Distribution of Uric Acid in the Fractions Obtained from Experimental Milling of Wheat Infested with Granary Weevil Larvae[1]

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ABSTRACT

To determine the fate of uric acid during wheat milling, samples of hard red winter wheat were inoculated with kernels containing late instar larvae of the granary weevil (*Sitophilus granarius*), such that the samples had a uric acid content of about 25 μg of uric acid per gram of wheat. The samples were then milled on a Buhler experimental mill to produce three break flours, three reduction flours, and two millfeed fractions. Uric acid in individual milled fractions was quantified by reversed-phase high-performance liquid chromatography, using ion-pairing with tetrabutylammonium phosphate and ultraviolet detection. After milling, over 90% of the uric acid was found in the flour, with approximately 50% of the total uric acid in the first break fraction. Only about 10% of the original uric acid was distributed into the millfeed.

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Of all insects that infest stored grains, granary weevil (*Sitophilus granarius* (L.)), rice weevil (*S. oryzae* (L.)), maize weevil (*S. zeamais* Motsch.), and lesser grain borer (*Rhyzopertha dominica* (F.)) are the most damaging species. These, the primary infesters, develop inside the kernel as larvae, where they cannot be completely removed by ordinary grain-cleaning operations. Grain infested by larvae of the primary infesters can be mistaken for uninfested grain and cause serious problems at later steps in marketing channels (Vick et al 1988). Primary infesters may cause the grain to be unsuitable for human food due to the feeding activity of the adults and the presence of the developing larvae inside the kernels (Russell 1988). Detection of insects in stored grain products is, therefore, essential to prevent economic losses during storage, to prevent production of contaminated products, and also to ensure that pesticides are used only when necessary.

At this time, the most widely accepted means for the detection of insects relies on the traditional method of sampling, followed by sieving and visual inspection (FGIS 1987). This method is based on the presence of visible insects and does not detect internal infestation by larvae of hidden insects that have not emerged. Existing methods for detecting internally infesting insects in whole grain include staining of the egg plugs (Goossens 1949), flotation of damaged kernels (Apt 1952), and X-ray analysis. Contamination of flour after milling is usually determined by counting insect fragments (FDA 1980). This procedure is, however, time-consuming and subject to human error, as identification of insect fragments from grain materials can be subjective and tedious. For these reasons, objective alternatives to the counting of insect fragments, such as the measurement of insect muscle protein by immunoassay (Kitto 1991), have been sought.

Among methods reported for objectively assessing contamination from hidden insects in grain, is determination of uric acid from the excreta of insects (Pachla and Kissinger 1977, Roy and Kvenberg 1981, Galacci 1983, Wehling and Wetzel 1983, Lamkin et al 1991). One problem with most techniques used for uric acid analysis, such as early colorimetric (Venkat Rao et al 1957), semi-automated colorimetric (Laessig et al 1972), and ultraviolet spectrophotometric (Farn and Smith 1963) procedures, is the lack of sufficient sensitivity to detect the low levels of contamination commonly occurring in stored grain and grain products. However, the measurement of uric acid from low-level infestation in commercial grain samples has been made possible by using high-performance liquid chromatography (HPLC). This improved sensitivity is shown by the work of Pachla and Kissinger (1977), who developed a method for uric acid determination in cereal products using HPLC coupled with thin-layer electrochemical detection. They reported a detection limit of 2 μg of uric acid per gram of sample. Wehling and Wetzel (1983) used HPLC with ultraviolet detection to determine the uric acid content of infested wheats. Their method was found to have a good correlation between numbers of infested kernels per 100 g of wheat and uric acid content (ppm) of the infested grain, with a correlation coefficient of 0.99 (Wehling et al 1984). They also reported an effective uric acid detection limit of 1.0 μg/g of wheat. This limit is capable of detecting the infestation levels commonly encountered in commerce. However, little is known about the fate of uric acid during processing. Much work has been done to determine the distribution of constituents in the wheat kernel (Pomeranz 1988), but no work has been done to determine how uric acid is distributed into the various fractions obtained from dry milling. Since wheat is primarily used for human consumption in a variety of foods, and uric acid has the potential of being adopted as a marker of insect contamination in flour and flour-based foods, how uric acid distributes from wheat into flour during milling needs to be determined.

The purpose of this study, therefore, was to determine the fate of uric acid in granary weevil infested wheat during the milling process.

MATERIALS AND METHODS

Preparation of Insect-Infested Wheat Samples

The insect of interest for this study was the granary weevil (*S. granarius*). A parent culture was obtained from the stored-product research laboratory of the Department of Entomology at Kansas State University, Manhattan. New cultures were prepared by placing approximately 100 adult insects into samples of hard red winter (HRW) wheat. Four wide-mouth, pint canning jars were used to hold the insect cultures. These jars were covered with 40-mesh window screen over a circle of Whatman no. 4 filter paper, so that the wheat and insects would be protected from outside contamination and have a source of oxygen while incubating. Each culture contained 250 g of wheat to provide enough infested wheat to prepare samples for the milling process. The cultures were kept at 27 ± 3°C and 65 ± 5% rh, which are favorable conditions (Pedersen 1992) for hatching and development of the

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insects. The female insects deposited eggs into the wheat kernels over a period of four days, after which the adults were removed by sieving. The cultures were incubated for four weeks to achieve the late instar stage of development. At this point, cultures were frozen at −18°C overnight to stop further growth of the larvae. The uric acid content of each culture was determined using the HPLC procedure described later. The uric acid levels of the individual cultures, based on the average of duplicate determinations, were 103.5, 164.7, 164.7, and 197.6 μg /g of wheat. The four cultures were then blended to achieve uniformity. A portion of the blended culture was added to three lots of clean HRW wheat, such that the final uric acid concentration was about 25 μg of uric acid per gram of wheat. These three lots of infested wheat were then used for experimental milling.

Milling of Wheat

The three infested wheat samples, weighing 1535.0, 1520.0, and 1530.0 g, were tempered to 15.2% moisture with 20 hr of equilibrium time. Moisture was determined in duplicate by AACC method 44-15A (AACC 1983) following grinding on a burr mill. The samples were individually milled on a Buhler pneumatic laboratory flour mill (Buhler, Uzwil, Switzerland). The Buhler mill fractionates wheat into three break and three reduction flours, plus shorts and bran. Each fraction was collected from the mill and weighed separately. The flour and millfeed fractions obtained from milling were then analyzed in duplicate for uric acid.

Standards

Stock uric acid standards were made by dissolving 0.050 g of uric acid (Sigma Chemical Co., St. Louis, MO) in a 1.0% aqueous solution of sodium acetate and diluting to 500 ml in a volumetric flask to give a concentration of 100 μg/ml. The stock solution was made weekly and kept refrigerated. Working uric acid standards were made freshly just before use by diluting 1 ml of the stock solution to 100 ml with 1% aqueous sodium acetate.

Extraction of Uric Acid from Milled Fractions

Uric acid was extracted from milled fractions by a procedure previously shown to give quantitative recoveries (Wehling and Wetzel 1983). Each fraction was extracted in duplicate. A 5.000-g sample of each milled fraction was placed into a 100-ml centrifuge tube, followed by addition of 10 ml of 1*M* hydrochloric acid to the sample. The tube was then placed in a 55–60°C water bath for 15 min to denature uricase enzyme. After removal from the water bath, 30 ml of distilled water was added to the sample. The extract was neutralized using 5*M* sodium hydroxide, with final adjustment of pH to 9.0–10.0 using 0.5*M* sodium hydroxide solution. After pH adjustment, two drops of carbon disulfide were added. The tube was capped and shaken vigorously on a Burrell wrist action shaker (Burrell Corp., Pittsburgh, PA) for 5 min, then centrifuged for 15 min at 4,500 × *g*. The supernatant was decanted into a 50-ml volumetric flask and diluted to volume with distilled water. An aliquot of the extract was filtered through a 0.45-μm microporous cellulose acetate filter (Alltech Associates, Inc., Deerfield, IL), using a Swinny adapter fitted to a 10-ml hypodermic syringe, before injection into the chromatograph.

Chromatography

Liquid chromatography was used for uric acid determination. The technique was adapted from the method of Wehling and Wetzel (1983). The HPLC separation was performed with a solvent delivery system (Beckman 110 B, Fullerton, CA) and injection valve (100-μl injection loop) (Rheodyne 7010, Cotati, CA). A reversed-phase C18 Econosil column (250 × 4.6 mm i.d., 5-μm particle size) from Alltech Associates was used. A 30 × 2.1 mm i.d. Brownlee C18 guard cartridge (Rainin Instrument Co., Woburn, MA) was used to prevent HPLC column degradation caused by particulate matter and reactive or corrosive reagents in the sample or solvent. The mobile phases were 95:5 water-methanol solution for the analysis of flour fractions, 96.5:3.5 for bran, and 100% water for the analysis of shorts. Water used in the liquid chromatographic mobile phase was redistilled. The pH of the mobile phase solution was adjusted to 6.6–6.7 by the addition of 0.560 g of KH_2PO_4 and 0.480 g of Na_2HPO_4 in 1 L of the solution. An amount of 1.697 g (0.005*M*) of tetrabutylammonium dihydrogen phosphate (Sigma Chemical Co.) was added to each liter of solution as the ion-pairing agent. The solution was filtered through a nylon membrane filter with a pore size of 0.45 μm (Alltech Associates, Inc.) before use.

A model 440 ultraviolet absorbance detector (Waters Associates, Milford, MA) was used. The wavelength was set at 280 nm and the detector sensitivity at 0.05 absorbance units full scale. Chromatograms were recorded, and peak areas were integrated using a model HP 3395 A integrator (Hewlett-Packard, Wilmington, DE). The solvent flow rate was 1 ml/min, and the analyses were made at ambient temperature. Duplicate injections of each extract were made.

Identification of Uric Acid Peaks

Uric acid peaks were identified on the basis of retention times and comparison with standards. Samples were also spiked with uric acid, and an increase in the area was taken as additional confirmation of the peaks. Peaks were further characterized by the use of uricase enzyme. Samples were treated with uricase enzyme (Sigma Chemical Co.), which resulted in the disappearance of the uric acid peak.

Cumulative Uric Acid Curves

To evaluate the distribution of uric acid into flour millstreams during milling, cumulative uric acid curves were developed. First, flour streams were arranged from lowest to highest uric acid concentration on a micrograms-per-gram basis. Second, the total amount of uric acid (μg) in each fraction was calculated by multiplying the weight of each fraction by the uric acid concentration (μg/g) of the respective fraction. Cumulative uric acid was then calculated on the arranged data by dividing the sum total (μg) of uric acid of the two lowest flour streams by the sum of weights of the two flour streams. This procedure was used to sequentially calculate the cumulative uric acid contents of combined flour streams by including the next flour fraction with the immediately higher uric acid level. The percent flour yields were calculated on a starting material basis. The cumulative uric acid contents (μg/g) were then plotted versus percent flour yield.

Statistical Analyses

The experiment was conducted using a randomized complete block design, with three replications. Significant differences at the 5% level of probability were tested by analysis of variance computed by a Statistical Analysis System (SAS 1986) computer program with the general linear model procedure. Comparisons of means, when required ($P < 0.05$), were made using Fisher's protected least significant difference test (Steele and Torrie 1980).

RESULTS AND DISCUSSION

Chromatography

The eight fractions produced by the mill were analyzed for uric acid by a modification of the ion-pair HPLC method reported by Wehling and Wetzel (1983). The separation was optimized by evaluating different column packings and different concentrations of organic modifiers in the mobile phase to achieve baseline separation in the shortest time possible. The best results were obtained when the concentration of methanol was adjusted to 5% in the mobile phase for the flour fractions, and 3.5% for bran, with the previously described analytical column. For the analysis of shorts, methanol was not used in the mobile phase. Analyses were

done using two extracts of each fraction with duplicate injection of extracts onto the chromatographic column. Chromatograms of standards were obtained, and the peak areas were averaged from duplicate runs, then used for comparison with the averaged peak area of milled-fraction extracts. Uric acid had a retention time of approximately 16 min. A chromatogram of an extract of a first break flour fraction is presented in Figure 1.

The uric acid contents of the first break samples were significantly greater than those of the other milled fractions; therefore, the extracts of the first break samples were diluted into a 250-ml flask rather than a 50-ml flask, and the dilution factor was considered in the calculation of the uric acid contents.

In extracts of shorts and bran, interferences were encountered due to the presence of other compounds that eluted with uric acid. The composition of shorts and bran varies greatly from that of endosperm. They are high in indigestible fiber and fat, whereas endosperm is high in starch and protein. To resolve the uric acid peak, the concentration of methanol in the mobile phase was lowered to 3.5% for the analysis of bran and was completely excluded from the mobile phase for the analysis of shorts.

Distribution of Uric Acid in Milling Fractions

Uric acid was found throughout the milled fractions. Levels of uric acid, on a per-gram basis in individual fractions, are shown in Table I. The highest ($P < 0.05$) concentration of uric acid was found in the first break flour. Most of the uric acid was readily extracted from the wheat kernels and was released into the flour as soon as the first corrugated rolls broke open the kernels. There were no differences ($P > 0.05$) in uric acid concentrations of second break, third break, second reduction and third reduction flours. The uric acid concentration was lower in the first reduction flour as compared to the other fractions. No significant difference ($P > 0.05$) was found in the uric acid levels of the shorts and bran on a microgram-per-gram basis. The relative order of uric acid concentration in the fractions was therefore first break followed by second break, third break, second reduction, and third reduction, followed by the first reduction, and finally the shorts and bran.

The distribution of uric acid in milled fractions as a percent of the total uric acid present did not follow the same pattern as that for uric acid per gram of sample, because each fraction obtained from the mill varied in weight. The product yield and percent of total uric acid present in individual fractions is also shown in Table I. The maximum ($P < 0.05$) amount of uric acid was in the first break, as it contained 51.90% of the total uric acid, followed in order by first reduction and second break. There was no difference in the percent of total uric acid in the third break flour, third reduction flour, and shorts, as they had the lowest uric acid content of all. The uric acid contents of various fractions, as a percent of total uric acid in decreasing order, were therefore first break flour, first reduction flour, second break flour, second reduction flour and bran, followed by third break flour, third reduction flour, and shorts.

The four major products produced by the mill (break flour, reduction flour, shorts, and bran), contained widely different quantities of uric acid. The break flour contained 63.64% of the total uric acid present, followed by reduction flour with 27.31%, bran with 7.09%, and shorts with 1.97%. Uric acid was more concentrated in the flour than in the feed, as over 90% of the uric acid was in the flour. This is due to the feeding activity of the larvae of granary weevils, which consume primarily endosperm and germ tissues and often leave the bran intact (Campbell et al 1976, Singh et al 1976). Also, the feeding location of the larvae of granary weevil in the endosperm suggests that there should be more uric acid in the flour than in the feed.

Cumulative Uric Acid Curves

Figure 2 shows plotted cumulative uric acid (μg/g) versus percent cumulative flour yield for the three replications of granary weevil infested wheat milled on the experimental Buhler mill. The first data points on the graph are the first reduction flours and the last points are the first break flours. The cumulative distributions

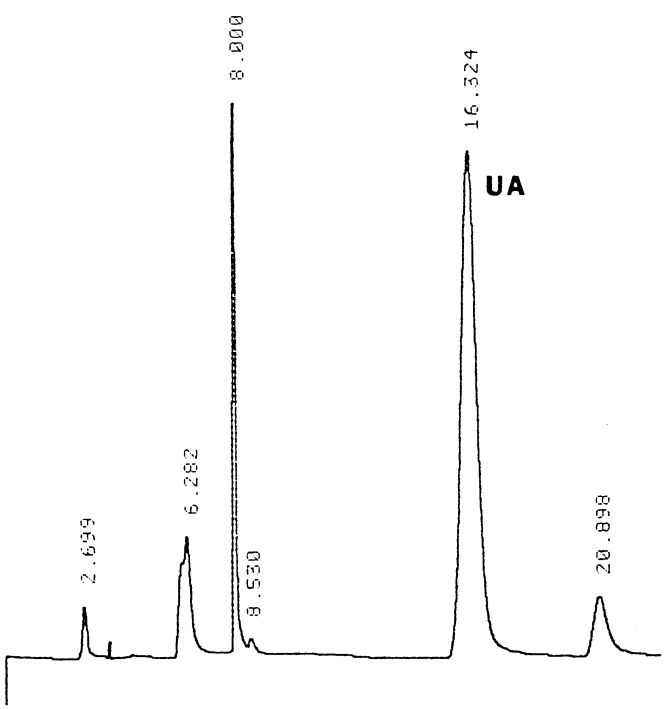

Fig. 1. Chromatogram from extract of the first break flour obtained from wheat infested with granary weevil larvae. UA = uric acid.

TABLE I
Mean Uric Acid (UA) Concentrations, Product Yield, and Percent of Total Uric Acid in Milled Fractions of Granary Weevil Larvae Infested Wheat

Milled Fractions	UA[a] (μg /g)	Product Yield (%)	UA[a] (% of total)
1st break	234.76 a	6.97	51.90 a
2nd break	26.22 b	11.89	9.86 b
3rd break	29.71 b	2.02	1.88 c
1st reduction	14.56 c	41.62	19.08 d
2nd reduction	25.95 b	8.28	6.71 e
3rd reduction	28.50 b	1.71	1.52 c
Shorts	7.50 d	8.26	1.97 c
Bran	11.70 cd	19.26	7.09 e

[a] Means in a column not followed by the same letter differ ($P < 0.05$).

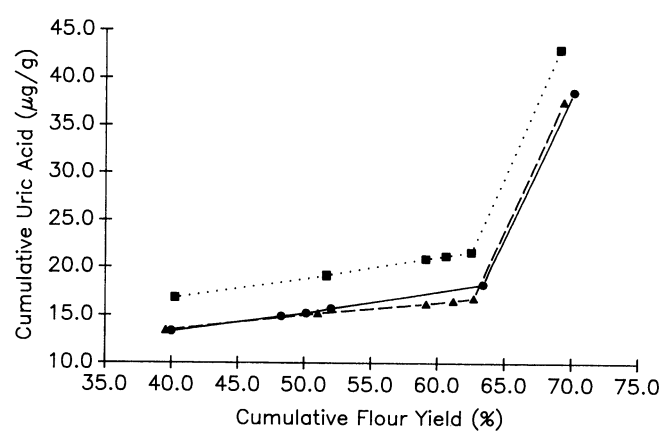

Fig. 2. Cumulative uric acid plots of flour fractions contaminated by granary weevil larvae, obtained from milling the three infested wheat samples. Each curve represents one wheat sample.

of uric acid in milled fractions were similar for the three replications. For any selected cumulative flour yield, the corresponding cumulative uric acid content can be calculated. For example, the average cumulative straight-grade flour yield of the three replications is 69.58%, with an average cumulative uric acid content of 39.67 μg/g. If the first break flour is eliminated, the cumulative uric acid content of the remaining flour is 18.90 μg/g, which represents a 47.64% reduction in uric acid content with only a 6.69% decrease in yield.

In summary, the results presented here show that uric acid was distributed through all the milled fractions of granary weevil larvae infested wheat. The first break flour had the highest and the first reduction flour had the lowest concentrations of uric acid. Over 90% of the uric acid was found in the flour, with approximately 50% of the total in the first break fraction. Only about 10% of the original uric acid was distributed into the millfeed. Although a small amount of uric acid was found in millfeed fractions, the conclusion from this study is that uric acid is closely associated with the endosperm of granary weevil infested wheat.

Should uric acid be used as a marker of insect infestation in flour and flour-based foods, the knowledge of how it distributes during milling can be helpful in selecting uses of different milled fractions. Our results from experimental milling indicate that uric acid can be successfully used to detect insect contamination in milled flour, as well as in whole grain. Further research is needed using larger-scale milling operations to determine how uric acid distributes in systems with a greater number of break and reduction operations.

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