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Note

High-performance liquid chromatography of azodicarbonamide

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The breadmaking quality of freshly milled flour tends to improve with storage for a period of up to two months but this process may be accelerated by addition of chemical substances called improvers. Among other substances, The Bread and Flour Regulations 1984[1] permit the use of azodicarbonamide (ADA) up to a maximum level of 45 mg kg^{-1} on flour weight. ADA may be added to flour at the mill at levels of 5–10 mg kg^{-1} or at the bakery in the form of a compound bread improver containing 0.05–0.20 g/100 g.

ADA has previously been determined at levels up to 10 g/100 g in bulk concentrates by iodometric titration[2], and by visible[3], infrared[4] and near-infrared reflectance[5] spectroscopy; none of these methods is, however, sufficiently sensitive for compound improvers or flour. A colorimetric method[6] for ADA in flour has been published but it is slow (3 h) and has poor precision. There do not seem to be any reports on the determination of ADA by chromatographic methods.

This paper describes the results of experiments into the use of silica gel and reversed-phase C_{18} high-performance liquid chromatography (HPLC) with ultraviolet (UV) and electrochemical detectors for the determination of ADA in compound improvers and flour.

EXPERIMENTAL

Apparatus

A high-performance liquid chromatograph comprising Altex 110 pump set at 1.0 ml min^{-1} and Rheodyne injector with 20-μl loop was used with (A) a 250 × 4.6 mm I.D. Partisil 5 μm silica column and a Pye-Unicam LC-UV detector set at 275 nm and (b) a 110 × 4.7 mm I.D. Partisphere 5 μm C_{18} column and a Coulochem 5100A electrochemical detector/5011 analytical cell set at $E_1 = -0.15$V and $E_2 = -0.35$V.

The mobile phase for system A was 20 ml l^{-1} methanol in diethyl ether (HPLC grade, degassed by ultrasonication prior to use) and for system B 50 m*M* sodium dihydrogen orthophosphate in deionised water (filtered through a 0.45-μm Millipore membrane filter and degassed by ultrasonication prior to use).

Sample preparation

Model compound improvers were prepared by adding weighed amounts of

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ADA to a base comprising per 100 g: 25 g enzyme-active soya flour, 17.5 g calcium sulphate, 10 g fat, 1 g calcium propionate, 0.3 g DATA ester (a complex mixture of diacetyl tartaric acid esters of mono- and di-glycerides), 0.13 g fungal α-amylase and 46.07 g untreated, unbleached wheat flour.

A 5-g sample of model improver was extracted with a 10 ml N,N-dimethylmethanamide (DMF) (A.R., dried over sodium sulphate anhydrous) in a centrifuge tube using a Vortex mixer. The mixture was centrifuged at 1150 *g* for 5 min and the supernatant transferred to a 50-ml volumetric flask. Three further extractions were carried out in the same way and the combined extracts diluted to 50 ml with DMF. HPLC was carried out using system A.

A 5-g sample of flour was extracted with 25 ml deionized water in a screw-capped bottle for 5 min with a wrist-action shaker. The mixture was transferred to a centrifuge tube, centrifuged at 1150 *g* for 5 min and the supernatant filtered through a Whatman No. 1 paper. HPLC was performed on the filtrate using system B.

Recovery experiments were carried out in duplicate with duplicate injections of each extract, *i.e.* four measurements. Reproducibility was assessed by making 12 duplicate injections of standard solutions or extracts and calculating the standard deviation of replicates $s_r = \sqrt{(\Sigma d_i/2n)}$ where d_i are the individual differences between n duplicates ($n = 12$).

The limit of detection was defined as $y_B + 3s_B$ where y_B and s_B are the mean and standard deviation, respectively, of the normal distribution of the blank response. These terms were calculated from the calibration data by taking the intercept of the calibration graph as an estimate of y_B and the residual standard deviation as an estimate of s_B[7].

RESULTS AND DISCUSSION

ADA has a solubility of 6 g l^{-1} in DMF[6] and 0.035 g l^{-1} in water[2] and its structure is $H_2N \cdot CO \cdot N{=}N \cdot CONH_2$. These facts suggest that normal-phase

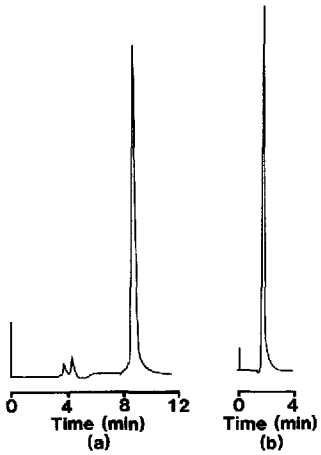

Fig. 1. (a) HPLC chromatogram of 0.5 g l^{-1} ADA in DMF on Partisil 5 μm silica with mobile phase 20 ml l^{-1} methanol in diethyl ether at 1 ml min^{-1} and UV detection at 275 nm (0.08 a.u.f.s.). (b) HPLC chromatogram of 10 mg l^{-1} ADA in water on Partisphere 5 μm C_{18} with mobile phase 50 m*M* sodium dihydrogen orthophosphate at 1 ml min^{-1} and electrochemical detection at $E_1 = -0.15$ V and $E_2 = -0.35$ V (gain 1 × 10).

adsorption chromatography would be the most appropriate mode of separation. The mobile phase for the silica gel column was selected by reference to graphs of solvent strengths for various binary mixtures of solvents[8] in which a parameter E_3 is defined as the solvent strength required to give $k'=3$. Amides have E_3 values in the range 0.45–0.60 and $E_3=0.50$ corresponding to the binary solvent 20 ml l^{-1} methanol in diethyl ether was found experimentally to give $k'=2.98$ for ADA. ADA has a UV absorption with a maximum ($A_{max.}$) at 245 nm but detection at 275 nm corresponding to 0.50 $A_{max.}$ was selected because the solvent ADA dissolved in (DMF) has a UV cutoff at 260 nm. The chromatogram obtained for a 0.5 g l^{-1} solution of ADA in DMF is shown in Fig. 1a and the column efficiency (N) for ADA was 9020. The response was linear in the range 0–0.5 g l^{-1} and s_r was 0.005 g l^{-1}. The recovery of ADA from a model compound improver containing 0.2 g/100 g was 99.6 ± 1.1% with four DMF extractions and 90.0 ± 2.7% with only two DMF extractions.

The limit of detection of the method was 1 mg l^{-1}; yet a flour extracted in the same manner as the bread improver may only contain 1 mg l^{-1} in the extract. Attempts to concentrate the DMF extracts to increase sensitivity led to losses of ADA and recoveries of only 30.5 ± 5.4% were obtained. ADA, however, is an oxidising agent which is readily reduced in the presence of water:

$$H_2N \cdot CO \cdot N=N \cdot CO \cdot NH_2 + 2\,H^+ + 2\,e^- \rightarrow H_2N \cdot CO \cdot NH \cdot NH \cdot CO \cdot NH_2$$

so electrochemical detection[9] may be possible. However, electrochemical detection is not compatible with adsorption chromatography so attention was directed to reversed-phase chromatography.

The voltammogram in Fig. 2 demonstrates that ADA can indeed be reduced at a graphite electrode in an electrochemical detector. Since ample reserves of sensitivity are available, it was decided to operate the detector test electrode at a potential (E_2) on the rising part of the voltammogram rather than on the limiting current

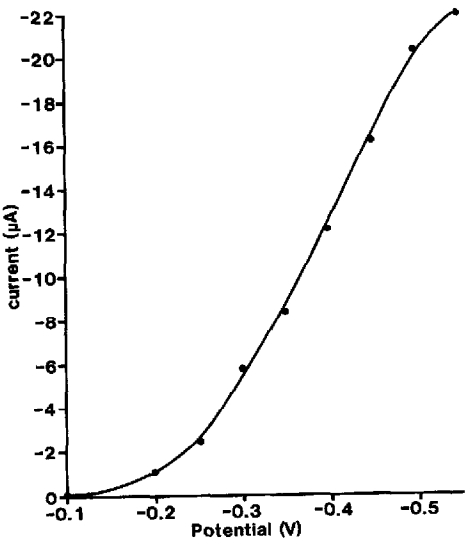

Fig. 2. Voltammogram for the reduction of azodicarbonamide at a graphite electrode.

plateau. This confers better selectivity which was even further enhanced by operating the screen mode of the detector at a potential (E_1) near the foot of the voltammogram.

The chromatogram obtained for a 10 mg l^{-1} solution of ADA in water is shown in Fig. 1b. As might be expected, ADA has little affinity for the C_{18} stationary phase and is virtually unretained ($k' = 0.1$); nevertheless, the selectivity of the electrochemical detector is such that the chromatogram obtained from an aqueous extract of untreated, unbleached flour did not contain any significant peaks. The recovery of ADA from flour was 87.3 ± 1.5% with only a single extraction with water, the response was linear in the range 0–10 mg l^{-1} and s_r was 0.12 mg l^{-1}. Since the calibration was linear and no unretained peak was obtained for the blank, it may be assumed that the peak observed in sample extracts is pure. In this case it is arguable whether the chromatographic column is serving any useful purpose and it might be preferable to perform the determination by flow injection analysis. On the other hand, ion-pairing with an alkanesulphonate may give rise to increased retention. Clearly further work would be needed to resolve these questions.

These studies have shown that a satisfactory chromatographic separation of ADA may be achieved using a silica gel HPLC column and UV detection allows sufficient sensitivity for the analysis of compound bread improvers. In order to achieve the sensitivity necessary for the determination of ADA in flour, the feasibility of electrochemical detection was demonstrated but this had to be used in conjunction with a less satisfactory separation on a C_{18} columns.

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