Use of 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole for Determining Cysteine and Cystine in Cereal and Legume Seeds

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A new colorimetric method has been developed for the determination of chemically available cysteine and half-cystine in maize and legume seeds. The method is based on the reduction of cystine with aqueous $NaBH_4$/urea/EDTA solution and the reaction of cysteine with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) in 0.2 M sodium acetate/HCl buffer (pH 2.0) to form a greenish product showing maximum absorbance at 410 nm. The method is simple, accurate, and highly specific for cysteine, in the presence of other naturally occurring amino acids. The method was applied to the determination of cysteine and of cysteine plus half-cystine in some seed meals. There was a good correlation between the results obtained using this method and those obtained using Ellman's reagent [5,5-dithiobis(2-nitrobenzoic acid)]. There was also a good correlation between the results obtained using this method and cysteic acid values calculated from amino acid analysis of the samples.

Keywords: *NBD-Cl; chemically available cysteine; cereals; legume seeds*

INTRODUCTION

The sulfur amino acids cysteine (and cystine) and methionine are recognized as the limiting essential amino acids in legume seeds. Owing to prevailing economic conditions, particularly in developing countries, the dependency on legumes in meeting human protein requirements is on the increase and so is the need to improve the protein quality of legumes through breeding and adequate processing.

During processing, especially when heat is involved, cysteine is readily converted to lysinoalanine (Ziegler, 1964; Mauron, 1990) or oxidized to cysteine sulfonic acid, whereas cystine is readily oxidized to cystine disulfoxide and or cystine disulfone (De Man, 1980). Some of these oxidized forms of cysteine and cystine are nutritionally unavailable. Common methods for determination of cysteine (and half-cystine) are based on performic acid oxidation (Moore et al., 1958), which converts the cysteine (and cystine) to cysteic acid. These methods give total cysteine plus half-cystine and cannot distinguish the proportion of cysteine (and half-cystine) that may have been oxidized during processing and, therefore, do not measure chemically available cysteine (and half-cystine). Published methods for chemically available cysteine (and half-cystine) are few and either are too elaborate and time-consuming or have not been fully tested on cereal and legume seeds. These include reactions with vinyl pyridine (Inglis et al., 1976), Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] (Peniazek et al., 1975; Felker and Waines, 1978), sodium iodoacetate (Moodie and George, 1976), and phenylisothiocyanate in the presence of dithiodiglycolic acid and high-performance liquid chromatography (HPLC) separation of the derivatized amino acids (Hoogerheide and Campbell, 1992). There is therefore further need for rapid and accurate methods for available cysteine and half-cystine for selecting cereal and legume seeds of high protein quality and for monitoring changes in protein quality during cooking/processing of the seeds.

Studies involving 7-halogenated-4-nitrobenzo-2-oxa-1,3-diazoles (Ghosh and Whitehouse, 1968; Watanabe and Imai, 1981; Toyo'oka et al., 1989) showed that these compounds could be used in the quantitative estimation of thiols and amines. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) has been used for complete amino acid analysis of protein hydrolysates by HPLC with fluorometric detection (Watanabe and Imai, 1983; Imai et al., 1988). 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) has been used for chromatographic determination of hydroxyproline in collagen hydrolysate (Ahnoff et al., 1981) and of proline and hydroxyproline (Gramova et al., 1992). Although NBD-F has been more widely applied probably because it was proposed to be superior to NBD-Cl with regard to reactivity and fluorescence yield (Watanabe and Imai, 1981), NBD-Cl has the advantage of solubility in aqueous solutions (Ghosh and Whitehouse, 1968). Birkett et al. (1970) reported that the site of attack of NBD-Cl on the cysteine molecule is pH dependent and the composition of the product of the reaction varied with varying cysteine/NBD-Cl ratio. In this study, the pH-dependent reaction of NBD-Cl with the thiol group of cysteine (Birkett et al., 1970; Nitta et al., 1979) to produce NBD−*S*-cysteine was investigated to ascertain its suitability for determining chemically available cysteine plus half-cystine in cereals and legumes and their products.

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Table 1. Chemically Available Cysteine and Cysteine plus Half-Cystine Determined in Seed Meals

sample	% crude protein, N × 6.25	g of cysteine/16 g of N[a] (± SD)		g of cysteine plus half-cystine/16 g of N[a] (± SD)		g of cysteine/16 g of N determined using performic acid oxidation and amino acid analysis (E)
		NBD-Cl (A)	Ellman's reagent (B)	NBD-Cl (C)	Ellman's reagent (D)	
cowpea	27.6 ± 0.27	0.330 (± 0.010)	0.271 (± 0.005)	1.134 (± 0.036)	1.367 (± 0.002)	1.54
maize	10.8 ± 0.31	0.521 (± 0.067)	0.519 (± 0.018)	3.703 (± 0.032)	3.526 (± 0.030)	3.70
soybeans	44.2 ± 0.30	0.165 (± 0.009)	0.128 (± 001)	1.249 (± 0.008)	1.138 (± 0.049)	1.61
black-eyed beans	23.5 ± 0.17	0.353 (± 0.007)	0.328 (± 0.004)	1.339 (± 0.033)	1.486 (± 0.016)	1.64
white kidney beans	21.7 ± 0.14	0.235 (± 0.011)	0.307 (± 0.003)	1.741 (± 0.010)	1.371 (± 0.049)	1.83
butter beans	21.4 ± 0.12	0.229 (± 0.011)	0.203 (± 0.002)	1.563 (± 0.011)	1.487 (± 0.068)	1.53

[a] Values are means of quintuplicate determinations. Correlation coefficients (r) = 0.9396 between A and B, 0.9750 between C and D, 0.9850 between C and E, and 0.9802 between D and E.

EXPERIMENTAL PROCEDURES

Materials. Dried cowpea seeds (variety Ife Brown) and maize (variety TZB SR-Y) were obtained from the Institute of Agricultural Research and Training (IAR&T), Moor Plantation, Ibadan, Nigeria. Other legumes were purchased from a supermarket. Total amino acid analysis was carried out by chromatography in a Technicon model NC-2P amino acid analyzer using C2 resin, a 75 cm column, and sodium citrate buffer (pH 2.5). Norleucine was used as an internal standard. Nitrogen contents of the samples were determined using a Tecator Digestion System 20 1015 digester and a Tecator Kjeltec Auto 1030 analyzer. NBD-Cl, Ellman's reagent′, amino acids, and all other reagents were obtained from Sigma Chemical Co. Ltd., Dorset, England, and used as supplied. Buffer solutions tested were 0.2 M CH_3COONa/HCl buffer (pH 1.0, 2.0, 3.0, 4.0); 0.2 M CH_3COONa/CH_3COOH buffer (pH 5.0); 0.066 M KH_2PO_4/Na_2HPO_4 buffer (pH 6.0); 0.05 M borax/boric acid buffers (pH 7.0, 8.0, 9.0). Absorbance spectra of NBD derivatives were determined using a Phillips PU 8740 UV−vis scanning spectrophotometer. Fluorescence measurements were carried out using a Perkin-Elmer LS 50 luminiscence spectrometer. Absorbance measurements were made on a Cecil CE 1020 spectrophotometer. The model of centrifuge used was the MSE Centaur 2. Seed meals were prepared by grinding the seeds in a Glen Creston microhammer mill through a 0.5 mm stainless steel sieve.

Reaction of Amino Acids with NBD-Cl. Half a milliliter of each of the solutions of the amino acids L-cysteine, L-cystine, L-alanine, L-valine, L-leucine, L-phenylalanine, L-tryptophan, L-proline, l-serine, L-threonine, L-tyrosine, L-aspartic acid, L-glutamic acid, L-lysine, L-methionine, L-histidine, l-arginine in each of the various buffers tested was mixed with 0.5 mL of a solution of NBD-Cl in 95% ethanol. Each mixture was made up to 9.5 mL with the appropriate buffer solution. The tubes were covered with Parafilm and kept in a thermostat-controlled shaking water bath at various temperatures for varying periods of time. To stop the reaction, the mixture was cooled in ice (2 min) and acidified with 0.5 mL of 3 M HCl. The mixture was subjected to spectrocolorimetric and fluorometric analyses.

Determination of Cysteine and Cysteine plus Half-Cystine Using NBD-Cl. To determine available cysteine, 9.0 mL of 0.2 M $NaOOCCH_3$/HCl buffer (pH 2.0) was added to 0.1 g of each seed meal in a test tube, followed by 0.5 mL of 0.1 M NBD-Cl in 95% ethanol and mixed thoroughly. The test tubes were covered with Parafilm and were left in a thermostat-controlled shaking water bath at 70 °C for 30 min. The reaction was stopped by cooling in ice (2 min) and adding 0.5 mL of 3 N HCl. The mixture was centrifuged at 2000 rpm for 15 min, and the absorbance of the supernatant was measured at 410 nm. A blank containing 0.1 g of seed meal but no NBD-Cl was prepared in exactly the same manner as for the sample. Absorbance values were converted to millimoles of cysteine per assay using the linear regression equation $y = 161.97x - 0.001$ calculated from a standard curve based on L-cysteine solutions of various concentrations (0.0005−0.01 mmol/assay).

To determine available cysteine plus half-cystine, 0.05 g of seed meal was treated with 1.2 mL of $NaBH_4$/8 M urea/EDTA solution according to the method of Felker and Waines (1978). The pH of the $NaBH_4$-treated mixture was adjusted to 2.0 by adding concentrated HCl, the mixture was made up to 9.0 mL by adding 0.2 M $NaOOCCH_3$/HCl buffer (pH 2.0), and 0.5 mL of 0.1 M NBD-Cl in 95% ethanol was then added. The rest of the procedure was as described above for available cysteine.

Determination of Cysteine and Cysteine plus Half-Cystine Using Ellman's Reagent. For chemically available cysteine determination (Table 1), 9.8 mL of 0.05 M $NaHPO_4$/HCl buffer (pH 8.2) was added to 0.1 g of seed meal followed by 0.2 mL of 0.01 M Ellman's reagent in 0.05 M $NaHPO_4$/HCl buffer. The mixture was vortexed and then centrifuged at 2000 rpm for 15 min. The absorbance of the supernatant was read at 411 nm. A blank containing 0.1 g of seed meal but no Ellman's reagent was prepared in the same manner as for the sample. Absorbance values were converted to millimoles of cysteine per assay using the linear regression equation $y = 1336.866x + 0.0348$, calculated from a standard curve based on l-cysteine solutions of various concentrations (0.0001−0.0075 mmol/assay). For the determination of chemically available cysteine plus half cystine, 0.05 g of seed meal was weighed into a test tube. The cystine in the sample was reduced according to the method of Felker and Waines (1978). The content of each test tube was made up to 9.8 mL with 0.05 M Na_2HPO_4/HCl buffer (pH 8.2), and 0.2 mL of Ellman's reagent was then added. The rest of the procedure was as described above for chemically available cysteine.

Determination of Protein-Bound Cysteine in Selected Feedstuffs. Samples (10 g/assay) were pretreated with performic acid as described by Moore (1963) and then hydrolyzed in 6 N HCl by refluxing under nitrogen for 24 h. The hydrolysates were subjected to amino acid analysis, and cysteine contents were manually calculated from the chromatograms by the height × width method.

Statistical Analysis. The correlation between the results obtained using NBD-Cl and Ellman's reagent was evaluated by correlation analysis according to the procedure of Christian (1980).

RESULTS AND DISCUSSION

Effect of pH on Reaction of NBD-Cl with Amino Acids. Cysteine is known to form three derivatives with NBD-Cl, the NBD-S, the NBD-N and the N,S-bis-NBD derivatives, which can be readily distinguished from their spectroscopic properties, the NBD-S and the NBD-N derivatives showing absorption maxima at 425 and 475 nm, respectively (Birkett et al., 1970). The absorption spectra, color, and spectrofluorometric properties of NBD derivatives of L-cysteine observed in this study show that different derivatives were formed at different pH values. At pH 1, the product was almost colorless, the absorption spectrum showed no peak, and the fluorescence intensity was negligible, suggesting that no colored derivative was formed at pH 1. This is possibly because at very low pH, the NH_2 group of cysteine is protonated and unavailable for attack by NBD-Cl. At pH 2, the product was a bright green color

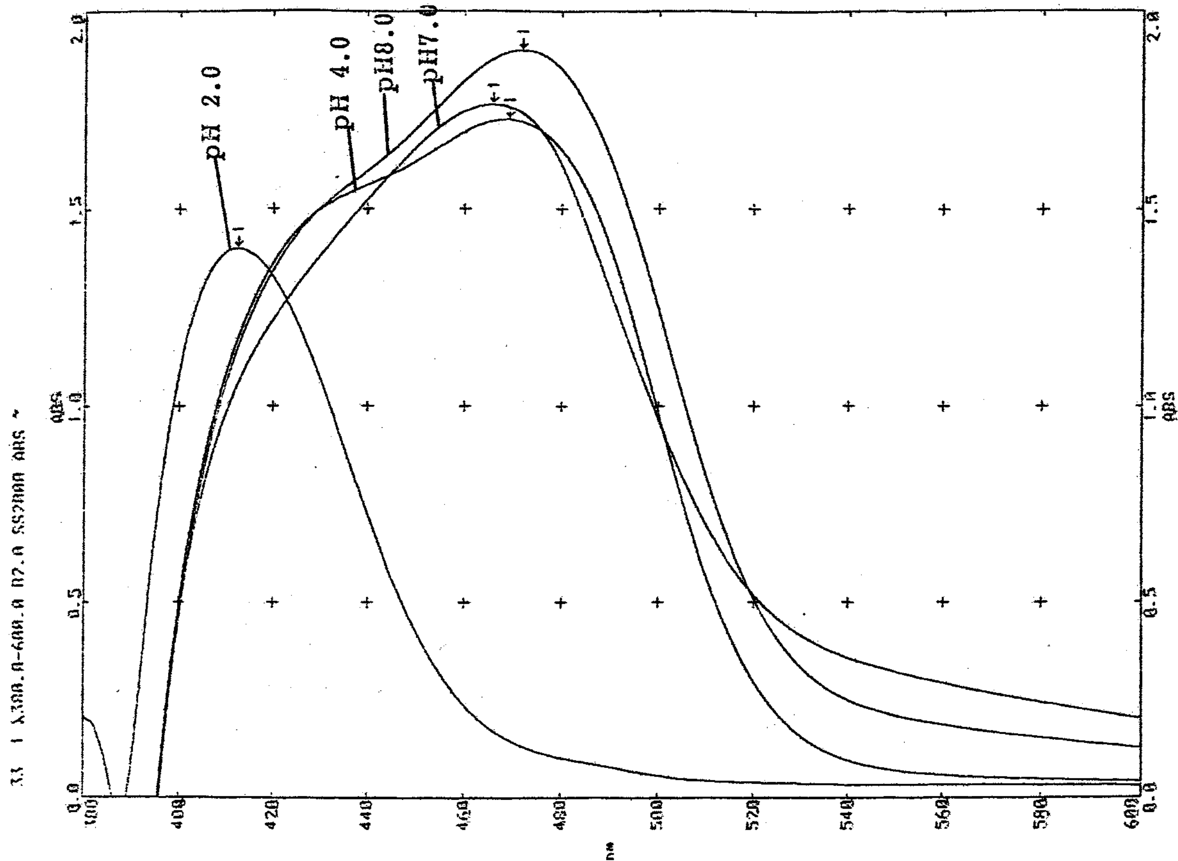

Figure 1. Absorption spectra of NBD derivatives of cysteine at pH 2−8.

and the absorption spectrum showed one peak at 410 nm (Figure 1), suggesting the formation of a single derivative, possibly the NBD-S derivative, showing a shift from the expected 425 nm due to a high hydrogen ion concentration in the medium. The absorption spectra of the products confirm that above pH 2, a mixture of NBD−cysteine were formed. The color of the products varied from light yellow to deep orange as the pH of the reaction medium increased from 3 to 9.

It is believed that NBD-Cl will not react directly with NH_2 groups of cysteine but will rapidly form the N,S-bis-NBD derivative indirectly by intramolecular S → N transfer (Birkett et al., 1970). This transfer possibly could not occur at pH 2, due to protonation of the NH_2 groups, caused by high hydrogen ion concentration, but could occur slightly at pH 3, giving rise to the S-NBD and N-NBD derivatives, which showed absorption maxima at 417 nm (showing a shift from the expected 425 nm) and 475 nm, respectively. At pH 4 and above, the intramolecular S → N transfer was possibly favored, and the three derivates N-NBD−cysteine, S-NBD−cysteine, and N,S-bis-NBD−cysteine were formed, with the concentration of the N-NBD and N,S-bis-NBD derivatives increasing with increasing pH at the expense of the concentration of S-NBD−cysteine, until at pH 7, the shoulder at 425 nm representing S-NBD−cysteine had completely disappeared. The absorption spectra of the NBD derivatives of cysteine formed at different pH values (Figure 1) show that the derivative formed at pH 2 was different from those formed above pH 2.0 and could only be the NBD-S derivative, showing a shift to 410 nm from the expected 425 nm, due to high concentration of hydrogen ions in the medium. It can therefore be concluded that at pH 2.0, it is possible to selectively estimate cysteine spectroscopically as the NBD-S derivative. Cysteine thus measured would be chemically available because the thiol group is the reacting group. Reaction would be impossible if the thiol group had been oxidized and rendered chemically unavailable.

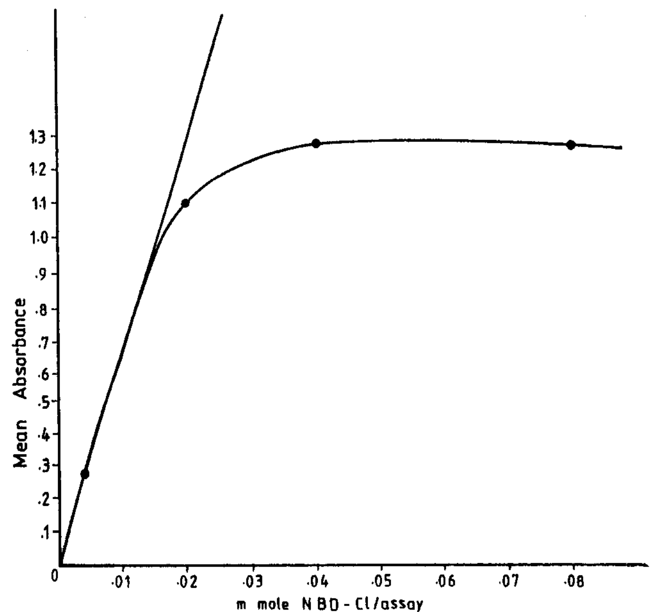

Figure 2. Reaction of 0.001 mmol of L-cysteine with various concentrations of NBD-C1 at pH 2.0 for 1 h at 80 °C. NBD-C1 was present in excess.

Effect of NBD-Cl Concentration, Temperature, and Time. At pH 2.0, NBD-Cl is expected to react only with the −SH group of cysteine and to do so on a 1:1 molar ratio. The results obtained in this study did not

Table 2. Reaction of Excess NBD-C1 with 0.005 mmol of L-Cysteine at 80 °C for 1 h at pH 2.0

mmol of NBD-C1/assay	mean absorbance at 410 nm[a] ± SD
0.04	4.945 ± 0.023
0.08	4.842 ± 0.129
0.12	4.944 ± 0.039
0.16	4.947 ± 0.035
0.20	4.908 ± 0.007

[a] Values are means of quintuplicate determinations.

conform to this expectation. Consequently, an investigation of the excess of NBD-C1 required to achieve maximum derivatization of cysteine was conducted. Various amounts of excess NBD-Cl (0.005−0.1 mmol/assay) were reacted with 0.001 mmol of L-cysteine at 80 °C for 1 h at pH 2.0. The absorbance of the reaction mixtures increased with increasing NBD-Cl concentration up to a maximum, which was reached at an L-cysteine/NBD-Cl ratio of 1:25 (Figure 2). Again, various amounts of excess NBD-Cl (0.04−0.2 mmol/assay) were reacted with 0.005 mmol of L-cysteine (representing a cysteine/NBD-Cl ratio ranging from 1:8 to 1:40), at 80 °C for 1 h at pH 2.0 (Table 2). The absorbance of the reaction mixtures did not increase beyond the value obtained for a cysteine/NBD-Cl ratio of 1:8, suggesting that the requirement for maximum derivatization of cysteine was a cysteine/NBD-Cl ratio of 1:8 or less at these higher levels of cysteine and NBD-Cl concentrations/assay. In view of these observed deviations from expectation, the fact that in previous studies involving the −SH group a minimum molar ratio of −SH to NBD-Cl of 1:2 was recommended (Nitta et al., 1979) and in a study involving amino acids a molar ratio of amino acid/NBD-Cl of 1:10 was used (Ahnoff et al., 1981), a minimum molar ratio of 1:10 was used in this study to determine the optimum reaction time and temperature for the reaction of cysteine with NBD-Cl at pH 2.0.

The relationships between NBD−cysteine measured by absorbance at 410 nm, reaction time, and reaction temperature are illustrated in Figure 3. It can be seen that absorbance increased linearly with increase in reaction time even up to 90 min at 40, 70, and 80 °C and that absorbance increased with increase in temperature for each reaction time tested. These results suggest that the time and temperature that will give maximum derivatization of cysteine could be far greater than 100 min and 80 °C, respectively. Such conditions may lead to excessive denaturation of the test material. To avoid this, reaction time and temperature of 30 min at 70 °C were adopted, because the relationships between absorbance and reaction time and between absorbance and temperature are linear over the time and temperature ranges tested (Figure 3). It can also be seen from Figure 3 that for reactions carried out at 70 °C, absorbance values obeyed Beer's law very closely, up to 30 min, after which time some of the values failed to fall on the line. This suggests that after 30 min, the reaction mixture may contain species other than NBD-S−cysteine.

Proportionality between Cysteine Concentration and Color Formation. The absorbance values obtained when various amounts (0.005−0.01 mmol) of L-cysteine were reacted with 0.1 mmol of NBD-Cl in 95% ethanol showed that absorbance increased linearly with increase in L-cysteine concentration ($r = 0.9987$), indicating that Beer's law was obeyed even at a concentration of 0.01 mmol of L-cysteine/assay.

Stability of Reaction Product. The absorbance of the product formed between L-cysteine and NBD-Cl using the adopted procedure, when read 1 h after the reaction was stopped, was 98.5% of that when the absorbance was read immediately after the reaction was stopped. Therefore, reliable results could be obtained, provided absorbance measurements are carried out within 1 h of color formation.

Specificity of the Adopted Procedure for Available Cysteine. When 0.005 mmol of each of L-Ala, L-Val, L-Leu, L-Phe, L-Trp, L-Pro, L-Ser, L-Thr, L-Tyr, L-Asp, L-Glu, L-Lys, L-Met, L-His, L-Arg, and L-cystine was reacted with NBD-Cl using the adopted procedure and the absorbance spectra of the products were obtained, no absorption peaks were observed for any of

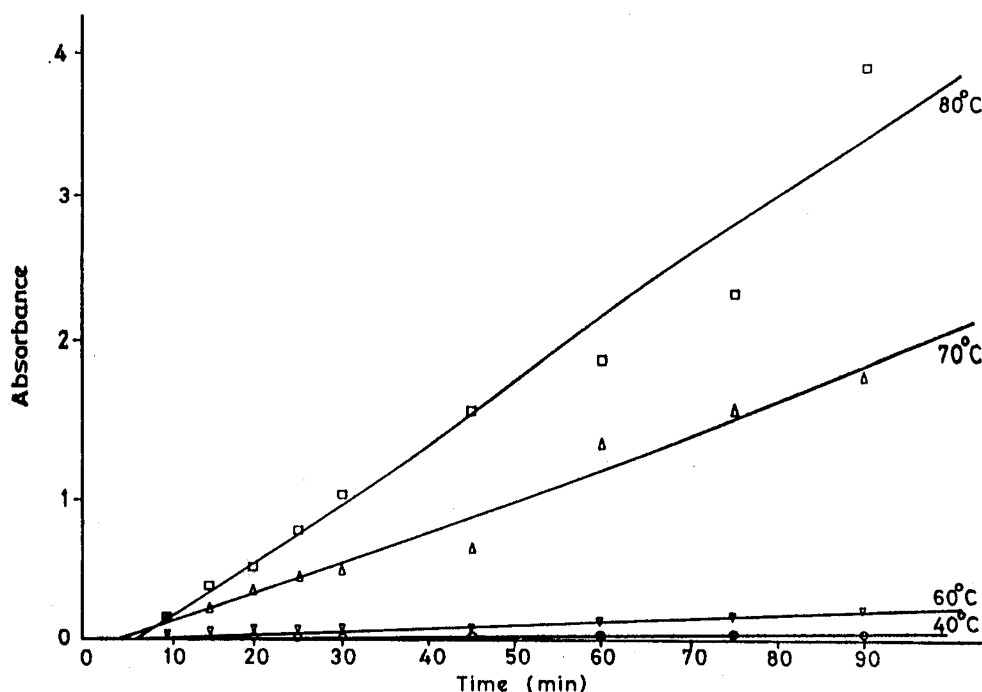

Figure 3. Effect of temperature and time on the reaction of NBD-Cl (0.05 mmol) and L-cysteine (0.0025 mmol).

the products, showing that none of these amino acids would interfere in the adopted procedure.

Recovery of Cysteine in the Presence of Other Amino Acids. When 0.005 mmol of L-cysteine containing 0.005 mmol of each of the amino acids mentioned above was reacted with NBD-Cl, the results obtained indicate that provided the pH of the reaction mixture was maintained at 2.0 before the addition of NBD-Cl, interference from any of the amino acids tested was negligible, contributing only 1.3% of the observed value. When the pH was not adjusted, the absorbance values were almost doubled.

Application of the Adopted NBD-Cl Procedure to Maize and Legume Seeds. The results obtained when chemically available cysteine and cysteine plus half-cystine were determined on seed meals using the adopted NBD-Cl procedure and Ellman's reagent are shown in Table 1. There was a good correlation between chemically available cysteine values of seed meals determined using NBD-Cl and those determined using Ellman's reagent ($r = 0.9396$) and between chemically available cysteine plus half-cystine values of seed meals determined using the two methods ($r = 0.9750$). There was also a good correlation ($r = 0.9850$) between chemically available cysteine plus half-cystine values of seed meals determined using NBD-Cl and cysteine values determined using performic acid oxidation followed by amino acid analysis. It can therefore be concluded that the NBD-Cl procedure can complement the few known published procedures for available cysteine and available cysteine plus half-cysteine.

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