Analysis of Azodicarbonamide in Wheat Flour by Liquid Chromatography

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ABSTRACT

A liquid chromatographic procedure was developed for the analysis of azodicarbonamide (ADA) in wheat flour. Presence of either $KBrO_3$ or KIO_3 did not interfere with the analysis. ADA was extracted with acetone from flour. An aliquot of the extract was evaporated to dryness (23–25° C). The ADA in the residue was extracted with 0.5% K_2HPO_4 buffer (pH 8.40) and the lipids with *n*-hexane, simultaneously. The ADA was analyzed by liquid chromatography, using an external flour standard of known treatment. The method was developed for the quantitation of 1.0–45.0 ppm ADA in wheat flour. A detailed description of the method is given.

The functional role of flour improvers has been recognized for many years by both the milling and baking industries. In 1962, azodicarbonamide (ADA) was introduced as a maturing agent for wheat flour. At that time, a gaseous maturant (chlorine dioxide) was being used. As one would expect, ADA was quickly adopted because it could be added to flour as a powder, while still providing the benefits of chlorine dioxide. Joiner et al (1963) demonstrated that ADA remains fairly inert in flour until the dough-mixing stage. The authors further recommended a procedure for analysis of the improver, which involved extracting flour with water, fermenting interfering carbohydrates, and converting biurea to hydrazine. Method reliability was estimated at ±20%. Weak et al (1976) made some revisions, although the procedure remained lengthy and laborious. The AACC approved method for ADA (method 48-71A) is specific for premix concentrates but not for flour (ppm range). Furthermore, the simultaneous presence of $KBrO_3$ or KIO_3 interferes with the analysis.

This research was undertaken to develop a simple procedure for the analysis of ADA in wheat flour.

MATERIALS AND METHODS

Chemicals

Acetone, *n*-hexane, chloroform, methanol, isopropanol, and *N,N*-dimethylformamide (DMF) were analytical grade. Potassium phosphate, dibasic, was obtained from J.T. Baker Chemical Co. Ferrous sulfate (bakery grade) was received from Mallinckrodt, Inc. Wheat flour was enriched with type 540 enrichment premix (Pennwalt Corp.).

Powdered ADA was washed with 50% aqueous methanol (4×) at pH 4.00–4.50, then given a rinse with absolute methanol, air-dried for 3 hr, followed by final drying overnight at 50° C. The washed ADA was 99.7% pure.

Maturox (10.0–10.5% ADA in wheat starch) is a flour maturant from Pennwalt Corporation, Lucidol Division. The ADA concentration of Maturox was verified with washed ADA as 10.3%.

Commercially milled flour used for all experiments was unbleached, unmalted, and untreated. The flour had 12.8% protein, 13.2% moisture, and 0.45% ash.

Preparation of ADA-Treated Flour

Both enriched and unenriched flour samples were treated with Maturox corresponding to 0–100.0 ppm of ADA (stock treated flour). Stock treated flour was added subsequently to untreated flour to obtain ADA from 1.0–45.0 ppm. Typical powder blending techniques were followed to achieve product uniformity.

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Extractability of ADA from Flour

ADA-treated flour (0–100.0 ppm) in 5.0–10.0 g quantities was weighed into 100-ml volumetric flasks, followed by additions of 60–70 ml of solvent (acetone, chloroform, isopropanol, *n*-hexane, methanol, DMF, and water). Flasks were attached to a Wrist-Action shaker (Burrell Corp.) and shaken for 5–30 min, diluted to the mark with the solvent, and mixed by inversion. Flour extracts were filtered through Whatman No. 1 filter paper into 125-ml Erlenmeyer flasks. Precautions were taken to minimize solvent evaporation during filtration. A 50-ml aliquot was pipetted into another 125-ml Erlenmeyer flask and evaporated to dryness with the flash evaporator assembly (Buchler Instruments) at 40–45° C (water extract was not evaporated to dryness but analyzed as-is by liquid chromatography). Twenty milliliters of 0.3–0.5% K_2HPO_4 was pipetted into the Erlenmeyer flask, and the residue was dissolved by shaking for 15 min as described previously. The extract was filtered through Whatman No. 42 filter paper. One milliliter of the filtrate was injected in the liquid chromatograph.

Liquid Chromatograph

The liquid chromatograph (LC) used for the analysis of ADA was a Perkin-Elmer series 10, equipped with an LC-95 ultraviolet-visible variable wavelength detector and LCI-100 computing integrator.

LC Operating Conditions

Conditions for the LC analysis of ADA were as follows: mobile phase, 0.02% K_2HPO_4 adjusted to pH 2.50 with 85% H_3PO_4; column, C_{18} (15 cm) cartridge type (catalog no. 0258-0169); sample loop, 20 μl (Rheodyne); syringe 1 ml (B-D Yale tuberculin); flow rate, 0.5–0.7 ml/min (adjusted to obtain retention times of 3–4 min); absorbance, 260 nm; attenuation, programmable, 16 (first 6 min), then 128 (6–10 min), to clear column for next run.

ADA is poorly soluble in most solvents at gram quantity levels. Wheat flour can be treated with ADA to a maximum of 45.0 ppm; however, the generally accepted practice by the industry has been at levels of 2.0–8.0 ppm. Therefore, there is no reason for one to be concerned with gram quantity solubilizations. A solvent capable of extracting to 100.0 ppm ADA from flour ought to be adequate for the LC analysis.

Development of Methodology—Extraction Solvent

The extraction potential of each solvent (acetone, chloroform, isopropanol, *n*-hexane, methanol, DMF, and water) was evaluated with flour treated with 100.0 ppm ADA. The extraction efficiency was determined by comparing the solvent-extracted ADA with pure ADA by liquid chromatography. A pure solution of ADA was prepared by dissolving in 0.1*N* NaOH for 3 min at 23–25° C. The clear solution was adjusted to pH 6.00 with 0.1*N* HCL, diluted to a known volume and mixed. The obtained ADA solution was injected in the LC and used as a reference standard.

Water was not acceptable as the extracting solvent for ADA for at least two reasons: certain flour components had the same retention time as ADA, and water-soluble components continued to elute past 40 min running time.

Weak et al (1976) found ADA to be readily extractable with DMF. However, this solvent could not be used under prevailing LC conditions for several reasons: DMF (extracts) could not be injected directly because it had the same retention time as ADA. Its high boiling point required increasing the bath temperature to at least 60–65°C for effective removal. Trace levels of DMF interfered with the analysis. ADA decomposes in strongly basic pH media, and flour in DMF was in the range of pH 10.50–11.20. In addition to ADA, DMF also extracted a flour component that eluted close to and at times with ADA.

Chloroform, hexane, and isopropanol extracted trace amounts of ADA that were difficult to measure, whereas acetone and methanol were more effective extractants. Methanol also removed a large amount of other flour components. Acetone was selected as the solvent of choice.

Development of Methodology—Extraction Time

Flour treated with 45.0 ppm ADA was extracted with acetone for 5–30 min (Wrist-Action shaker). Extraction of ADA as a function of time is represented in Figure 1. Complete extraction was accomplished in 10 min. For most flours, 15 min would be enough time for complete extraction of ADA.

Effect of Flour Lipids

For many years, acetone has been used in cereal research to extract lipids. Unlike other extracting solvents investigated, acetone extracts were free of proteins or other flour components, and therefore would not require removal before LC analysis. When the acetone extract was evaporated to dryness, ADA became entrained in the lipids. This entrainment of ADA could cause variability in the amount of extractable oxidant from the residue with water (K_2HPO_4 buffer). It should be noted that the LC analysis requires ADA to be in an aqueous medium at the point of injection. This problem was resolved by using a binary solvent system (*n*-hexane/K_2HPO_4 buffer). At the extraction step, both hexane and buffer were pipetted into the flask (residue). During shaking, hexane selectively extracted the lipids from the residue while the buffer extracted ADA. After shaking, the solvents separated into two layers. The lower aqueous layer (ADA) was isolated with a separatory funnel and analyzed by LC.

Stability of ADA Extract

ADA is readily soluble in a base at 23–25°C. The rate of dissolution is proportional to the strength of the base. During the early phase of this research, it was found that ADA also decomposed in proportion to the strength of the base. Since the K_2HPO_4 buffer (pH 8.20–8.40) was used to extract ADA, it seemed advisable to test its stability at ambient temperature (23–25°C), immediately after completing the shaking step of the procedure. Flour treated with 5.0 ppm ADA was extracted with acetone, as described previously. The ADA in 0.3–0.5% K_2HPO_4 buffer (pH 8.20–8.40) was kept at 23–25°C in amber bottles. Each solution was analyzed by LC after 1, 2, 3, 4, 5, and 24 hr. Preliminary testing showed no difference in the results between 0.3 and 0.5% K_2HPO_4 buffer extracts. Stability (activity) of the ADA extract relative to storage time is shown in Figure 2. The extract remained stable at pH 8.20–8.40 during the first 3 hr after shaking. Minor changes (lower) appeared from 4 to 5 hr; however, degradation was appreciable after 24 hr.

Often, enriched flour needs to be analyzed for ADA. The B vitamins had no effect, whereas $FeSO_4$ exerted a destabilizing effect on ADA in K_2HPO_4 buffer (pH 8.20–8.40) at 23–25°C. Other sources of iron (reduced, electrolytic) normally used to enrich flour were considerably less reactive than $FeSO_4$. Good ADA stability with $FeSO_4$ was obtained to 1 hr, whereas drastic degradation occurred after 2 hr. Stability data obtained with enriched and unenriched flours suggest analyzing ADA within 60 min after

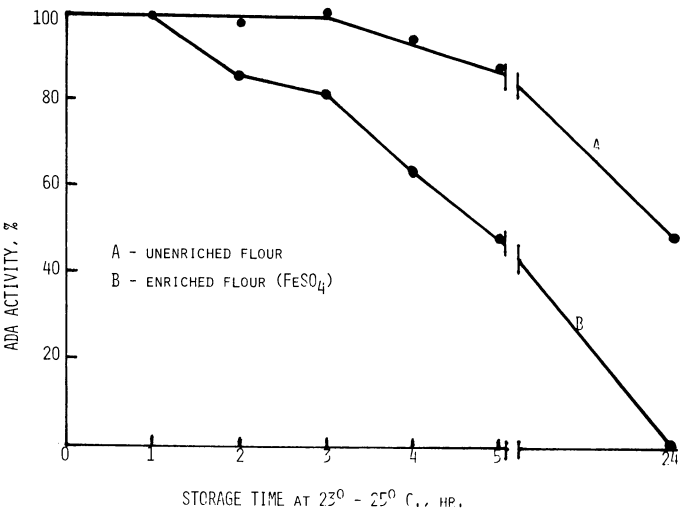

Fig. 2. Effect of storage time on stability (activity) of azodicarbonamide (ADA) in K_2HPO_4 buffer (pH 8.40). ADA extracts were kept at 23–25°C in stoppered amber bottles. ADA (5.0 ppm) was extracted from both unenriched (**A**) and enriched ($FeSO_4$) (**B**) wheat flour with acetone. Data points are the mean of triplicate extractions.

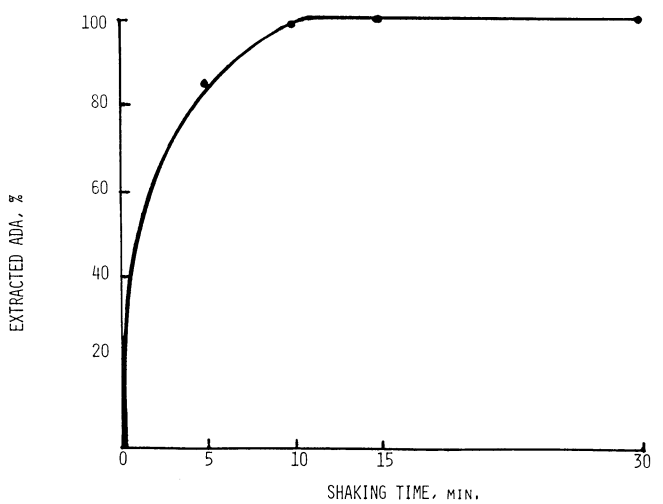

Fig. 1. Effect of shaking time on the extraction of azodicarbonamide (ADA) (%). Wheat flour was treated with 45.0 ppm ADA (Maturox). ADA was extracted with acetone. Data points represent the mean of triplicate extractions.

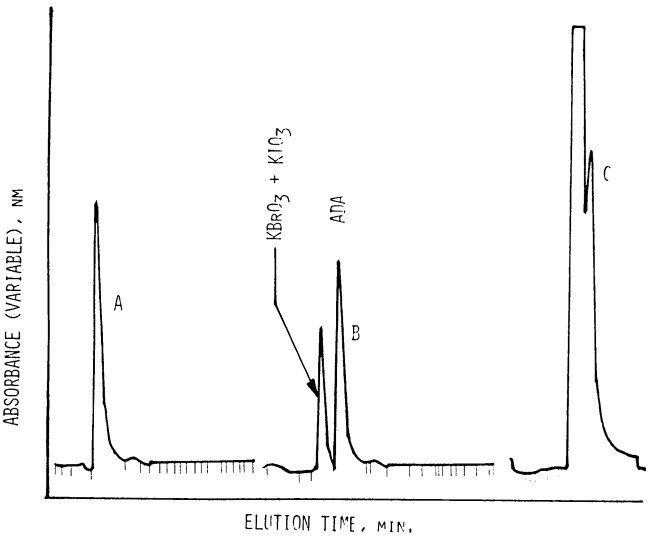

Fig. 3. Liquid chromatographic separation of azodicarbonamide (ADA) from $KBrO_3$ using different absorbances: **A**, ADA only at 260 nm; **B**, ADA, $KBrO_3$, and KIO_3 at 260 nm; **C**, ADA, $KBrO_3$, and KIO_3 at 220 nm. ADA elutes in 3–4 min.

completion of extraction (Wrist-Action shaking) with K_2HPO_4 buffer, especially when dealing with unknown flours.

Analysis of ADA in the Presence of Other Oxidants

Wheat flour is often treated with either $KBrO_3$ or KIO_3, or both. The question is raised whether these oxidants affect the analysis of ADA. This problem was circumvented by operating with the absorbance most favorable to ADA (Fig. 3).

Detailed Working Procedure

Preparation of reference standards. Pure ADA (e.g., Alfa Products)(0.100 g) was dissolved in 300–350 ml of 0.1*N* NaOH for 3 min at 23–25° C. This solution was neutralized to pH 6.00–6.50 with 0.1*N* HCl, transferred to a 1,000-ml volumetric flask, and diluted to volume with water. Then 0.100 g of Maturox was transferred to a 100-ml volumetric flask; 40 ml of 0.1*N* NOH was added and shaken for 3 min. This solution was neutralized to pH 6.00–6.50 with 0.1*N* HCL and diluted to volume with water. The exact concentration of ADA in Maturox was determined (10.0–10.5%).

Working reference standard (stock). For a working reference standard, an exact amount of Maturox was weighed out so that when diluted to 100.0 g with untreated flour, it would provide 100.0 ppm of ADA. This standard was refrigerated when not in use.

Dilute working reference standards. For weaker treatments, 10.0 g of the stock standard and 90.0 g of untreated flour were mixed for a treatment resulting in 10.0 ppm ADA. To achieve 20.0 ppm ADA, 20.0 g of stock standard and 80.0 g of untreated flour were mixed. The 10.0-ppm ADA standard was used on unknown flours for treatments between 1.0 and 10.0 ppm ADA. For treatments between 11.0 and 45.0 ppm ADA, the 20.0 ppm standard was used. All dilute working reference standards were refrigerated when not in use.

Extraction of ADA with acetone. When flour samples contained 10.0 ppm ADA or less, 10.0 g of flour was used for the acetone extraction; for flours with more than 10 ppm of ADA, 5.0 g was used. To perform the extraction, either 5.0 g (treatments of 10.0–45.0 ppm) or 10.0 g (treatments of 1.0–10.0 ppm) of flour was transferred to a 100-ml volumetric flask. From 60 to 70 ml of acetone was added and the suspension was shaken for 15 min, then diluted to the 100-ml mark and mixed. Both unknown flour and dilute working reference standard flour were handled in the same manner throughout the entire procedure. Extracts were filtered through Whatman No. 1 filter paper into a 125-ml Erlenmeyer flask (effort was made to minimize evaporation), and 50 ml of the filtrate was pipetted into another 125-ml Erlenmeyer flask and evaporated to dryness at 23–25° C with the Flash Evaporator (Buchler Instruments).

Purification of ADA extract. Twenty milliliters of *n*-hexane and 20 ml of 0.5% K_2HPO_4 were pipetted and the residue in each flask dissolved by shaking for 15 min (Burrell Corp.). The contents were transferred to a 125-ml separatory funnel. After phase separation, the lower layer (ADA in K_2HPO_4 buffer) was filtered through Whatman No. 42 paper.

LC Analysis. Each filtrate was analyzed within 30 min after completion of the extraction with 0.5% K_2HPO_4 buffer. For chromatography, 1 ml of the filtrate was injected and analyzed by the LC method described under Materials an Methods.

TABLE I
Replicate Analyses of One Flour Treated with 5.00 ppm Azodicarbonamide

Sample No.[a]	Recovered Azodicarbonamide[b] (ppm)
1	4.98
2	4.91
3	4.88
4	4.94
5	4.90
Mean	4.92
Standard deviation	0.04

[a] Separate weighings of the flour.
[b] Each sample extract was injected twice and each value is the mean of the two injections.

TABLE II
Application of Liquid Chromatography Procedure to Flour Treated with Different Levels of Azodicarbonamide (ADA)

ADA Added (ppm)	ADA Determined[a] ($\bar{x}$) (ppm)	Number of Samples[b]	Recovery (%)	Standard Deviation (ppm)
1.0	1.1	3	110	0.22
5.0	4.9	3	98	0.42
10.0	10.6	3	106	0.45
15.0	15.7	3	105	0.37
30.0	29.8	3	99	0.88
45.0	44.8	3	100	0.72

[a] Each treated flour sample was analyzed in duplicate on three different days. Results are the means.
[b] Treated flour was prepared in triplicate with each level of ADA (1.0–45.0 ppm).

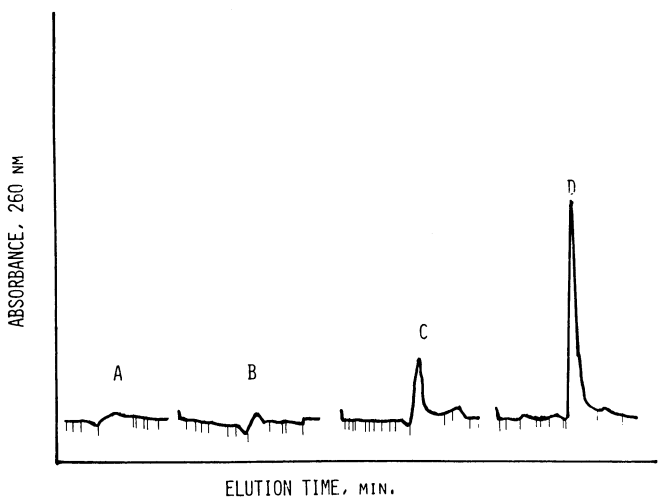

Fig. 4. Typical chromatograms of flour treated with different levels of ADA: **A,** none; **B,** 1.0 ppm; **C,** 5.0 ppm; **D,** 20.0 ppm. ADA treatments were made on unenriched flour.

RESULTS

When this procedure was followed step by step, as described above, typical chromatograms of flour treated with 0, 1.0, 5.0, and 20.0 ppm ADA were as shown in Figure 4.

Table I lists results of replicate analyses conducted on a single sample (flour treated with 5.0 ppm ADA). The results are the mean of duplicate injections per extract. Flour sample was weighed separately five times, yielding five different extracts. The low standard deviation indicates that ADA analysis can be conducted with good precision.

The applicability of the developed procedure was tested on three samples of flour (unenriched) with treatments between 1.0 and 45.0 ppm ADA. Each prepared test sample was analyzed in duplicate on three different days (Table II). Good recoveries were obtained throughout the entire ADA range (1.0–45.0 ppm). Also, relatively good precision was obtained with the developed method.

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