DETERMINATION OF AZODICARBONAMIDE IN WHEAT FLOUR[1]

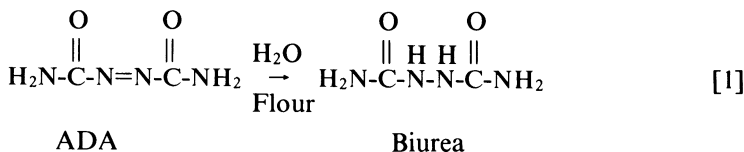E. D. WEAK[2,3], R. C. HOSENEY[3], and P. A. SEIB[3]

ABSTRACT   Cereal Chemistry 53(6): 881-889

Azodicarbonamide (ADA) in flour was extracted with N,N-dimethylformamide (DMF). After DMF was removed, the residue was redissolved in dimethyl sulfoxide (DMSO) and treated with strong alkali and a reducing agent (D-glucose). Acidification released hydrazine, which was determined colorimetrically (a_{max} = 460 nm) after it coupled with p-dimethylaminobenzaldehyde (PDAB). A linear, reproducible relationship was found between absorbance at 460 nm and the concentration of ADA in flour. The procedure detected ADA as low as 2.5 ppm in flours.

In 1962, azodicarbonamide (ADA), a yellow crystalline solid that decomposes at 180°C, was introduced as a maturing agent for flour under the trade name "Maturox." Joiner et al. (1) showed ADA was converted to biurea in wetted flour, as shown in equation 1.

$$\underset{\text{ADA}}{\overset{\displaystyle O \qquad O}{\underset{}{\overset{\parallel \qquad \parallel}{H_2N\text{-}C\text{-}N\text{=}N\text{-}C\text{-}NH_2}}}} \quad \overset{H_2O}{\underset{\text{Flour}}{\longrightarrow}} \quad \underset{\text{Biurea}}{\overset{\displaystyle O \qquad O}{\underset{}{\overset{\parallel \ H \ H \parallel}{H_2N\text{-}C\text{-}N\text{-}N\text{-}C\text{-}NH_2}}}} \qquad [1]$$

In a comparative study of iodate, ADA, and acetone peroxides, Tsen (2) found that ADA increased dough's resistance to extension more rapidly than either of the other oxidants. Reportedly, ADA is an excellent oxidant when added to the brew of continuous processes, giving reduced mixing requirements, improved mixing tolerance, better machinability, and overall better bread quality (3).

Only two methods to assay for ADA were found in the literature. The quantitative determination of ADA in flour and premixes, AACC Method 48-71 (4), is an iodometric procedure suitable for premixes but not sensitive enough for the levels of ADA used in flour, nor specific for ADA.

The other method, briefly referred to by Joiner et al. (1), indirectly measured ADA by determining biurea. The procedure is long and reliable to ±20%. It consists of extracting biurea with water, removing carbohydrates by fermentation, and converting biurea with acid to hydrazine, which is determined with p-dimethylaminobenzaldehyde (PDAB)[4].

This study was undertaken to develop an analytical method to determine ADA at levels normally used in flours.

MATERIALS AND METHODS

Chemicals and Wheat Flours

Powdered ADA was obtained from the Pennwalt Company and recrystallized from water three times, then dried at 56° C. Hydrazine sulfate (Sigma Chemical Co.) and biurea (Eastman Organic Chemicals) were purified in a similar manner. Combustion analysis (Huffman Laboratories, Wheatridge, Colo.) showed the three standards to be analytically pure. Maturox (10% ADA in starch) obtained from the Pennwalt Co. was used for making mixes. The percentage of ADA was confirmed by the detection procedures we developed.

Other chemicals used were reagent grade. Dimethylformamide (DMF) was vacuum-distilled as described by Faulkner and Bard (5).

Flours for the experiments were untreated, commercially milled samples. They were assayed by AACC methods (4). The low-protein flour had 9.5% protein, 11.4% moisture, and 0.57% ash; the high-protein flour had 12.1% protein, 13.2% moisture, and 0.49% ash.
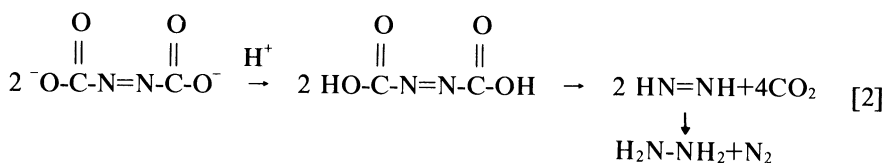
Solubility Methods

Solubility tests were run on ADA and biurea in various solvents. Solubility was determined visually by observing solid disappearance upon shaking. Quantitative solubilities of ADA and biurea were determined in DMF. Individual samples (1.0 g/ 100 ml DMF) of ADA and biurea were shaken 60 min each, filtered, and the solid air-dried and weighed. Three determinations were made on each compound.

RESULTS AND DISCUSSION

The initial problem in determining ADA in flour is the selective extraction of ADA. Both ADA and biurea are soluble in hot water or strong base at 25° C. However, aqueous solvents are not desirable for extracting ADA from flour because ADA converts to biurea in wetted flour (1). The only other solvents we found for ADA and biurea were dimethyl sulfoxide (DMSO) and N,N-dimethyl-formamide (DMF). However, DMSO (90%) is an excellent solvent for starch (6,7); a slurry containing soluble starch would be difficult to use. Also, DMSO has a high boiling point, which makes concentrating extracts difficult. We used DMF because it is more selective (solubilities ADA 0.6 g/ 100 and biurea 0.2 g/ 100 in DMF), easily concentrated, and does not dissolve starch. An aprotic dipolar solvent, such as DMF, is desirable for ADA extraction because such solvents resist oxidation (8).

Converting ADA to Hydrazine

Urea and hydrazine have been determined (9,10) by their reaction with PDAB. However, ADA, which is structurally similar to urea, produced no colored product with PDAB. In 1892, Thiebe (11) showed that potassium azodicarbonate quantitatively decomposed through an unstable diimide (HN=NH) intermediate into hydrazine, nitrogen, and carbon dioxide under acid conditions. A possible chemical reaction for the decomposition of azodicarbonate is shown in equation 2.

$$2 \ ^-O\text{-}C\text{-}N\text{=}N\text{-}C\text{-}O^- \xrightarrow{H^+} 2 \ HO\text{-}C\text{-}N\text{=}N\text{-}C\text{-}OH \rightarrow 2 \ HN\text{=}NH\text{+}4CO_2 \quad [2]$$

$$\downarrow$$

$$H_2N\text{-}NH_2\text{+}N_2$$

Because that salt could be produced by the alkaline deamidation of ADA, ADA was reacted with a mixture of NaOH and Ba(OH)$_2$ and the solution was acidified with H$_2$SO$_4$. The precipitate (BaSO$_4$) was removed and the liquid concentrated to give crystalline hydrazine sulfate which, after recrystallization from water, melted at 235°C—compared with 240°C for known hydrazine sulfate (N$_2$H$_4$·HSO$_4$). Infrared spectra for known hydrazine sulfate and the recrystallized product were essentially identical (Fig. 1). The spectrum for the product of reaction between PDAB and hydrazine sulfate was identical to that between ADA first treated with base-acid and then reacted with PDAB. This was taken as further evidence that hydrazine is released by acidification of the base-hydrolysate of ADA. The amide groups in ADA are hydrolyzed in strong alkali, as shown in equation 3. Biurea is less susceptible to nucleophilic attack by the hydroxide ion because the electron density around the carbon atoms is higher in biurea than ADA. Alkaline deamination of ADA to form the salt, sodium azodicarbonate, explains the apparent solubility of ADA in base. Higher concentrations of base are needed to dissolve biurea.

$$H_2N\text{-}C\text{-}N\text{=}N\text{-}C\text{-}NH_2 \xrightarrow{OH^-} \ ^-O\text{-}C\text{-}N\text{=}N\text{-}C\text{-}O^-\text{+}2NH_3 \quad [3]$$
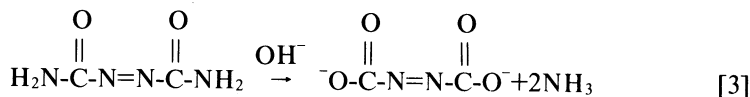
Development of an Analytical Procedure

No-Flour System. Based on the above results, a procedure could involve extracting ADA from flour with DMF, converting ADA to hydrazine, and determining hydrazine with PDAB. The colored azine, which results from hydrazine reacting with PDAB, is pH-sensitive (Fig. 2). Maximum absorbance (460 nm) was obtained at about pH 1.0. The other conditions used were essentially those described by Watt and Chrisp (10).

Conversion of ADA to hydrazine, as measured by the reaction with PDAB, was affected by continuously shaking the reaction mixture (Table I), the concentration of base, the reaction time, and temperature. Reaction time and base concentration seemed to interact, with longer reaction times at higher base concentrations detrimental to color formation.

Effect of Reducing Agents. Hydrazine sulfate contains 24.6% hydrazine, while ADA potentially could release 24.1% hydrazine. Thus, equal weights of each compound should release almost equal amounts of hydrazine. Comparing the standard curve for ADA in DMF and a plot for hydrazine sulfate (Fig. 3) shows that hydrazine sulfate gives a greater absorbance than an equal weight of ADA. But, if equations 2 and 3 are correct, then 50% or more of the azine nitrogen in ADA is lost as a gas. Absorbance values could be increased by forming hydrazine from all the azine nitrogen in ADA. The chemical reaction for conversion of ADA to hydrazine requires that the proposed diimide intermediate be reduced to
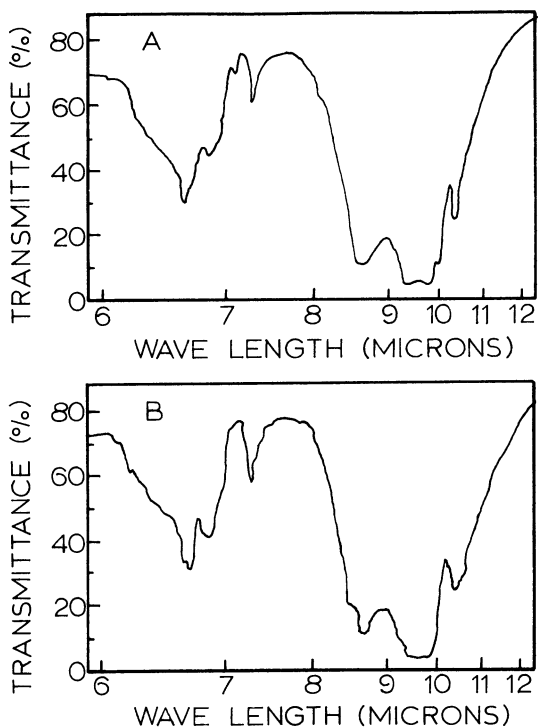
Fig. 1. Infrared spectra of standard hydrazine sulfate (A) and of the product prepared from ADA (B).
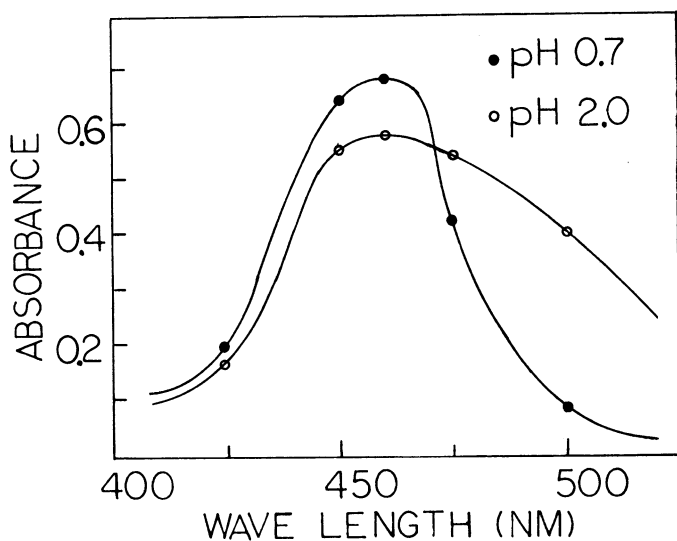

Fig. 2. Effect of pH on the spectra of the hydrazine-PDAB reaction product.

produce hydrazine. If that assumption is correct, then a reducing agent should improve ADA's conversion to hydrazine and, thus, improve the yield of the azine chromophore. Adding cysteine to the alkaline reaction mixture after 1 hr gave increased and reproducible absorbance values.

A possible action of the reducing agent may be expressed chemically as shown in equation 4.

TABLE I
Effect of Base (NaOH) Concentration and Type
of Shaking on Conversion of ADA to Hydrazine

Base Normality	Limited Shaking Absorbance 460 nm[a]	Continuous Shaking Absorbance 460 nm[a]
2N	0.410	0.324
3N	0.330	0.398
4N	0.270	0.441
5N	0.152	0.545
6N	0.222	0.609
7N	0.167	0.625
8N	0.140	0.560
10N	0.112	0.071

[a]Standard deviation for absorbance = $\pm$ 0.020.
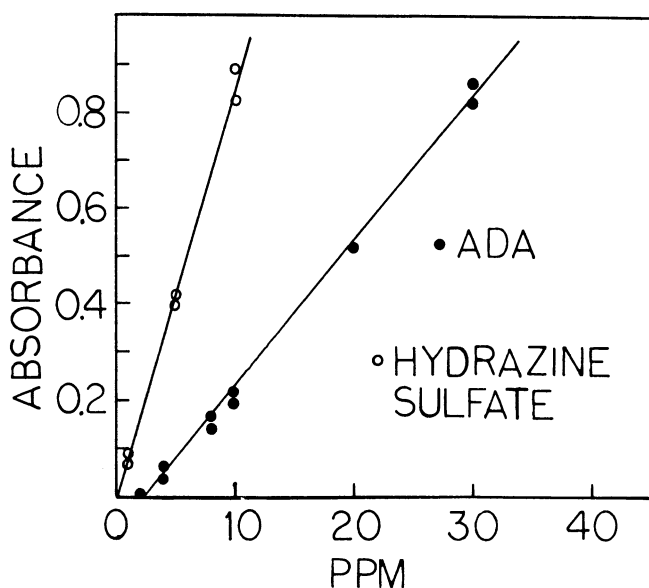
Fig. 3. Comparison of the absorbance produced with standard hydrazine sulfate and hydrazine produced from ADA.

$$\begin{array}{ccccc} O & O & & O & O \\ \| & \| & 2RSH & \| & H\ H\ \| \\ {}^-O\text{-}C\text{-}N{=}N\text{-}C\text{-}O^- & & \rightarrow & {}^-O\text{-}C\text{-}N\text{-}N\text{-}C\text{-}O^- & +R\text{-}S\text{-}S\text{-}R \end{array} \qquad [4]$$

$$H^+$$

$$H_2N\text{-}NH_2^+ + 2CO_2$$
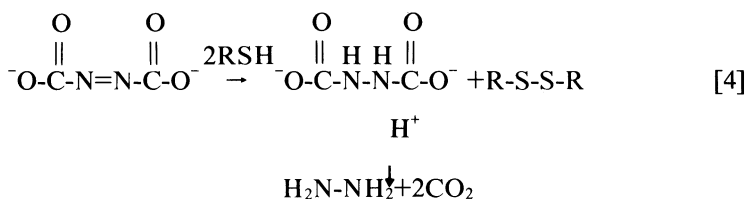
Flour System. However, when ADA was extracted from flour, higher concentrations of base were required (Table II) to obtain absorbances similar to those for the same quality of pure ADA. Addition of cysteine no longer increased absorbance. Presumably something extracted from flour consumed part of the base and also served as a reducing agent. However, including cysteine gave more reproducible results. Therefore, a base concentration of $8N$ and 0.01 g cysteine was used with flour extracts.

The effectiveness of DMF in extracting ADA from flour was studied. Absorbances obtained from control samples with ADA dissolved in DMF and from flour samples extracted with DMF gave nearly equal results (Fig. 4). A second extraction of the flour sample with fresh DMF gave negligible absorbance.

Procedure for Determining ADA in Flour

Although the above method was satisfactory, it did not give the desired sensitivity. Further experiments showed that DMSO was more effective than DMF as a solvent for the deamination reaction. The previous procedure to determine ADA in flour required concentrating the DMF extract to 25 ml and using 2 ml of the concentrate. Thus, a dilution factor of $2/25$ was always involved. The DMSO system requires no dilution because DMSO carried more solids than DMF did. Therefore, DMF extracts of flour were evaporated to dryness, then dissolved in DMSO, and used for the ADA determination. Glucose was substituted for cysteine as the reducing agent. It was found that 0.001 g of glucose was as effective as 0.01 g of cysteine. That is important because it decreases the solid-to-solvent ratio.

With DMSO as a solvent, base hydrolysis reaction time of 1 hr gave optimum color production, provided 0.3 ml concd. H_2SO_4 was used in addition to the acid in the PDAB solution. Residual DMF interfered in the procedure. As little as 0.5

TABLE II
Effect of Base (NaOH) Concentration on Absorbance
of DMF Extract of Flour Containing ADA

	Absorbance (460 nm)[a]		
Concentration NaOH	4 ppm ADA	12 ppm ADA	16 ppm ADA
$4N$	0	0.040	0.070
$6N$	0	0.090	0.200
$8N$	0.050	0.175	0.250
$10N$	0.063	0.195	0.260
$12N$	0.051	0.150	0.205

[a]Standard deviation for absorbance $= \pm 0.020$.

ml DMF flour extract remaining after drying decreased absorbance. The decreased absorbance cannot be accounted for by dilution; it must result from a chemical interaction.

At least 6 ml of DMSO at 75°C was required to dissolve the dried DMF extract (4 g flour/60 ml DMF) and obtain reproducible results. However, care
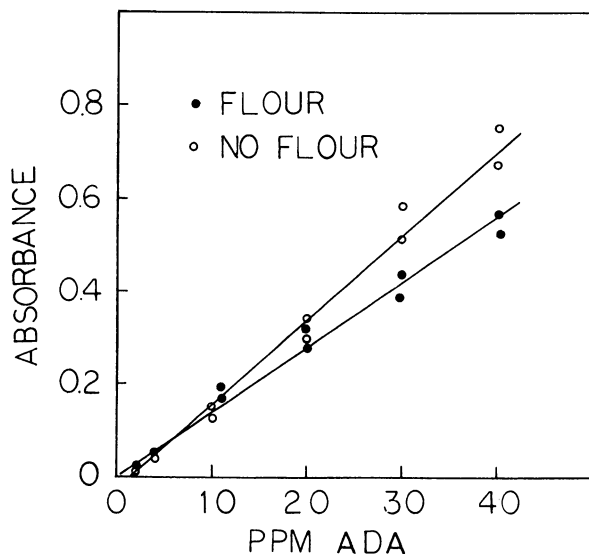

Fig. 4. Standard curve of absorbance *vs.* ppm ADA dissolved in DMF (no flour) and extracted from flour with DMF (flour).
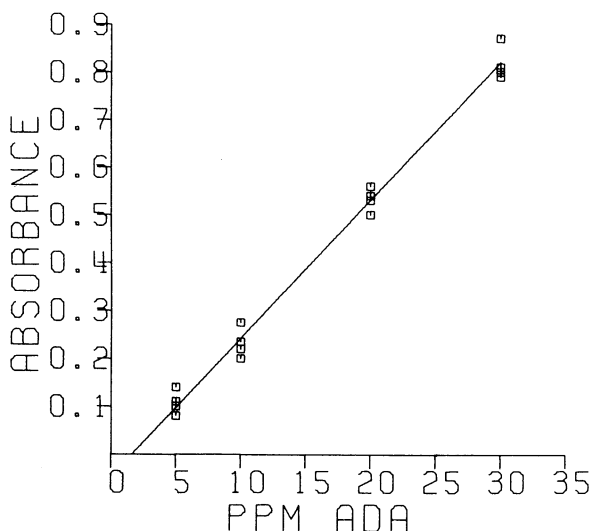

Fig. 5. Standard curve of absorbances and ppm ADA extracted from flour.

must be taken to have the DMSO solution at room temperature before adding base.

We evaluated the procedure by mixing flour with four different levels of Maturox and determining the ADA present. A plot of ppm ADA and absorption at 460 nm is shown in Fig. 5 for 10 samples for each amount of ADA. Regression analysis was run on the data and an equation for the line was developed (Fig. 5). The y-intercept is negative, so the regression line does not pass through zero. The plot shows the limit of detection is 1.5 ppm ADA. Goodness-of-fit and lack-of-fit tests were run for the model; both were highly significant relationships. The R^2 value for the model was 0.992 or 99.2%. The simple correlation coefficient between absorbance at 460 nm and ADA concentration was 0.996.

Table III shows means and standard deviations for absorbances determined at each amount of ADA on a low (9.5%) and a higher (12.1%) protein flour. Absorbances of the two flours showed no differences. The observations for the two flours are combined in Table IV, with a 95% confidence interval for absorbance calculated for each mean. The confidence interval for absorbance can be converted into ppm of ADA by using Fig. 5, as shown in Table IV. Less than 100 ppm of biurea was not detectable with the method.

Procedure for Determining ADA in Flour, Step-by-Step

1. Extract 4-g flour sample (containing 2.5–50 ppm ADA) by shaking 30 min with 60 ml of DMF.

2. Filter through Whatman No. 1 filter paper and evaporate to dryness (rotary evaporator) at 75°C.

TABLE III
Absorbance Values for ADA Determination in Flour

ADA ppm	Number of Samples	Low-Protein Flour		Number of Samples	High-Protein Flour	
		$\overline{X}$	s		$\overline{X}$	s
5	7	0.104	0.021	3	0.110	0.020
10	7	0.237	0.025	3	0.235	0.042
20	7	0.535	0.021	3	0.520	0.010
30	7	0.820	0.035	3	0.830	0.005

TABLE IV
Combined Averages and Confidence Intervals
for 10 Samples for Each Amount of ADA

ADA ppm	$\overline{X}$	s	95% Confidence Interval			
			Absorbance		ppm ADA	
			Lower	Upper	Lower	Upper
5	0.105	0.020	0.060	0.150	3.50	6.75
10	0.236	0.029	0.170	0.302	7.45	12.00
20	0.528	0.022	0.478	0.578	18.10	21.60
30	0.825	0.029	0.759	0.891	27.80	32.20

3. Add 6 ml of DMSO to the flask and completely dissolve solids by rotating the flask at 75° C 2 min.

4. Cool sample at room temperature 30 min, add 2 ml of 8 N NaOH, then shake continuously 1 hr at 25° C.

5. Add 1 ml of glucose solution (0.25 g glucose/25 ml DMF, prepared daily) then shake 30 min more.

6. Add 4 ml of the PDAB solution and 0.3 ml concd. H_2SO_4 to the sample mix, and let stand 30 min.

7. Filter the mixture through Whatman No. 1 filter paper and measure absorbance at 460 nm.

To 2 g PDAB dissolved in approximately 75 ml of 95% ethanol, slowly add 20 ml of concd. H_2SO_4 with continuous cooling. Make to 100 ml with 95% ethanol. Store solution at room temperature no longer than 2 days.

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