major polypeptides in G_1 and WI-ASG differ from zein and WS-ASG and can be classified as a water-insoluble, high-methionine, M_r 14000 and 10000 protein. Extraction with 63% EtOH plus 0.5% NaOAc plus 0.7% ME, followed by dialysis against water, is a simple method to effectively separate the high-proline–high-histidine and the high-methionine bands as WS-ASG and WI-ASG fractions. This is verified by WS-ASG and WI-ASG having one of the highest proline–highest histidine and highest methionine content, respectively, compared to those of the other protein fractions.

WS-ASG and acid-soluble G_2 glutelins have properties very different from zein and WI-ASG, as demonstrated in this and other investigations. After glutelin disulfide bonds are broken with reductant, WS-ASG or G_2-Ac glutelins become soluble in water; aqueous alcohol, neutral, acidic, or basic salt solutions, and dilute HOAc. They are high-proline–high-histidine proteins with predominant M_r 25000. According to Osborne–Mendel solubility schemes, these polypeptides could be classified as either albumins, globulins, prolamins, or glutelins. They are commonly classified as glutelins because they are soluble only after disulfide bond disruption. The term "reduced–soluble proteins", used by Wilson et al. (1981) for these subunits, is not specific enough to define the solubility of this fraction. Likewise, the term "prolamin-like" (Landry and Moureaux, 1981) stresses only the resemblance of G_2 subunits to prolamins. The term WS-ASG best describes this protein as being water soluble, alcohol soluble, and associated by disulfide bonds in the water glutelin protein.

The presence of cereal proteins with multiple solubility characteristics demonstrates once again that there are no clear-cut boundaries between Osborne and Mendel (1914) protein classes. The combined data from amino acid analyses, SDS–PAGE, IEF, and acidic PAGE have been useful in classifying these proteins according to their physical characteristics. On the basis of these data, a defined system for naming the alcohol-soluble glutelins is one using WS-ASG and WI-ASG. There is still need for total sequence analysis to establish the precise nature of the differences in these proteins.

Registry No. 2-PrOH, 67-63-0; EtOH, 64-17-5; ME, 60-24-2; LiCl, 7447-41-8; sodium acetate, 127-09-3.

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A New Procedure for Specific Determination of β-Amylase in Cereals

Paul R. Mathewson* and Bradford W. Seabourn

A new method for specific determination of β-amylase in cereals has been developed. This method utilizes a commercially available colorimetric substrate consisting of *p*-nitrophenyl oligosaccharides. Results are linear with β-amylase concentration over a wide range of activity. Tests in which increasing amounts of cereal α-amylase were added to a constant amount of β-amylase showed no significant increase in absorbance using this substrate. Under the same conditions, the commonly used Bernfeld assay for β-amylase activity showed an increased response. Using a new substrate, we observed a constant response for wheat samples with increasing degree of sprouting. This substrate was also found to be useful for the determination of fungal α-amylase activity.

The amylase enzymes in cereal grains play vital roles in the growth and development of the plant as well as in processing plant material for food use. In cereals, α-amylase has received considerable attention due to its dextrinogenic activity on starch and the detrimental results in terms of food processing. Since the saccharogenic activity of β-amylase is much less detrimental to processing grains, this enzyme has been viewed as relatively unimportant. However, there are other situations, physiological studies in particular, in which the ability to measure β-

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amylase could be of great use. In the past, determination of β-amylase has been complicated by the presence of α-amylase, which, while primarily dextrinogenic, can also produce small oligosaccharides. As a result, methods for determining β-amylase by measuring formation of reducing sugars in fact measure total saccharifying activity. Perhaps the most common β-amylase method is that reported by Bernfeld (1955). This method measures the production of reducing sugars by reaction with alkaline 3,5-dinitrosalicylic acid to form a colored complex. While this test responds primary to β-amylase, it is also affected by α-amylase activity. In a modification of the technique, Warchalewski and Tkachuk (1978) negated the α-amylase response by conducting the test at pH 3.4, where α-amylase was assumed to be inactive. However, under those conditions the β-amylase was determined far from its optimal pH, and hence, sensitivity is poor. We here present a new, simple assay procedure utilizing a commercially available colorimetric substrate consisting of p-nitrophenyl oligosaccharides that responds to β-amylase but is unaffected by α-amylase in cereals.

MATERIALS AND METHODS

Hard red winter and western white wheat samples were obtained at the U.S. Grain Marketing Research Laboratory. Barley malts were from the Malt and Barley Laboratory, Madison, WI.

Pantrak E. K. reagent, p-nitrophenyl oligosaccharides, microbial α-glucosidase, and one β-amylase preparation were from Calbiochem, La Jolla, CA.

Malted barley α-amylase (Type VII-A) and barley β-amylase (Type II-B) were from Sigma Chemical Co., St. Louis, MO. Pure barley α-amylase was a gift from Dr. L. C. Davis and Dr. S. Muthukrishnan, Department of Biochemistry, Kansas State University, Manhattan, KS. Barley β-amylase, fungal α-amylase (24 400 SKB/g), and fungal α-amylase (Millers Fermex, 115 000 SKB/g) were from Wallerstein Co., Morton Grove, IL. Fungal α-amylase (5000 SKB/g) was from Penwalt Corp., Plymouth, MN.

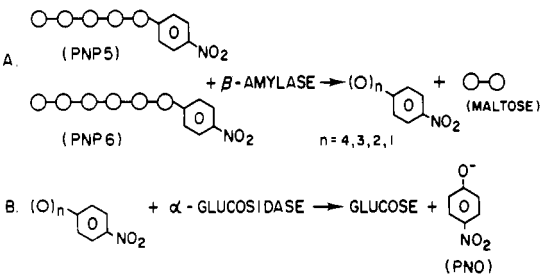
Enzyme Assays. α-Amylase was measured by the colorimetric method reported by Mathewson and coworkers (Mathewson and Pomeranz, 1979; Mathewson et al., 1982). β-Amylase was measured according to Bernfeld (1955).

The Pantrak assay was performed by dissolving the lyophilized substrate powder in the appropriate quantity of 47 mM monobasic sodium phosphate (5.96 g/L) to give a final pH of 6.0. The substrate is available in two sizes of sealed vials, requiring either 6.5 or 15.0 mL for reconstitution. One milliliter of this solution was incubated at 40 °C and an aliquot of the sample to be assayed added at zero time. The reaction was allowed to proceed for 3.0 min at which time 1.0 mL of 0.25 M Na_2CO_3 was added to terminate the reaction. The absorbance of the solution was then determined in a 1-cm cell at 405 nm.

When reconstituted, the substrate contains 8.5×10^{-4} M p-nitrophenyl α-maltopentaoside (PNP5), 6.5×10^{-4} M p-nitrophenyl α-maltohexaoside (PNP6), and ~800 units/L microbial α-glucosidase. Units of activity were defined as the amount of enzyme required to provide 1 μmol of p-nitrophenol (PNO)/min under test conditions. A molar extinction factor for PNO of 18.8×10^3 at pH 10.0 was used ("Pantrak E. K. Product Pamphlet", 1981).

Extracts were prepared by stirring 2.0 g of flour or ground wheat in 0.05 M sodium phosphate buffer at pH 6.0 for 30 min and filtering.

Scheme I



RESULTS AND DISCUSSION

The hydrolysis of starch by cereal α-amylase proceeds generally, from the production, first, of relatively large dextrins, and then slowly to production of small oligosaccharides, indicating a binding preference for polysaccharides over oligosaccharides. β-Amylase, however, produces only maltose regardless of the size of the glucose polymer and does not exhibit this binding preference. We took advantage of this difference to develop a test that could distinguish between α- and β-amylase activity, by utilizing p-nitrophenyl oligosaccharides as the substrate.

Pantrak reagent is a commercially available product normally used to assay for α-amylase in human serum and urine. The reagent consists of PNP5, PNP6, a microbial α-glucosidase, phosphate buffer, and stabilizers. To change the pH from its normal value of 7.0 to 6.0 for assaying cereal β-amylase, the reagent was reconstituted in monobasic phosphate rather than in distilled water. The α-glucosidase has a restricted specificity for oligosaccharides of 4 glucose units or less and so reacts very slowly with PNP5 and PNP6. However, a blank was always run and used as a reference for assaying samples. After cleavage of PNP5 and PNP6, the α-glucosidase begins to hydrolyze the remaining PNO-oligosaccharides to glucose and p-nitrophenol. These reactions are illustrated in Scheme I.

Since the p-nitrophenol must be in the phenoxide form to be colored, the pH must be raised from 6.0 to a basic pH. Because we used additional monobasic phosphate to reconstitute the reagent, a stronger solution than the 0.1 M Na_2CO_3 supplied by the manufacturer was required to terminate the reaction. The final pH chosen was 10.0 in order to quickly stop enzymatic activity and to correspond to the reported molar extinction factor for PNO at pH 10.0. A 0.25 M Na_2CO_3 solution was adequate for this purpose.

Preliminary data using a malt extract showed a pH optimum for Pantrak of approximately 6.0 and a temperature optimum at 40 °C. When extracts of wheat flour and malted barley were assayed, strong responses were observed with both the Bernfeld test and Pantrak. Since the wheat flour contained little or no α-amylase, indications were that both tests respond to β-amylase, although the observed response could also have resulted from α-amylase or amyloglucosidase activity, which could convert PNP5 and/or PNP6 to glucose and PNO.

Two approaches were used to determine whether the Pantrak response was due to a glucosidase. First, the reconstituted Pantrak reagent was boiled for 10 min to destroy the glucosidase activity. When this reagent was then used to test flour and malt extracts, no color development was observed. Second, we used both p-nitrophenyl α-D-glucopyranoside (PNP1) and p-nitrophenyl α-maltoside (PNP2) as substrates in place of Pantrak. If an α-glucosidase were present in the sample extract, it should hydrolyze both PNP1 and PNP2 to glucose and PNO. When extracts of sound wheat, sprouted wheat, and barley malt were used, no response was observed with PNP1 or PNP2 as the substrate. Addition of 5 μL of α-glucosidase

to the reaction mixture resulted in immediate production of PNO. Since Pantrak reagent does produce PNO, but boiled reagent does not, it is apparent that the PNP5 and PNP6 are being hydrolyzed, probably to PNP1 and PNP2. Since the boiling has destroyed α-glucosidase activity, and no color was then observed, the enzyme responsible for hydrolyzing PNP5 and PNP6 to PNP1 and PNP2 cannot take the latter substrates to glucose and PNO. Thus, there are no α-glucosidase enzymes in the extracts capable of producing PNO from either PNP5/PNP6 or PNP1/PNP2. The observed production of color must result from β- and/or α-amylase.

Inactivation studies were conducted to determine the effect of α- and β-amylase on Pantrak activity. Sulfhydryl reagents such as iodoacetamide, *N*-ethylmaleimide, *p*-(hydroxymercuri)benzoate, and 5,5′-dithiobis(2-nitrobenzoic acid) were effective inhibitors of β-amylase activity. However, inactivation was slow (15–18 h), and α-amylase was also partially inactivated. Hg^{2+} was an effective, rapid inhibitor of β-amylase and was relatively ineffective against α-amylase in the same period of time. These results agree with those reported previously (Tkachuk and Tipples, 1966). A total of 100 mg of a crude preparation of barley α-amylase [Sigma Chemical Co. No. A-2771, containing 1.5 units/mg α-amylase activity and 2.5 units/mg β-amylase activity (Bernfeld results, reported by Sigma Chemical Co.)] was dissolved in 10.0 mL of 0.05 M sodium phosphate buffer, which was 1.36×10^{-3} M in $CaCl_2 \cdot 2H_2O$, at pH 6.0. Ten microliters of Hg^{2+} (1000-ppm solution) was added to the enzyme solution and stirred for 2 h. Two milliliters of the treated solution was then passed over a 0.9×30 cm column of Sephadex G-50 and eluted with 0.05 M phosphate to remove excess Hg^{2+}. Treatment of the enzyme solution in this manner resulted in a complete loss of activity toward the Pantrak reagent while α-amylase activity (measured by colorimetric method) was almost fully retained. There was a substantial drop in the Bernfeld response to the treated enzyme solution to about 10% of the control response.

The failure of Pantrak to respond to the Hg^{2+}-treated enzyme may have been due to Hg^{2+} inactivation of the α-glucosidase in Pantrak rather than to inactivation of β-amylase. To test this, an aliquot of PNP2 was added to a Hg^{2+}-treated enzyme–Pantrak mixture. While no color was produced with the Pantrak after 5 min, an immediate response was observed upon addition of PNP2, thus showing that the α-glucosidase was active and capable of hydrolyzing PNP2, had it been produced. Thus, the lack of Pantrak response to Hg^{2+}-treated enzyme was due to β-amylase inactivation. Further tests showed that the α-glucosidase is susceptible to Hg^{2+} inactivation at higher concentrations of Hg^{2+}.

Another solution of barley α-amylase was prepared as before except that 0.25 M phosphate buffer containing no $CaCl_2 \cdot 2H_2O$ was used. Enzyme assays using these extracts showed a significant loss of α-amylase activity, but both Pantrak and Bernfeld response remained essentially unchanged from the control response in 0.05 M phosphate plus $CaCl_2 \cdot 2H_2O$.

These data strongly indicate that Pantrak responds to β-amylase only and is unaffected by cereal α-amylase. However, because the inactivation techniques employed are not truly specific and because the Bernfeld test responds to both α- and β-amylase, it was felt that some ambiguity remained. To remove this ambiguity, a preparation of barley α-amylase was used that had been purified by affinity chromatography on cycloheptaamylose–Sepharose. The purification scheme and determination

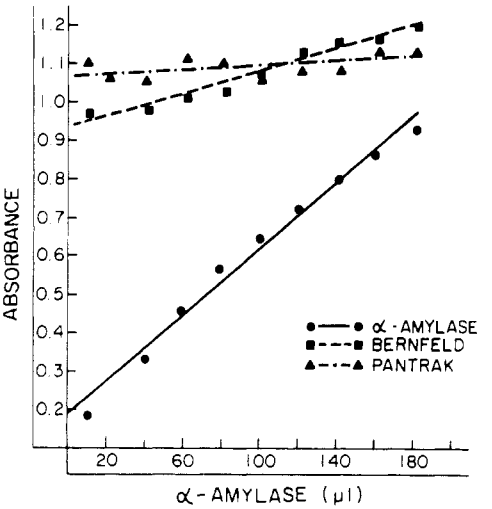


Figure 1. Bernfeld, Pantrak, and colorimetric α-amylase results on sound wheat extract with added pure α-amylase.

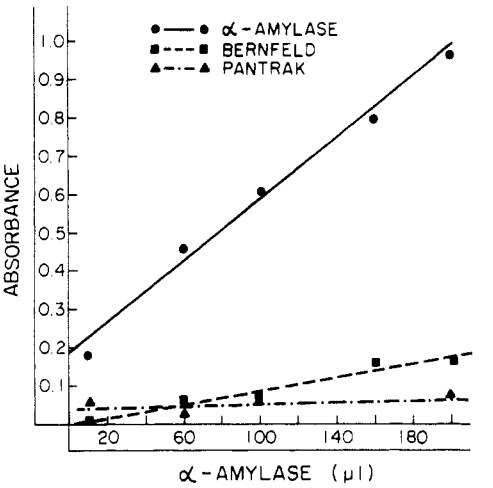


Figure 2. Bernfeld, Pantrak, and colorimetric α-amylase results on pure α-amylase dilutions.

of homogeniety were essentially identical with those described by Jacobsen and Higgins (1982). This α-amylase was pure by both two-dimensional electrophoresis and immunological criteria. To test the effect of α-amylase with Pantrak, increasing amounts of this enzyme preparation were added to an extract of sound wheat (containing a constant amount of β-amylase). The α-amylase was prepared by dilution so that a constant volume was added to a constant volume of wheat extract. These α-amylase dilutions were also tested for Pantrak activity. The results of these tests using Pantrak, the Bernfeld assay, and colorimetric α-amylase determination are shown in Figures 1 and 2. Figure 1 shows the results for the wheat extract plus increasing α-amylase activity. These data clearly show that while α-amylase activity increases, the Bernfeld response also increases, verifying that this test does respond to α-amylase. The Pantrak response is almost flat, showing a response to β-amylase but not to α-amylase activity. Relative to the slope of the α-amylase increase, the Bernfeld response is about 35% and Pantrak about 5%. The range of α-amylase used in this experiment ranged from 5 to 50 DU/g. Figure 2 shows the response of the α-amylase dilutions. Again, while α-amylase activity increases, the Bernfeld response, while much lower, does increase whereas the Pantrak slope is essentially zero. Statistical data for these regression equations are shown in Table I.

Table I. Statistical Data for Regression Equations from α-Amylase Additions to Wheat Extract[a]

		α-amylase plus extract	α-amylase alone
Bernfeld (A_{500}):	r	0.4600	0.5110
	m	0.0014	0.0009
	b	0.9335	0.0228
Pantrak (A_{405}):	r	0.0912	0.1682
	m	0.0003	0.0001
	b	1.0670	0.0440
α-amylase (A_{620}):	r	0.9833	0.9895
	m	0.0040	0.0037
	b	0.1934	0.1880

[a] r = correlation coefficient; m = slope; b = y intercept.

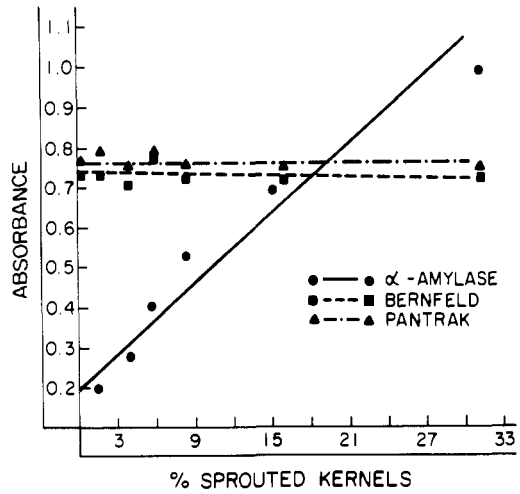


Figure 3. Bernfeld, Pantrak, and colorimetric α-amylase results on sprouted wheat.

As a model system, these data clearly show that Pantrak reagent is a good substrate for cereal β-amylase and is unaffected by cereal α-amylase. To verify that the model is applicable to real grain samples, we tested a series of sound hard red winter and soft white wheats from Kansas and California. The β-amylase content, determined by both Pantrak and Bernfeld methods was relatively constant. A series of soft white wheats ranging in degree of sprouting from 0 to 32%, were then tested by the Bernfeld and Pantrak methods and by the colorimetric α-amylase method. As in the model system, Figure 3 shows that while α-amylase activity increased relative to the degree of spouting, both the Bernfeld and Pantrak responses were virtually constant. The α-amylase activity of these samples ranged from 0 to 2.0 DU/g.

The pH and temperature optima of the Pantrak response were reinvestigated by using a commercial sample of β-amylase (Wallerstein). Data in Figure 4 show a pH optimum at 6.05 and temperature optimum at 40 °C. Under these conditions the assay can be used to measure β-amylase activity over a wide range of activity. A standard curve for the Pantrak reagent was developed by using commercial β-amylase. The results were linear to an absorbance of 1.5, which is equivalent to about 60 units of β-amylase activity. The regression equation for the standard curve of β-amylase (x) vs. absorbance at 405 nm (y) was $y = 0.0262x$ ($r = 0.9960$).

In the course of this work we also investigated other enzymes that might give a Pantrak response. It was found that fungal α-amylase also responds to Pantrak reagent. Using three commercial sources of fungal α-amylase (fungal source unknown), standard curves were run on the basis of reported activity (SKB units). Each fungal α-amylase

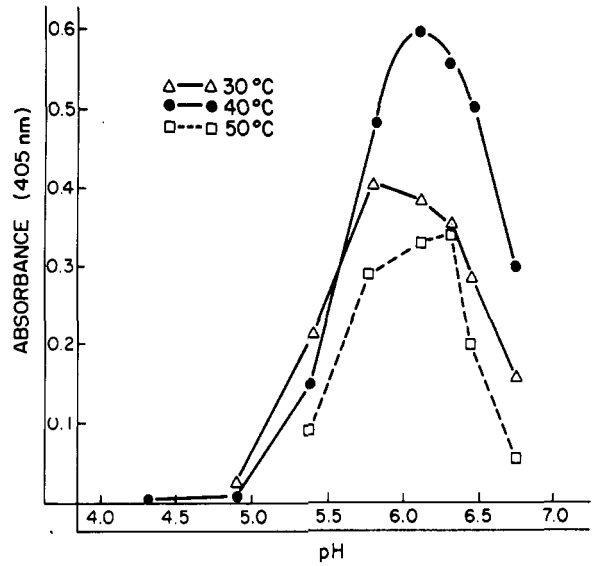


Figure 4. Temperature and pH data for Pantrak response to β-amylase.

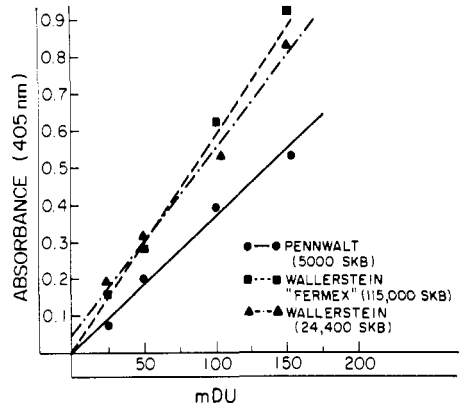


Figure 5. Pantrak response using commercial fungal α-amylase preparations.

solution was prepared to contain 200 mDU/mL (assuming 1 SKB = 2 DU), and dilutions of this solution were then tested with Pantrak. As shown in Figure 5, the responses of the three fungal α-amylases to Pantrak are not equivalent. The difference in absorbance at a given mDU value may simply reflect activity loss over storage time such that the reported activities were no longer accurate. However, the difference in slope, or rate of change in absorbance at 405 nm, suggests a kinetic difference. This difference is most apparent in comparing samples from different manufacturers.

These data demonstrate that the Pantrak reagent affords a simple and rapid analytical procedure for determining cereal β-amylase or fungal α-amylase with no interference from cereal α-amylase. This assay should prove valuable for physiological and other studies on cereal grains. This test can easily be performed on a commercially available instrument (Demaray Scientific Instrument Co., Pullman, WA) previously reported for α-amylase testing (Mathewson et al., 1982) by using a 405-nm interference filter (Edmund Scientific, Barrington, NJ). Thus, a technique is now available with which simple colorimetric assays can be performed on one instrument providing data on the quantitation of both cereal α- and β-amylase, independent of each other.

Registry No. β-Amylase, 9000-91-3; α-amylase, 9000-90-2; PNP5, 66068-38-0; PNP6, 74173-30-1.

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Separation and Quantitation of Red Pepper Major Heat Principles by Reverse-Phase High-Pressure Liquid Chromatography

Patrick G. Hoffman,* Mary C. Lego, and William G. Galetto

A rapid, reverse-phase high pressure liquid chromatographic method is presented that will identify and quantitate the major heat principles (capsaicinoids) in red pepper products. The capsaicinoids are extracted from the ground spice with ethanol, separated by high-pressure liquid chromatography, and detected by ultraviolet absorption at 280 nm. The concentration of the individual capsaicinoids is determined relative to that of a commercially available external standard, *N*-vanillyl-*n*-nonamide (*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-*n*-nonamide). The data obtained by this method can be mathematically transformed into a sensory value.

The compound primarily responsible for the pungency of the capsicums is capsaicin (*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonenamide). Other structurally similar heat contributors in red pepper include dihydrocapsaicin (*N*-[(4-hydroxy-3-methoxyphenyl)-methyl]-8-methylnonanamide), and, to a lesser extent, norcapsaicin (*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-7-methyl-5-octenamide), nordihydrocapsaicin (*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-7-methyloctanamide), homocapsaicin (*N*-[(4-hydroxy-3-methoxyphenyl)-methyl]-9-methyl-7-decenamide), and homodihydrocapsaicin (*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-9-methyldecanamide). Additional related capsaicinoids have been identified as trace constituents of these products (Jurenitsch et al., 1979a,b).

A review of the literature reveals a variety of wet chemical and instrumental methods to identify and quantitate these compounds in natural products. Thin-layer chromatography (TLC), TLC coupled with ultraviolet (UV) absorbance detection at 280 nm, gas chromatography (GC), GC coupled with mass spectrometry (MS), the combination of high-pressure liquid chromatography (HPLC), GC, and MS as well as mass fragmentation with quantitation by computer analysis (Todd and Perun, 1961; Hartman, 1970; Masada et al., 1971; Tirimanna, 1972; Govindarajan and Ananthakrishna, 1974; Todd et al., 1975; DiCecco, 1976; Lee et al., 1976; Polesello and Pizzocaro, 1976; Kosma-Kovacs et al., 1977; Palacio, 1977; Pankar and Magar, 1977; Todd et al., 1977; Pankar and Magar, 1978; DiCecco, 1979; Heresch and Jurenitsch, 1979; Iwai et al., 1979; Bajaj, 1980; Suzuki et al., 1980; Rajpoot and Govindarajan, 1981) are all significant efforts toward this endeavor. Most of these procedures require derivatization of the capsaicinoids prior to analysis and/or complex instrumentation, involved preparation time, and specialized knowledge. The most effective methods quantitate the individual capsaicinoids in order to account for the different heat contributions.

Table I. Sensory Values of the Individual Capsaicinoids ($\times 10^6$)[a]

	sensory values
nordihydrocapsaicin	9.3 ± 0.4
capsaicin	16.1 ± 0.6
dihydrocapsaicin	16.1 ± 0.6
homocapsaicin	6.9 ± 0.5
homodihydrocapsaicin	8.1 ± 0.7
N-vanillyl-*n*-nonamide	9.2 ± 0.5

[a] Todd et al. (1977).

One objective method that separates and quantitates the capsaicinoids is presented by Todd et al. (1977). This method correlates a sophisticated sensory analysis with an equally complex GC separation and quantitation of the individual capsaicinoids. The capsaicinoids—nordihyrocapsaicin, capsaicin, dihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin—are synthesized and the pungencies determined for each compound by a series of carefully controlled, sensory triangle tests. The threshold pungencies of the heat compounds, as determined by this sensory method, are reported in Table I.

High-pressure liquid chromatographic methods, however, have a strong advantage over GC approaches in that derivatization is not required in order to quantitate the individual capsaicinoids (Sticher et al., 1978; Johnson et al., 1979; Jurenitsch et al., 1979a; Woodbury, 1980). Advances in both the instrumentation and methodology allow routine application of HPLC analysis.

A reproducible method that uses common reagents to separate, identify, and quantitate individual capsaicinoids on a routine basis was the objective of our research. Like Sticher et al. (1978), Jurentisch et al. (1979a), and Woodbury (1980), we chose HPLC as the analytical tool. As noted earlier, HPLC methods do not require derivatization and, therefore, are simple and direct.

Our research involved the synthesis, isolation, separation, and quantitation of the capsaicinoids.

Research and Development Laboratories (P.G.H. and M.C.L.) and McCormick/Stange Flavor Division (W.G.G.), McCormick and Company, Inc., Hunt Valley, Maryland 21031.

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