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

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SUMMARY

Uric acid, excreted by insects as an end product of nitrogen metabolism, is measured, with a detection limit of 1.0 μg uric acid/g sample, in grain and cereal products as an indication of contamination by stored product insects. The uric acid extracted from a cereal product sample into alkaline aqueous solution is analytically separated from other components in the extract by high-performance liquid chromatography. Ion-pairing with tetrabutylammonium ion in methanol–water is used on a reversed-phase column with detection at 280 nm. A column switching technique is employed to minimize analysis time. The method is linear over the range of interest, allowing quantitative and objective detection of infestation levels which may be encountered in commerce.

INTRODUCTION

A major concern in the storage of grain and cereal products is insect infestation and the resulting contamination. Currently, the most widely accepted means for assessing the level of insect contamination is the counting of insect fragments isolated from a sample of grain or cereal product. However, the determination of insect fragments can be subject to a large degree of human error, as visual separation of the insect material from plant material is required. For this reason, the measurement of uric acid in grain has been proposed as an alternative method for detecting insect contamination.

Uric acid is excreted by various species of stored product insects as a primary end product of nitrogen metabolism, and measurement of uric acid content can serve as an integrating indicator of past infestations. However, up to this time, the use of uric acid as an index of insect contamination has not found widespread acceptance primarily due to the lack of sensitivity and long analysis times of analytical procedures currently available.

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Manual colorimetric procedures suffer from lack of reproducibility and high blank values due to poor specificity of the reaction, and have sensitivity sufficient only to detect very high infestation levels[1–6]. Semi-automated adaptations of these colorimetric procedures have improved sensitivity, but frequently suffer from severe background interferences requiring application of difference techniques following enzymatic treatment[7,8]. Enzymatic–ultraviolet (UV) procedures allow for some improvement in both sensitivity and specificity[9], but generally cannot detect infestation levels normally encountered in commerce. Those UV procedures utilizing sample concentration and clean-up steps, which are capable of detecting low-level infestations[10], have been found to be too time-consuming to be used for routine analysis. A fluorescence procedure making use of the native fluorescence of uric acid has also been reported[11]. Fewer interferences are obtained than with UV absorption, but lack of sensitivity remains a problem.

In recent years, high-performance liquid chromatography (HPLC) has been used to determine uric acid in plasma and urine[12–16], and to determine uric acid levels in animal feedstuffs containing added poultry waste[17]. Pachla and Kissinger[18] have also reported a procedure for determination of low levels of uric acid occurring in grain from insect infestation, using ion-exchange chromatography with thin-layer electrochemical detection. In this paper we describe the development of an alternative method for linear determination of low levels of uric acid in grains and cereal products by HPLC, using a reversed-phase separation mode with ion-pairing, and detection by absorption of UV radiation.

EXPERIMENTAL

Standards

A 100 μg/ml uric acid stock solution was prepared by dissolving 50.0 mg of uric acid (Fisher Scientific, Fair Lawn, NJ, U.S.A.) 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 were prepared by adding uric acid to a 500-g flour or grain sample, and mixing in a mechnical tumbler for 24 h.

Extraction of uric acid from cereal products

Whole grain samples were ground through a Wiley mill, equipped with a 2-mm diameter screen, prior to analysis. A 5.000-g sample of flour or ground grain was weighed into a 100-ml centrifuge tube; 10.0 ml of 1 *M* hydrochloric acid were added to the sample, and the sample was slurried with the acid. The tube was then placed in a 55–60°C water bath for 15 min. Upon removal from the heating bath, 30 ml of distilled water were added to the sample slurry.

The extract was neutralized by addition of 5 *M* sodium hydroxide solution. Final adjustment of the pH of the extracting solution, which should be in the range of 9.0–10.0, was made by the dropwise addition of 0.5 *M* sodium hydroxide solution. After adjustment of pH, two drops of carbon disulfide were added. The tube was then stoppered and shaken vigorously on a wrist action shaker for 5.0 min, then centrifuged for 15 min at a minimum of 2000 *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, Milipore, Bedford, MA, U.S.A.), by use of a Swinny adapter fitted to a 10-ml hypodermic syringe.

Chromatography

Water for use in the liquid chromatographic mobile phase was redistilled in an all-glass apparatus from alkaline permanganate solution. Dry methanol was prepared by distillation over Grignard reagent. Amounts of 1.697 g of tetrabutylammonium dihydrogen phosphate (Eastman Organic Chemical No. 17524, Eastman Kodak, Rochester, NY, U.S.A.), 0.560 g of KH_2PO_4 and 0.480 g of Na_2HPO_4 were placed in a 1-l volumetric flask, and dissolved in a small amount of redistilled water. After 110 ml of methanol had been added, the solution was diluted to volume with water. The final pH of the mobile phase should be 6.6–6.7.

The chromatographic apparatus consisted of a Waters M6000A dual-piston reciprocating pump (Waters Assoc., Milford, MA, U.S.A.), a Waters M45 dual-piston reciprocating pump, a Rheodyne Model 7120 six-port injection valve (Rheodyne, Cotati, CA, U.S.A.) equipped with 100-μl injection loop, a Rheodyne Model 7010 six-port switching valve and a Waters Model 202 fixed-wavelength UV 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 irradiated by the standard mercury source lamp (General Electric G4T4). The detector sensitivity was set at 0.02 absorbance units full scale. Chromatograms were recorded on a Hewlett-Packard 3385A recording integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The entire system was operated at ambient temperature.

The analytical column selected was a commercial 25 cm × 4.6 mm I.D. Alltech RP600 column (Alltech, Deerfield, IL, U.S.A.), packed with a fully silanized, highly loaded 10-μm diameter octadecylsilane (ODS) material. A column switching tech-

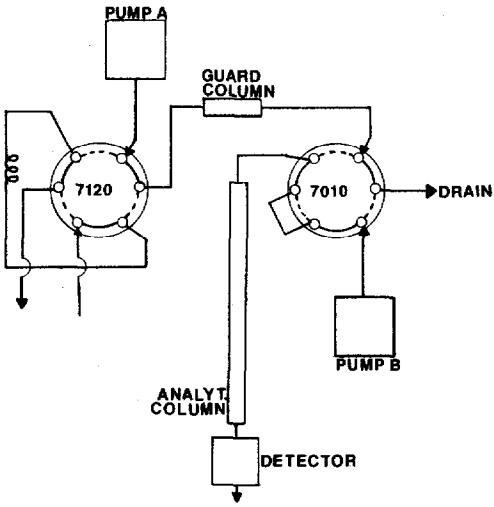

Fig. 1. Flow diagram of HPLC system in column switching mode as used for determination of uric acid in grain and cereal products.

nique was employed utilizing a 5-cm column packed with 10-μm diameter octadecylsilane material and the six-port switching valve, in conjunction with the analytical column (Fig. 1). Each pump was set to deliver mobile phase at 1.0 ml/min. Samples were injected via the 7120 injection valve onto the short column. At time of injection, the switching valve was set so that eluent from the short column was diverted directly to waste. Solvent flow through the analytical column and detector was maintained independently by the second pump. At 1.00 min after injection, the switching valve was rotated so that components eluting from the short column were directed onto the analytical column. At 2.50 min into the chromatographic run, after uric acid had eluted onto the analytical column, the valve was switched back to its initial position. During the remaining time that uric acid was eluting from the analytical column, other more highly retained components were eluted from the short column directly to waste.

RESULTS AND DISCUSSION

Chromatography

Initial attempts to separate uric acid with a reversed-phase separation mode were unsuccessful, as the highly polar uric acid eluted immediately following the void volume of the column, and could not be separated from other UV-absorbing components. Chromatography of uric acid on an amine polar bonded phase column was also unsuccessful, as the uric acid was so tightly bound to the polar column that it could not be eluted at a reasonable retention time.

An ion-pairing separation mode using an ODS column was found to be most satisfactory for separation of uric acid from interfering substances in the sample. A mobile phase containing 11% methanol and 0.005 *M* tetrabutylammonium dihydrogen phosphate yielded the best separation when the pH was adjusted to the optimum range of 6.6–6.7 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 separation of uric acid from other components difficult.

Use of the optimized conditions resulted in a baseline separation with good freedom from interferences. Uric acid has a retention time in the region of 12 min and a capacity factor (k') of *ca.* 4.5. Good reproducibility of retention time has been obtained during the course of analysis of several hundred samples. Freedom of uric acid from interfering substances in extracts of grain infested by major stored product insects, including granary weevil, rice weevil, and lesser grain borer, was confirmed. Sample extracts were analyzed, treated with uricase to break down the uric acid, then reanalyzed. No co-eluting interferences were encountered. The selectivity of the HPLC technique eliminates the need for enzymatic treatment and use of difference techniques. The separation has been used successfully for wheat, wheat flour, sorghum, and milled rice extracts.

Certain samples were found to contain highly retained components having retention times greater than 30 min. In order to minimize the required analysis time, a column switching technique was adopted. Use of the column switching technique allowed the required chromatographic time to be limited to *ca.* 20 min (Fig. 2). In

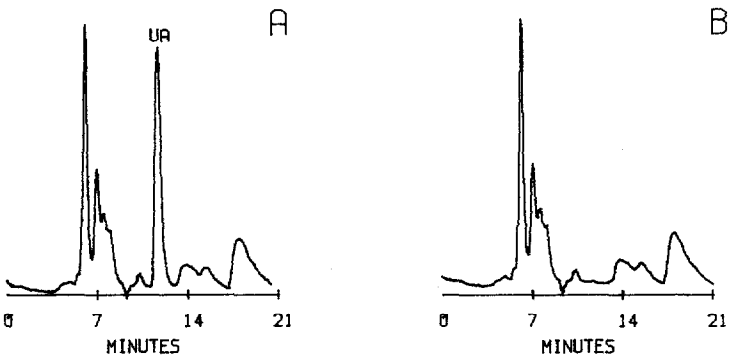

Fig. 2. Chromatogram from extract of granary weevil infested wheat (A), and that extract after treatment with uricase to break down uric acid (B).

addition, column switching prevents early eluting sample components, such as inorganic ions, from reaching the analytical column, thereby prolonging the column lifetime. Over 100 sample injections have been made onto this system, without requiring change of the short column or alteration of switching times.

Detection of uric acid at 280 nm, rather than the standard 254 nm, allowed measurements to be taken closer to the λ_{max} of uric acid of 292 nm. Use of the 280-nm wavelength enhanced the signal-to-noise ratio, and allowed the detection limit to be lowered by a factor of two.

Extraction of uric acid

Samples to be extracted were first treated with 1 *M* hydrochloric acid at elevated temperatures, as recommended by Laessig *et al.*[7]. Many samples were found to exhibit uricase activity, which tended to degrade uric acid while in solution. Treatment with hydrochloric acid prior to extraction denatures uricase, or other enzymes present, that may otherwise break down uric acid.

After the hydrochloric acid treatment, previous procedures recommended extracting uric acid in solutions of 3.0–5.0% sodium acetate following the neutralization with sodium hydroxide. However, we found that when sodium was already present in solution as a result of the neutralization step, the addition of sodium acetate to the extracting solution decreased the recovery of uric acid (Fig. 3). As the pH of the extracting solution is adjusted upward by the addition of sodium hydroxide, the sodium salt of uric acid is formed. Addition of sodium ion to the extracting solution, in the form of sodium acetate, suppresses the extraction of the uric acid salt by the common ion effect. Recovery was maximized by the use of distilled water as the extractant.

In working 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[10]. It was postulated that uric acid is adsorbed onto the surface of flour particles, and that added sulfides act to block the sites responsible for binding the uric acid. We noted the same effect for aqueous extracts of uric acid. The addition of very small amounts of carbon disulfide to the extracting solution resulted in a significant improvement in recovery; an 18%

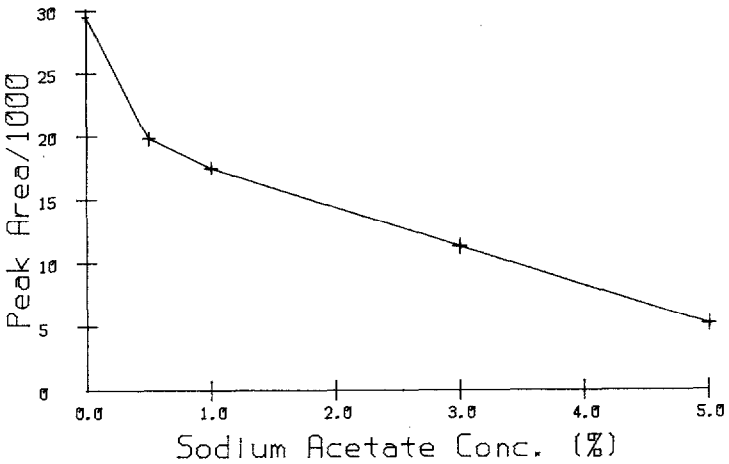

Fig. 3. Effect of sodium acetate concentration on recovery of uric acid from replicate wheat flour samples containing 10 μg uric acid/g. All samples extracted for 5.0 min at pH 9.0.

increase in recovery was obtained for wheat flour extracts. Carbon disulfide was added to the extracting solution rather than dimethyl disulfide, as the former is slightly soluble in aqueous solution whereas the latter is not.

Uric acid is highly soluble in basic aqueous solution above pH 8.0. The initial pH of the extracting solution was allowed to range from 9.0 to 10.0 with no effect on recovery, and increasing the pH further did not improve recovery. Increasing the pH of the extracting solution resulted in a more highly colored extract. However, these pigments eluted off the column early in the chromatographic run and did not interfere with the quantitation of uric acid. In general, the pH of the extracting solution was kept below 10 to minimize deterioration of the column packing material.

Uric acid was found to extract rapidly into solution, as maximum recovery was obtained after only 5.0 min of extraction on a wrist action shaker.

Reproducibility and linearity of uric acid determination

The reproducibility of the chromatography and integration was evaluated by making a series of seven injections of a given extract, and determining the relative standard deviation of peak areas. A relative standard deviation of 2.4% was obtained for these replicate injections. A series of wheat flours spiked with low levels of uric acid (2–10 μg/g) were analyzed, and a linear response (correlation coefficient = 0.99) obtained even at these low concentrations. The method was found to have an effective detection limit of 1.0 μg uric acid/g sample, a detection limit capable of detecting infestation levels which may be routinely encountered in commerce. A series of ground wheat samples spiked with somewhat higher levels of uric acid (10–30 μg/g) were also analyzed, and again a linear response for uric acid was obtained.

A more complete extraction of uric acid is obtained from ground whole 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 adsorbed onto the surface of the sample matrix, with the more finely granulated flour presenting a much larger surface area for this effect to take place. Because of these differences in rates of recovery, standards were prepared by spiking quantities of the sample matrix of interest with known amounts of uric acid.

TABLE I

ANALYSIS OF REPLICATE SAMPLES

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
Means	10.7
Standard deviation	0.27
Relative standard deviation	2.52%

To determine the reproducibility of the overall analytical procedure, seven replicate flour samples were analyzed. The average value from duplicate injections of each sample was used to calculate the precision (Table I). A relative standard deviation of 2.5% was obtained for the overall procedure.

This procedure offers improvement in sensitivity over colorimetric and fluorimetric procedures. Detection limits rival those of the UV spectrophotometric procedure with sample concentration, while providing significant time saving. Also, an alternative to existing HPLC procedures is made available, with extended linear range and the ability to employ the more widely used UV detector and reversed-phase column. HPLC determination of uric acid provides a practical, more objective method for assessing insect contamination in cereal products than the currently used insect fragment count.

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