Houpeline-De Cock, H. J., Vindevaghel-De Bacquer, Y. C. and Fockenier, G. (1980) *Phytochemistry* **19**, 704.
5. Vande Casteele, K., De Pooter, H. and Van Sumere, C. F. (1976) *J. Chromatogr.* **121**, 49.
6. Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911.
7. Aulin-Erdtman, G. and Sandén, R. (1968) *Acta Chem. Scand.* **22**, 1187.
8. Van Sumere, C. F., Cottenie, J., De Greef, J. and Kint, J. (1972) *Recent Advances in Phytochemistry* (Runeckles, V. C. and Watkins, J. E., eds.), p. 165. Appleton-Century-Crofts, New York.
9. Van Brussel, W., Tavernier, D. and Van Sumere, C. F. (1978) *Bull. Soc. Chim. Belg.* **87**, 403.
10. Pagani, F. and Romussi, G. (1967) *Farmaco, Ed. Parat.* **22**, 553
11. Pagani, F. and Romussi, G. (1969) *Farmaco, Ed. Sci.* **24**, 257.
12. Pagani, F. and Romussi, G. (1970) *Farmaco, Ed. Sci.* **25**, 727.
13. Gmelin, R. and Kjaer, A. (1970) *Phytochemistry* **9**, 667.
14. Psarréa-Sandris, A. (1974) *Ann. Pharm. Fr.* **32**, 541.
15. Loomis, W. D., Lile, J. D. Sandstrom, R. P. and Burbott, A. J. (1979) *Phytochemistry* **18**, 1049.
16. Rhodes, M. J. C. (1977) *Regulation and Enzyme Synthesis and Activity in Higher Plants* (Smith, H., ed.) p 245. Academic Press, London.
17. Synge, R. L. M. (1976) *Qual. Plant.* **25**, 9.
18. Laird, W. M., Mbadiwe, E. I. and Synge, R. L. M. (1976) *J. Sci. Food Agric.* **27**, 127.
19. Jennings, A. C. and Watt, W. B. (1967) *J. Sci. Food Agric.* **18**, 527.
20. Van Sumere, C. F., Wolf, G., Teuchy, H. and Kint, J. (1964) *Arch. Int. Physiol. Biochim.* **72**, 709.
21. Van Sumere, C. F., Wolf, G., Teuchy, H. and Kint, J. (1965) *J. Chromatogr.* **20**, 48.