Skrypec, D. J.; Gray, J. I.; Mandagere, A. K.; Booren, A. M.; Pearson, A. M.; Cuppett, S. L. *Food Technol. (Chicago)* **1985**, *39*(1), 74.
Sleeth, R. B.; Theiler, R. F.; Rendek, R. B. U.S. Patent 4314948, 1982.
Theiler, R. F.; Sato, K.; Asperlund, T. G.; Miller, A. F. *J. Food Sci.* **1984**, *49*, 341.
Toth, L.; Potthast, K. *Adv. Food Res.* **1984**, *29*, 87.
Walker, E. A.; Pignatelli, B.; Friesen, M. *J. Sci. Food Agric.* **1982**, *33*, 81.

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Solid-Phase Extraction and HPLC Determination of β-Cryptoxanthin and α- and β-Carotene in Orange Juice

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A simplified method is presented to determine three important orange juice carotenoids. Nonaqueous reversed-phase (NARP) high-performance liquid chromatography (HPLC) employing acetonitrile–methylene chloride–methyl alcohol (65:25:10, v/v) and a C-18 column was used to separate β-cryptoxanthin and α- and β-carotene from other juice components. Detection was at 450 nm. A photodiode array detection (PDAD) system was used to obtain UV–visible spectra for chromatographic peak identification and to determine chromatographic peak purity. Methanol was used to extract the carotenoids from centrifuged juice solids. A C-18 solid-phase extraction column was used to clean up the saponified sample prior to HPLC analysis. Ratios of β-carotene to α-carotene and β-cryptoxanthin to β-carotene from repeated (n = 5) injections were determined with relative standard deviations of 3.3 and 2.6%, respectively. Average carotenoid ratios for Valencia orange juice were 1.08 and 2.31, respectively. Murcott juices had average carotenoid ratios of 18.1 and 5.8, respectively.

INTRODUCTION

Certain carotenoids such as β-cryptoxanthin and α- and β-carotene are highly colored compounds that also exhibit provitamin A activity. β-Carotene has the highest vitamin A activity because it can be split through central enzymatic cleavage (at least in theory) into two molecules of vitamin A. Due to structural differences in part of the molecule, only half of each molecule of α-carotene, γ-carotene, and β-cryptoxanthin has the required intact β-ionone ring structure for vitamin A activity (Goodwin, 1980). Therefore, these carotenes exhibit lower vitamin A activity. Nevertheless, these carotenes are important dietary sources of vitamin A.

Carotenoids, located in juice vesicle plastids, are also the major source of color in orange juice and as such are an important quality factor. Orange juices with the expected deep yellow–orange color are preferred by most consumers and may even be perceived as being sweeter. The U.S. Department of Agriculture has set minimum color standards for orange juice and allots 40 of 100 quality points to juice color (USDA, 1983). In this grading system, color is weighted just as heavily as flavor.

Specific carotenoid patterns can be used to characterize different citrus cultivars and hybrids (Gross, 1977). Carotenoid patterns may have potential for use by citrus taxonomists to differentiate between different cultivars. However, before these patterns can be used with any certainty, a simple, accurate analytical method must be developed so that large numbers of samples can be analyzed and the natural variation in carotenoid levels determined.

Due to the relatively recent increase in the price of orange juice, certain juice processors have been tempted to dilute orange juice with inexpensive components such as sugars, citric acid, or washed orange solids (pulp wash) (Attaway, 1982). The net effect of these additions would be to reduce the natural yellow–orange color of orange juice. In order to mask the adulteration of orange juice, a coloring agent must also be added. Since β-carotene occurs naturally in orange juice, is commercially available, and is relatively inexpensive, it is a natural candidate to increase the color of adulterated juice to that of natural levels. At present, no rapid, simple method exists to determine this type of adulteration.

Whether an individual is interested in citrus carotenoids due to nutrition, taxonomy, or regulatory or quality control concerns, the need exists for a relatively simple, rapid procedure for determining citrus carotenoids that would lend itself to routine analysis.

The chromatographic isolation and identification of carotenoids are well represented in the literature (Nells and De Leenheer, 1983; Ritacco et al., 1984; Stewart and Wheaton, 1973; Calabro et al., 1978; Hsieh and Karel, 1983; Noga and Lenz, 1983; Will and Ruddat, 1984; Kamikura, 1961; Bushway and Wilson, 1982; Stancher and Zonta, 1982; Tsukida et al., 1982 and references therein). However, these procedures are not well suited for routine analysis often due to lengthy sample preparation procedures and the use of solvent gradients.

Sample preparation is the current bottleneck in citrus carotenoid analysis. All of the current procedures require lengthy liquid–liquid and or liquid–solid extractions. Artifact formation is a concern in any sample preparation

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procedure. Stewart and Wheaton (1973) reported that acetone used to extract carotenoids, particularly in a basic medium, tends to form Aldol condensation artifacts with aldehydic and ketonic carotenoids. Some procedures such as that of Noga and Lenz (1983) use an overnight saponification and an extraction system employing acetone and diethyl ether. It is well-known that diethyl ether can contain peroxide impurities that would rapidly react with carotenoids to produce artifacts.

Therefore, the object of this work was twofold. The first object was to develop a relatively simple, rapid sample preparation procedure that would minimize artifact formation. The second objective was to determine the optimum chromatographic conditions to separate the major nonpolar carotenoids in processed orange juice that would be suitable for routine analysis.

MATERIALS AND METHODS

Apparatus. A Model ALC 202 high-performance liquid chromatograph with a Model 6000 A pump, automated sample injector (Wisp, Model 710B), data module (Model 730), and a system controller (Model 720) (Waters Associates, Milford, MA) were used. A Spectro Monitor D variable-wavelength detector (Laboratory Data Control, Rivera Beach, FL) was used for routine peak detection. A Hewlett-Packard Model 1040A HPLC photodiode array detector system with a plotter (HP 7470A) and flexible disk drive (HP 89201M) (Hewlett-Packard, Palo Alto, CA) were used for real-time spectral data acquisition. A Beckman spectrophotometer (Model UV-5260) was used to obtain absorbance spectra of collected peaks and standards. An ultrasonic cleaner (Cole-Parmer, Model 8845-6), an international clinical centrifuge (Model CL), and a Sorvall Omni-Mixer (Model 17150) (Newton, CT) with a 6 mL microattachment and a 6 mL C-18 extraction column (J. T. Baker Chemical Co., Phillipsburg, NJ) were used in sample preparation.

Standard Carotenoids. Authentic α- and β-carotenes (Sigma, St. Louis, MO) were recrystallized from benzene–methanol (1:3). β-Cryptoxanthin was isolated from citrus juice (Stewart and Wheaton, 1973). The above three carotenoids were examined in the visible region on an HP 1040A to verify purity and identity.

Column and Eluting Systems. A Du Pont Zorbax ODS analytical column (4.6 mm × 250 mm) (Wilmington, DE) was used with the following solvent systems: (A) acetonitrile–methylene chloride–methanol (65:25:10); (B) acetonitrile–methylene chloride–methanol (70:20:10); (C) acetonitrile–tetrahydrofuran–methanol (70:20:10); (D) acetonitrile–ethyl acetate–methanol (70:20:10). Solvents were HPLC grade purchased from J. T. Baker Chemical Co. Solvent systems were prepared on a volume to volume basis and degassed with an ultrasonic cleaner.

Chromatographic Conditions. Sample injection volume was usually 50 μL. The eluant was monitored at 450 nm with an attenuation of 0.005 AUFS. Recorder chart speed was 0.50 cm/min. Column temperature was 22–24 °C. Flow rate was 1.5 mL/min. After several injections, the column was washed with methylene chloride and then equilibrated with the mobile phase.

Sample Preparation. A 10-mL sample of single-strength orange juice was centrifuged for 5 min at 3170*g*. This produced a pellet containing the carotenoids. After the clarified juice was decanted and discarded, the wet pellet was stirred with 2.0 mL of methanol. The slurry was recentrifuged as described above and the supernatant discarded. Using the Omni-Mixer at a setting of 3.5 for 1 min at 10 °C, the pellet was extracted with a second portion of methanol (3.0 mL). The resulting slurry was centrifuged as above and the supernatant saved. This operation was repeated two more times, and the methanolic extracts were combined. All 9.0 mL from the latter methanol extractions was used in the saponification reaction. All work with extracted carotenoids was done under nitrogen in subdued light.

Saponification. Methanolic potassium hydroxide (4.5 mL; 10.0 g/100 mL methanol) was added to the 9.0 mL of methanolic extract and the resultant mixture allowed to stand in the dark without stirring for 1.0 h at 23 °C. The yellow reaction mixture was transferred to a 125-mL separatory funnel with 30 mL of methylene chloride. The potassium hydroxide was removed by washing with 4 × 20 mL of water. The yellow organic layer containing the carotenoids was evaporated to dryness under nitrogen at 35 °C.

Sample Cleanup. The above residue was dissolved in 2.0 mL of methanol and placed on the top of a 6-mL C-18 extraction column preconditioned with 5 mL of methanol. A syringe with an adapter was used to push the liquid slowly through the column. The absorbed carotenoids were then eluted with 4.5 mL of methanol–water (95:5, v/v; fraction 1) followed by 3.0 mL of methylene chloride (fraction 2). Each fraction was separately evaporated to dryness. The resulting two residues were each redissolved in 2.0 mL of the chromatographic mobile phase and placed in an amber 4-mL vial prior to injection.

Recovery and Precision. Six identical samples of Natal orange juice from Brazil were fortified with sufficient β-carotene to provide concentrations of from 1 to 6 μg/100 mL of juice in 1-μg increments. Each sample was analyzed in triplicate. Five replicate 10-mL samples from the same orange juice were analyzed to determine analytical precision on a given day. Both the β-carotene to α-carotene and β-cryptoxanthin to β-carotene ratios were calculated for each of the five samples.

Chromatographic Peak Purity and Identification. The photodiode array detector was used to determine the purity of chromatographic peaks of interest during the entire chromatogram. Every 320 ms the spectrum from 350 to 550 nm was recorded and stored. Optical bandwidth was 4 nm. Peak purity was established by recalling the spectra that were obtained at the upslope (at peak half-height), apex, and downslope (at peak half-height) against a base-line reference. The spectra were normalized and overlaid for comparison. Peak purity was further established by obtaining multiple signal plots at 480, 450, 420, and 400 nm against a 570-nm optical reference with spectral bandwidths of 20 nm.

RESULTS AND DISCUSSION

HPLC Procedure. The isocratic separation of Nells and De Leenheer (1983) was modified by increasing the solvent strength and solvent flow rate. Methylene chloride content was increased from 20 to 25% and acetonitrile content decreased from 70 to 65%. Flow rate was increased from 1.0 to 1.5 mL/min. Excessive solvent back-pressure, usually associated with increased flow rate, was not a problem due to the relatively low viscosity of this solvent system. At a flow rate of 1.5 mL/min, the solvent back-pressure is normally only 1200 psi. The net effect of these changes was a slight (but acceptable) loss of resolution in order to achieve an adequate separation in the shortest time possible.

Since the carotenoids of interest were of similar polarity and since our goal was to develop a method suitable for routine use, we elected to employ an isocratic rather than a gradient system for our HPLC analysis. Using an isocratic system, we were able to maximize resolution between

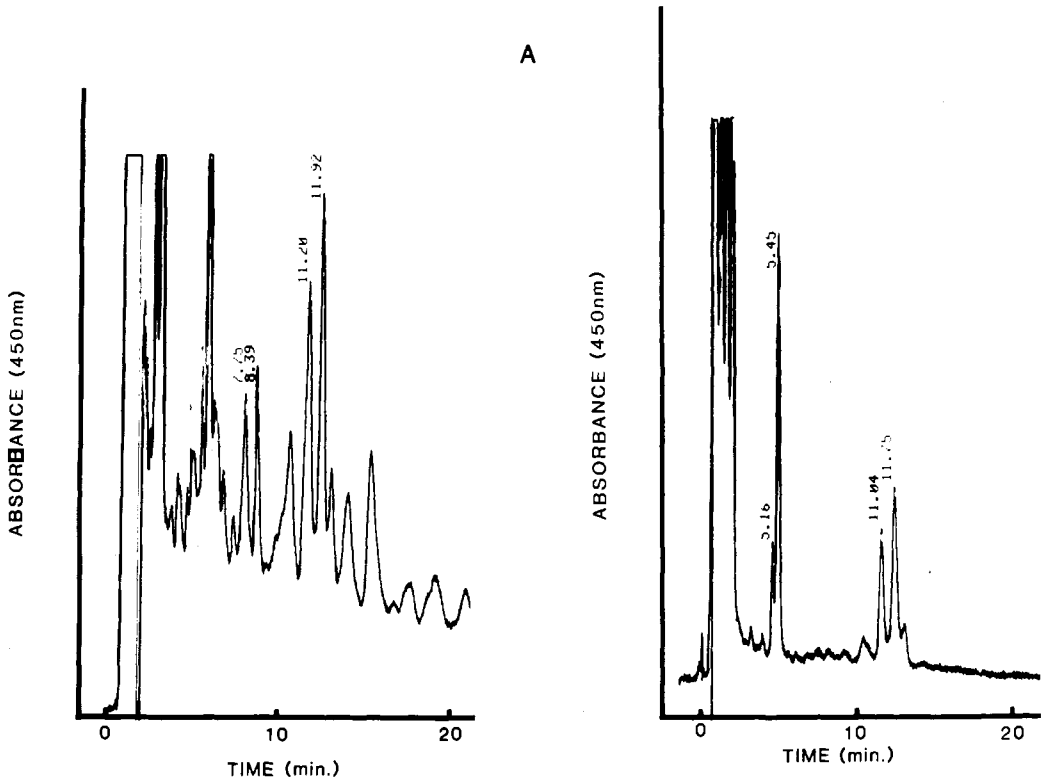

Figure 1. Chromatogram A: Fraction 1 from a juice sample showing the elution of the more oxygenated xanthophylls. For experimental details, see the text. Chromatogram B: Fraction 2 showing the elution of the less oxygenated xanthophylls including β-cryptoxanthin at a retention time of 5.79 min and the hydrocarbon carotenoids such as α- and β-carotene at 11.68 and 12.45 min, respectively.

the nonpolar carotenoids of interest and still minimize overall analysis time as it was not necessary to equilibrate the column after each injection. Using the identical column, we observed better resolution between α- and β-carotene with the current isocratic system than with the gradient solvent system of Noga and Lenz (1983). Furthermore, gradient systems are subject to greater fluctuations in retention times and require additional hardware. Neither factor is desirable for a routine procedure.

Sample Preparation. A sample preparation procedure was developed to maximize recovery and still remove as many interferences as possible. The first washing of the wet pellet with methanol removes most of the water and only the most polar materials. Cryptoxanthins or carotenes were not extracted in the first methanol extraction apparently due to the presence of water associated with the pellet. The second methanol extract solubilized the carotenoids.

Saponification was used early in the sample preparation to simplify the observed chromatograms. Figure 1 compares chromatograms from the same sample before and after saponification (compare parts A and B). Reaction times from 0.5 to 2.0 h in 0.5-h increments showed that under the above conditions 1.0 h was the optimum saponification time. Saponification times greater than 1.0 h produced smaller carotenoid peaks, thus suggesting decomposition was occurring. Saponification times less than 1 h failed to adequately reduce extraneous late eluting peaks, which are thought to be esters.

The first water wash from the saponified sample is yellow. This is probably due to the presence of chalcones from the reaction between the potassium hydroxide and juice flavanones, which is essentially the Davis test (Davis, 1947). Acidification of this yellow fraction resulted in a colorless solution, which is typical of chalcones but not of carotenoids.

All of the colored material from the saponified sample was eluted from the solid-phase extraction column in two fractions. Since the first wash eluted some of the colored material using a very polar solvent (95% methanol–5% water), the eluted material must also be very polar. The colored compounds in this fraction probably contains the very polar xanthophylls. The remaining colored material was eluted with methylene chloride. This latter fraction contained both the less polar xanthophylls such as the cryptoxanthins and the hydrocarbon carotenoids such as α- and β-carotene. If desired, it was found that the cryptoxanthins could be separated from the carotenes on the solid-phase extraction column by eluting with 97% methanol–3% water instead of methylene chloride.

Total Analysis Time. The time required from the collection of a single juice to final carotenoid concentration values is approximately 4 h. If additional samples are run concurrently, the analysis time *per sample* can be greatly reduced. This is a considerable improvement over existing methods. The majority of the time savings is realized from the 1-h saponification step as most of the current methods employ overnight saponification. Additional time savings are realized from the employment of an isocratic chromatographic system. With this system, several injections may be made before the column is washed and reequilibrated. Most existing chromatographic systems employ a solvent gradient that requires a 10–15-min reequilibration period between each injection.

Recovery and Precision. Natal orange juice β-carotene to α-carotene ratio values observed in the five reproducibility experiments ranged from 1.18 to 1.30 with a mean value of 1.23 and a standard deviation of 0.04. β-Cryptoxanthin to β-carotene ratios ranged from 1.48 to 1.53 with a mean value of 1.51 and a standard deviation of 0.04.

The six triplicate orange juice samples that were fortified with 1–6 μg of β-carotene in 1-μg increments gave average recoveries of 0.8, 1.7, 2.8, 3.6, 4.9, and 5.6 μg of β-carotene, respectively. Average recovery was 90% and ranged from

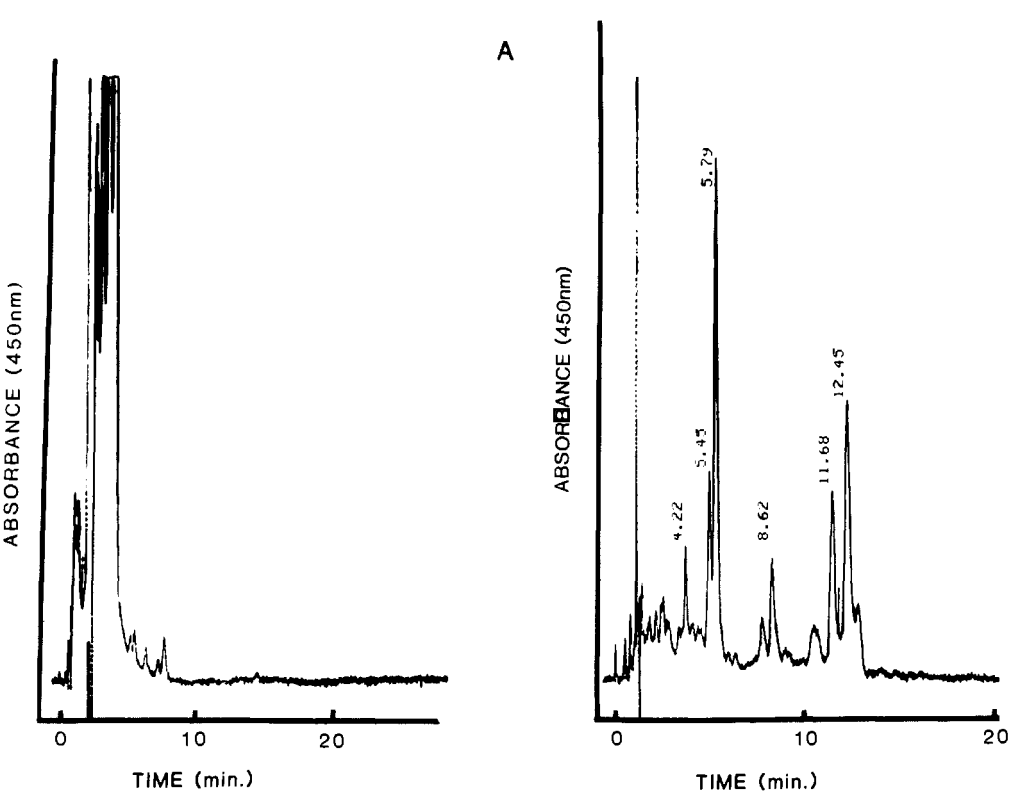

Figure 2. Chromatogram A: Methanolic extract of the pellet prior to saponification. Chromatogram B: Sample after saponification showing β-cryptoxanthin at 5.45 min and α- and β-carotene at 11.04 and 11.75 min, respectively. The attenuation and volume injected were the same for both chromatograms. Other experimental details are in the text.

80% at the lower concentrations to 98% at the higher concentrations.

Carotenoid Ratios from Different Juices. In three Valencia juices, β-carotene to α-carotene ratios ranged from 1.04 to 1.11 with an average of 1.08. β-Cryptoxanthin to β-carotene ratios of 2.28–2.38 with an average of 2.31 was observed. Five Murcott juices (a tangerine hybrid) had an average β-carotene to α-carotene ratio of 18.1, and individual values ranged from 17.6 to 18.9. β-Cryptoxanthin to β-carotene ratios of 5.3–6.2 with an average of 5.7 were observed.

Peak Purity and Identification. Recent reports (Miller et al., 1982; Elgass et al., 1983; George and Maute, 1982; George and Elgass, 1984; Martin et al., 1984; Jaffe, 1983) have described the application of PDAD for peak purity determination and peak identification in HPLC. In Figure 3, a comparison of the three spectra that were obtained at half upslope, apex, and half downslope of the peak eluting at 5.79 min in Figure 2B is shown. Since there is no change in spectral characteristics, it must be assumed that the chromatographic peak is due to a single compound or that an impurity would have the identical spectral characteristics. Multiple-signals plots for the same peak showed nonshifting retention times at four different wavelengths, which further supports the supposition of peak purity (Miller et al., 1982). Similar results were obtained for the peaks eluting at 11.68 and 12.45 min (Figure 2B). Therefore, these three peaks may be considered chromatographically pure.

Chromatographic peaks were identified by two independent means, chromatographic characteristics, and spectral characteristics. Samples fortified with known β-cryptoxanthin increased the peak height of the peak at 5.79 min in Figure 2B. Similar results were obtained with alternate solvent systems. The compounds responsible for the peaks at 11.68 and 12.45 min (Figure 2B) had coincident retention times, with authentic α- and β-carotene, respectively, in solvent systems A–D. Fortification experiments increased the peak heights of these peaks without altering respective retention times.

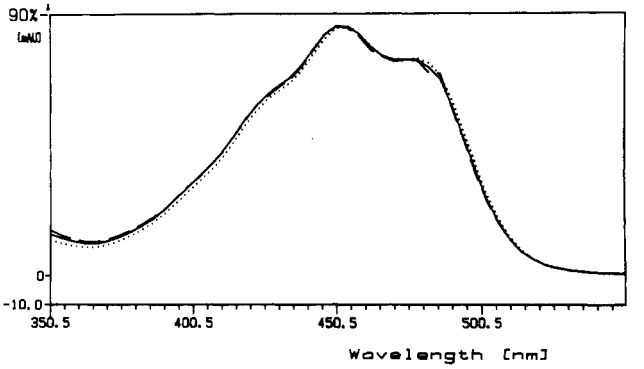

Figure 3. Peak purity experiments showing the three spectra obtained from the β-cryptoxanthin peak from Figure 2B at the upslope (···), apex (—), and downslope (– –) employing the photodiode array detector. For experimental details, see the text.

Identification of β-cryptoxanthin and α- and β-carotene was established from the wavelength of maximum absorbance, shape, and detail of the absorbance spectra. The photodiode array detector allows the entire visible spectra to be acquired in 10 ms, thus permitting qualitative characterization at different points in a single chromatograph peak. All spectra agreed exactly with those reported in the literature (DeRitter and Purcell, 1981) for these three carotenoids and with those obtained from purified standards.

Cis–Trans Isomers. A small absorbance band at 365 nm was observed in some β-carotene scans, indicating the minor presence of a cis isomer (Goodwin, 1980). Since all samples are exposed to the same conditions, any induced

cis–trans isomerization would be consistent from sample to sample and would be minimized due to the rapid sample preparation procedure.

Uses. This method is ideally suited for routine analyses of the major nonpolar carotenoids in orange juice. It is rapid and accurate and can be applied with a minimum amount of equipment. This method can be used to rapidly obtain nonpolar carotenoid data bases for orange juice nutrition, taxonomy, and regulatory or quality control studies.

Registry No. β-Cryptoxanthin, 472-70-8; α-carotene, 432-70-2; β-carotene, 7235-40-7.

LITERATURE CITED

Attaway, J. A. Proceedings of the New England Food and Drug Officials Association Conference, Hartford, CT, Nov 1982.
Bushway, R. J.; Wilson, A. M. *Can. Inst. Food Sci. Technol. J.* **1982**, *15*, 165.
Calabro, G.; Micali, G.; Curro, P. *Essenze Deriv. Agrum.* **1978**, *48*, 359; *Chem. Abstr.* **1980**, *93*, 6195*j*.
Davis, W. B. *Anal. Chem.* **1947**, *19*, 476.
DeRitter, E.; Purcell, A. E. In *Carotenoids as Colorants and Vitamin A Precursors*; Bauernfeind, J. C., Ed.; Academic: New York, 1981; p 883.
Elgass, H.; Maute, A.; Martin, R.; George, S. *Am. Lab.* (*Fairfield, Conn.*) **1983**, *15*(9), 71.
George, S. A.; Elgass, H. *Am. Lab.* (*Fairfield, Conn.*) **1984**, *16*, 24.
George, S. A.; Maute, A. *Chromatographia* **1982**, *15*, 419.
Goodwin, T. W. *The Biochemistry of the Carotenoids*; Chapman and Hall: New York, 1980; Vol. 1.
Gross, J. In *Citrus Science and Technology*; AVI Publishing: Westport, CT, 1977; Vol. 1, p 302.
Hsieh, Y. C.; Karel, M. *J. Chromatogr.* **1983**, *259*, 515.
Jaffe, H. *LC Mag.* **1983**, *1*(7), 418.
Kamikura, M. *Bull. Natl. Inst. Hyg. Sci.* (*Tokyo*) **1981**, *99*, 147.
Martin, R.; Elgass, H.; George, S. A. *Am. Lab.* (*Fairfield, Conn.*) **1984**, *16*(9) 34.
Miller, J. C.; George, S. A.; Willis, B. G. *Science* (*Washington, D.C.*) **1982**, *218*, 241.
Nells, H. J. C. F.; De Leenheer, A. P. *Anal. Chem.* **1983**, *55*, 270.
Noga, G.; Lenz, F. *Chromatographia* **1983**, *17*, 139.
Ritacco, R. P.; Britton, G.; Simpson, K. L. *J. Agric. Food Chem.* **1984**, *32*, 301.
Stancher, B.; Zonta, F. *J. Chromatogr.* **1982**, *238*, 217.
Stewart, I.; Wheaton, T. A. *Phytochemistry* **1973**, *12*, 2947.
Tsukida, K.; Saiki, K.; Takii, T.; Koyama, Y. *J. Chromatogr.* **1982**, *245*, 359.
USDA *United States Standards for Grades of Orange Juice*; ARS, Fruit and Vegetable Division, Processed Products Branch: Washington, DC, Amended 1983.
Will, O. H.; Ruddat, M. *LC Mag.* **1984**, *2*, 610.

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Production of Oxalic Acid from Dry Powder of *Parthenium hysterophorus* L.

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The process of preparing oxalic acid from dry powder of *Parthenium hysterophorus* L. is described. Favorable conditions for the production of oxalic acid in moderately high yield include conducting the oxidation of the plant material with a mixture of concentrated nitric and sulfuric acids (50:50) at a solid to liquid ratio of 1:10 (w/v), addition of ammonium vanadate catalyst at 0.005% of the oxidizing mixture, careful temperature control at 75 °C, and reaction period of 4 h. The purity of recovered oxalic acid was around 98%.

INTRODUCTION

Oxalic acid has a widespread industrial applications. It is extensively used as a souring agent to neutralize the residual alkalanity of washed fabrics. It is included in cleaning solutions intended for the removal of ink markings, stains, and discolorations caused by bleeding of dyes. The textile industry makes use of it for dye stripping of wool, degumming of silk, printing of cotton, cleaning and dyeing of fabrics, and bleaching plant fibers. A large-scale use for oxalic acid is in acidic formulations for removal of rust and scale. Other applications include whitening of leather, formulation of metal polishes, refining of tall oil, manufacture of dyes and numerous other chemical products as an intermediate, and many more.

Oxalic acid can be manufactured by four general methods that are based on the nature of raw material selected (Kirk and Othmer, 1967). These methods are (i) alkali fusion of cellulose, (ii) fermentation process for carbohydrates, (iii) synthetic process from formates, and (iv) oxidation by nitric acid. Among these methods, the latter two have been considered important for the commercial route to oxalic acid. However, the most widely acceptable commercial method for the manufacture of

Table I. Effect of Reaction Conditions on Yield of Oxalic Acid from *P. hysterophorus* L. as Raw Material

variable		yield,[a] g
nitric to sulfuric acid ratio,[b] mL	80:20	4.85 ± 0.08
	70:30	5.60 ± 0.12
	60:40	5.92 ± 0.10
	50:50	6.18 ± 0.17
reactn time,[c] h	2	5.20 ± 0.13
	4	5.80 ± 0.11
	6	5.47 ± 0.08
	8	5.32 ± 0.10
reactn temp,[d] °C	55	5.10 ± 0.12
	65	5.30 ± 0.15
	75	5.84 ± 0.11
	85	4.40 ± 0.15

[a] Average of four experiments using 10 g of plant material. [b] Reaction conditions: vanadate catalyst, 75 °C, 4 h. [c] Reaction conditions: vanadate catalyst, acid ratio 60:40, 75 °C. [d] Reaction conditions: vanadate catalyst, acid ratio 60:40, 4 h.

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