Phytochemistry, 1973, Vol. 12, pp. 1141 to 1147. Pergamon Press. Printed in England.

METHODS FOR THE ISOLATION AND PURIFICATION OF ETHANOL-INSOLUBLE, PHENOLIC ESTERS IN *MENTHA ARVENSIS*

W. MAJAK* and G. H. NEIL TOWERS

Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada

(*Received* 4 *October* 1972. *Accepted* 17 *November* 1972)

Key Word Index—*Mentha arvensis*; Labiatae; ethanol-insoluble phenolic esters; purification methods; anthocyanins.

Abstract—Previous kinetic, isotopic studies have suggested that 'insoluble' phenolic esters may be precursors of lignin. Heretofore, the 'insoluble' esters have been detected by the chromatographic examinations of gross hydrolysis products of ethanol-insoluble resides and/or acetone powders. We have developed new methods for the isolation and purification of certain of the ethanol-insoluble, phenolic esters of *Mentha arvensis*. 'Insoluble' conjugates of caffeic, ferulic and *p*-coumaric acids were purified and were shown to be electrophoretically and chromatographically homogeneous. These compounds were distinguished on the basis of their anionic mobility at pH 1·9. A second pool of caffeic acid was associated with a high MW fraction. Two acylated anthocyanins containing *p*-coumaric acid and caffeic acid were also obtained from acetone powders.

INTRODUCTION

THE UBIQUITY of phenolic acids in higher plants has been established in the last decade[1–3] and they have been shown to be directly involved in the biosynthesis of coumarins, flavonoids and lignins.[4,5] Investigations have centered on the hydroxylated and methoxylated cinnamic acids which occur, in combined form as glycosides or esters in practically every higher plant.[1] Caffeic acid esters such as chlorogenic acids[6] and/or rosmarinic acids[7] are often the major phenolic esters encountered in ethanolic extracts of plants. An aqueous ethanolic extract of fresh plant material is the classical starting point for the isolation of such low MW, phenolic acid derivatives. However, phenolic acids can also be liberated by hydrolysis of ethanol-insoluble residues[8–10] or autolysis of acetone powders.[11] On the basis of kinetic studies, it has been suggested that these 'insoluble' esters may be natural intermediates in the biosynthesis of lignins[12] which remains a major, unresolved problem in plant biochemistry.

* Present address: Canada Department of Agriculture, Research Station, Kamloops, British Columbia, Canada.

[1] BATE-SMITH, E. C. (1962) *J. Linn. Soc. (Bot.)* **58,** 95.
[2] IBRAHIM, R. K., TOWERS, G. H. N. and GIBBS, R. D. (1962) *J. Linn. Soc. (Bot.)* **58,** 223.
[3] TOMASZEWSKI, M. (1960) *Bull. Acad. Polon. Sci.* **8,** 61.
[4] HARBORNE, J. B. (1964) *Biochemistry of Phenolic Compounds*, Academic Press, New York.
[5] MCCALLA, D. R. and NEISH, A. C. (1959) *Can. J. Biochem. Physiol.* **37,** 531.
[6] SONDHEIMER, E. (1958) *Arch. Biochem. Biophys.* **74,** 131.
[7] HARBORNE, J. B. (1966) J. *Naturforsch.* **21b,** 604.
[8] EL-BASYOUNI, S. Z. and TOWERS, G. H. N. (1964) *Can. J. Biochem.* **42,** 203.
[9] GLASS, A. D. M. and BOHM, B. A. (1969) *Phytochemistry* **8,** 371.
[10] FUCHS, A., ROHRINGER, R. and SAMBORSKI, D. J. (1967) *Can. J. Botany* **45,** 2137.
[11] EL-BASYOUNI, S. Z. and NEISH, A. C. (1966) *Phytochemistry* **5,** 683.
[12] EL-BASYOUNI, S. Z., NEISH, A. C. and TOWERS, G. H. N. (1964) *Phytochemistry* **3,** 627.

Heretofore, the study of insoluble esters has been confined to chromatographic examinations of gross hydrolysis products derived from crude ethanol-insoluble residues and acetone powders. The purpose of this study was to examine the ethanol-insoluble fraction of *Mentha arvensis* in some detail and to develop practical and reliable methods for resolving the various pools of phenolic acids. Three 'insoluble' pools of caffeic acid were resolved: a caffeoyl conjugate exhibiting anionic mobility at pH 1·9, a high MW conjugate(s) and a caffeic acid ester of a cyanidin glycoside. Anionic conjugates of *p*-coumaric acid and ferulic acid were also purified. A *p*-coumaric acid ester of a cyanidin glucoside was found to be the major anthocyanin in non-flowering shoots of *M. arvensis*.

RESULTS

The Derivation of Major Phenolic Acid Pools in M. arvensis

Two parallel schemes were developed for the isolation of 'insoluble', phenolic conjugates (Fig. 1). In these flow diagrams the acetone- and ethanol-soluble fractions represent free phenolic acids, glycosides and esters such as rosmarinic acid and chlorogenic acid (Fractions IA and IIA). Aqueous extracts of the acetone- or ethanol-insoluble residues contain the acylated anthocyanins and a pool of phenolic conjugates (compounds I, II and III) which exhibit anionic mobility at pH 1·9 (Fractions IB and IIB). The PAW-soluble fraction yields a pool of caffeic acid associated with a high molecular fraction (Fractions IC and IIC). The amount of phenolic acids that can be liberated from Acetone Powder IC and Residue IIC is negligible. These are designated as the lignin-cell wall fractions. They yield *p*-hydroxybenzaldehyde, vanillin and syringaldehyde on alkaline nitrobenzene oxidation.[13]

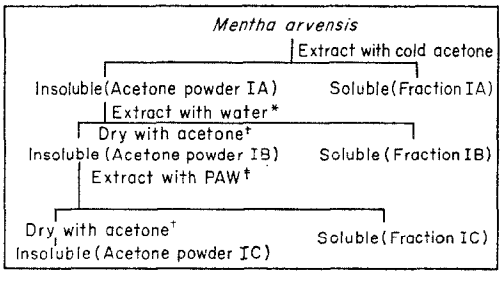

FIG. 1(a). THE DERIVATION OF MAJOR PHENOLIC ACID POOLS BY METHOD I.

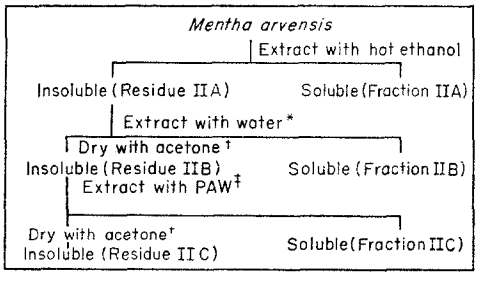

FIG. 1(b). THE DERIVATION OF MAJOR PHENOLIC ACID POOLS BY METHOD II.

* Water containing 0·1% acetic acid and 0·1% mercaptoethanol. † The extracted acetone powder is combined with acetone, pulverized in a Waring blender and collected on a Buchner funnel. ‡ Phenol–acetic acid–water (1:1:1).

Although acetone powders (Method I) were used as the starting point for large-scale isolations, the alternate scheme (Method II) was pursued to discover whether Fractions IIB and IIC respectively contained the same phenolic conjugates as Fraction IB and IC.

Spectroscopic, Electrophoretic and Chromatographic Properties of Compounds I, II and III

These conjugates are characterized by their fluorescence, anionic mobility at pH 2 and insolubility in apolar solvents. Their wavelength of maximum absorbance, bathochromic shifts and color under UV light are listed in Table 1. Compound I, occurring as 0·005% of the fresh weight, was the major constituent and at least 10 times more concentrated than

[13] STONE J. E. and BLUNDELL, M. J. (1951) *Anal. Chem.* **23**, 771.

compound II or III. Compounds I, II and III respectively yielded caffeic acid, ferulic acid and *para*-coumaric acid after alkaline hydrolyses.

TABLE 1. UV SPECTRA AND FLUORESCENT COLORS OF ISOLATED CONJUGATED COMPOUNDS

Compound	λ_{max} (nm) Water	Bathochromic shift (nm) +NaOH	+H_3BO_3/ NaOAc	Colors UV	UV/NH_3
I	325	59	19	Sky-blue	Blue-white
II	322	49	0	Blue	Blue-green
III	312	46	0	Absorbs	Dark-blue

The fluorescence under UV light and the bathochromic shifts in the UV spectra (Table 1) of compounds I, II and III indicated that caffeic acid (in compound I), ferulic acid (in compound II) and *p*-coumaric acid (in compound III) were linked via their respective carboxyl groups. This was confirmed by the absence of a hypsochromic shift in the presence of sodium acetate.

Compounds I, II and III migrated as discrete bands toward the anode under the following conditions of high voltage paper electrophoresis (HVPE): pH 1·9 (acetate–formate buffer); 3000 V; initial current of 100 mA; 40 min; initial temperature 16° increasing to 21°. Each compound migrated 3·2 cm as compared to zero for umbelliferone and caffeic acid and 10·8 cm for cysteic acid.

TABLE 2. CHROMATOGRAPHIC PROPERTIES OF ISOLATED CONJUGATES

Compound	BAW	BzAWE	$R_f \times 100$ Solvents* Tol	$HCOOH^{+++}$	Pyr
Caffeic acid	91	60	86	20^{++}	58
No. I	73 74†	17 39+	26 5+	68	4
No. II	80 75†	34 58+	34 10+	68	4
No. III	80 77†	34 58+	39 22+	68	4

* *Key to solvents.* BAW, butanol–acetic acid–water (4:1:5, upper phase); BzAW, benzene–acetic acid–water (10:7:3, upper phase); BzAWE, BzAW–water–ethanol (50:5:20, monophasic system); Tol, toluene–butanol–pyridine (containing 5% acetic acid)–water (1:4:2:1); HCO_2H, 2% formic acid; Pyr, butanol–pyridine–benzene–water (5:3:1:3). AnAW, anisole–acetic acid–water (70:30:2); BBz, butanol–benzene–formic acid–water (100:19:10:25). R_f's were determined on Whatman No. 3 MM chromatography paper unless otherwise indicated.

† On MN Cellulose 300G.

+ On Avicel.

++ Refers to *trans*-caffeic acid. R_f *cis*-caffeic acid = 55.

+++ $R_f \times 100$ chlorogenic acid = 58. $R_f \times 100$ rosmarinic acid = 34.

The purified compounds migrated as single spots on paper chromatograms and on cellulose TLC plates in a variety of solvent systems (Table 2). Compound I was found to be strongly adsorbed in a variety of gel filtration systems including Sephadex G25, G10 and Bio-gel P-2 equilibrated with various solvents such as 7 M urea:0·1% acetic acid, PAW, and 0·1 M pH 7 phosphate buffer. The departure from true gel filtration was manifested by the extent of zone broadening in these systems which resulted in a 50- to 100-fold dilution of the sample size as compared to a five fold dilution of the ion exclusion standards (Blue Dextran and horse heart cytochrome-*c*).

When compound I was applied to a polyamide (Woelm) column equilibrated in water, it was strongly adsorbed and could not be eluted with water, aqueous ethanol or acetone. High concentrations of salt, however, could effect the elution. The compound was completely recovered from polyamide by eluting with acetone–acetic acid–water (1:1:1) and freeze-drying. The IR spectrum of compound I (1–2 mg/100 mg KBr) revealed the following bands (tentatively assigned): *b*, 3000–3500 (OH stretching); *m*, 1725 (ester, C=O stretching); *s*, 1610, *w*, 1530 and sharp, 1490 (aromatic C=C in-plane vibrations); *s*, 1390; *m*, 1270 (ester, C–O stretching); *m*, 1160; *w*, 1120, 1060, 980, 810, 750 and 680 cm^{-1}.

Hydrolyses of Compounds I, II and III

Equal quantities of compound I were subjected to partial acid hydrolyses (90°, 2 N HCl) for 10, 20, 40 and 60 min, and each hydrolyzate was freeze-dried and subjected to HVPE at pH 1·9. The developed electrophoretogram revealed the gradual appearance of a neutral, fluorescent band and the concomitant disappearance of compound I which is anionic. The fluorescent neutral band was excised and stitched to a strip of chromatography paper and by co-chromatography with authentic standards in five solvent systems was identified as caffeic acid.

Alkaline hydrolysis was carried out in the presence of excess $NaBH_4$ which stabilizes caffeyl compounds in alkali.[14] The hydrolyzate was resolved on DEAE-cellulose (see Experimental) and the concentration of caffeic acid was determined spectrophotometrically. Caffeic acid was identified by the UV spectral shifts with sodium acetate[15] (hypsochromic) and sodium acetate–H_3BO_3[16] (bathochromic) as well as by co-chromatography with authentic caffeic acid in five solvent systems. The yield of caffeic acid after mild alkaline hydrolysis was 22% (±2%) based on the average of four spectrophotometric determinations. Interference by esculetin, (the coumarin derived by the light-activated cyclization of *cis*-caffeic acid[17]) was negligible as judged by its absorbance at 380 nm.

Compound I was subjected to alkaline hydrolysis (N NaOH, 25°, 6 hr) in the absence of $NaBH_4$, and the Na^+ ions were removed on CM cellulose (H^+). The gel was eluted with water and the freeze-dried eluate was subjected to HVPE (3kV) at pH 1·9 for 60 min. The developed electrophoretogram revealed 3 bands: a minor, fluorescent, anionic band comprised of unhydrolyzed compound I, a neutral fluorescent band identified as caffeic acid, and an anionic, periodate-positive (yellow) band, the *R* band, situated slightly ahead but overlapping compound I. The *R* band was purified by excising this anionic region and developing the strip by PC in BAW (Table 2). In BAW, *R* moves with an $R_f = 0{\cdot}17$. Similarly, alkaline hydrolyzates of compounds II and III yielded respectively a periodate-positive band with mobilities and R_fs identical to *R*. Two features of the *R* band should be pointed out: since it exhibits anionic mobility after alkaline hydrolysis, this fragment must contain the anionic, acidic group which imparts the characteristic electrophoretic pattern at pH 1·9. Compounds II and III do not give a positive periodate test, and compound I yields only a weak reaction (brown), therefore, alkaline hydrolysis must result in the production of hydroxyl groups on adjacent carbon atoms or an adjacent hydroxyl-amino system. The purified *R* band was subjected to acid hydrolysis (3 N HCl, 12 hr, 100°) and the freeze-dried

[14] SCHROEDER, H. A. (1967) *Phytochemistry* **6,** 1589.
[15] KRUPNIKOVA, T. A., DRANIK, L. I. and SHKOL'NIK, M. (1968) *Dokl. Akad. Nauk SSSR* **180,** 1497.
[16] JURD, L. (1956) *Arch. Biochem. Biophys.* **63,** 376.
[17] KAGAN, J. (1966) *J. Am. Chem. Soc.* **88,** 2617.

hydrolyzate was submitted to HVPE at pH 1·9. The developed electrophoretogram showed that the *R* band was strongly resistant to hot, acidic conditions. Chromatographic examinations for 6-sulfoquinovose, quinic acid or simple sugars showed that none of these compounds were produced. The electrophoretogram of the acid-treated *R* band, however, did reveal the presence of eight ninhydrin-positive bands in low amounts. Hydrolysis (5·7 N HCl, 24 hr, 110°) of 1–5 mg of compound I followed by amino acid analysis disclosed the presence of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, isoleucine and leucine. The yield of amino acids, however, was extremely low accounting for less than 1% by weight of compound I. Compound I did not give an immediate, positive test with ninhydrin but a violet color appeared 6 months later. This delayed ninhydrin reaction suggests that an amino group(s) of No. I undergoes gradual unmasking. The delayed ninhydrin reaction is completely superimposable with the fluorescent position of No. I.

The electrophoretic behaviour of compound I at pH 1·9 suggested the presence of phosphate, sulfate or sulfonate groups. Elemental analysis gave C, 33·85, 34·10%; H, 5·10, 5·17%; N, 6·45, 6·71%; S, 2·29, 2·44% and P, 2·69%. The sample contained less than 1% ash after combustion.

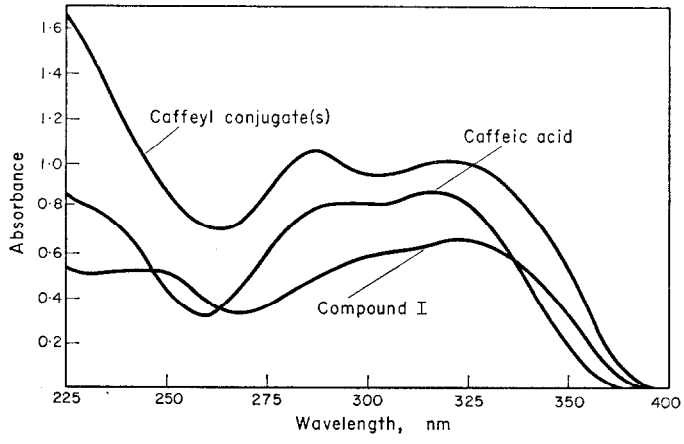

FIG. 2. UV ABSORPTION SPECTRA OF THE HIGH MW CAFFEYL CONJUGATE(S), CAFFEIC ACID AND COMPOUND I IN DISTILLED WATER.

The High MW Caffeyl Conjugate(s)

The caffeyl components from fractions Ic or IIc had a high MW, deduced from chromatography on G100 and dialysis, and lacked fluorescence. These two features distinguish the conjugate(s) of caffeic acid from compound I. The UV adsorption spectra of these compounds along with caffeic acid are shown in Fig. 2. The absence of fluorescence indicates that the caffeyl moiety is substituted on the phenolic hydroxyls. This is supported by the small borate shift (3 nm as compared to 19 nm for compound I). However, since a bathochromic shift (57 nm) was observed in alkali this would suggest that only the *para* position was occupied. The high absorbance at 280 nm may reflect a protein-type association with caffeic acid. The rigorous method of isolation (i.e. chromatography in 7 M urea: 0·1% acetic acid) argues against the possibility of non-covalent bonding between the caffeyl

moiety and the macromolecular complement. The macromolecular fraction containing bound caffeic acid is strongly adsorbed on paper electrophoretograms, DEAE-cellulose (acetate counter ion) and Sephadex G10 and G15. It is stable indefinitely when stored as a freeze-dried powder at 0° and under nitrogen. In neutral solution it is gradually oxidized by air to a brown suspension. Electrophoresis (3 kV, pH 2, 45 min) of the isolated compound(s) revealed a series of anionic bands migrating 0–6 cm. These bands are detected under UV light by their yellow coloration in the presence of ammonia.

The Acylated Anthocyanins

Two acylated anthocyanins were found in the PAW eluate and separated electrophoretically. One contained *p*-coumaric acid and the other caffeic acid. The esterified acids were released by mild alkaline hydrolysis and identified by the procedures already described. Both anthocyanins yielded cyanidin and glucose on acid hydrolysis. Cyanidin was identified by its UV spectrum, bathchromic shift with $AlCl_3$, color and R_fs in four solvent systems. Glucose was identified by its color reaction with *p*-anisidine phosphate and by co-chromatography with authentic standards.

The *p*-coumaroyl anthocyanin exhibits the following absorption maxima: 528, 315 (shoulder), 295 (shoulder) and 277 nm. The absorption maxima of the caffeoyl anthocyanin are as follows: 526, 327, 295 (shoulder) and 277 nm.

EXPERIMENTAL

Plant material. Mentha arvensis L. was grown under greenhouse conditions supplemented with a 16-hr photoperiod. Non-flowering shoots that were at least 3 months old were harvested.

Adsorbents. DEAE-cellulose (Whatman, DE-32, microgranular form) was precycled with 0·5 N NaOH and washed with H_2O until the eluate was neutral; converted to the acetate form batchwise with 10% HOAc, and finally washed with H_2O to neutral pH. For preparative isolations, Whatman No. 3 MM chromatography paper was washed with H_2O for 24 hr. The paper was handled with surgeon's gloves to avoid contamination. Electrophoretograms and chromatograms were cross-stitched with transparent nylon thread. When an excised band was sewn to a strip of chromatography paper it was cross-stitched on both sides, the blank underlay was removed and the flaps were cross-stitched to the band.

PAW reagent. Liquified phenol, U.S.P., redistilled under N_2 was combined with HOAc (1:1) and stored in amber glass blottles at 4°. The PAW reagent[18,19] (phenol–HOAc–H_2O, 1:1:1) was freshly prepared before use.

Spray reagents. The Cd–ninhydrin reagent was prepared according to the method of Heilmann *et al.*[20] Sugars were detected by the *p*-anisidine–phosphate reagent.[21,22] Diazotized *p*-nitroaniline, for phenols, was used according to the procedure of Bray *et al.*[23] The method of Gordon *et al.*[24] was used for the preparation of the benzidine–periodate reagent.

Analysis of phenolic acids. Methods for the analysis of phenolic acids by PC and UV absorption spectroscopy have been described by others.[8,25]

Apparatus for electrophoresis. High voltage paper electrophoresis (HVPE) was performed in a modified Michl[26] apparatus. The buffer solutions employed were formulated by Ambler.[27]

Elemental and amino acid analyses. Elemental analyses were accomplished by Dr. C. Daesslé (Organic Microanalyses, Montreal). Compound I (1 mg) was hydrolyzed *in vacuo* with 5·7 N HCl for 24 hr at 110°

[18] SYNGE, R. L. M. and YOUNGSON, M. (1961) *Biochem. J.* **78,** 31.
[19] BAGDASARIAN, M., MATHESON, N. A., SYNGE, R. L. M. and YOUNGSON, M. A. (1964) *Biochem. J.* **91,** 91
[20] HEILMAN, J., BARROLLIER, J. and WATZKE, E. (1957) *Z. Physiol. Chem.* **309,** 219.
[21] BRYSON, J. L. and MITCHELL, T. J. (1951) *Nature* **167,** 864.
[22] MUKHERJEE, S. and SRIVASTAVA, H. C. (1952) *Nature* **196,** 330.
[23] BRAY, H. G. THORPE, W. V. and WHITE, K. (1950) *Biochem. J.* **46,** 271.
[24] GORDON, H. T., THORNBURG, W. and WERUM, L. N. (1956) *Anal. Chem.* **28,** 849.
[25] IBRAHIM, R. K. and TOWERS, G. H. N. (1960) *Arch. Biochem. Biophys.* **87,** 125.
[26] MICHL, H. (1951) *Monatsch. Chem.* **82,** 489.
[27] AMBLER, R. P. (1963) *Biochem. J.* **89,** 349.
[28] SPACKMAN, D. H., STEIN, W. H. and MOORE, S. (1958) *Anal. Chem.* **30,** 1190.

and the hydrolyzate was dried in a vacuum desiccator over KOH pellets. Amino acid analyses were performed by Dr. I. E. P. Taylor and co-workers at the Botany Department, University of British Columbia. The method of Spackman *et al.*[28] was employed using a Beckman 120C amino acid analyzer.

Detailed procedures devised for the preparative isolation of the anionic 'insoluble' phenolic conjugate. From acetone powders. Preparation of acetone powder IA. Shoots were diced into a chilled, Waring blender, cold acetone (−20°) added and the mixture comminuted at high speed for 45 sec. The extract was filtered (Buchner) and washed exhaustively with cold acetone. The powder was air-dried for 10 min and then for 1 hr *in vacuo*. The dried acetone powders were stored at −20°.

Preparation of Fraction IB. The temp. was maintained at 2° unless otherwise indicated. 50 g of acetone powder were combined with 2000 ml H_2O containing 0·1% HOAc and 0·1% mercaptoethanol. The mixture was stirred for 1 hr, squeezed through fine nylon netting and centrifuged for 10 min in a Sorvall GSA rotor at 16000 *g*. The supernatant was decanted and freeze-dried.

Precipitation of high MW components. The freeze-dried extract was diluted to 400 ml with H_2O containing 0·1% mercaptoethanol. While stirring rapidly, dioxane (15°) was added to give a final concentration of 33% and the stirring continued for 15 min. The mixture was centrifuged at 27000 *g* for 2 min and the supernatant decanted and freeze-dried. The freeze-dried supernatant was dissolved in 250 ml H_2O containing 0·4% mercaptoethanol and 0·4% HOAc. While stirring rapidly, acetone was added to give a final concentration of 50%. The mixture was stirred, centrifuged and freeze-dried.

Adsorption by DEAE-cellulose. The freeze-dried supernatant from the above treatment was dissolved in a minimum amount of 2% HOAc and combined with 50 g DEAE-cellulose (OAc′ form) suspended in 500 ml H_2O. The mixture was stirred for 40 min, the gel collected and washed with 3 × 500 ml H_2O and then with 10 × 500 ml 5% HOAc (Fraction 1). The washed gel was suspended in 2 × 250 ml PAW, stirred for 40 min and filtered. The filtrates were combined and freeze-dried (to completely remove phenol it was necessary to add H_2O, melt and shell-freeze the fractions at least 5× during the freeze-drying procedure). Washing was terminated when the filtrate gave only a pale yellow color (*o*-dihydroxy groups) with NaOH. The yield is increased if Fraction 1 is recycled with fresh DEAE-cellulose.

Resolution by HVPE, PC and *TLC.* The freeze-dried PAW eluate from above was dissolved in H_2O and electrophoresed as follows: pH 1·9 (acetate–formate buffer); 3000 V; initial current of 100 mA; 60 min; initial temp. 16° increasing to 21°. The principle, fluorescent band migrated 3–5 cm in the direction of the anode. This band was excised, stitched to chromatography paper and developed in BzAWE. The chromatogram yielded pure compound I and a mixture of compounds II and III. The mixture was eluted, freeze-dried and separated by multiple development on Avicel plates in AnAW (see Table 2). Pure compound I was isolated as a powder after the final freeze-drying step. On prolonged exposure to air it was gradually converted to a pale green color and this oxidized component was removed by TLC chromatography (Avicel, 2% HCO_2H) in a N_2 atmosphere.

Preparation of residue II*A. Mentha*, 100 g fr.wt. was homogenized with 2 l. 95% EtOH and thoroughly extracted with hot 80% EtOH. The insoluble residue was collected and extracted with 500 ml H_2O containing 0·1% HOAc and 0·1% mercaptoethanol. The filtrate was freeze-dried and treated according to the steps described above.

Purification of anthocyanins. The cationic, magenta band obtained by electrophoresis of the PAW eluate above was stitched to chromatography paper and developed in BAW. Two magenta bands were resolved and each was subsequently purified by PC in 2% HCO_2H and TLC on Avicel in BBz (see Table 2).

Alkaline hydrolysis of compound II was with $NaBH_4$ (0·5 g) and compound I (0·5 mg in 5 ml), at 50° under N_2 plus 1 ml 2 N NaOH for 1 hr. After dilution with H_2O, the soln. was adjusted to pH 7 (2 N HCl) and applied to a DEAE-cellulose column (OAc′; 3·5 × 3 cm). The column was washed with 50 ml H_2O and the filtrate discarded. Caffeic acid was eluted with 100 ml 10% HOAc, the eluate freeze-dried and the concentration determined spectrophotometrically.

Partial purification of the high MW conjugate(s) of caffeic acid. Acetone powder IB or Residue IIB (Fig. 1) (10 g) were extracted for 1 hr with 150 ml PAW, filtered through Miracloth and the filtrate freeze-dried. The freeze-dried extract was dissolved in 7 M urea: 0·1% HOAc, filtered and the filtrate applied to a Sephadex G100 column (2·5 × 15 cm) equilibrated with the same solvent. The elution pattern revealed a single, major fraction absorbing at 325 nm. This fraction was dialysed against H_2O containing 0·01% mercaptoethanol. The dialysis proceeded for 48 hr with 3 × 4 l. changes H_2O and the bag contents were freeze-dried. A pale yellow powder was obtained (yield 0·1% of acetone powder IB or Residue IIB).