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Separation and Quantification of Red Beet Betacyanins and Betaxanthins by High-Performance Liquid Chromatography

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A high-pressure liquid chromatographic method has been developed for betacyanin and betaxanthin pigments in red beet extracts and liquid and powdered products. The method employs a reverse-phase microparticular C_{18} column, ion pairing, and a methanol–water mobile phase. Column chromatographic data indicates that configurational isomers of the principal betacyanins, betanin and betanidin, can be resolved through solvent gradient programming. The quantitative method for betanin, using prepared standards, has shown good linearity of response over a concentration range of 0.005 to 0.030%. Concentrations as low as 10–4% w/v are detectable. Recoveries of betanin were typically in the 95–99% range with relative standard deviations of 2% for replicate determinations.

The synthetic food colorants have come under considerable scrutiny lately by regulatory agencies as a result of the well-publicized studies questioning the safety of some of them. Red beet color extracts are currently under development as alternatives to certain of the FD&C red food color dyes for application in food systems. A number of processing modifications including conversion of beet juice to powders and concentrates and fermentation procedures for concentrating the colorants have enhanced the potency and applicability of beet extract as a food colorant (Adams and von Elbe, 1976). To assist in this development program, qualitative and quantitative methods for monitoring dyestuff composition in raw and finished products were sought.

Methods to date for separating and/or quantifying the major pigments or dyestuffs in beets have involved electrophoretic techniques (Nilsson, 1970; von Elbe et al., 1972), mainly for qualitative work, and spectrophotometry at multiple wavelengths, mainly for quantitative work (Nilsson, 1970). The high loading capacities available with large packed polyamide and polyacrylamide gel columns have made them particularly attractive for preparative separation of beet colorants; quantitative analysis with gel columns has been carried out successfully as well (Adams and von Elbe, 1977). High-performance liquid chromatography (LC) was investigated as a means of improving the resolution of beet color components, speed of analysis, and the quality of data presentation over other techniques.

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MATERIALS AND METHODS

Sample Preparation. Beet juice was expressed from raw milled beets and filtered and diluted as necessary. Red beet powder and yellow beet powder derived from fermented beet juice (J. H. von Elbe, University of Wisconsin, Madison, Wis.) were dissolved in water. All samples were filtered through a 0.45-μm MF-Millipore filter (Millipore Corp., Bedford, Mass.) prior to chromatography.

Partially purified crystalline betanin was prepared by the method of Adams and von Elbe (1977) and dissolved in water for quantitative analysis calibration standards.

Method and Apparatus. High-pressure liquid chromatography was conducted with two Model M6000 pumps and a Model 660 solvent programmer from Waters Associates (Milford, Mass.), together with a Model 7120 syringe loading sample injector (Rheodyne, Berkeley, Calif.), a Vari-Chrom UV-visible variable wavelength detector (Varian Instruments, Palo Alto, Calif.), and a 100-mV single channel strip recorder (Sargent-Welch Scientific Co., Skokie, Ill.). The bonded reverse-phase column was a μ-Bondapak/C_{18} column (Waters Associates, Milford, Mass.).

Detailed conditions for the qualitative solvent gradient procedure are shown in Table I along with conditions for the quantitative isocratic procedure.

It was discovered that the 90:10 water–methanol mobile phase containing PIC A reagent, premixed and delivered through one pump (Table I), is not strictly comparable to a 90:10 mixture delivered through a mixing chamber from

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Table I

Conditions for Analysis of Betalaines by Solvent-Gradient LC
Column, μ Bondapak C_{18} (Waters Associates), 30 cm × 4 mm i.d.
Mobile phase and gradient
Initial: 90% solvent A/10% solvent B
Final: 70% solvent A/30% solvent B
Gradient: curve 10 on Model 660 Solvent Programmer (Waters), 20-min duration
Solvent A: 0.005 M PIC reagent A in water
Solvent B: 0.005 M PIC reagent B in methanol
Column temperature, ambient
Flow rate, 60 mL/h
Inlet pressure, 1500-3000 PSI
Chart speed, 0.2 in./min
Detector, Varian Vari-Chrom, ca. 476 and/or 538 nm
Sample injection amount, 20 μL
Sample description, aqueous betacyanin/betaxanthin mixtures, 10^{-4}-1.00%

Conditions for Analysis of Betalaines by Isocratic LC
Column, Bondapak C_{18} (Waters Associates), 30 cm × 4 mm i.d.
Mobile phase, 0.005 M PIC reagent A in 90:10 water-methanol
Column temperature, ambient
Flow rate, 60 mL/h
Inlet pressure, 1500 PSI
Chart speed, 0.2 in./min
Detector, Varian Vari-Chrom, ca. 476 and/or 538 nm
Sample injection amount, 20 μL
Sample description, aqueous betacyanin/betaxanthin mixtures, 10^{-4}-1.00%

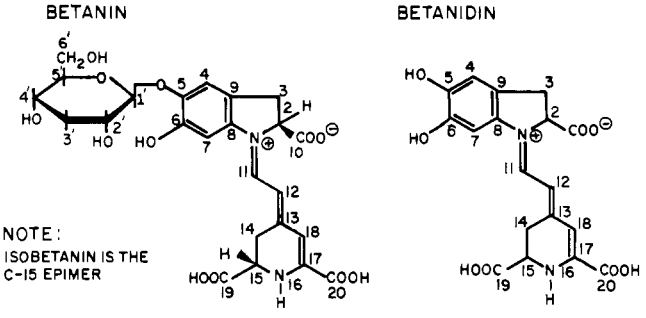

Figure 1. Principal red pigments in beet color.

two separate solvent pumps using two separate solvents each with PIC A reagent; therefore, it is not necessarily accurate to suggest that the isocratic conditions correspond to the initial conditions from the gradient procedure (Table I). Slight differences in pump delivery rates, the dynamics of flow, mixing, and pulsation, and effective pHs of the mobile phase can significantly alter the actual final separations obtained. The chromatographer should be forewarned of these considerations before attempting to strictly adapt the conditions described to his system.

Paired ion solvents should not remain static in the C_{18} column for overnight periods or longer. Either five or more column volumes of a 50:50 methanol–water solvent (free of PIC reagent) should be pumped through the column or, alternatively, a slow flow of mobile phase should be maintained during the unattended period (0.1–0.3 mL/min).

RESULTS AND DISCUSSION

Structural Considerations. Structures of the principal red betacyanin pigments, betanin and betanidin, are represented in Figure 1. The free phenolic and carboxyl groups (carbons 6, 10, 19, and 20) actually make them organic acids with complete ionization of all these groups occurring in the 7.5–9.5 pH range. Acid hydrolysis can

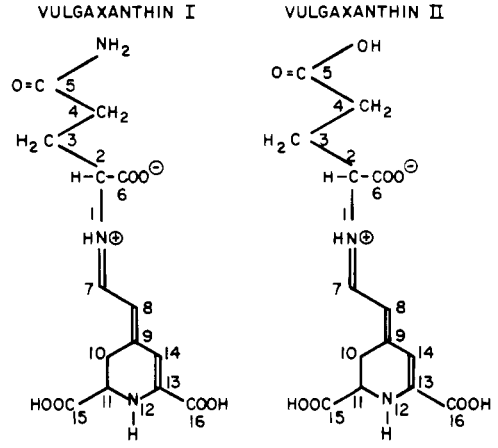

Figure 2. Principal yellow pigments in beet color.

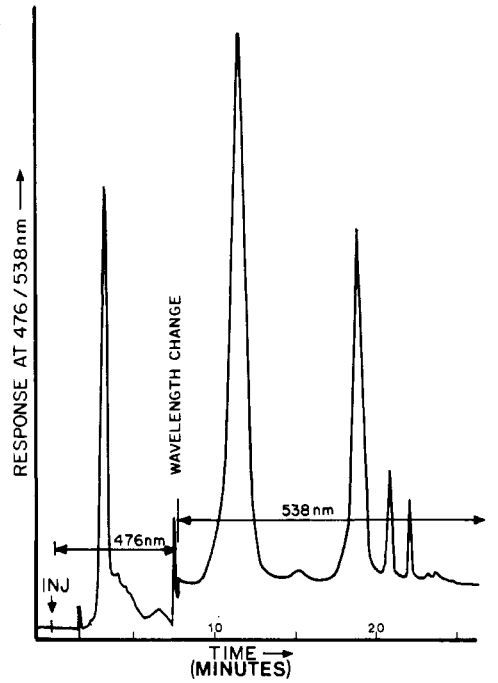

Figure 3. Gradient paired-ion LC separations of beet red and yellow color extract. Conditions are shown in Table I.

convert the glycon (betanin) to the aglycon (betanidin). Each species is known to exist in equilibrium with one or more of its epimers, the principal one being isobetanin (and isobetanidin) which is formed at the C-15 asymmetric carbon locus. The betacyanins display absorbance maxima in the 537–538-nm region.

Structures corresponding to the principal yellow betaxanthin pigments, vulgaxanthin I and vulgaxanthin II, are shown in Figure 2. These pigments are present in lower quantities and possess less potency as colorants, but come into play as secondary dyestuffs and background colorants in achieving color balance in raw and finished products. Their absorbance maxima are in the 476–477-nm region.

Development of the Qualitative Method. The ion pairing approach was adopted as the method of choice in order to exploit the highly ionic character of these compounds and the durability of the reverse-phase "C_{18}" column. Using a pH of 7.5 and the tetrabutylammonium ion in the mobile phase in the form of the PIC reagent A, nonpolar unprotonated complexes with the solute molecules were generated.

For qualitative separations, the solvent gradient conditions of Table I were used to obtain chromatograms such

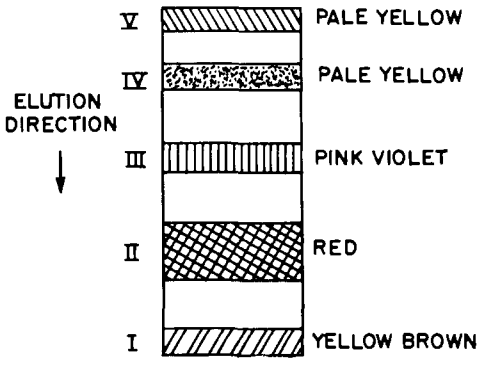

Figure 4. Column chromatographic band identification of beet pigments on Bio-Gel P6.

as the one shown in Figure 3 for a mixture of red and yellow beet powders. Two wavelength settings are used in the course of the chromatogram to selectively monitor the red and yellow dyestuffs which elute far enough apart to permit this change in the middle of the chromatogram. A single wavelength setting may be used to selectively screen out either the red or yellow dyestuffs, although the reds do absorb to a certain degree at 476 nm, making 476 nm the wavelength of choice for a comprehensive screening of dyestuffs for fixed wavelength (or unattended) instruments.

The vulgaxanthins (yellows) elute early because of their lack of bulky fused ring and sugar side chains in relation to the number of charged sites, rendering them more polar with low retention even in the paired ion mode. Because of the predominant interest in the betacyanin composition, optimal retention of the vulgaxanthins was sacrificed somewhat in favor of resolution of the betacyanins when the final operating parameters for the separation were selected.

Identification of Betacyanins. Some circumstantial evidence as to the identity of the betacyanin peaks was obtained through column chromatography and LC. The column chromatographic procedure was undertaken to recover concentrated betacyanin pigment for calibration in quantitative work (Adams and von Elbe, 1977).

Figure 4 depicts the separation of colored bands obtained with the Bio-Gel P6 columns with vulgaxanthins and high molecular weight tannins eluting early with the internal elution volume of the gel bed (fraction I), a major deep red band containing betanin and betanidin eluting next (fraction II), followed by a narrower red violet band (fraction III), and finally by some strongly adsorbed trace yellows (fractions IV and V).

A typical gradient separation of the dark-red fraction (fraction II) is shown in Figure 5 (top). Speculation on the basis of relative abundance of pigments indicated that the major peak (A) which runs off scale is probably betanin and the second largest peak (B) is probably betanidin. The ring OH group on carbon 6 (Figure 1) can be expected to be more available for ion pairing in the aglycon because of the absence of steric hindrance from the attached glucose, hence its increased retention relative to the glycon.

To strengthen the speculated identification, the bottom chromatogram in Figure 5 represents the same fraction (fraction II) which was freeze-concentrated, treated with HCl, and held at 10 °C for several hours. The partial hydrolysis of the glycon to the aglycon has resulted in an increase in the size of peak B relative to peak A, as predicted by the tentative identification of these peaks. Of further interest are the simultaneous reduction in peak D and the enlargement of peak E, suggesting that these components might represent isomers or epimers of betanin

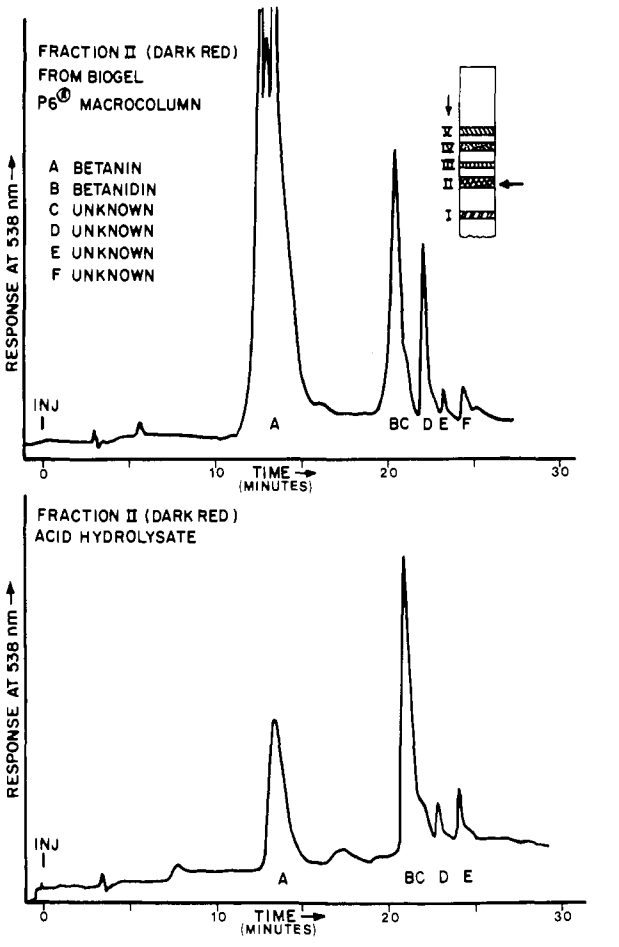

Figure 5. Gradient paired-ion LC separation of fraction II from Bio-Gel P6 column.

and betanidin, respectively, that are also interconverted by the gain or loss in a sugar.

Other column chromatographic separations (Nilsson, 1970) of the pigments giving bands similar to those reported here (Figure 4) using polyamide gels have led to the identification of the fraction in position III as the C-15 epimer or isobetanin/isobetanidin fraction from red beets. Its greater retention was attributed to less steric hindrance of the C-15 carboxyl group by the attached sugar and indole moieties. If this mechanistic argument were generally valid, the "iso" components might be expected to elute closer to betanidin than betanin in HPLC for the same reasons.

The gradient, paired-ion separation of fraction III from the gel column isolation and cleanup was conducted, giving the chromatogram shown in Figure 6. The peak size distribution shows an inverted pattern from that in Figure 5, with the betanin and betanidin peaks A and B present in only trace amounts. By inference from the column chromatographic data discussed, the later peaks, D and E, probably represent the most abundant "iso" epimers of the parent compounds, betanin and betanidin. Further characterization of these components by optical activity, NMR, and the like are needed to establish definite structures. Such characterization was not required for the practical application of the method, however.

Development of the Quantitative Method. A chromatogram corresponding to the quantitative isocratic separation of vulgaxanthins and betacyanins is shown in Figure 7 using the conditions shown in Table I. Only a small amount of red dyestuff is resolved from the major peak, and there is virtual assurance that the major red

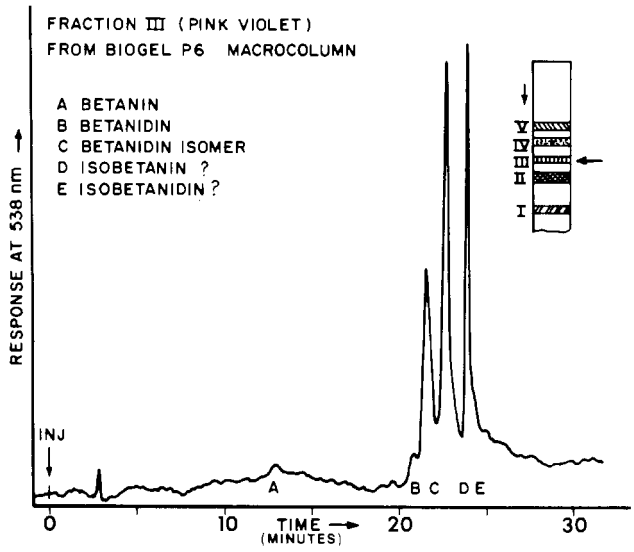

Figure 6. Gradient paired-ion LC separation of fraction III from Bio-Gel P6 column.

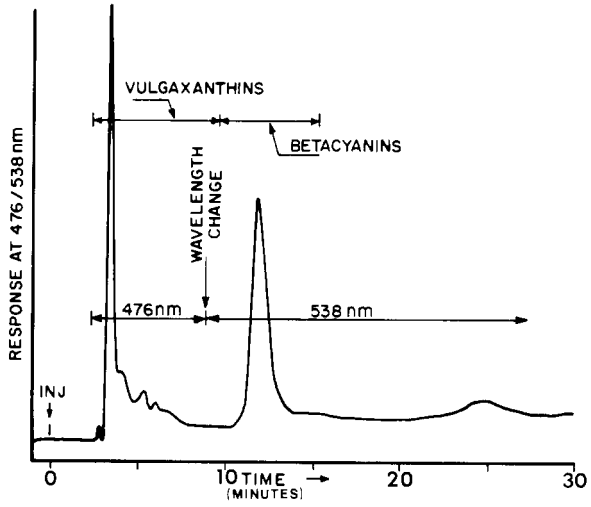

Figure 7. Isocratic paired-ion LC separation of beet red and yellow color extract. Conditions are shown in Table I.

dyestuff (betanin) will be included in the major peak. Also, since betanin comprises at least 80% of the red pigment and is so structurally similar to most of the remaining red pigments, the analyst can assume that the extinction coefficient of pure betanin is the same as that for the aggregate of betacyanins. It is then possible to prepare a standard from purified freeze-dried material, measure its extinction coefficient spectrophotometrically, calculate the real concentration of betanin using the ideal extinction coefficient of 1120%$^{-1}$ cm^{-1} (Nilsson, 1970), and to calibrate the LC system using known betanin concentrations.

Using the isocratic LC method, good linearity was obtained over a practical working range of 0.005–0.03% w/v betacyanin. The detectability limit is approximately 10–4%. Relative standard deviations among replicate samples is typically about 2.0%. Table II shows statistical data for the calibration lines obtained over a 3-day period from the same series of solutions with refreezing in between. The data show that the "fit" of points to the calibration line degraded somewhat to the point where the standard deviation of the slope is only marginally acceptable on the third day. A fourth day's calibration did not produce a satisfactory calibration line for analytical use. It was deemed advisable to prepare fresh standards after every three days in view of the apparent variability

Table II. Standard Curve Equation Parameters and Statistics Using Five Serial Dilutions of Betanin on Three Successive Days[a]

Day	a	b	Correlation coeff.	SD a	SD b
1	21.763	-0.053	0.994	1.35 (6.2%)	0.026
2	68.39	-0.057	0.998	4.47 (6.5%)	0.55
3	149.83	-0.537	0.992	13.61 (9.1%)	0.21

[a] $Y = aX + b$, Y = peak height, and X = betanin concentration. Solutions (% betanin): (1) 0.00575, (2) 0.0115, (3) 0.017, (4) 0.023, (5) 0.029. SD = standard deviation.

Table III. Recovery of Betanin from Spiked Gelatin Samples

Sample	Endogenous betanin, mg	Added betanin, mg	Total betanin, mg	Betanin found mg	Recovery
1	1.0	1.0	2.0	2.11	105.5
2	1.0	2.0	3.0	3.75	125.0
3	1.0	3.0	4.0	4.17	104.3
4	1.0	4.0	5.0	5.54	110.9

Standard addition curve data (peak height vs. added betanin)				
Correlation coeff.	Slope	y intercept	SE of estimates[c]	x intercept = mg of betanin
0.991	5.13	5.00[a]	6.1%	-0.97[b]

[a] Equal to units of height due to endogenous betanin. [b] Milligrams of endogenous betanin; recovery (overall) = (0.974/1.0) × 100 = 97.4%. [c] SE = standard error.

in the rate of betacyanin degradation over time among solutions of different concentrations.

Besides measuring levels in expressed beet juice, concentrates and beet color powders, the method can measure quantities of beet color added to formulated mixes. Over 90% of the color was recovered quantitatively from a powdered beverage mix without any interferences from the matrix ingredients.

The method of standard additions was employed in extending the method to powdered gelatin dessert products (Table III) as a means of obviating any sampling or recovery problems with this matrix. Here the amount of endogenous color bound to an inert soluble carrier and the amount of added color were both known. The system was calibrated with a known beet powder solution. Fixed recoveries for each level of added color were somewhat variable (104–125%), but the statistically determined weight of betanin in the sample is close to the known or nominal amount (determined 0.97 vs. 1.0 mg known).

A final example of the utility of the quantitative LC method is in providing a direct index of the color quality of a liquid sample by measuring the ratio of concentrations of betacyanin (B) to vulgaxanthin (V) in solutions. Higher ratios accompany deeper, grape-like hues, whereas lower ratios are associated with brighter, more orange-red solutions. This can be done by a direct peak height ratioing technique, correcting for the differing response factors of the betacyanin peak at 538 nm and the vulgaxanthin peak at 476 nm as follows:

α = betacyanin concentration/vulgaxanthin concentration = $\frac{Ht_B\ (538)}{Ht_V\ (476)} \times \frac{RF_V\ (476)}{RF_B\ (538)}$

The quotient of response factors reduces to a constant for fixed conditions. The advantage of using LC over straight spectrophotometry at multiple wavelengths to measure this

ratio (Nilsson, 1970) is in its clean resolution of colors and elimination of both overlap in red and yellow spectral bands and interference in the measurements by decomposition products.

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Isolation and Identification of Volatile Components from Wild Rice Grain (*Zizania aquatica*)

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The isolation and identification of flavor compounds characteristic of the popular gourmet item, Wild Rice, have heretofore been unreported. Wild rice volatiles were isolated using vacuum steam distillation followed by solvent extraction. The isolate was fractionated by preparative gas chromatography, and the fractions were subjected to gas chromatography–mass spectrometry using 500 ft × 0.03 in. i.d. Carbowax 20M wall-coated open-tubular columns. Identification were based upon flame ionization and alkali flame ionization detector responses, retention indices (I_E), and GC–MS analyses in conjunction with the specific detectors. This first analysis of wild rice resulted in the identification of 112 compounds reported herein.

Wild rice (*Zizania aquatica*) is one of four species of distinct types of annual grasses which grow in shallow moving water near Lake shores, shallow streams, or ponds in the upper Great Lakes region of the United States and Canada. Other species have been identified in North America (*Zizania palustris* and *Zizania texana*) and in Manchuria and the Far East (Rossman et al., 1973).

Wild rice has been harvested by American Indians for centuries as a staple of their diets. Within the past decade the grain has achieved wide recognition for its distinctive flavor by the gourmet food market and more recently has been increasingly used in rice mixes and casserole items which feature wild rice as an ingredient. Native wild stands of the grain once served as the sole supply, but these stands now contribute approximately 1 million pounds of grain annually compared to 5 million pounds from the commercial cultivation of nearly 20 000 acres of wild rice paddies (Lund et al., 1977).

Unlike other cereal grains, will rice undergoes a series of postharvest processing steps which contribute to the development of a commercially acceptable commodity. The mature kernel is harvested as a moist, metabolically active seed which is piled approximately 18 in. deep to undergo a biochemical and microbiological fermentation during which time the flavor develops and the kernel becomes darkly pigmented. Following fermentation, the grain is dried or parched to reduce the kernel moisture to approximately 7%, hulled, and separated into various grades of finished product. The flavor characteristics of commercial wild rice are dictated by variations of these processes and can be broadly described as either "tea-like", "grainy", "earthy", or "toasted". The grainy flavor and at least part of the tea-like flavor is inherent to the grain at harvest time. A lighter grassy flavor is more pronounced in immature grain while starchy flavor variations are more apparent in mature grains. The tea-like flavor is enhanced during fermentation. It is characteristic of high-quality grain and contributes to the overall, full, hearty flavor. It is frequently accompanied by varying degrees of earthy and moldy or musty flavors reflecting the product moisture level during fermentation. Variation of toasted flavor results from the specific design of parching equipment with the direct-fired surface parchers providing a more burnt and smoky character while the forced-pair parchers produce a milder toasted or roasted flavor. The unique processes involved with the production of wild rice contribute not only to its distinctive flavor, but also to the flavor variations of commercial wild rice (Lund et al., 1977).

Literature concerned with the flavor chemistry of wild rice is nonexistent and that for cereal grains is limited. Wheat has been studied most extensively (Hougen et al., 1971; McWilliams and Mackey, 1969; Okada, 1969; El-Basyouni and Towers, 1964) with the flavor chemistry of white bread receiving the greatest attention (Johnson and Sanchez, 1973; Coffman, 1967a,b; Johnson et al., 1966; Collyer, 1964; Kobayasi and Fujimaki, 1965; Zyuz'ko et al., 1973, 1974; Lorenz and Maga, 1972: Mulders, 1973a,b; Mulders and Dhont, 1972; Mulders et al., 1972, 1973). Roasted barley (mugi-cha), used in the production of dark beer and a tea-like beverage, has received attention (Shimizu et al., 1967; Shimizu et al., 1970a,b; Wang et al., 1968, 1969; Collins, 1971) while the volatile carbonyls and amines of malted barley have been identified by several investigators concerned with their contribution to the flavor of beer (Arkima and Ronkainen, 1971; Hrklicka and Dyr, 1968; Damm and Kringstad, 1964; Wagner, 1971; Baerwald Niefind, 1969; Drews et al., 1957; Palamand et al., 1969; Slaughter, 1970; Slaughter and Uvgard, 1971, 1972; Steinke and Paulson, 1964). The volatile components of cooked rice have been studied (Ayano and Furuhashi, 1970; Tanaka, 1972) with specific interest in the factors

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