(Nickerson, 1974). If the solubility is a major factor, this difference should cause appreciable differences in the amount of lactose precipitated at 5 and 25 °C. This was not the case as shown in Table IV.

The texture of the precipitate, slimy and gelatinous, and the large amount of water in it imply that the precipitate is actually a colloid—with the nature of a heavy suspension at nonoptimal conditions and approaching gel-like properties at optimal conditions for lactose precipitation. The relatively increased solubility of $Fe(OH)_2$ in solution does indicate that a soluble adduct lactose·$Fe(OH)_2$ exists with a solubility greater than that of $Fe(OH)_2$ and less than that of lactose. This argues that the precipitate consists primarily of a lactose·$Fe(OH)_2$ adduct, free $Fe(OH)_2$, and water.

The increased recovery of lactose in the precipitate using $Fe(OH)_2$ when compared with the results using $Ca(OH)_2$ (McCommins et al., 1980) and $Mg(OH)_2$ (Kwon, 1979) indicate that the amount of complex precipitated may be a function of the relative insolubility of the metal hydroxide. The percentage of lactose recovered decreases in the order $Fe(OH)_2 > Mg(OH)_2 > Ca(OH)_2$, which can be correlated with increasing solubilities through this series [$Fe(OH)_2 < Mg(OH)_2 < Ca(OH)_2$]. From this one can argue that the best metal hydroxide for lactose precipitation would be the one least soluble in water.

The lack of precipitation reported by Charley et al. (1962) with fructose and iron(II) even at highly alkaline pHs after addition of NaOH may be due to the difference in binding reported by Davis and Deller (1966). The 100-fold stronger iron binding ability of fructose may in some manner enable the complex to remain soluble after the addition of NaOH as compared with the relatively small chelating ability of lactose in which case the insolubility of $Fe(OH)_2$ predominates—forcing the lactose out of solution with the $Fe(OH)_2$.

The adduct model of Moulik and Khan (1975) is compatible with the findings of this study. Using their model, the incomplete conversion to $Fe(OH)_2$ at molar ratios of $NaOH/FeCl_2 < 2$, could be rationalized for there is a variety of iron(II) species in solution. This could account for the additional time necessary for the reaction to reach completion at these ratios.

The total amount of lactose precipitated is a function of the solubility of the metal hydroxide and the solubility of the adduct formed. This is in agreement with the work of McCommins et al. (1980). They found that the increased surface area provided by the addition of $CaCO_3$ for possible adsorption in the reaction mixture did not increase the amount of lactose recovered when using $Ca(OH)_2$.

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Received for review August 17, 1979. Accepted December 26, 1979. This research was supported by a grant from the Dairy Council of California.

# Quantitative Determination of Individual Betacyanin Pigments by High-Performance Liquid Chromatography

Steven J. Schwartz and Joachim H. von Elbe*

A preparatory high-performance liquid chromatography (LC) method is employed to obtain crystalline betacyanin pigments. The betanin and betanidin hydrochloride molar absorptivities ($\epsilon$) were determined to be 65 000 and 54 000, respectively. Spectrophotometric evidence and an enzymatic method were used to unequivocably identify peaks obtained from LC chromatograms. Pure solutions of pigment were used to calibrate an analytical LC apparatus to quantitate pigment mixtures. By employing visible detection at 535 nm, colorants are selectively screened for the red pigments. This method can accurately quantitate individual pigments rapidly without interference from other components.

Concern over the questionable safety of synthetic red food dyes as additives has prompted research in the use of natural pigments as food colorants. Water soluble extracts or powders of the red beet (*Beta vulgaris*) have been shown to be useful alternatives (Pasch et al., 1975; von Elbe, 1977).

Methods for the production of highly pigmented con-

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0021-8561/80/1428-0540$01.00/0 

centrates by fermentation of beet juice and/or purification of beet juice by gels have increased the feasibility of betalains as food dyes (Adams et al., 1976; Adams and von Elbe, 1977). Techniques to identify degradation products and quantify pigment components rapidly are needed to supplement further research in this field.

Previous methods for isolation of betalains involve paper electrophoresis with quantification by spectrophotometry (Nilsson, 1970) or densitometry (von Elbe et al., 1972). Larger amounts of betalains have been obtained and quantified by chromatography on gels (Adams and von Elbe, 1977). A computer aided determination of beet pigments has been developed by Saguy et al. (1978). Separation and quantification by high-performance liquid chromatography (LC) has also been shown to be a feasible method (Vincent and Scholz, 1978).

The purpose of this paper is to establish a rapid LC method to separate betacyanin pigments and quantify individual components. This technique will monitor changes in pigment composition and quantitatively account for pigment content in commercial colorant concentrates, purified mixtures, and partially degraded samples.

## MATERIALS AND METHODS

**Preparation of Betanin and Betanidin.** Betanin and betanidin were prepared by a modified method of Adams and von Elbe (1977). Following chromatography with 1% acetic acid on Sephadex G-25, the betacyanin fractions were freeze-dried. The resulting material was dissolved with a minimum volume of doubly distilled water, filtered through a 0.45-$\mu$m-HA-Millipore filter (Millipore Corp., Bedford, MA), and chromatographed by preparatory LC. The betanin and betanidin fractions (50 mL each) were collected. The betanin fraction was placed in centrifuge tubes and held at −15 °C for 2.5 h to initiate crystallization. The crystals were collected by centrifugation after the samples were thawed at 25 °C. Recrystallization in 0.1% HCl at 25 °C gave needles of betanin hydrochloride.

The betanidin fraction was lyophilized, dissolved in 0.1% HCl, and held at −15 °C, and the microcrystals were collected by centrifugation. Recrystallization in 0.1% HCl at 25 °C gave pure betanidin hydrochloride. The LC chromatograms of betanin and betanidin exhibited a single peak when monitored at either 535 or 210 nm. UV-visible spectra and molar absorptivity values for each pigment were determined and compared with known data (Wyler and Dreiding, 1959; Wilcox et al., 1965).

**High-Performance Liquid Chromatography.** All LC separations were run at ambient temperature. Solvents and reagents were of analytical grade, and buffers were prepared in doubly distilled water.

Preparatory apparatus consisted of a Model 6000A pump, a fixed wavelength (254 nm) UV detector, and a Model U6K injector equipped with a 10-mL injection loop (Waters Associates, Milford, MA). Ten milliliter samples were chromatographed on four 7.8 mm i.d. × 61 cm Bondapak $C_{18}$/Porasil B columns, particle size 37–75 $\mu$m, (Waters Associates, Milford, MA), connected in series. The eluant was $CH_3OH$/0.05 M $KH_2PO_4$/HOAc (17.8:81.2:1.0, v/v/v), adjusted to pH 2.75 with phosphoric acid. The flow rate was set at 8.0 mL/min.

Analytical apparatus consisted of two Model 6000A pumps, solvent programmer Model 660 (Waters Associates, Milford, MA), a Model 70–10 injector (Rheodyne, Berkley, CA) with a 20-$\mu$L injector loop, and a variable wavelength detector fitted with a 8-$\mu$L flow cell, Model 55 (Perkin Elmer). The chromatograms were monitored at 535 nm. The column ($\mu$Bondapak $C_{18}$, particle size 10 $\mu$m) was

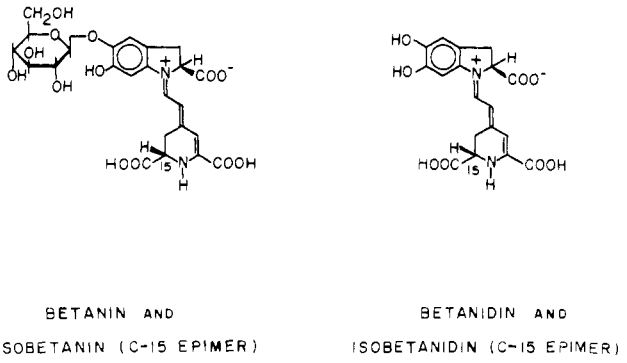


**Figure 1.** Structure of betanin, betanidin, and their C-15 epimers.

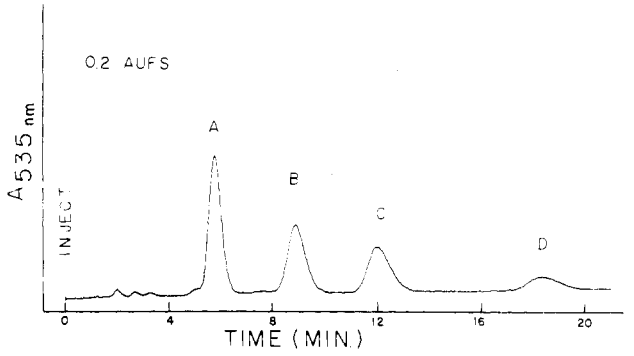


**Figure 2.** Isocratic LC chromatogram of betacyanin pigments after gel filtration.

developed isocratically with $CH_3OH$/0.05 M $KH_2PO_4$ (18:82, v/v), adjusted to pH 2.75 with $H_3PO_4$ (solvent A). The solvents for gradient elution separations were as follows: initial, 100% solvent A; final, 80% solvent A, 20% $CH_3OH$ solvent B. Curve 8 on the programmer was followed for a duration of 9 min.

## RESULTS AND DISCUSSION

**Identification of Pigment Peaks.** The structure of betanin, betanidin, and their C-15 epimers are shown in Figure 1. The chemistry and natural abundance of the betalains in plants has been reviewed by Mabry (1970). Although betanidin and isobetanidin are minor pigments in the beet root, greater quantities of the aglycons may be present in purified beet juice mixtures. Presently, beet colorants are produced by countercurrent extraction of beet slices or pressing of beet pulp. Fermentation of beet juice solids to obtain more highly pigmented colorants has been suggested (Adams et al., 1976). Monitoring all betacyanin components during these processes is important to determine whether or not changes in composition do occur.

A typical analytical LC separation of the betacyanin fraction after gel filtration is shown in Figure 2. Resolution of these four pigments is complete in 20 min. A more rapid separation can be achieved by gradient elution, shown in Figure 3. Increasing $CH_3OH$ concentration will speed the analysis and achieve elution of the pigments in 9 min. The smaller peaks in both chromatograms, eluting before betanin (peak A), are believed to be degradation products. These should not be confused with the vulgaxanthines, the yellow pigments of the red beet, which comprise approximately 30% of the total pigment and elute at similar retention times (Singer, 1978). Peaks A and B (Figure 2) are the more polar betacyanin glucosides, followed by the less polar aglycons (peaks C and D). This result is anticipated with reverse-phase chromatography. The configuration of the C-15 epimers, isobetanin (peak B) and isobetanidin (peak D) allows greater interaction

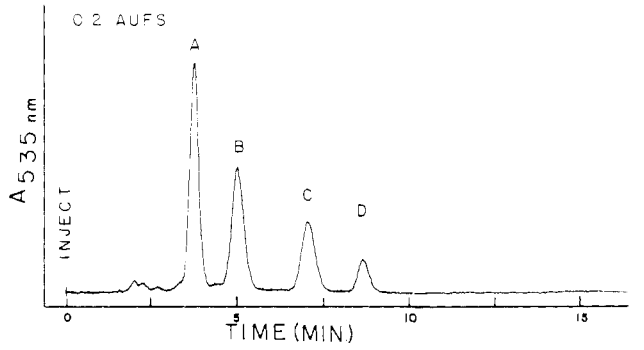


**Figure 3.** Gradient elution LC chromatogram of betacyanin pigments after gel filtration.

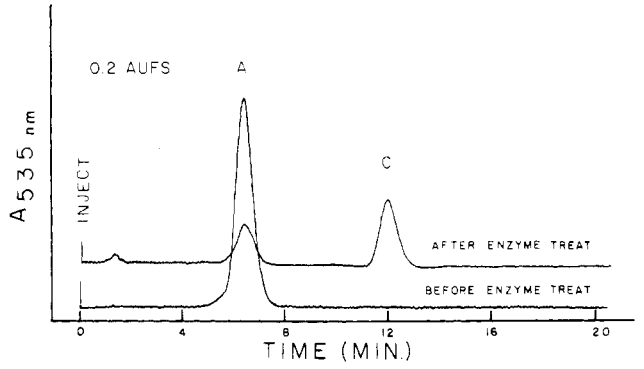


**Figure 4.** LC chromatograms of betanin before and after treatment with β-glucosidase.

with the stationary phase and therefore has a greater retention value relative to their parent.

The evidence in support of this identification is as follows. A pure solution of betanin (peak A, obtained by preparatory LC, $A_{max}$ = 535 nm), when subjected to enzymatic hydrolysis with β-glucosidase (Figure 4), at pH 5.0 and 37 °C, yields betanidin exclusively (peak C, $A_{max}$ = 542 nm). Isobetanin (peak B) if identically treated, gives rise to peak D (Figure 5). The specificity of almond emulsion (β-glucosidase) to the betacyanin glycons has been reported previously by Piattelli and Minale (1964a). These authors also found that epimerization to the C-15 isomer does not take place under the conditions for enzymatic hydrolysis.

Identical samples were chromatographed using the above method and that described by Vincent and Scholz (1978) using tetrabutylammonium phosphate (Pic A, Waters Associates, Milford, MA) buffer as solvents. The elution patterns of the chromatograms in Figures 2 and 3 and those developed with Pic A buffer were similar. Vincent and Scholz speculated the order of elution as betanin, betanidin, isobetanin, and isobetanidin, respectively. Their evidence was based on an acid hydrolysis of the glucosides to yield the aglycons. Previous authors have obtained mixtures of betanidin and isobetanidin when betanin was subjected to acid hydrolysis conditions (Piattelli and Minale, 1964a; Wyler and Dreiding, 1961). Observations in our laboratory have indicated that isomerization of betanin to isobetanin can occur. This isomerization has been reported previously by Piattelli and Minale (1964b), when a solution of betanin in 5% citric acid was allowed to stand at room temperature for 36 h. The conversion of the parent betacyanin to its C-15 epimer under acid conditions as performed by Vincent and Scholz (1978) may account for the discrepancy between our results and theirs.

**Quantitation of Betacyanins.** Quantitative methods

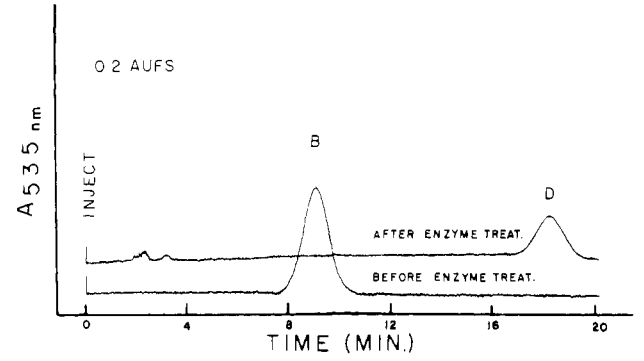


**Figure 5.** LC chromatograms of isobetanin before and after treatment with β-glucosidase.

for estimation of pigment content in beet extracts have been carried out successfully. Nilsson's method (1970) is designed to measure the pigment content of fresh beet root spectrophotometrically without separation of pigments. The betacyanin measurement is based on the 1% absorptivity value of 1120 for betanin, and further corrections are made for small amounts of interfering substances. This technique is not intended to measure pigment content in heat-treated, partially degraded, or stored colorant mixtures. Large amounts of degradation products will interfere with the spectrophotometric method. Since no prior separation of the pigments are made, this method includes all the minor betacyanins and reports these as betanin. Betanin and isobetanin usually comprise 95% of the betacyanin content in fresh beet juice (Nilsson, 1970); therefore for this purpose the technique presents little error. von Elbe et al. (1972) estimated the betanin content of beets by electrophoretic separation of the betacyanins with subsequent quantification employing densitometry. This method is time-consuming and requires several hours. Vincent and Scholz (1978) separated the pigments by LC and assumed a 1% absorptivity value ($E_{1cm}^{1\%}$) of 1120 for all betacyanins. Results are reported in terms of milligrams of betanin. All betacyanin pigments were measured spectrophotometrically at 538 nm in a single peak as they coeluted from the LC column, disregarding separation capability.

We propose a quantitative method utilizing separation of betacyanin pigments by LC and the application of their molar absorptivity values. This method will provide quantitation of individual pigment components and a more accurate determination of the total betacyanin content.

In previous estimations of total betacyanin content, authors have assumed the absorptivity value of betanidin to be equal to that of betanin. Our experiments determined molar absorptivity (ε) values for betanidin hydrochloride and betanin hydrochloride as 54 000 and 65 000. These results are in close agreement with reported values (Wyler and Dreiding, 1959; Wilcox et al., 1966). Converting the ε to $E_{1cm}^{1\%}$ results in values of 1275 and 1120. It is, therefore, more appropriate for quantitative purposes to apply the individual absorptivity values of each pigment component, particularly when betanidin comprises a significant portion of the pigment mixture. Isobetanidin and isobetanin are assumed to have identical molar absorptivity values as betanidin and betanin, respectively. Pure solutions of betanin and betanidin were appropriately diluted for injection on the LC. The isocratic LC conditions were employed for optimum reproducibility in detector response. A standard linear response of detector peak area vs. concentration was checked. The LC detector was then calibrated with known concentrations of betanin and betanidin vs. peak area.

This method may be extended to include quantification of the vulgaxanthines, the yellow pigments of the beet root. Singer and von Elbe (1980) have resolved the vulgaxanthines from interfering components using a strong anion-exchange (SAX) LC column. The chromatographer may envision the quantification of all betalain pigments, by coupling a $C_{18}$ reverse-phase column with an SAX column. This method may then be employed to analyze many commodities after sufficient sample cleanup, such as commercial colorant concentrates, fresh beet cultivars, or betalain colored food products.

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Received for review October 11, 1979. Accepted January 28, 1980. Research supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and with funds made available by General Foods Corporation, White Plains, NY.

# Radioimmunoassay of Limonin Using a Tritiated Tracer

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A radioimmunoassay is described for the determination of nanogram quantities of limonin in crude tissue extracts or juice. The detection limit of this assay, using tritium tracer, is 0.22 ng or 2.2 parts per billion of limonin and the processing capacity is several hundred samples per working day. Standard curves are linear over the wide range from 0.5 to 100 ng of limonin. No prior purification of extracts is necessary.

Limonin is the intensely bitter triterpenoid lactone present throughout the Rutaceae (Goodwin and Goad, 1970, and references therein) but is of major economic concern in certain citrus fruits and their processed products. The major contribution of limonin to deterioration of fruit and juice quality has been observed in California Navel Oranges, grapefruit, and certain other oranges (Maier and Dreyer, 1965; Wilson and Crutchfield, 1968; Barton et al., 1961). In addition, there are a large number of other limonoid compounds present in citrus (Dreyer, 1968; Bennett, 1971).

There have been a number of analytical methods developed for limonin (Fisher, 1978, 1973 and references therein) but in general they are comparatively slow, some are based on subjective evaluation and none are sensitive below the microgram range (ppm).

Recently we have developed and described a radioimmunoassay (RIA) which has a high specificity for limonin and a detection level of 0.071 ng which corresponds to 150 fmol or less than 1 ppb. This original test system was developed using an $^{125}I$ tracer which was stable for about 10–12 weeks (Mansell and Weiler, 1979). The object of the present work was to develop an alternative assay utilizing tritium tracer which has a much longer half-life. This alternative method would lessen the number of radioactive syntheses necessary but would retain the sensitivity and accuracy of the test system.

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## MATERIALS AND METHODS

**Apparatus.** A Model BF 5000 scintillation counter (Berthold Co., Wildbad) with 200 sample capacity and punched tape output was used for radioactivity determinations. All calculations were done on a HP 9825/9871 desk-top calculator/printer-plotter (Hewlett-Packard). Centrifugations were performed in a cooled Heraeus Cryofuge 6-6 with Analmatic 100 centrifuge head (Heraeus Co., Osterode). Pipetting was done using Eppendorf pipets.

**Materials.** Radioimmunoassays were performed in 63 × 12 mm glass tubes. For radioactivity determinations, standard scintillation vials modified to accomodate the 63 × 12 mm tubes were used.

**Chemicals.** Sodium [$^3H$]borohydride, sp act. 11.1 Ci/mmol, was purchased from New England Nuclear. The scintillator used was PCS (Amersham-Searle). Bovine serum was supplied by Mediapharm, Aschaffenburg (cat. no. SER Bo 1000). Phosphate-buffered saline (PBS, 0.1 M potassium phosphate, 0.15 M NaCl, pH 7.4) was used as RIA buffer. Concentrated ammonium-sulfate solution was prepared by stirring 800 g of $(NH_4)_2SO_4$ and 1000 mL of water overnight. After filtration, the pH was adjusted to 7.0 with concentrated NaOH. Standard limonin was a gift from Dr. James Fisher, Florida, Department of Citrus. Ten milligrams of limonin was dissolved in 1 mL

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