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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presented with the advent of modern high-performance liquid chromatography (HPLC). Although flavonols from a number of plant sources have been separated by HPLC (Court, 1977; Asen, 1977; Niemann and Koerselman-Kooy, 1977; Schuster, 1980; Wulf and Nagel, 1980) the flavonols of hops have not attracted much attention.

Hops are used by brewers mainly to impart bitterness and aroma to their products. Humulone and its derivatives obviously give rise to most of the bitterness, but it is not known fully to what extent other hop components may also contribute. For instance, polyphenols and their oxidation products are generally associated with harsh flavor (Bruckner et al., 1970). When added to beer, both kaempferol and quercetin tasted bitter but their flavor thresholds were relatively high at 10–50 mg/L (Dadic and Belleau, 1973). Although data on the levels actually occurring in beer are rare at least one report (Drawert et al., 1977) indicates that the concentrations of individual flavonols and their glycosides were between one-hundredth and one-tenth of the reported flavor thresholds.

The objective of this study was to determine the contents of flavonols in several batches of hops so that the range of possible variation could be assessed. Furthermore, it was of interest to study the fate of hop flavonols during the brewing process and determine their concentrations in the finished product. Methods for measuring polyphenols in brewing materials by HPLC have already been described (McMurrough, 1981). In this report results are given in detail for the application of several chromatographic methods, including HPLC, for the separation and measurement of hop flavonols.

MATERIALS AND METHODS

Chemicals and Equipment. Flavonol standards, solvents, and other chemicals used were as described (McMurrough, 1981). High-pressure liquid chromatography was performed on a Waters Associates liquid chromatograph using a hydrocarbon-bonded silica column in the reversed-phase mode (McMurrough, 1981). Equipment for gradient elution of dextran–gel columns or for cellulose thin-layer chromatography was as used previously (McMurrough and McDowell, 1978; McMurrough, 1979).

Extraction and Analysis of Hops. Samples of named varieties of hops were taken from bulk shipments made by commercial suppliers in England, Ireland, the United States, Germany, and Australia and were stored at 4 °C in sealed bags. Extracts were prepared in a nitrogen atmosphere from ground hops (10 g) in 3:1 acetone–water (150 mL) as described previously (McMurrough and McDowell, 1978). These extracts were either used directly for HPLC or two-dimensional TLC analysis or fractionated further (McMurrough, 1981), by first saturating with sodium chloride and then allowing the separation of two liquid phases. The upper liquid phases obtained were vigorously mixed with equal volumes of hexane, and after the phase separation the lower liquid phases obtained contained the bulk of the flavonol glycosides. This fractionation accomplished a concentration of flavonol glycosides and removed unwanted phenolic acids, depsides, and polymeric flavanols. For some purposes flavonols were simultaneously extracted from ground hops (10 g) and hydrolyzed to aglycons by refluxing for 2 h in 150 mL of methanol–4 N HCl (3:1 v/v) as described previously (McMurrough, 1981).

Isolation and Characterization of Flavonol Glycosides. The aqueous acetone extract from 100 g of hops provided yields of the major hop flavonols sufficient to permit their provisional identifications. This extract was first concentrated and then chromatographed on a column (1.5 cm × 100 cm) of Sephadex G-25 Superfine by a modification of the method described by Vancraenenbroeck et al. (1969). The dextran–gel was swollen in water prior to packing, and after applying the sample the column was eluted with a gradient of methanol (0–50% v/v) in water. This gradient was generated by continuous addition of 50% aqueous methanol (2.0 L total) through a pump to a mixing chamber containing degassed water (2.0 L total).

Eluent was supplied to the column at a rate of 50 mL/h, and the elution of flavonols was monitored by absorbance measurements at 350 nm. No flavonols were detected in the eluate after collection of the first 150 fractions (10 mL each). Flavonol glycosides were recovered from the appropriately pooled fractions and were further purified when necessary by either column or paper chromatography (Harris, 1956).

Identities of the major components were assigned on the basis of established tests (Vancraenenbroeck et al., 1969). Accordingly, the natures of the aglycons and sugars were determined after acidic hydrolysis and TLC of the hydrolysates. The positions of the sugars on the aglycons were determined by spectrophotometric behaviors in the presence of certain reagents and by hydrolysis with β-glucosidase. The 3-rhamnoglucosides of quercetin and kaempferol so identified were assigned either rutinoside (3,6-*O*-α-L-rhamnosyl-D-glucoside) or neohesperidoside (3,2-*O*-α-L-rhamnosyl-D-glucoside) structures from their relative stabilities when refluxed in 10% aqueous acetic acid.

Preparation and Analysis of Worts and Beers. Worts and beers were prepared in a miniature brewery as described previously (McMurrough, 1979). A standard pretreatment was used on all samples (100 mL) to remove unwanted materials and to concentrate the flavonols. In this pretreatment large moleuclar weight components were precipitated by adding acetone (300 mL) and then chilling (4 °C) for 2 h. After centrifugation (15000*g* for 30 min) at 4 °C the clear supernatant obtained was evaporated to about 10 mL under vacuum and then freeze-dried. The solids obtained were dissolved in methanol (5–10 mL), and insoluble matter was removed by centrifugation. The extract so obtained was suitable for analysis by HPLC.

RESULTS AND DISCUSSION

TLC of Hop Flavonols. Twenty-four hop components were recognized as flavonols by their fluorescence in UV light after spraying developed TLC plates with AEDBE (Figure 1), but attention was concentrated on component groups K_1–K_5 and Q_1–Q_5 that fluoresced green and yellow, respectively. The mobilities of these components on two-dimensional TLC were similar to those of the glycosides of kaempferol and quercetin isolated from hops and already characterized (Vancraenenbroeck et al., 1969).

HPLC of Hop Flavonols. Gradient elution of samples of crude acetone–water extracts from nine varieties of hops satisfactorily separated individual hop flavonols [see McMurrough (1981)] that were detected by their absorbances at 365 nm. Phenolic acids and polyhydroxy flavans were eluted early in the chromatographic run (1–12 min) and were seen by detection at 254 or 280 nm. Nonflavonoid material that eluted late in the run (14–35 min) absorbed strongly at 365 nm and consisted mainly of esterified hydroxycinnamic acids. Flavonol glycosides from hop extracts were invariably separated as 16 peaks, but of these 6 peaks attributable to components K_1–K_3 and Q_1–Q_3 were consistently bigger than the others.

Preparative Separation of Hop Flavonols on Dextran–Gel. Chromatography of concentrated acetone–

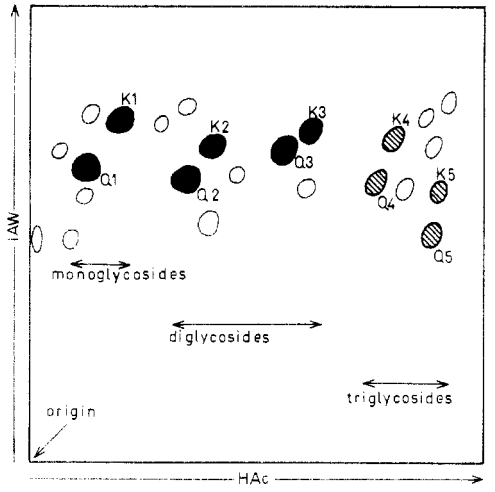

Figure 1. Two-dimensional cellulose TLC separation of flavonol glycosides from hops. Chromatograms were developed in the first dimension with isoamyl alcohol–acetic acid–water (2:1:1, IAW) and in the second dimension with 6% aqueous acetic acid (HAc). For visualization of major (solid areas), minor (shaded areas), and trace (open areas) components chromatograms were sprayed with 2-aminoethyl diphenylborate ester (AEDBE, 1% in ethanol) and viewed under UV light. Glycosides of kaempferol (K_1–K_5) fluoresced green whereas glycosides of quercetin (Q_1–Q_5) fluoresced yellow.

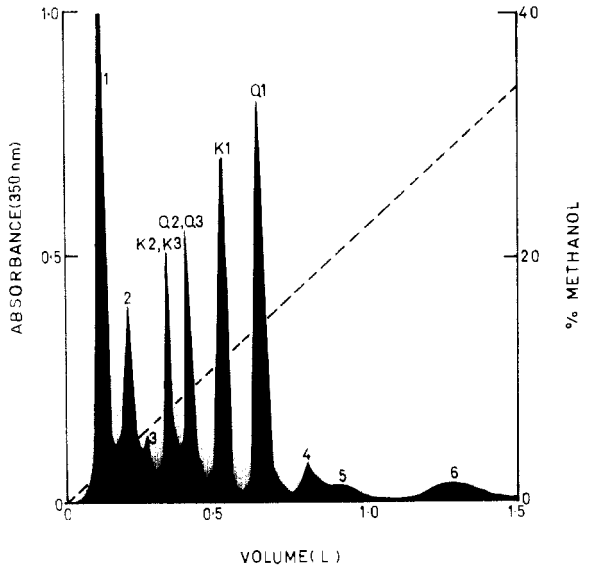

Figure 2. Preparative separation of major flavonol glycosides (K_1–K_3, Q_1–Q_3) from phenolic acids and esters (peak 1), flavonol triglycosides (peak 2), and trace flavonol components (peaks 3–6). Material extracted from Challenger hops (100 g) with 75% acetone–water was eluted from a column (100 cm × 1.5 cm) of Sephadex G-25 Superfine with a gradient of methanol in water, pumped through at 50 mL/h.

water extracts from hops on columns of Sephadex G-25 Superfine eluted with a methanol–water gradient produced the separation shown in Figure 2. Peak 1 contained cinnamic acid derivatives and was discarded. The major flavonol glycosides of hops were located in four resolved peaks while the minor triglycosides were located together in peak 2. Peaks 3–6 contained only traces of flavonols and were not retained for characterization. The main components of peaks K_1 and Q_1 were the β-3-glucosides of kaempferol and quercetin, commonly termed astragalin and isoquercitrin, respectively. Individual flavonol glucosides were freed from contaminants by repeated chromatography on Sephadex LH-20 eluted with methanol.

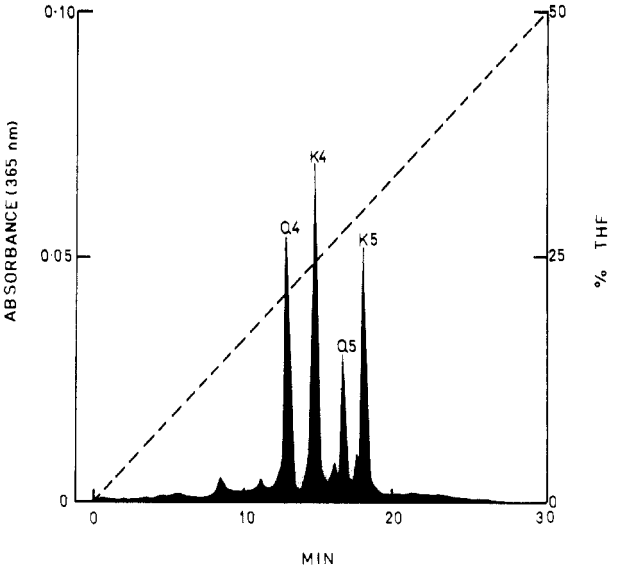

Figure 3. HPLC separation on μBondapak C_{18} (Waters Associates, Milford, MA) of the triglycoside fraction obtained by chromatography on Sephadex G-25 of the acetone–water extract from Challenger hops. The reversed-phase column (30 cm × 3.9 mm i.d.) was eluted with a gradient of increasing tetrahydrofuran (THF) concentration in aqueous acetic acid (2.5% v/v).

The flavonol diglycosides were located in the G-25 eluate in two peaks, each peak containing diglycosides of either kaempferol (K_2, K_3) or quercetin (Q_2, Q_3). Peak 2 of the G-25 eluate contained eight flavonol components with the mobilities on cellulose thin layers (Figure 1) characteristic of flavonol triglycosides (Vancraenenbroeck et al., 1969). Four of these components were present in sufficient quantities to permit their isolation by preparative paper chromatography and for their provisional identification as two different triglycosides of both kaempferol and quercetin (K_4, K_5, Q_4, and Q_5). The components of the triglycoside fraction (peak 2) were well separated by HPLC (Figure 3), and from the retention times of the individual components the positions of the triglycosides in the HPLC profiles of hop flavanols were gauged.

Quantification of Flavonol Glycosides. Although the hop flavonols isolated did not fully account for the 16 flavonoid substances separated by HPLC, it was calculated from peak height measurements that the flavonol glycosides K_1–K_5 and Q_1–Q_5 constituted more than 80% of the total yield of flavonols from hops. Moreover, these components were structurally identifiable with certain flavonol glycosides described previously (Vancraenenbroeck et al., 1969). The monoglucosides (K_1 and Q_1), rutinosides (K_2 and Q_2), and neohesperidosides (K_3 and Q_3) were recovered in sufficient quantities to permit calibration of high-performance chromatograms by measurement of peak heights and areas. The analytical hydrolysis of flavonol glycosides in extracts to their parent aglycons was also quantified by using quercetin and kaempferol from commercial sources as standards. A selection of the results obtained is given in Table I. Whereas most of the kaempferol present in hops was accounted for by the presence of monoglucosides and diglycosides, this was not true for quercetin. Only about half to about three-quarters of the total quercetin extracted was measured as identifiable glycosides. Invariably a large shortfall was associated with increased complexity in the HPLC profile obtained, with a greater than average abundance of peaks due to triglycosides or unidentified minor components.

Total Flavonols in Hops. The extraction of flavonols in their native forms and their subsequent assay by gra-

Table I. Percentages of Kaempferol and Quercetin Measured as Identifiable Monoglucosides and Diglycosides in Bullion, Challenger, and Talisman Hops

hop variety	kaempferol glycosides measured, % total kaempferol			quercetin glycosides measured, % total quercetin		
	glucoside	rutinoside	neohesperidoside	glucoside	rutinoside	neohesperidoside
Bullion	46	39	12	12	19	7
Challenger	30	30	10	27	14	5
Talisman	22	61	14	37	33	8

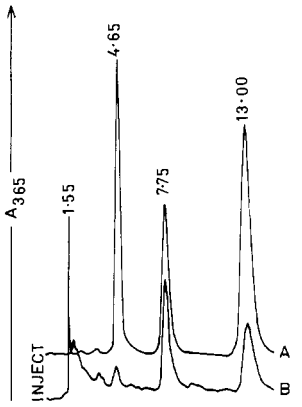

Figure 4. HPLC profiles of aglycon standards (A) and extract of hydrolyzed Challenger hops (B). The standard mixture contained myricetin (R_t = 4.65 min), quercetin (R_t = 7.75 min), and kaempferol (R_t = 13.00 min), and the eluting solvent contained 2.5% acetic acid (v/v) and 50% methanol (v/v) in water.

dient-elution HPLC were not conducive to rapid throughput of samples. A simpler and less time consuming method was developed (McMurrough, 1981) by which flavonols were simultaneously extracted and hydrolyzed to aglycons and then measured by isocratic HPLC. By use of the isocratic system coupled to a data-handling system, the analysis time was decreased to about 15 min/per sample. No interrun equilibration period was required, and the uncomplicated chromatographic profiles obtained (Figure 4) facilitated accurate measurement. Invariably the chromatographic profiles of hydrolyzed hops displayed peaks corresponding to a preponderance of quercetin and kaempferol and a minor peak with the same retention as myricetin (Figure 4). Since no myricetin-containing flavonol glycoside was isolated from hops, this aglycon was not included in the summation for total flavonols. Nevertheless, it seems probable that myricetin-containing glycosides do occur in hops in small amounts.

The contents of kaempferol and quercetin in samples of nine varieties of hops harvested in 1980 are given in Table II. Whereas most varieties contained either more kaempferol than quercetin or almost equal amounts of both, Talisman hops contained more quercetin than kaempferol. The presence of free flavonol aglycons in one variety (Northern Brewer) of hops was examined after extraction with hot 75% methanol–water (v/v). From this it was concluded that only small amounts of quercetin (7% total) and kaempferol (8% total) occur in hops in a nonglycosylated form. Possible differences between varieties were further explored by using hops that had not only been harvested in different years but also been stored for different times prior to analysis. The results of these analyses did not permit any definite conclusions on possible relationships between hop variety, year of harvest, or storage time on flavonol contents. For samples of Northern Brewer hops harvested on successive years from 1977 to 1980, the contents of kaempferol varied from 0.11 to 1.50 mg/g and averaged 0.79 mg/g while those of quercetin

Table II. Contents of Flavonol Aglycons in Hops Harvested in 1980

hop variety	flavonol aglycons,[a] mg/g	
	kaempferol	quercetin
Brewer's Gold	0.82	0.52
Bullion	0.84	0.32
Challenger	0.89	0.85
Comet	1.25	0.79
Fuggles	1.32	1.44
Northern Brewer	1.53	0.86
Pride of Ringwood	1.62	0.88
Talisman	0.87	1.32
Wye Target	1.63	1.30
mean	1.20	0.92

[a] Measured after hydrolysis of flavonol glycosides.

varied from 0.26 to 0.93 mg/g and averaged 0.75 mg/g. It was established, however, with five samples from a batch of Northern Brewer hops (1980) that intrabatch variations in flavonol contents were within the limits of accuracy of the assay method. Discounting the possibilities for bona fide varietal differences, it is clear that different batches of hops did differ in flavonol contents. In many breweries it is the custom to blend different batches of hops to maintain consistency in the raw materials used. From this it follows that less variation may be encountered in the hop grists actually used for brewing than would be indicated by Table II.

Flavonols in Worts and Beer. After sweet worts were boiled with a mixed hop grist (2 g/L) for 2 h, the resulting hopped wort contained flavonol glycosides that after hydrolysis yielded a total flavonol content of 1.2 mg of quercetin/L and 2.0 mg of kaempferol/L. Boiling in wort, therefore, extracted 88% of the quercetin glycosides and 91% of the kaempferol glycosides present in the hops. Fermentation of the worts decreased the contents of flavonol glycosides as quercetin and kaempferol to 0.5 and 0.95 mg/L, respectively, but storage of beer in bottles for up to 2 months produced no further losses in flavonols. The contents of flavonols in different beers would seemingly depend, therefore, largely on the rates of hop addition to worts, a specificaion that can vary widely throughout the world.

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Analysis of Oxalic Acid in Carambola (*Averrhoa carambola* L.) and Spinach by High-Performance Liquid Chromatography

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Oxalic acid was quantified in 15 Florida-grown carambola cultivars by a simple and rapid high-performance liquid chromatographic (HPLC) method using a propylamine column and aqueous sodium dihydrogen phosphate as the eluant. Oxalic acid levels varied almost 10-fold (from 0.08 to 0.73 g/100 g of fruit) among the cultivars tested. The generality of the method for determining oxalic acid in other food products was shown by analysis of fresh spinach.

The carambola is grown in South Florida primarily as an ornamental dooryard tree that produces fruit of unique flavor and relatively high ascorgic acid content. Smaller amounts of carambola are grown for the fresh fruit market and for export to Europe (Wagner et al., 1975). Commercial acceptance has been limited because the fruit is susceptible to shipping damage, it requires storage below 70 °F to maintain optimum quality during shipment (Grierson and Vines, 1965; Vines and Grierson, 1966), and it has an oxalic acid content that is comparable to that of spinach, rhubarb, and other foods known to have relatively high levels of this acid (Wagner et al., 1975; Singh, 1973; Zarembski and Hodgkinson, 1962b). There is a need for more information on the oxalic acid content of different carambola cultivars to see if certain cultivars might combine low oxalic acid content with other favorable marketing characteristics.

Several lengthy methods for determining oxalic acids in foods have been reported. These methods all involve the same basic steps [summarized by Zarembski and Hodgkinson (1962a)]: extraction of oxalic acid from the plant material with hydrochloric acid (HCl) or with sodium carbonate and overnight precipitation of calcium oxalate, followed by treatment of the precipitate with dilute sulfuric acid to form a solution of oxalic acid. The oxalic acid was then quantified by one of the following methods: potentiometric with a quinhydrone electrode (Pucher et al., 1934), titration with standard potassium permanganate (Baker, 1952), or colorimetric by conversion of oxalic acid to glycolic acid and derivitization with 3,6-dihydroxynaphthalene-2,7-disulfonic acid (Zarembski and Hodgkinson, 1962a).

In the above methods conflicting results for complete oxalic acid extraction were found, depending on whether hot or cold HCl was used to extract plant material and whether fresh plant material or dry finely divided sample was extracted. Baker (1952) used hot 1 N HCl to extract oxalic acid from plant material, and he confirmed a previous observation by Hoover and Karunairatnam (1945) that oxalic acid was stable for several hours in hot 1.5 N HCl. He also concluded that drying plant material at 100 °C for 24 h resulted in appreciable loss of oxalic acid. Conversely, Zarembski and Hodgkinson (1962a) reported abnormally high oxalic acid values from foods extracted with hot rather than cold HCl and proposed the major source for increased oxalic acid was carbohydrate degradation. These authors recommended extraction with cold HCl and showed that at least 3 N HCl was required for complete extraction of oxalic acid from most foods. They also reported oxalic acid recoveries of 96–100% from dry finely divided foods with negligible conversion of carbohydrates and other components to oxalic acid.

High-performance liquid chromatography (HPLC) using reverse-phase or anion-exchange columns with organic buffers affords a rapid and sensitive method for analyzing certain mixtures of organic acids in foods (Buslig et al., 1982; Shaw and Wilson, 1981). Oxalic acid, in particular, was well separated from other organic acids normally found in foods on a propylamine column with an aqueous buffer (Buslig et al., 1982).

The current study reports a rapid and simple method for quantification of oxalic acid in foods using HPLC with a propylamine column and sodium dihydrogen phosphate buffer as the eluent. The method was used to determine oxalic acid content in 15 carambola cultivars and in one fresh spinach sample as well.

EXPERIMENTAL SECTION

Carambolas were obtained from the U.S. Subtropical Horticultural Research Station, Miami, FL. Samples were picked in Sept 1981, and the fruit were ripened for 1 week to a uniform yellow color at 21 °C and then kept at −18 °C until extracted. Fresh spinach was purchased at a local market.

Preparation of Samples. Carambola fruit or spinach leaves (500–600 g from five to six fruit) were cut into small pieces and the pieces thoroughly mixed. Then, duplicate 100-g portions were removed from the composite sample, and each 100-g sample was mixed in a blendor with 100

U.S. Citrus and Subtropical Products Laboratory, Southern Region, U.S. Department of Agriculture, Agricultural Research Service, Winter Haven, Florida 33880 (C.W.W. and P.E.S.), and U.S. Subtropical Horticultural Research Station, Southern Region, U.S. Department of Agriculture, Agricultural Research Service, Miami, Florida 33158 (R.J.K.).