HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF URIC ACID IN GRAINS AND CEREAL PRODUCTS AS A MEASURE OF INSECT INFESTATION

by

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INTRODUCTION

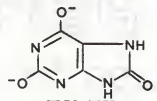
A major concern in the storage of grains and cereal products is insect infestation, and the contamination that occurs as a result of this infestation. When receiving wheat or other grains for processing into flour or feeds, it is desirable to have some index by which past infestations can be detected, in order to prevent the production of a contaminated product. Bakeries and other food processors also must have a means for analyzing ingredients to assure that they have been produced from raw materials free of insect contamination. Currently, the most widely accepted means for making these assessments is the counting of insect fragments isolated from a sample of the grain or grain product. However, the determination of insect fragments can be subject to large degrees of human error, as a highly skilled analyst is required to visually separate the insect fragments from plant material. X-raying of grain can also be used to detect internal infestations, but cannot be used on flour or milled products to determine whether infested grain was used to produce these products.

The measurement of uric acid in grains and cereal products has been proposed as an alternative method for detecting insect contamination (1). Uric acid is excreted by insects as the primary end product of nitrogen metabolism. Measurement of uric acid content will not give an indication of the future storability of a given lot of grain, but serves as an integrating indicator of past infestations.

Up to this time, the use of uric acid as an index of insect contamination has not found widespread acceptance. Most techniques currently available for uric acid analysis, including manual and semi-automated

colorimetric and ultraviolet spectrophotometric procedures, lack sufficient sensitivity to detect contamination levels normally encountered in commercial channels. Those techniques yielding sensitivities sufficient to detect low level infestations have been found to be too time consuming or inconvenient to be used in routine quality control operations. However, the development of high performance liquid chromatography (HPLC) has presented a new and promising technique for the analysis of uric acid in cereal products, as evidenced by the work of Pachla and Kissinger (22).

The purpose of this work is the development of a rapid and relatively simple procedure for analysis of uric acid in grain and cereal products by high performance liquid chromatography, with sufficient sensitivity to detect uric acid levels that may be commonly encountered in commerce, and without the inconveniences encountered with prior procedures. Also, the relationship of uric acid content to levels of insect damage in grain for various stored-product insects is investigated.



URIC ACID

LITERATURE

COLORIMETRIC PROCEDURES:

Uric acid was first suggested as a measure of insect infestation in the 1950's. Venkat Rao, et al. (1) developed a colorimetric determination of uric acid in insect damaged grain. Samples with insect damage levels of 8-50 percent were extracted with water in a Waring Blendor, and the protein precipitated with a sulfuric acid-sodium tungstate solution. The uric acid in the extract was then measured by the procedure of Benedict and Franke (2), in which uric acid is reacted with a solution containing sodium tungstate in phosphoric and arsenic acid, in the presence of sodium cyanide, to form a colored complex. The uric acid content of the grain was found to increase with increasing kernel damage. Using the previous method, Venkat Rao, et al. (3) made more extensive studies of the relationship of uric acid content to insect damage. Uric acid content was found to be better correlated to the number of insect exit holes in the sample, than to percent kernel damage by weight.

A modification of the colorimetric procedure was reported by Venkat Rao, et al. (4). Since the reaction of Benedict's uric acid reagent is not entirely specific for uric acid, a blank was prepared by treating an aliquot of the sample extract with the enzyme uricase. The uricase destroys any uric acid present in the extract, prior to reaction with the color reagent. By subtracting the blank value from the "total" uric acid value, a "true" uric acid value was obtained. Gupta and Sinha (5) used this colorimetric procedure to determine the uric acid content of insect excreta. Approximately 18 percent of the total frass of the confused flour beetle (Tribolium confusum) was found to be uric acid.

Pixton (6), and Pillai, et al. (7) assessed the value of the colorimetric procedure for determining uric acid in grain products, and found it of limited value. The lack of sensitivity of the colorimetric procedure allowed only very high infestation levels to be detected. Pixton reported that it required two weeks for four adult confused flour beetles per gram of food to produce a measurable amount of uric acid; an infestation level that should never be encountered in properly stored grain in developed countries.

Paper chromatography has been used by Venkat Rao, et al. (8) to improve selectivity by separating uric acid from other interfering sample components prior to colorimetric determination. Samples were extracted with water in a Waring Blendor, centrifuged, and concentrated by evaporation on a steam bath. The extracts were spotted on Whatman No. 1 paper, and chromatographed for 48 hrs. in a descending mode using 1-butanol- acetic acid - water (4:1:5). After drying, the chromatograms were sprayed with the arsenophosphotungstic (Benedict) reagent. Blue spots were obtained where uric acid was present. These spots could then be cut out, extracted into phosphate buffer, and the uric acid determined colorimetrically.

ULTRAVIOLET ABSORPTION PROCEDURES:

Improvements in sensitivity and specificity were obtained by use of enzymatic - ultraviolet absorption procedures. Farn and Smith (9) extracted samples of wheat flour for 1.0 hr. with a 5% sodium acetate solution. One aliquot of each extract was treated with uricase enzyme for two hours. Uricase breaks down uric acid, which absorbs ultraviolet radiation, into allantoin, a compound which does not absorb in the ultraviolet region. The absorbance of the original extract and the enzyme treated extract were then

measured at 284 nm. The difference in absorbance between these two solutions was related to the uric acid content of the sample. A detection limit of 100 µg uric acid/gram sample was reported. The current official A.O.A.C. method for uric acid in wheat flour (10) is based on a similar procedure, utilizing extraction into aqueous sodium acetate and determination by absorption of ultraviolet radiation at 292 nm. The reported detection limit is 40 µg uric acid/g flour.

Using the UV spectrophotometric procedure, Farn and Smith (11) studied the rate of excretion of uric acid by the red flour beetle (Tribolium castaneum). Both the larvae and adult insects were found to produce uric acid. The rate of excretion of uric acid per insect was found to decrease with increasing population pressure, ranging from 0.08 mg/larva/day at low populations, to 0.04 mg/larva/day at high population levels. Since relatively low population levels are normally encountered in commerce, an average figure of 0.1 mg uric acid per insect per day for both larvae and adults was taken. Using this value, the lower limit of infestation measurable by the ultraviolet - enzymatic procedure was found to be the amount excreted into 100 g of flour by one red flour beetle in 100 days, or any other combination which works out to 100 insect-days.

Bhattacharya and Waldbauer (12) used an ultraviolet absorption procedure to measure uric acid in the feces of various insects. The uric acid was extracted into aqueous lithium carbonate, and the absorbance of the extract measured before and after the addition of uricase at 292 nm, which was found to be the wavelength of maximum absorption for uric acid. A similar method of extraction and quantitation was used by Booth (13) to evaluate alfalfa seed chalcid infestation in alfalfa seed.

In order to improve the sensitivity of the ultraviolet absorption method, Sen (14) developed a procedure involving a concentration step. After pre-extraction with hexane, flour samples were extracted for 3.5 hrs. with a solution of lithium hydroxide in methanol (pH 11.5). The methanol was then evaporated under vacuum, and the residue taken up into aqueous solution. Extraction with very alkaline solutions resulted in a highly colored extract, which interfered with the spectrophotometry. A cation exchange resin was used to decolorize the extracts. Following this treatment, the absorbance of the extracts at 292 nm was measured, with and without the addition of uricase. The difference between absorbance readings was related to the uric acid concentration. A detection limit of 0.5 µg uric acid/g flour was reported for this method. Sen found that recovery of uric acid increased with increasing pH; also that recovery of uric acid could be improved by the addition of small amounts of an organic sulfide, such as dimethyl disulfide, to the extracting solution. It was postulated that uric acid was adsorbed onto the surface of the flour, and that the addition of an organic sulfide acted to block the adsorption sites.

Using this procedure, Sen studied the rate of excretion of uric acid by adults and larvae of the red flour beetle. Average excretion rates of 39.6 µg uric acid/larvae/day and 12.2 µg uric acid/adult/day were obtained. These rates are significantly lower than those reported by Farn and Smith (11). Sen and Vazquez (15) also correlated uric acid content to other methods of assessing insect damage in wheat and wheat flour. In whole grain wheat, uric acid content was compared to a) percent of damaged kernels, b) whole or equivalent insects, and c) insect damage as determined by X-ray. The best correlation was obtained between uric acid and X-ray data. Naturally infested wheat samples were milled on a Buhler experimental mill, and the

correlation between uric acid content and insect fragment count determined. The best correlation between uric acid and insect fragments was obtained for first break flours.

SEMI-AUTOMATED PROCEDURES:

In the 1970's, semi-automated colorimetric determination of uric acid in food products was introduced. Procedures of this type offered improved sensitivity over the earlier manual procedures. Laessig, et al. (16) used a Technicon AutoAnalyzer system to analyze cereal product extracts for uric acid. The methodology involves reaction of uric acid with phosphotungstic acid in the presence of cyanide:urea to form a colored complex, with subsequent colorimetric determination (660 nm). The incorporation of a dialysis step in the AutoAnalyzer flow eliminates turbidity problems frequently encountered in manual procedures. A "uricase-like" activity was noted in some sample extracts; therefore, samples are treated with 1N HCl at elevated temperature to denature any enzyme activity prior to extraction. The reported detection limit for this method is 40 µg uric acid/g sample.

A second AutoAnalyzer method for uric acid in food products (17) involves the reaction of uric acid with phosphotungstic acid and sodium tungstate to form a colored phosphotungstite complex (Fig. 1). Since the reaction is non-specific, sample blanks are determined by re-analyzing each sample extract with an immobilized enzyme coil placed into the system flow prior to dialysis. Sensitivity is enhanced by using a long-path flowcell in the colorimeter. The reported detection limit is 20 µg uric acid/g sample.

FLUORESCENCE PROCEDURES:

Fluorescence spectroscopy has also been used in the determination of

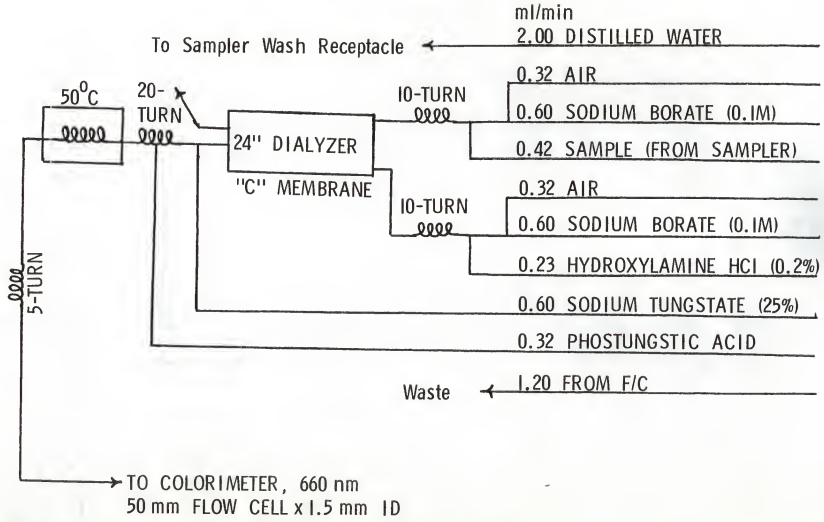
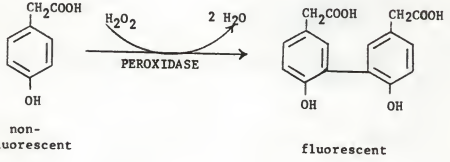


Figure 1. Flow of Technicon AutoAnalyzer for Uric Acid Analysis.

uric acid. Uric acid exhibits a relatively weak native fluorescence, with a reported excitation maximum of 330 nm, and emission maximum of 420 nm. Holmes (18) extracted flour samples in sodium acetate solution, adjusted to pH 12.0 with sodium hydroxide. After centrifugation and filtration of the supernatant through a microporous filter, the fluorescence of the extract was measured using an excitation wavelength of 330 nm, and a 390 nm emission cut-off filter. Fewer interferences from the sample matrix were found using fluorescence, than were encountered using ultraviolet absorption spectroscopy. A linear response was obtained for solutions containing 40-480 µg uric acid/ml. Samples of flour were infested with various levels of confused and red flour beetles, and these samples analyzed for uric acid. The uric acid content was found to increase with increasing insect population and residence time.

Clinical procedures for uric acid determination in plasma using fluorescence have been developed, in which a more highly fluorescent fluorophor is produced (19,20). Plasma extracts are treated with uricase enzyme. One of the products of the uric acid degradation is hydrogen peroxide, which in the presence of peroxidase couples two molecules of p-hydroxyphenylacetic acid as follows:



non-fluorescent

fluorescent

The fluorescent product is then measured, using a 317 nm excitation wavelength, and 414 nm emission wavelength. Quantities of uric acid as low as 10 ng have been detected by this method.

CHROMATOGRAPHIC PROCEDURES:

High performance liquid chromatography has been used in recent years in the determination of uric acid. Kissinger, et al. (21) used HPLC with thin layer electrochemical detection to separate uric acid from ascorbic acid in urine. Pachla and Kissinger (22) have also used this technique to determine uric acid in cereal products. Cereal product extracts were chromatographed using a pellicular strong anion exchange resin with a 0.1M acetate buffer (pH 5.25) mobile phase. Detection was obtained using a thin-layer amperometric detector with carbon paste electrode, set at +0.8 V vs. a Ag/AgCl reference. A linear response was obtained down to a level of 10 µg uric acid/g sample, with a detection limit of 2 µg/g.

Cox, et al. (23) used HPLC to determine uric acid in animal feeds containing dried poultry waste. These feeds have relatively high levels of uric acid (0.2-1.0%). Samples were extracted into a lithium carbonate-boric acid buffer solution, then chromatographed on a strong anion exchanger operating at 45° C, using a sodium acetate (0.1M) - acetic acid (0.04M) mobile phase. An ultraviolet absorption detector operating at 290 nm was used. The ultraviolet detector was found to perform satisfactorily; the high selectivity of the electrochemical detector not being required for high levels of uric acid in a feed matrix.

Reverse phase HPLC has been used for the determination of uric acid in a few selected applications. Krstulovic, et al. (24) used a reverse phase mode to separate nucleosides and bases in plasma and serun samples. Sample

components were separated on an octadecylsilane (ODS) column, using a linear gradient from 60/40 methanol/water to 0.02M KH_2PO_4. Uric acid was found to elute soon after the void volume of the column, and was not separated from other ultraviolet absorbing components. However, uric acid exhibits fluorescent properties while the interfering substances do not, allowing use of a fluorescence detector to selectively measure uric acid. Rustum (25) separated nucleosides and bases on an ODS column by eluting with a series of buffer solutions at varying pH (pH 3.0, 6.6, 9.0). Uric acid eluted soon after the void volume, but other components were sufficiently retained so that a separation was effected.

A technique which has found success as an alternative to ion-exchange for separation of ionic and highly polar organic molecules is that of ion-pairing. In ion-pairing chromatography, an ionic compound with a large, non-polar R-group, and of opposite charge from the analyte, is introduced into the mobile phase. Under proper conditions of pH, so that the sample is in ionic form, the presence of this counter-ion will allow a normally polar analyte to be retained on a non-polar column packing. Several hypotheses have been proposed for the ion-pairing mechanism. One view involves the formation of an ion-pair in the mobile phase, prior to absorption onto the bonded phase (26). This ion-pair appears as a single, non-polar molecule to the non-polar bonded phase. A second view involves an ion-exchange mechanism, with the unpaired counter-ion adsorbing onto the bonded phase surface, and causing the column to behave as an ion-exchanger (27).

The most current theory of the ion-pairing mechanism is that proposed by Bidlingmeyer, et al. (28). This theory proposes that ion-pairs are not formed in the mobile phase, but rather that counter-ions present in the mobile phase are adsorbed to the surface of the bonded phase. The presence

of the counter-ion molecules on the surface of the bonded phase creates a more favorable environment for the oppositely charged analyte molecules to adsorb to the surface; the capacity factor for the analyte is increased. In turn, the adsorption of an analyte molecule shifts the equilibrium so that the adsorption of an additional counter-ion is favored. In this manner, a pair of ions is adsorbed, but not necessarily an "ion-pair." This process occurs in a dynamic equilibrium throughout the length of the column.

Ion-pairing chromatography has been used successfully for the separation of organic acids, using various organic cations as the counter-ion. Sood, et al. (29) determined ascorbic acid in pharmaceuticals and food extracts using an ODS column and methanol/water mobile phase containing tridecylammonium formate as the ion-pairing agent. Another compound that has been used successfully in separating organic acids by ion-pairing is tetrabutylammonium dihydrogen phosphate. Wahlund (30) used tetrabutylammonium (0.03M) in phosphate buffer (pH 7.4) to separate aromatic sulfonates and benzoic acid derivatives on a silica column coated with 1-pentanol as stationary phase. Willis, et al. (31) separated water soluble vitamins, including ascorbic acid and folic acid, on reverse phase and polar bonded phase columns, using water/methanol mobile phases containing tetrabutylammonium phosphate as the ion-pairing reagent. The use of reverse phase chromatography with ion-pairing for the separation of uric acid from cereal extracts is to be investigated.

MATERIALS AND METHODS

MATERIALS:

Insect Damaged Wheat Samples:

Cultures of hard red winter (HRW) wheat infested with larvae of three of the most serious internally infesting stored product insects were obtained. The insects included for study were the granary weevil (Sitophilus granarius), the rice weevil (Sitophilus oryzae), and the lesser grain borer (Ryzopertha dominica). The cultures were maintained at 37° C and 55% relative humidity. The cultures were sieved daily to remove the adult insects as they emerged from within the kernels. This procedure was continued until a significant decrease in the rate of emergence was noted, indicating that the majority of insects had emerged. At this time, the cultures were frozen at -18° C to kill any forms that had not yet emerged.

A sample of clean HRW wheat, from the same stock as that used to prepare the cultures, was obtained. Insect damaged kernels (those kernels from which insects had emerged) were removed from the cultures and added to samples of the clean wheat, at various levels beginning at the current U.S. Food and Drug Administration defect action level of 32 damaged kernels/100 g, and at other lower levels. Each sample was ground through a Wiley mill equipped with a 2 mm screen, and mixed thoroughly prior to analysis for uric acid content.

Standards:

A 100 µg/ml uric acid stock solution was prepared by dissolving 500 mg of uric acid (Fisher Scientific, Fair Lawn, NJ) in a 1.0% aqueous solution

of sodium acetate, and diluting to 500 ml in a volumetric flask. Dilutions were made using the 1.0% sodium acetate solution to obtain standard solutions of the desired concentration.

Samples of flour and ground grain spiked with uric acid were prepared by adding uric acid to a 500 g flour or grain sample, and mixing in a mechanical tumbler for 24 hrs.

Packing of HPLC Columns:

The analytical column used consisted of a 4.1 mm I.D. by 25 cm, 316 stainless steel column (Handy and Harmon Tube Co., Norristown, PA). The exit end was milled to contain a two micron stainless steel frit (0.1875 in. dia. x 0.125 in. thick; Mott Metallurgical Corp., Farmington, CT). The column ends were fitted with 1/4 inch to 1/16 inch Swagelok low dead volume reducing unions (Crawford Valve and Fitting Co., Cleveland, OH).

The packing system consisted of a Haskel DSTV-122/CP4 pneumatic amplifier pump (Haskel, Inc., Burbank, CA), equipped with a two way valve for packing solvent flow selection, a manual override valve, a ball valve, and 25 ml slurry packing reservoir. The columns were slurry packed by the procedure of Gere (32) described below:

1) Weigh out 2.7 grams of packing material. Place in clean, dry screwcap bottle (50 ml) with plastic inner cap liner.

2) Add 10 ml of packing solvent consisting of 3 parts tetrachloroethane to 1 part dioxane. Packing solvent should be passed through an open silica column prior to use. Shake the bottle 10 times, and place in ultrasonic bath for 1.0 min.

3) Remove from ultrasonic bath and dry water adhering to bottle.

4) Pour suspension into packing apparatus, with 4.1 mm x 25 cm column attached. End terminator must be installed.

5) Top off suspension in reservoir with 10 ml of excess packing solvent.

6) Turn on high pressure pump loaded with degassed hexane. Allow hexane to flow until a minimum of 10 column volumes have passed.

7) Carefully remove excess packing material at top of column and insert upper terminator. A porous TFE disc, Ca. 100 μm thick, is used as the upper terminator.

Columns were packed at 8,500 psi. Guard columns with dimensions of 4.1 mm I.D. by 10 cm were packed using the same procedure, except that only 1.7 grams of packing material was used to pack each of these columns.

Liquid Chromatographic Mobile Phase:

Water for use in the mobile phase consisted of distilled water, which was then redistilled in an all glass apparatus from alkaline permanganate solution. Methanol was prepared using the Grignard reaction procedure described by Vogel (33). 1.697 g tetrabutylammonium dihydrogen phosphate (Eastman Organic Chemical #17524, Eastman Kodak Co., Rochester, NY), 0.560 g KH_2PO_4, and 0.480 g Na_2HPO_4 were placed in a 1 liter volumetric flask, and dissolved in a small amount of redistilled water. 100 ml of methanol were added, and the solution diluted to volume with water. Final pH of the mobile phase should be 6.6-6.7. Prior to use, the mobile phase was filtered through a 0.45 μm microporous filter (EHWP 047 00, Millipore Corp., Bedford, MA), using a Millipore filter holder.

Liquid Chromatograph:

The chromatographic apparatus consisted of a Waters M6000A dual piston reciprocating pump (Waters Associates, Milford, MA), a Rheodyne Model 70-10 six-port injection valve (Rheodyne Corp., Berkeley, CA) equipped with a Model 70-11 sample loader and 100 μl injection loop, and a Waters Model 202 fixed wavelength, differential ultraviolet detector. The detector was modified to monitor at 280 nm, rather than the standard 254 nm, by use of an adapter

utilizing a phosphor to produce radiation at 280 nm when radiated by the standard mercury source lamp (General Electric G4T4). For procedures requiring fluorescence detection, an Aminco Fluoro-Colorimeter (American Instrument Co., Silver Spring, MD) fitted with an 8 µl flowcell was used. The fluorometer was equipped with an F4T4/BL source lamp, and Corning 7-60 filter on the excitation side. This source and filter combination produces maximum excitation in the range of 360 nm. A Wratten 2A filter, with a cut-off of 414 nm was used as the emission filter.

Chromatograms were recorded on a Houston Instruments Omniscribe strip chart recorder (Houston Instruments, Austin, TX), or on a Hewlett-Packard 3385A electronic integrator (Hewlett-Packard Corp., Avondale, PA).

METHODS:

Extraction of Uric Acid from Cereal Products:

Whole grain samples were ground through a Wiley mill, with a 2 mm diameter screen, prior to extraction. A 5.000 g sample of flour or ground grain was weighed into a 100 ml centrifuge tube. 10.0 ml of 1N HCl were added to the sample, and the tube stoppered and shaken to slurry the sample with the acid. The tube was then placed in a 55-60° C water bath for 15 min. Upon removal from the heating bath, 35 ml of distilled water were added to the sample slurry.

The extract was neutralized by the addition of 5N NaOH. Final adjustment of the pH of the extracting solution, which should be in the range of 8.5-9.5, was made by the dropwise addition of 0.5N NaOH. After adjustment of pH, the tube was stoppered and shaken vigorously on a wrist-action shaker (Burrell Corp., Pittsburgh, PA) for 5.0 min, then centrifuged for 15 minutes at a minimum of 2000 x g. Following centrifugation, the supernatant was

decanted into a 50 ml volumetric flask, and diluted to volume with distilled water.

Prior to injection into the chromatograph, an aliquot of the extract was pressure filtered through a 0.45 µm microporous filter (HAWP 013 00, Millipore Corp., Bedford, MA), by use of a Swinney adapter fitted to a 10 ml hypodermic syringe. Filtration through a microporous filter removes particulate matter from the extract which may otherwise tend to clog the analytical column.

Chromatography of Sample Extracts:

The solvent pump was set to deliver mobile phase at a rate of 1.0 ml/min. The sample injection size used was 100 µl. A 10 cm guard column was placed between the sample injection valve and the analytical column. Both the guard column and the analytical column were packed with the same material, specifically Partisil ODS-2 (Whatman, Inc., Clifton, NJ), with a mean particle diameter of 10 µm. The system was operated at ambient temperature. The UV detector sensitivity was set at 0.02 absorbance units full scale.

A Hewlett-Packard 3385A integrator was used to quantitate the data. Integrator parameters used included:

chart speed:	0.5 cm/min
attenuation:	4
slope sensitivity:	0.1
area reject:	1000

The integrator was set to re-establish the baseline at the valley following each peak of the chromatogram. Quantitation was obtained by use of an external standard.

RESULTS AND DISCUSSION

Because of the subjective nature of the insect fragment count, and the wide variability in results that is frequently experienced between laboratories, an alternative method of assessing insect contamination is desirable. Uric acid content appears to hold promise as an alternate, and less subjective method. Initial attempts in our laboratory to measure uric acid in grain products involved the use of a Technicon AutoAnalyzer (Technicon Instrument Corp., Tarrytown, NY), with current AutoAnalyzer methodology (17). However, this procedure was found to be less than satisfactory. The color producing reaction involving phosphotungstic acid is not specific to uric acid. In many cereal product extracts, the blank was found to yield a response of much greater magnitude than the response due to uric acid in the sample. This frequently made reproducibility of results very poor. Also, a detection limit of 20 µg uric acid/g sample could be obtained at best. Data of previous investigators tended to indicate that significantly lower detection limits were needed for a method to provide useful information.

High performance liquid chromatography appeared to present the best opportunity for a sensitive yet rapid procedure for analysis of uric acid in grain products. However, we believed that for a method to be accepted as a routine quality control procedure, it would be desirable to eliminate the ion-exchange separation mode, with its harsh solvents and need for close thermal regulation, and inconvenient electrochemical detector used in existing methods (22).

CHROMATOGRAPHY OF URIC ACID:

Initial attempts to separate uric acid with a reverse phase separation

mode were unsuccessful. Uric acid, being a highly polar compound, could not be retained on a non-polar ODS column. Uric acid eluted immediately following the void volume of the column, and could not be separated from other UV absorbing components.

UV absorption had been selected as the preferred method of detection, as uric acid is a relatively strong absorber in the UV region, and also because the UV detector is the easiest to operate and most widely used HPLC detector. However, since a fluorescence detector has been used successfully in clinical applications to selectively detect uric acid in the presence of other components (24), this method of detection was investigated for uric acid in cereal product extracts. However, the native fluorescence was not sufficiently strong so that uric acid could be detected at the low levels encountered in cereal product extracts, with the fluorescence detection system available to us. An attempt to enhance the sensitivity of fluorescence detection was made by preparation of the p-hydroxyphenylacetic acid derivative. This was also unsuccessful, as the use of organic solvent in the mobile phase quenched the fluorescence of this derivative, and it could not be separated from other fluorescing components in the sample using only aqueous solvents.

Because of the lack of success with fluorescence detection, UV absorption was returned to as the method of detection, and other separation modes were investigated to separate uric acid from UV absorbing interferences. Chromatography of uric acid on an amine polar bonded phase column was unsuccessful, as the uric acid was so tightly bound to the polar column that uric acid could not be eluted off the column at a reasonable retention time. An ion-pairing separation mode using an ODS column was then investigated, and found to be most satisfactory for separation of uric acid from other sample interferences.

Tetrabutylammonium dihydrogen phosphate was used as the ion-pairing reagent, and added to a water/methanol mobile phase at a concentration of 0.005M. The phosphate salt of tetrabutylammonium was selected, as it is not extremely basic as is the hydroxide form, and is less detrimental to the wetted surfaces of the chromatograph than are the halogen salts. Increasing the concentration of the ion-pairing reagent above 0.005M did not improve the separation.

Two factors which must be carefully controlled to obtain a successful separation are the pH of the mobile phase, and the percent of organic modifier in the mobile phase. The optimum pH of the mobile phase was found to be 6.6-6.7. The pH of the mobile phase was adjusted by use of a dilute inorganic phosphate buffer system. Decreasing the pH of the mobile phase below 6.6 tended to increase the retention time of uric acid, resulting in broad peaks with significant tailing. Increasing the pH decreased the retention time of uric acid, making the deparation of uric acid from other components difficult. It should be remembered that the presence of an organic solvent in the mobile phase will raise the pH above that of the parent aqueous fraction; this must be taken into consideration during preparation of a mobile phase.

Methanol was used as the organic modifier in the mobile phase, with the best separation obtained at a level of 10% methanol. In general, increasing the percentage of organic modifier in the mobile phase of an ion-pairing system will decrease the retention time of the analyte.

The column used with greatest success in the separation was a 25 cm column packed with Whatman Partisil 10 ODS-2. Partisil ODS-2 is a reverse bonded phase packing material, with a mean particle diameter of 10 μm. An ODS (octadecyl silane) column is formed by bonding a hydrocarbon chain 18

carbons in length to a silica support material (Fig. 2). Specifically, monochlorooctadecyl silane is reacted with silica gel to form the monomeric ODS bonded phase. Partisil-2 is what is known as a high load, medium coverage, reverse phase packing. The carbon load is expressed in percent carbon, as determined by microcombustion analysis. The carbon load of Partisil-2 is approximately 15%. Coverage is an indication of the percentage of hydroxy groups on the silica support to which an ODS molecule has been bonded. The percent coverage of Partisil-2 is reported as 75%. Increasing the carbon load tends to make a column more retentive. The carbon load and coverage also affects the elution order of acidic and basic analytes (34).

A 10 cm guard column also packed with Partisil-2 was used ahead of the analytical column to protect the analytical column. Uric acid is extracted into relatively basic solution (pH 8.5-9.5), and a relatively large sample injection volume (100 µl) is used to improve the sensitivity of the determination. The injection of this large of a volume of alkaline solution may tend to dissolve away a portion of the silica skeleton at the head of the column. By inserting the guard column, the guard column will be sacrificed and the solvent saturated with silicate ions prior to reaching the analytical column. Dissolution of the analytical column will be suppressed by the common-ion effect. This prevents the development of a void volume at the head of the analytical column, with resultant loss in efficiency. Also, the guard column serves to catch undesirable materials from the sample matrix, and prevents them from reaching and contaminating the analytical column.

Use of the aforementioned mobile phase and column conditions resulted in a near-baseline separation with good freedom from interferences (Fig. 3).

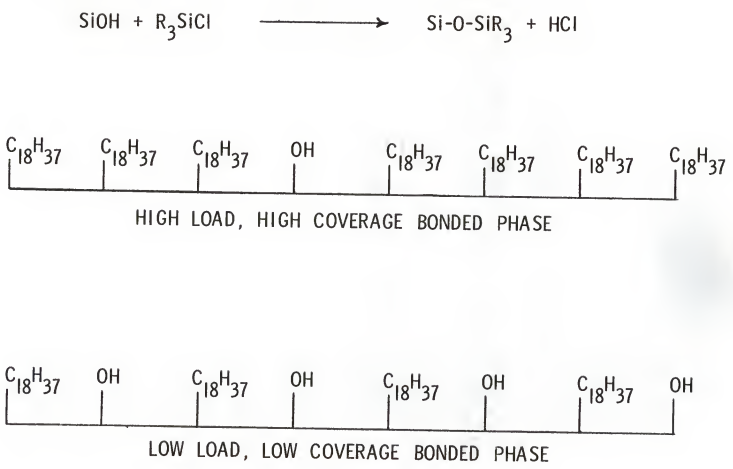
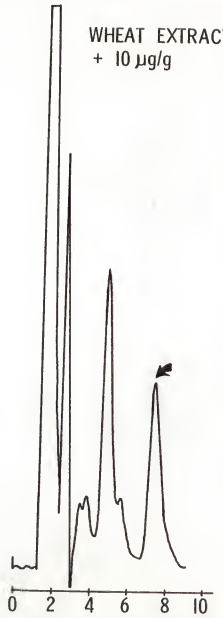


Figure 2. Preparation of ODS Bonded Phase Packing.



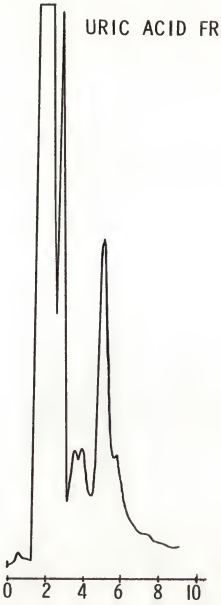


Figure 3. Chromatographic Separation of Uric Acid.

The separation has been found to be successful for wheat, wheat flour, sorghum, and milled rice extracts. No additional interferences were found in sample extracts containing uric acid as a result of insect infestation. Uric acid has a retention time in the range of 7 min, with good reproducibility of retention times. The k' value for uric acid is in the range of 5.0. k' is defined as the ratio of the retention time of the analyte of interest, to the residence time on the column of a non-retained compound. Concentration of organic modifier and pH can be adjusted slightly to optimize the system for any given column.

The Waters Model 202 UV detector was modified for measurement at 280 nm, rather than the standard 254 nm. Use of the 280 nm wavelength allowed measurements to be taken closer to the λmax of uric acid at 292 nm. The Model 202 uses a phosphor to obtain the 280 nm wavelength, requiring certain tradeoffs to be made. The intensity of radiation produced by the phosphor at 280 nm is significantly decreased from the intensity at 254 nm, yielding a poorer signal to noise ratio. Also, the radiation emitted from the phosphor is not monochromatic, and therefore Beer's law does not strictly apply. However, in this case, the increased molar absorptivity at 280 nm was sufficient to overcome the negative contributions of these other factors, and the instrument response was improved by approximately a factor of two (Fig. 4).

EXTRACTION OF URIC ACID FROM CEREAL PRODUCTS:

Samples to be extracted were first treated with 1N HCl at elevated temperatures, as recommended by Laessig (16). Many samples were found to exhibit the presence of uricase activity, which tended to degrade uric acid while in solution. The treatment with HCl prior to extraction denatures the uricase, or any other enzymes present, that may break down uric acid.

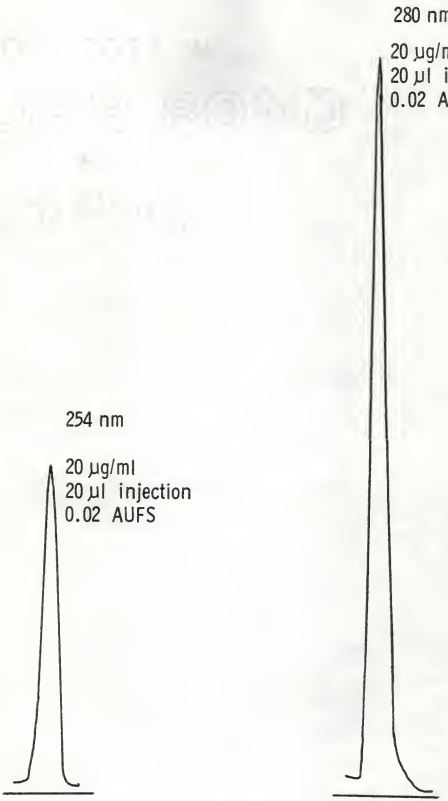


Figure 4. Comparison of Response at 280nm vs. 254nm.

After the HCl treatment, the slurry is diluted with distilled water, and the HCl neutralized with NaOH. Previous procedures recommended extracting the uric acid in solutions of 3.0-5.0% sodium acetate, following the neutralization with NaOH; however, it was found that when a sodium salt was already present in the solution as a result of the neutralization step, the addition of sodium acetate to the extracting solution decreased the recovery of uric acid (Fig. 5). As the pH of the extracting solution is adjusted upward by the addition of NaOH, the sodium salt of uric acid will be formed. Addition of sodium ion to the extracting solution, in the form of sodium acetate, will suppress the ionization of the sodium salt of uric acid by the common ion effect, thereby decreasing the amount of uric acid extracted into solution. Recovery can be maximized by the use of distilled water as the extractant, or by using potassium acetate, or other salt having a cation other than sodium, in the extracting solution.

In his work with alkaline methanolic extracts of uric acid, Sen reported that the addition of small amounts of organic sulfides, such as dimethyl disulfide, to the extracting medium improved the recovery of uric acid (14). It was postulated that uric acid is adsorbed onto the surface of flour particles, and that the added sulfides act to block the sites responsible for binding the uric acid. This same effect was noted for aqueous extracts of uric acid. The addition of very small quantities of carbon disulfide (1 drop, Ca. 0.05 ml) to the extracting solution resulted in a significant improvement in recovery; an 18% increase in recovery was obtained for wheat flour extracts (Fig. 6). Carbon disulfide was added to the extracting solution rather than dimethyl disulfide, as CS_2 is slightly soluble in aqueous solution while dimethyl disulfide is not.

The effect of pH of the extracting solution on recovery was studied.

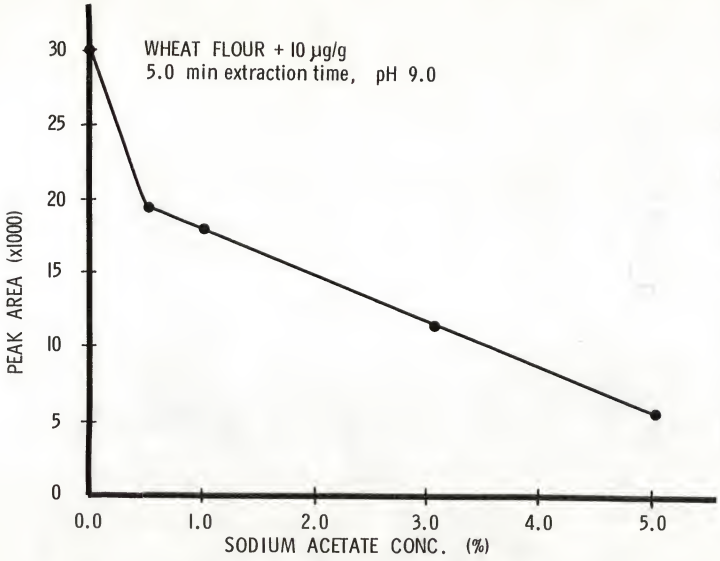


Figure 5. Effect of Sodium Acetate Concentration on Recovery of Uric Acid.

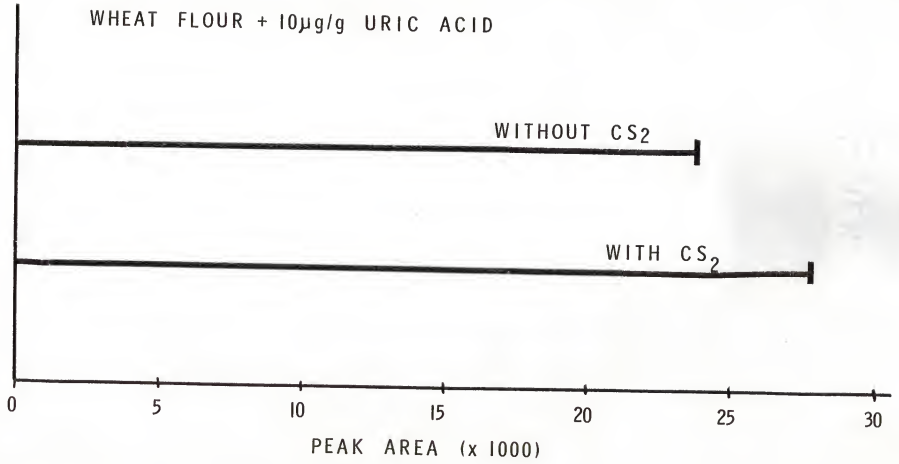


Figure 6. Effect of Addition of Carbon Disulfide on Recovery of Uric Acid.

Uric acid is highly soluble in basic aqueous solution above pH 8.0. It was found that the initial pH of the extracting solution can be allowed to range from 8.5 to 9.5 with no effect on the recovery (Fig. 7). Differences of response within this range were within the standard deviation of the method. Increasing the pH further did not improve recovery. As the pH of the extracting solution is increased, a more highly colored extract is obtained. The presence of these pigments in the extract presented no problem; however, as they eluted off the column very early in the chromatographic run and did not interfere with the separation or quantitation of uric acid. In general, the pH of the extracts was kept below ten. With the large sample size introduced onto the column, the injection of very basic extracts does affect the chromatographic separation. Also, the continued injection of very alkaline extracts may accelerate the rate of column deterioration, due to dissolution of the silica support skeleton.

The effect of extraction time on recovery was also investigated. Uric acid was found to extract rapidly into solution. Maximum recovery was obtained after only 5.0 min of extraction on a wrist action shaker (Fig. 8). Extracting for longer periods of time did not improve the recovery.

REPRODUCIBILITY AND LINEARITY OF URIC ACID DETERMINATION:

The reproducibility and linearity of the uric acid determination were evaluated. The reproducibility of the chromatography and integration was evaluated first. A series of seven replicate injections of a given extract were made, and the relative standard deviation of peak areas for these replicate injections determined (Table 1). A relative standard deviation of 2.47% was obtained for these replicate injections.

Fig. 9 shows the results obtained from analyzing a series of wheat flours spiked with low levels of uric acid (2-10 μg/g). A highly linear

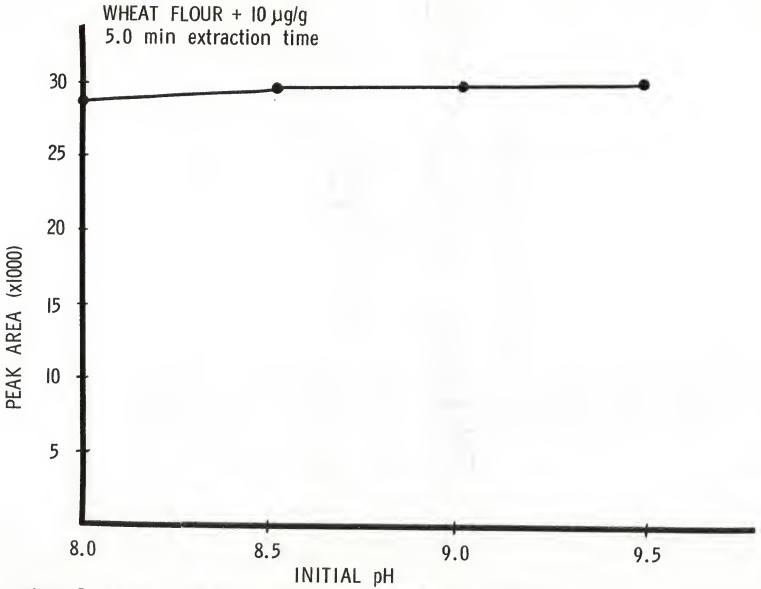


Figure 7. Effect of pH During Extraction on Recovery of Uric Acid.

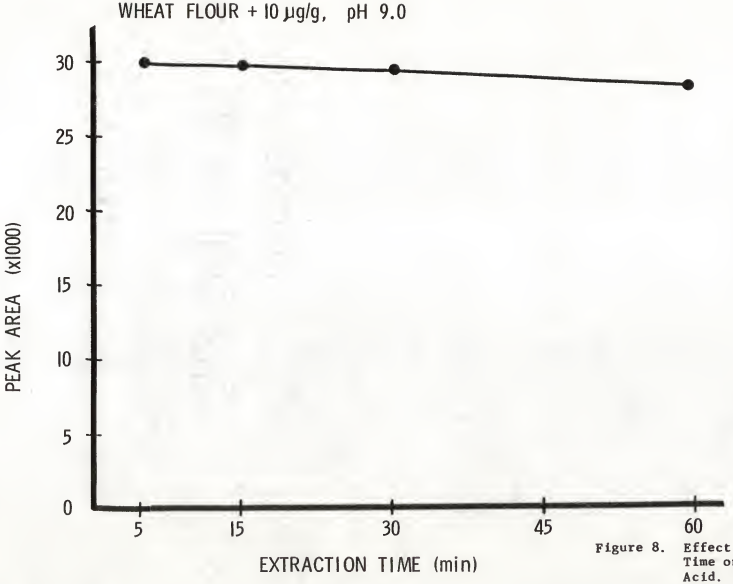


Figure 8. Effect of Extraction Time on Recovery of Uric Acid.

TABLE 1

REPRODUCIBILITY OF INJECTION
OF SPIKED WHEAT EXTRACT

Injection No.	Peak Area
1	24780
2	25620
3	25930
4	24920
5	24130
6	24920
7	24540

Mean Peak Area:	24977
Standard Deviation:	616
% Relative Standard Deviation:	2.47%

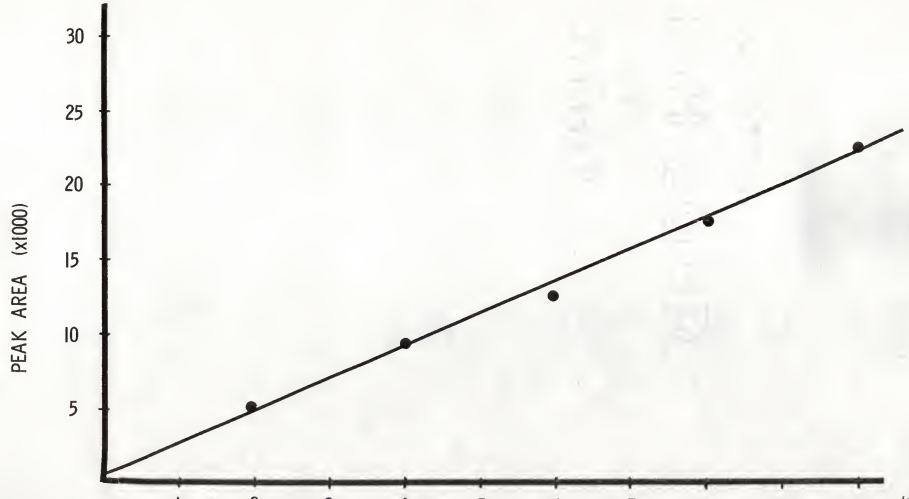


Figure 9. Peak Area vs. Concentration for Wheat Flour Extracts.

response was obtained even at these low concentrations. The method has an effective detection limit of 1.5 µg uric acid/g sample, as quantitation of peak areas from lower concentrations is extremely difficult. Fig. 10 shows the results of analyzing several ground wheat samples spiked with somewhat higher levels of uric acid. Again, a highly linear response was obtained.

A higher recovery of uric acid is obtained from ground wheat samples than from flour, yielding a different calibration slope for different sample matrices. An 80% recovery is obtained from wheat flour, while 95% of added uric acid can be recovered from ground wheat. The difference in recovery rates may be the result of uric acid being sorbed to the surface of the sample matrix, with the more finely granulated flour presenting a much larger surface area for this adsorption to take place. Because of these differences in rates of recovery, it is recommended that standards be prepared by spiking quantities of the sample matrix of interest with known amounts of uric acid.

To determine the reproducibility of the overall analytical procedure, extraction and chromatography, seven replicate flour samples were analyzed. Quantitation for each sample in µg uric acid/g sample was obtained by use of an external standard. The average value from duplicate injections of each sample was used to calculate the precision (Table 2). A standard deviation of 0.27 µg/g, with sample mean of 10.7 µg/g, was obtained for the overall procedure.

CORRELATION OF URIC ACID CONTENT TO INSECT DAMAGE:

Samples of HRW wheat, spiked at varying levels with wheat kernels damaged by granary weevil, rice weevil, and lesser grain borer, were analyzed for uric acid content by the previously described method. The data obtained for samples containing granary weevil damaged kernels is presented in

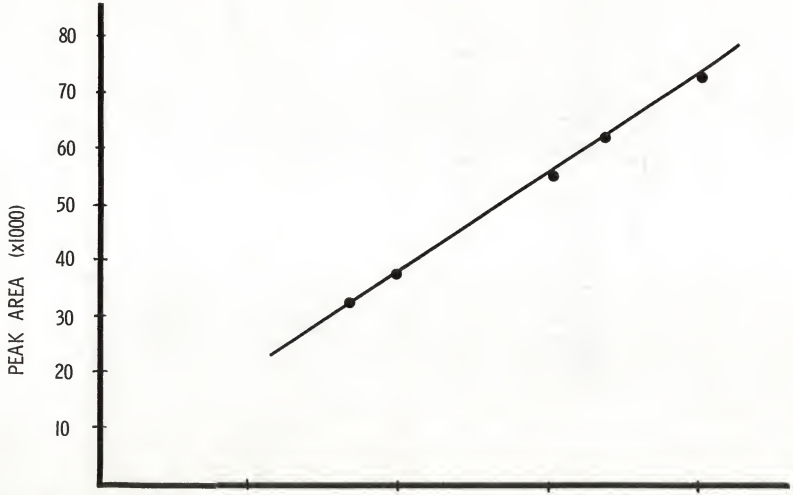


Figure 10. Peak Area vs. Concentration for Wheat Extracts.

TABLE 2

REPRODUCIBILITY OF ANALYSIS

Sample No.	Uric Acid (µg/g)
1	10.8
2	10.9
3	11.2
4	10.4
5	10.6
6	10.6
7	10.5

Mean:	10.7
Standard Deviation:	0.27
Relative Standard Deviation:	2.52%

Table 3. Duplicate wheat samples spiked with the FDA defect action level of 32 damaged kernels/100 g were found to contain 27.4 µg uric acid/g sample ±0.7 µg/g. Based on this data, each granary weevil damaged kernel contained an average of 85.6 µg uric acid ±2.4 µg, where a damaged kernel is defined as a kernel from which an internally infesting insect has emerged. The value of 85.6 µg should serve as a good representation of the quantity of uric acid produced during the development of a single granary weevil from egg to adult. The granary weevil produces very little dust that is ejected from the kernel; therefore, most of the uric acid and other products produced are retained within the kernel from which the adult has emerged.

Uric acid levels obtained from wheat samples containing varying levels of granary weevil damaged kernels indicate a high degree of linearity between uric acid content and levels of insect damaged kernels (Fig. 11). Given the detection limit of the method, it should be possible to detect granary weevil damage at the rate of 2 damaged kernels, or 2 kernels containing developing insects late in the life cycle, per 100 g of grain. Insect infestation levels in this range may routinely be encountered in commerce.

Table 4 presents the data obtained for wheat samples containing rice weevil damaged kernels. The rice weevil, being a smaller insect than the granary weevil, was also found to produce smaller quantities of uric acid. Duplicate wheat samples infested at a rate of 32 rice weevil damaged kernels/100 g yielded a uric acid content of 11.9 µg uric acid/g sample ±0.4 µg/g. This is equivalent to 37.2 µg uric acid ±1.1 µg per rice weevil damaged kernel. Again, the rice weevil produces very small amounts of dust, so the uric acid produced should be retained within the damaged kernel. The detection limit for rice weevil infestation would be an average of 5 damaged kernels/100 g.

TABLE 3

URIC ACID CONTENT OF GRANARY WEEVIL DAMAGED WHEAT

Damaged Kernels/100 g	Uric Acid (µg/g)
32	27.4 ± 0.7
16	14.2
4	3.2

µg Uric Acid/Damaged Kernel = 85.6 ± 2.4

Detection Limit = 2 Damaged Kernels/100 g

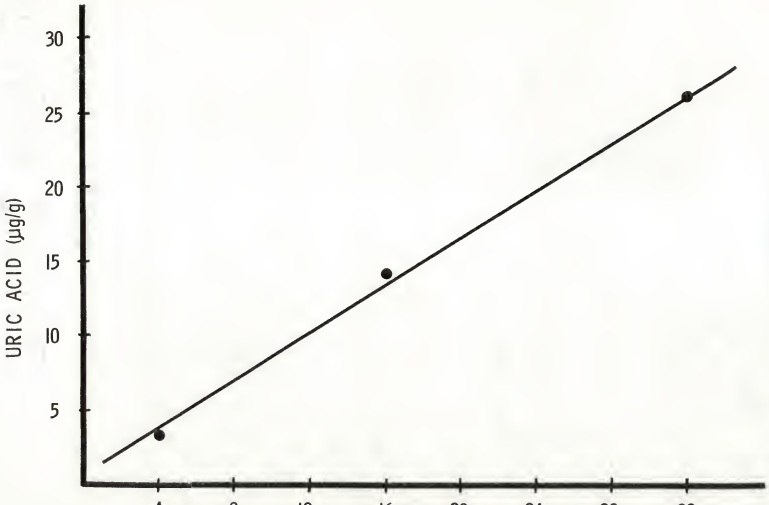


Figure 11. Relationship of Granary Weevil Damage to Uric Acid Content.

TABLE 4

URIC ACID CONTENT OF RICE WEEVIL DAMAGED WHEAT

Damaged Kernels/100 g	Uric Acid (µg/g)
32	11.9 ± 0.4
16	4.8

µg Uric Acid/Damaged Kernel = 37.2 ± 1.1

Detection Limit = 5 Damaged Kernels/100 g

Lesser grain borer differs from the two weevils, in the fact that the lesser grain borer produces a large amount of dust which is ejected out of the infested kernel. Most of the uric acid produced by the lesser grain borer is ejected out of the infested kernel in this dust. Wheat containing lesser grain borer damaged kernels at the rate of 32 damaged kernels/100 g yielded a uric acid concentration of only 3.9 µg uric acid/g sample ±0.2 µg/g. This is equivalent to only 12.2 µg uric acid ±0.4 µg per damaged kernel. A sample of the dust sieved from the lesser grain borer culture was analyzed, and found to contain very high levels of uric acid. The amount of uric acid present in a given lot of grain infested by lesser grain borer would depend on the handling of the grain prior to the time it was analyzed. If the grain underwent cleaning by aspiration prior to the time it was analyzed, much of the uric acid would probably be removed from the grain with the dust. However, if the grain was analyzed prior to cleaning, a uric acid value reflecting a more accurate measure of infestation levels would be obtained.

The data indicates that a good correlation exists between insect damage levels and uric acid content, especially for weevil infested grain. Detection limits are sufficiently sensitive to detect levels of insect damage that may be encountered in commerce in developed countries. The procedure provides a means for screening incoming grain for contamination from insect infestation, so that grain which has been heavily infested can be prevented from being processed into consumer food products.

CONCLUSION

A high performance liquid chromatographic procedure for analysis of uric acid in wheat, wheat flour, and other grains has been developed. The procedure involves extraction of uric acid into aqueous alkaline solution, following denaturation of uricase in the samples by acid treatment at elevated temperature. Separation of uric acid from other components in the sample extract is accomplished using a reverse phase separation mode with an ODS column, and ion-pairing with tetrabutylammonium ion. Detection is obtained by measurement of UV absorption at 280 nm. A detection limit of 1.5 µg/g is obtained with good linearity throughout the range of interest. Overall precision for the method is 0.27 µg/g.

This procedure offers significant improvement in sensitivity over colorimetric and fluorometric procedures. Detection limits are at least one order of magnitude lower than those of the colorimetric methods. Detection limits rival those of the UV spectrophotometric procedure with sample concentration, while providing significant time saving. Improvement over previous HPLC methods is also obtained, as the response is linear from 2-10 µg/g, and the ion-exchange separation mode, with its need for close temperature regulation, strong aqueous solvents, and frequent column regeneration, is eliminated. An ultraviolet absorption detector is also used in place of the electrochemical detector. The UV detector is the most widely used HPLC detector, and can in general be more easily operated and maintained by semi-skilled personnel, than can the electrochemical detector.

Analysis of wheat samples containing varying levels of kernels damaged by internally infesting insects, indicates a good correlation between uric

acid content and insect damage levels, especially for grain infested by weevil. Detection limits for the method are sufficiently low, so as to allow detection of infestation levels that may be encountered in commerce in developed countries.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF URIC ACID IN GRAINS AND CEREAL PRODUCTS AS A MEASURE OF INSECT INFESTATION

by

RANDY LEE WEHLING

B.S., Kansas State University, 1976

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1980

ABSTRACT

A relatively rapid method for uric acid analysis in grains and cereal products, with sufficient sensitivity to detect infestation levels frequently encountered in commerce, is presented. The measurement of uric acid in grain and cereal products is proposed as an alternative to insect fragment count for determination of insect contamination. Data obtained indicates good correlation between uric acid content and insect damage levels, particularly for weevil damaged grain. The procedure is sufficiently sensitive to detect infestation levels of less than five damaged kernels/100 g.

Uric acid was extracted in alkaline aqueous solution, following treatment of a sample with 1N HCl at 55-60° C to denature uricase enzyme. Uric acid was then separated from other sample components by high performance liquid chromatography (HPLC), using reverse phase separation with ion-pairing and detection by absorption of UV radiation at 280 nm. A 25 cm Partisil-2 ODS column was used, with a 90:10 water/methanol mobile phase adjusted to pH 6.6-6.7 containing 0.005M tetrabutylammonium dihydrogen phosphate as the ion-pair reagent.

The method gave a linear response even at low concentrations (<10 µg/g sample), and has a practical detection limit of 1.5 µg uric acid/g sample. A relative standard deviation of 2.47% was obtained for replicate injections, while the reproducibility for the overall procedure was found to be 0.27 µg/g (2.52% RSD). This new method offers significantly improved sensitivity over colorimetric procedures, and significant time savings over UV spectrophotometric procedures, while maintaining approximately the same detection limit. It also eliminates the inconvenience of ion exchange separation and electrochemical detection used with existing HPLC procedures.