High-Performance Liquid Chromatographic Determination of Hydroxycinnamic Acids in the Maize Mesocotyl

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Cinnamic, *o*-coumaric, *p*-coumaric, caffeic, ferulic, and sinapic acids were resolved in 30 min by using the liquid chromatographic system described here. The acids were separated on a reversed-phase column with a gradient of methanol and acetate buffer and quantitatively determined at 254 nm. Since alkaline hydrolysis gave better recoveries of phenylpropanoids than did acid hydrolysis, the soluble and insoluble esters of phenylpropanoids in maize mesocotyls were hydrolyzed under mild alkaline conditions to prepare them for chromatography. The concentrations of the phenylpropanoids in the maize mesocotyl were established for plants grown for various amounts of time in the light and in the dark. The extraction, hydrolysis, and separation methods described here should be applicable to qualitative and quantitative determination of phenylpropanoids in other tissues.

The phenylpropanoids cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid are precursors of more complex plant phenolics such as flavonoids (Hahlbrock and Grisebach, 1979). Rapid methods for determining the hydroxycinnamic acid precursors would facilitate studies of the regulation of phenol metabolism. Thin-layer chromatography (Newby et al., 1980; Vande-Casteele et al., 1981), which has been extensively used to separate phenolics, is difficult to quantitate, and gas chromatography (Salomonsson et al., 1978; Horvat and Senter, 1980; Krygier et al., 1982a) requires preliminary derivatization of the phenylpropanoids. High-performance liquid chromatography (HPLC) is a rapid and sensitive method for qualitative and quantitative analysis and seems suitable for analysis of plant phenolics.

Several methods for separating hydroxycinnamic acids by HPLC on reversed-phase columns have been proposed. These methods either involve complex solvent mixtures (Hardin and Stutte, 1980; Roston and Kissinger, 1981), do not separate all five of the naturally occurring phenylpropanoids (Billett et al., 1981; Charpentier and Cowles, 1981; Roston and Kissinger, 1981), or have not been tested with plant extracts (Price et al., 1979).

A new method for rapid analysis of the phenylpropanoids by HPLC and techniques for recovering soluble and insoluble forms of the phenylpropanoids from fresh or dried tissue are described here. The applicability of these methods is illustrated with a study of phenylpropanoid accumulation in the maize mesocotyl.

MATERIALS AND METHODS

HPLC Methods. Hydroxycinnamic acids were from Sigma Chemical Co. or from ICN Pharmaceuticals, Inc., and stock solutions were prepared in methanol and stored in amber bottles at 4 °C. Spectrophotometric-grade methanol and glass-distilled water were used. All other chemicals were reagent grade.

The chromatograph (Spectra-Physics 8000, Santa Clara, CA 95051) was equipped with a 254-nm detector. The reversed-phase column (Brownlee Laboratories RP-10A, 250 × 4.6 mm, Santa Clara, CA 95051) contained 10-μm particles of Lichrosorb C-8. The column was protected with a precolumn of similar composition (Bioanalytical Systems Guard Holder PN6020, Cartridge PN6019, West Lafayette, IN 47906).

Buffer A was a 1:100 water dilution of a stock pH 4.7 acetate buffer, prepared by adjusting 5 M acetic acid to pH 4.7 with sodium hydroxide. Buffer A was filtered through a 0.22-μm Millipore filter (Type GS), and methanol was filtered through a 0.2-μm Millipore filter (Type EG).

The initial solvent was 15% methanol and 85% buffer A. After 15 min of isocratic flow at 2 mL/min, a 5-min linear gradient was used to alter the solvent composition to 30% methanol and 70% buffer A. This mixture was maintained for 10 min, after which a final 5-min linear gradient returned the solvent to its original composition. A total of 45 min was required to run the entire solvent program and to reequilibrate the column in the initial solvent. The column was maintained at a constant temperature of 37 °C and run at a constant flow rate of 2 mL/min (pressure ~900 psi). The 254-nm detector was routinely set at a sensitivity of 0.04 absorbance unit full scale.

A mixture of standards composed of 200 nmol of each of the six phenylpropanoids (cinnamic acid, *p*-coumaric acid, *o*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid) in a total of 5 mL of 15% methanol and 85% buffer A was prepared daily. A 100-μL aliquot of this mixture, containing 4 nmol of each standard, was injected by using a 100-μL sample loop.

Maize Mesocotyl Growth. The mesocotyl or first internode of the maize (*Zea mays* L.) seedling is the unpigmented tissue between the coleoptile and the point of attachment of the embryo axis to the kernel. When the seedling is exposed to light, elongation of the mesocotyl ceases and phenol production in the mesocotyl is enhanced (Duke and Naylor, 1976). Seeds of the hybrid $B73_{Ht}$ × $Va26_{Ht}$ (MIGRO Hybrids; Mitchell, IN 47446) were imbibed in aerated water at 20 °C for 6 h and then incubated between layers of moist germination paper in the dark at 28 °C. Following 90 h of incubation, rolls of germination paper containing seedlings with mesocotyl lengths of about 70 mm were placed in clear plastic bags to maintain 100% relative humidity. The plants were then transferred to a 28 °C growth chamber and kept in complete darkness or in a 15-h light/9-h dark photoperiod initiated with 4-h light. After the first 24 h the plastic bags were removed so plants would be more completely exposed to the light. Seedlings were constantly supplied with water.

Tissue Extraction and Hydrolysis. At appropriate times seedlings were removed from the growth chamber, and mesocotyls were excised 5–10 mm above the point of attachment to the seed and 5–10 mm below the coleoptile. Samples of 10 mesocotyls (approximately 1.5 g) were cut into 2–3-mm segments, immersed in methanol (2 mL/g of

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tissue), and homogenized with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY 11590) for 60 s at medium speed. The homogenizer generator was rinsed with methanol for maximum recovery of the homogenate. The extract was filtered (5.0-μm Type LS or 0.2-μm Type EG Millipore filter) and the solid residue was rinsed with several volumes of methanol, which were combined with the filtrate. The volume of the filtrate was reduced from about 16 mL to about 1 mL under reduced pressure at 30 °C. The resulting cloudy extract was transferred to a hydrolysis tube. The white fibrous solid residue was also transferred to a hydrolysis tube and about 1 mL of water was added.

The extract and solid residue samples were chilled while 1 mL of 5 N sodium hydroxide was added and were then immediately sealed under vacuum. The yellow color characteristic of caffeic acid derivatives in alkaline solution frequently developed upon addition of base (Ribereau-Gayon, 1972). After hydrolysis for 1 h at 30 °C, the tubes were opened and 1 mL of 6 N hydrochloric acid was rapidly added.

Methanol was removed from the acidic solution by evaporation under reduced pressure, and the hydrolyzed extracts were partitioned 3 times against a small volume of diethyl ether. The aqueous phase was discarded, and the combined organic phases were extracted 3 times with 5% aqueous sodium bicarbonate. This aqueous phase was acidified to pH 1 with concentrated hydrochloric acid and was then partitioned 3 times against diethyl ether. The organic phase was saved for chromatographic analysis.

The acidified hydrolysates of the solid residues were filtered through Whatman No. 1 paper. The residue was washed several times with water, and the filtrate was then partitioned 3 times against diethyl ether. An emulsion that could be partially broken by low-speed centrifugation usually formed, preventing complete recovery of the organic phase. The ether extract was saved for chromatographic analysis.

So that only the unesterified hydroxycinnamic acids would be obtained, the original methanol extract of the tissue was acidified and a small volume of water was added. The methanol was removed by evaporation under reduced pressure, and the resulting acidic aqueous extract was partitioned successively against diethyl ether, aqueous sodium bicarbonate, and diethyl ether as described above.

The final ether solutions of all extracts could be stored for several days at 4 °C without change. For preparation of samples for analysis, 200 nmol of *o*-coumaric acid was added and the sample brought to a dryness under reduced pressure. It was then redissolved in 85% buffer A and 15% methanol. Cloudy samples were filtered through a 0.2-μm filter (Type RC 58) in the Bioanalytical Systems MF-1 centrifugal microfilter. For initial fresh tissue weights of 1–2 g, a final sample volume of 5 mL and an injection aliquot of 100 μL were appropriate.

RESULTS

Each of the standard phenylpropanoids except caffeic acid was recovered in good yield after mild alkaline hydrolysis but was destroyed by acid hydrolysis (Table I). The recovery of caffeic acid after base hydrolysis was variable, ranging from 45 to 90%. Rapid and complete exclusion of oxygen after the addition of base minimized destructive oxidation and maximized the recovery of caffeic acid.

A 1-h hydrolysis like that routinely used for tissue samples was sufficient to completely hydrolyze a commercial preparation of chlorogenic acid to caffeic acid. A 4-h hydrolysis of the mesocotyl extract or of the solid residue released no more hydroxycinnamic acids than were released by the routine 1-h treatment.

Table I. Recovery of Phenylpropanoids after Acid or Base Hydrolysis and Extraction into Ethyl Acetate or Diethyl Ether

compound	% recovery[a] acid hydrolysis[b]	% recovery[a] base hydrolysis[c]
cinnamic acid	68	100
p-coumaric acid	9	92
o-coumaric acid	56	93
caffeic acid	18	45
ferulic acid	14	92
sinapic acid	19	79

[a] The recoveries were calculated from peak heights or peak areas by using unhydrolyzed standards not extracted into organic solvents as 100%. [b] A mixture of the standards was treated with 2 N HCl for 1 h at 100 °C. The sample was extracted into ethyl acetate, dried, dissolved in a mixture of 85% buffer A and 15% methanol, and chromatographed. [c] A mixture of the standards was hydrolyzed with NaOH as described in the text. The acidified sample was extracted into diethyl ether, dried, and chromatographed as described above.

Table II. Relative Retentions of Phenylpropanoids Separated by Liquid Chromatography

compound	relative retention[a]
caffeic acid	0.32 ± 0.01
p-coumaric acid	0.61 ± 0.01
ferulic acid	0.79 ± 0.01
sinapic acid	0.90 ± 0.03
cinnamic acid	1.26 ± 0.01

[a] The relative retentions were calculated by dividing the retention time of the compound by that of the internal standard, *o*-coumaric acid, so that *o*-coumaric acid had a relative retention of 1.00. Values are the average of 11 determinations ± standard deviation. Data were taken from experiments performed on separate days.

Recovery of phenylpropanoids (except caffeic acid) from extracted and hydrolyzed tissue was estimated to range from 60 to 95% of the total, based on recovery of standards treated in a similar fashion. The most important losses occur during hydrolysis and during the diethyl ether extraction of the hydrolysate (Table I). Recovery of phenylpropanoids in the diethyl ether or ethyl acetate phase was always greater than 80%. The values reported here for accumulation of phenylpropanoids in the maize mesocotyl have not been corrected to account for these losses.

Constituents of the hydrolysates of the soluble and insoluble fractions from maize mesocotyls were changed if the mesocotyls were frozen before the compounds were extracted. The concentrations of components in the soluble phenylpropanoid fraction were much larger in extracts of frozen tissue than in extracts of fresh tissue. An unidentified component with relative retention (the ratio of retention time to the retention time of the internal standard, *o*-coumaric acid) of 0.74 was present in large quantities in extracts of frozen tissue. Concentrations of components in the insoluble hydroxycinnamate fraction were generally decreased in extracts of frozen tissue. In subsequent analyses, mesocotyls were always extracted without prior freezing to avoid such changes.

The five commonly occurring phenylpropanoids (cinnamic, *p*-coumaric, caffeic, ferulic, and sinapic acids) and the internal standard (*o*-coumaric acid) were completely resolved in 30 min (Figure 1; Table II). The subsequent 15 min of elution restored initial conditions so that the next

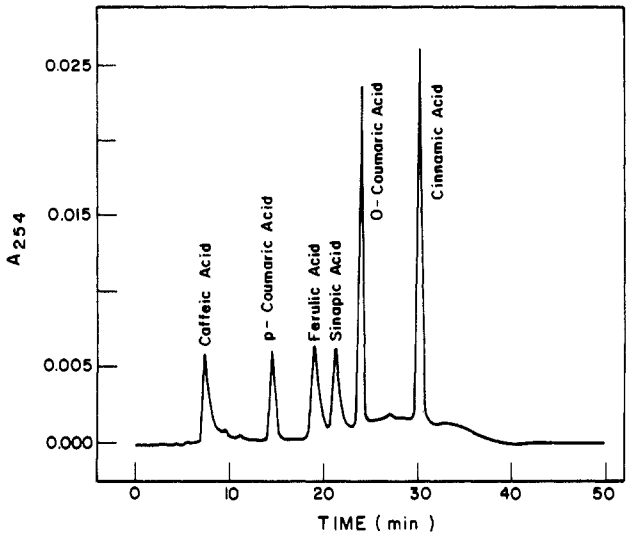

Figure 1. Separation of phenylpropanoid standards by liquid chromatography. A 100-μL aliquot containing 4 nmol each of caffeic, *p*-coumaric, ferulic, sinapic, *o*-coumaric, and cinnamic acids was injected. The conditions are described in the text.

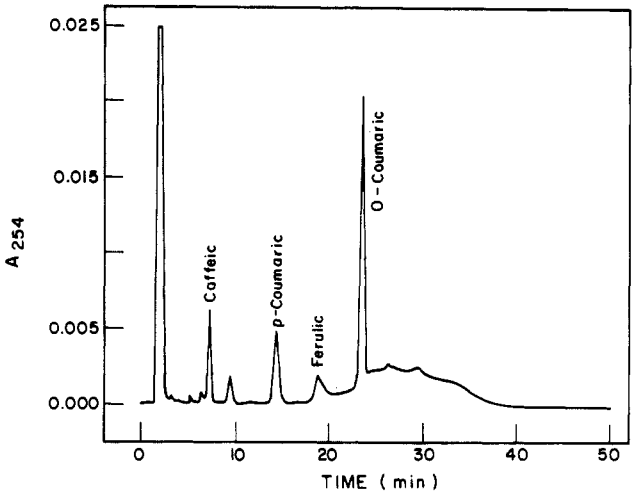

Figure 2. Separation of hydroxycinnamic acids in the hydrolyzed extracts of maize tissue. Peaks have relative retentions corresponding to those of caffeic, *p*-coumaric, and ferulic acid and the internal standard *o*-coumaric acid. The conditions are described in the text.

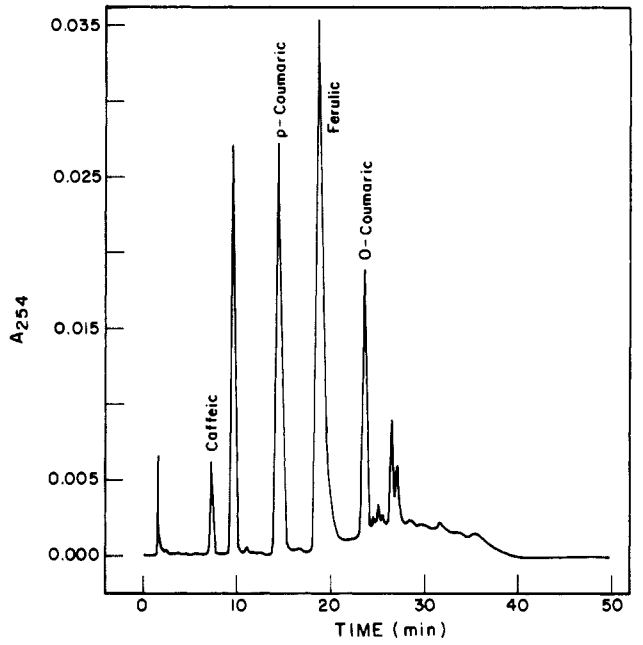

Figure 3. Separation of hydroxycinnamic acids released from the solid residue of maize mesocotyl by base hydrolysis. Peaks have relative retentions corresponding to those of caffeic, *p*-coumaric, and ferulic acid and the internal standard *o*-coumaric acid. The conditions are described in the text.

sample could be injected. The base-line shifts between 15 and 20 min and between 30 and 35 min are due to changes in solvent composition during the run. Peak height or peak area is a linear function of component concentration up to at least 15 nmol of each component. Compounds more polar than the phenylpropanoids, including sugar or organic acid esters of phenylpropanoids, benzoic acids and their esters, and anthocyanins, are eluted in the solvent front. Components less polar than the hydroxycinnamic acids, including cinnamic acid, were eluted with the final 30% methanol containing solvent or were retained on the precolumn. Peaks that could be attributed to cis isomers were never observed although spectral analysis (Ribereau-Gayon, 1972) of the standards indicated that both cis and trans isomers were present. Analysis of the phenylpropanoids in meal and flour samples using gas chromatography indicated that the trans isomers are the major species and that the cis isomers are minor constituents (Krygier et al., 1982b; Sosulski et al., 1982) probably formed by the action of light on the extracts (Fenton et al., 1978).

Chromatograms of the hydroxycinnamic acid containing extracts of maize mesocotyls were similar to those of the standard compounds (Figures 2 and 3). Peaks with relative retention times corresponding to *p*-coumaric, caffeic, and ferulic acids (Table II) were found in hydrolysates of the methanol extracts (Figure 2) and of the solid residue (Figure 3), but sinapic acid was not detected. Chemical analysis (Ribereau-Gayon, 1972) of hydrolysates showed that the total concentration of phenolics in the tissue was similar to the total phenylpropanoid concentration calculated from the areas of peaks corresponding to the standards. This suggests that peaks could be tentatively identified as individual phenylpropanoids on the basis of their retention time. Small peaks corresponding to cinnamic acid occurred infrequently and irreproducibly, perhaps because of low recovery from the tissue extracts. This may result from the low solubility of the compound and its tendency to adsorb to glass (Billett et al., 1981).

In preliminary experiments *o*-coumaric acid was not detected in extracts of the maize mesocotyl and was therefore useful as an internal standard.

There was no detectable change in the amount of caffeic, *p*-coumaric, or ferulic acid in the methanol extract of the mesocotyl during 48 h of growth in the photoperiod (parts a, b, and c of Figure 4). Caffeic acid is the major soluble phenylpropanoid and was present during the 48-h interval at an average concentration of 176 ± 60 (standard deviation) nmol/g fresh weight (g fr wt) of tissue, while *p*-coumaric and ferulic acids were present at average concentrations of 104 ± 28 (standard deviation) and 89 ± 38 (standard deviation) nmol/g fr wt (parts a, b, and c of Figure 4). During the 48-h interval less than 10% of the total phenylpropanoids in corn were present as soluble free acids.

Caffeic, *p*-coumaric, and ferulic acids were released from the solid residue of the mesocotyl by base hydrolysis. Ferulic and *p*-coumaric acids accumulated in the solid residue starting between 6 and 15 h after initiation of illumination (parts c and b of Figure 4), but caffeic acid did not accumulate (Figure 4a). After 47 h of growth in the photoperiod, ferulic and *p*-coumaric acids in the solid

Table III. Accumulation of Hydroxycinnamic Acids in Maize Mesocotyls after 24 Hours of Growth

	caffeic, nmol/g fr wt		p-coumaric, nmol/g fr wt		ferulic, nmol/g fr wt	
treatment	soluble	insoluble	soluble	insoluble	soluble	insoluble
photoperiod	240 ± 26[a]	208 ± 40[b]	102 ± 27[a]	393 ± 37[b]	115 ± 13[a]	630 ± 94[b]
dark	135 ± 45[a]	80, 87[c]	106 ± 21[a]	286, 368[c]	92 ± 13[a]	545, 702[c]

[a] Mean of four determinations ± standard deviation. [b] Mean of six determinations ± standard deviation. [c] Two determinations.

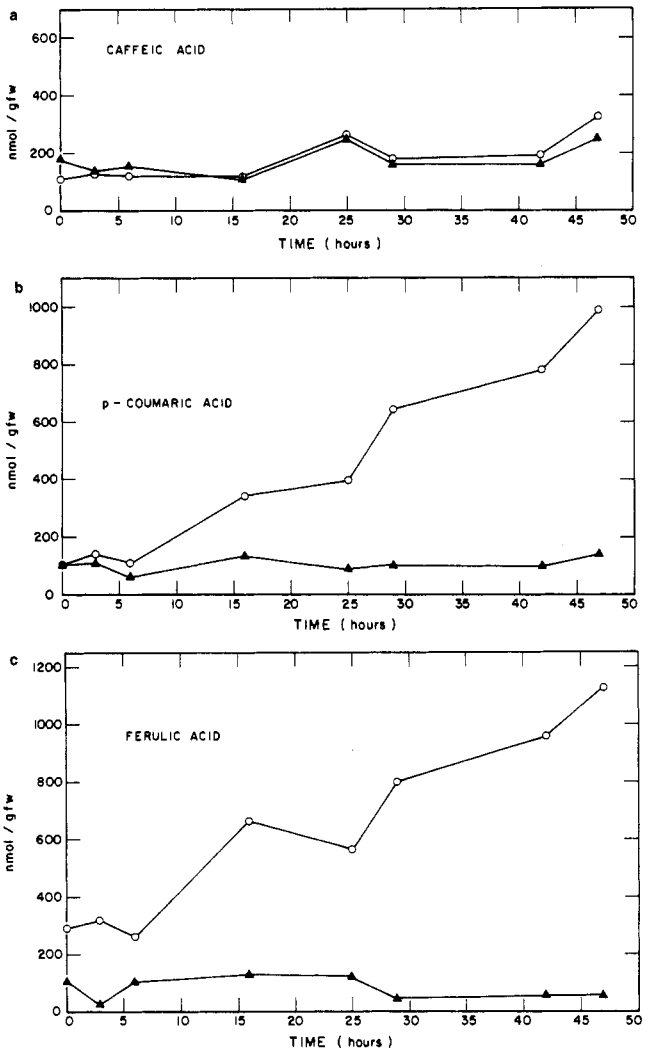

Figure 4. Accumulation of hydroxycinnamic acids by maize mesocotyls. Dark-grown mesocotyls were put into the light at time 0; after 4 h of illumination, a 9-h dark/15-h light photoperiod was initiated. Symbols: (▲) phenylpropanoids in the soluble pool; (○) phenylpropanoids released from cell walls. (a) Caffeic acid (relative retention = 0.32); (b) *p*-coumaric acid (relative retention = 0.61); (c) ferulic acid (relative retention = 0.79).

residue accounted for 75% of the total phenylpropanoids (Figure 4).

The amounts of caffeic, *p*-coumaric, and ferulic acid in the soluble extract of the maize mesocotyl were not altered when plants were grown in complete darkness rather than in the photoperiod (Table III). The accumulation of *p*-coumaric and ferulic acids in solid residue of the mesocotyl was not altered when plants were grown in the dark, but a smaller amount of caffeic acid was found in the solid residue of dark-grown plants (Table III).

DISCUSSION

Phenylpropanoids occur in plant tissue as free acids, as acid-labile glycosides, and as acid- or alkali-labile esters (Ribereau-Gayon, 1972). The hydroxycinnamic acids are partially destroyed (VanSumere et al., 1972; VanBrussel et al., 1978) in hot acidic medium (Table II). Thus, accurate determination of these compounds after acid hydrolysis is probably not possible, although it is sometimes attempted (Murphy and Stutte, 1978; Hardin and Stutte, 1980; Charpentier and Cowles, 1981). The mild alkaline hydrolysis described here gives good recovery of both soluble and insoluble forms of the hydroxycinnamic acids and is applicable to both fresh tissue like the mesocotyl and dry tissue like sorghum straw (Butler et al., 1982).

Qualitative and quantitative analysis of hydroxycinnamic acids from plant tissue is possible with the HPLC method described here. A 300–330-nm detector, which would respond only to phenylpropanoids and other compounds with absorption bands in this region, would allow analyses of more complex mixtures of natural products without interference. Further characterization of peaks including absolute identification by techniques such as mass spectrometry (Krygier et al., 1982a) would be possible if fractions were collected during chromatography.

In mesocotyls two pools of hydroxycinnamic acids were identified. The phenylpropanoids extracted from the tissue with methanol are the soluble pool, which is composed of a small amount of free acids and a larger quantity of alkali-labile esters of caffeic, *p*-coumaric, and ferulic acids. The coenzyme A thioesters of hydroxycinnamic acids, which are precursors of lignin and flavonoids (Hahlbrock and Grisebach, 1979), are probably included in this pool.

Phenylpropanoids not extracted from the solid residue by methanol but released from the residue by alkaline hydrolysis are insoluble esters of cell wall materials like cellulose (Hartley and Jones, 1977) or lignin (Smith, 1955). Ferulic, *p*-coumaric, and sinapic acids are components of the cell walls of straw from several grasses (Salomonsson et al., 1978). In the maize mesocotyl ferulic and *p*-coumaric acids are the major constituents of the pool of insoluble esters and caffeic acid is a minor constituent (Figure 4).

When the mesocotyl is illuminated, phenylalanine ammonia-lyase activity increases to a level 2–5-fold higher than that in unilluminated tissue (Duke and Naylor, 1974; Heim, 1981). Anthocyanin synthesis is induced in the epidermal and hypodermal cells of the mesocotyl after the plant is exposed to light (Duke and Naylor, 1974). It was expected that the concentration of the soluble phenylpropanoids, which are anthocyanin precursors, would be greater in the light-grown tissue than in the dark-grown tissue. However, similar amounts of soluble hydroxycinnamic acids were found in maize mesocotyls grown in the photoperiod and in the dark (Table III). We propose that the rate of flux of intermediates through the soluble pool, rather than their concentration in the pool, must change in response to stimuli that control anthocyanin synthesis.

The accumulation of insoluble esters of ferulic and *p*-coumaric acids is not regulated by light in the maize mesocotyl (Table III) or in the *Sorghum* mesocotyl (Stafford, 1967). However, incorporation of caffeic acid into the insoluble pool in maize or *Sorghum* is stimulated by light (Table III; Stafford, 1967). Distinct mechanisms,

perhaps involving isozymes or cellular compartmentalization (Hahlbrock and Grisebach, 1979), probably regulate the light-sensitive synthesis of anthocyanins and of insoluble caffeic acid esters and the light-insensitive synthesis of insoluble ferulic and *p*-coumaric esters.

The applicability of the simple chromatographic system described here to studies of phenol accumulation and metabolism is illustrated with the maize mesocotyl. Base hydrolysis in the absence of dissolved oxygen is used to cleave the soluble and insoluble esters of hydroxycinnamic acids, so that destruction by acid hydrolysis is avoided. Rapid qualitative and quantitative analysis of the phenylpropanoids from plant tissue is achieved with HPLC. This system for sample preparation and analysis should facilitate further studies of phenol metabolism in many tissues.

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Quantitative Analysis of Hop Flavonols Using High-Performance Liquid Chromatography

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Three chromatographic techniques were used in studying the flavonol composition of brewing hops. For qualitative separation two-dimensional chromatography on cellulose thin layers was used. The major flavonol glycosides of hops were recovered after preparative chromatography of extracts on dextran–gel columns. Quantitative measurements of flavonols, either as glycosides or as aglycons, were accomplished by using high-performance reverse-phase chromatography. The mean contents of flavonol aglycons measured as kaempferol and quercetin in nine varieties of hops were 1.20 and 0.92 mg/g, respectively. Variations in flavonol contents were found in samples of different varieties of hops and between samples harvested in different years. During the brewing process flavonols were incompletely extracted from hops and losses in extracted flavonols occurred during fermentation with the result that the contents of flavonols in an experimental beer were less than 1 mg/L.

Among the 2000 or more plant flavonoids that have been described, flavonols are one of the more important groups with significant physiological activities (Harborne, 1980). Flavonols are almost universally distributed in plants and are invariably conjugated as water-soluble *O*-glycosides. The three aglycons that occur most frequently differ only in number of hydroxyl groups [see McMurrough (1981)]. Whereas glycosides of kaempferol and quercetin are found in most angiosperms, myricetin glycosides occur mainly in the leaves of woody plants. Both the pattern of aglycon hydroxylation and the pattern of glycosylation are clearly of taxonomic significance (Harborne, 1967). The dried flower cones of hops (*Humulus lupulus*) used in brewing are not exceptional in their contents of flavonol glycosides. Several glycosides of both kaempferol and quercetin have been identified in hops (Harris, 1956; Vancraenenbroeck et al., 1969), but quantitative data are sparse. Attempts have been made to measure the relative proportions of the major flavonol glycosides in hops (Hubáček and Trojna, 1970) as a chemotaxonomical marker and index of quality. Doubtless, deficiencies in the methods of separation then available limited the extent to which quantitative studies on different hop varieties could be performed. The opportunity to extend the findings of early investigators was

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