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Determination of protein amino acids as benzylthiocarbamyl derivatives compared with phenylthiocarbamyl derivatives by reversed-phase high-performance liquid chromatography, ultraviolet detection and precolumn derivatization

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Abstract

All 22 protein amino acids were successfully derivatized to benzylthiocarbamyl (BZTC) derivatives by precolumn reaction at 50°C for 30 min with benzylisothiocyanate (BZITC) and separated in 35 min on a reversed-phase Nova-Pak C_{18} column (30 cm×3.9 mm, 4 μm). However, phenylthiocarbamyl-derivatives were not completely separated on this column; in particular cystine and cysteine were resolved into a single peak. The optimum wavelength for determination of BZTC derivatives was 246 nm although the strong absorption wavelengths were 220 nm and 238 nm. The relative standard deviations (R.S.D.) of the relative molar response to the internal standard (norleucine) were less than 5%, except for cysteine (7.22%); however for the PTC derivatives, glutamine, proline, threonine, alanine, leucine, cystine+cysteine and lysine the R.S.D. values exceeded 5%. Standard calibration curves showed good linearity in the measured range from 0.125 to 5 nmol. The correlation coefficient of cystine was the lowest, 0.952. The stability of BZTC derivatives of standard amino acids was about 120 h except for threonine, alanine, cystine and serine. The detection limit was about 3.9 pmol at 0.05 AUFS in both of BZTC and PTC derivatives. The compositions of amino acids of soybean and bovine serum albumin (BSA) analyzed with both derivatives were similar to the results found in the literature and food composition tables, except for cysteine. The BZTC derivatives of cystine and cysteine in soybean and BSA could be determined separately, but the PTC derivatives could not, nor could data on separate dertermination of cysteine and cystine be found in the literature studied.

Keywords: Derivatization, LC; Soybean; Food analysis; Benzylisothiocyanate; Isothiocyanates; Amino acids; Albumin; Phenylisothiocyanate

1. Introduction

According to a need for simple, sensitive, reproducible and stable precolumn derivatization for analysis of 22 protein amino acids by reversed-phase high-performance liquid chromatography (RP-HPLC), some methods have been intensively developed during the past decades. Dansyl [1,2], dabsyl [3,4], *o*-phthalaldahyde (OPA) [5,6], 9-fluorenylmethyl carbonyl chloride (FMOC-Cl) [7,8], phenylthiohydantoin (PTH) [9,10] and phenylthiocarbamyl (PTC) [11–13] amino acids have been widely adapted in RP-HPLC. Of those methods, PTC derivatization by phenylisothiocyanate (PITC) might

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be considered a comparatively successful and widely usable method that is exclusively coupled to UV detection, especially for the secondary amino acids proline and hydroxyproline. In the PTC derivatization, excess reagent and by-products produced during derivatization must be completely removed with a high vacuum to avoid interfering peaks. Moreover, cystine and cysteine could not be resolved separately by this method.

Recently, a butylthiocarbamyl (BTC) derivative of butylisothiocyanate (BITC) was developed and all 22 protein amino acids were resolved on a Nova-Pak C_{18} column; in particular cystine and cysteine were successfully separated with this derivative. The reagent peak appeared after all of the BTC derivatives had been resolved, so there was no risk of interfering peaks due to incomplete removal of excess reagent. The boiling point of BITC was much lower than that of PITC, so the excess BITC reagent could be more easily removed than PITC. However, asparagine and serine peaks overlapped completely and the stability of BTC derivatives at room temperature was estimated to be only ca. 8 h [14].

We think that there is still a need for a simple, sensitive and more stable precolumn derivatization method for all of the 22 protein amino acids for their separation by RP-HPLC and UV detection. In this work, benzylisothiocyanate (BZITC) was chosen for this purpose and applied it to the determination of 22 standard amino acids and samples of bovine serum albumin (BSA) and soybean protein.

2. Experimental

2.1. Materials

BZITC and PITC were obtained from Aldrich (Milwaukee, WI, USA), standard amino acids and bovine serum albumin (BSA) from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol and tetrahydrofuran were from Merck (Darmstadt, Germany). All other reagents and solvents were of analytical reagent grade.

2.2. Chromatography

The HPLC system consisted of a Spectra-Physics 8800 ternary solvent-delivery system and a Spectra 200 programmable-wavelength UV detector. The column temperature was adjusted to 40°C. The column was Nova-Pak C_{18} (300×3.9 mm I.D., 4 μm dimethyloctadecylsilyl-bonded amorphous silica, Waters). The solvents for separation were kept under a blanket of helium with a solvent stabilization system (Spectra-Physics). The solvent system consisted of three eluents: (A) 0.02 *M* NaH_2PO_4 containing 5% methanol and 1.5% tetrahydrofuran (pH 6.8, adjusted with phosphoric acid); (B) solvent A–acetonitrile (50:50): and (C) acetonitrile–water (70:30). The flow-rate was 1.2 ml/min. The solvent gradient was as follows: 0 min, 100% A; 10 min,80% A–20% B; 15 min, 76% A–20% B–4% C; 20 min 70% A–20% B–10% C; 30 min, 50% A–30% B–20% C; 40 min, 30% A–35% B–35% C. After this gradient program a washing step for about 20 min with solvent C substantially protected the column damage.

2.3. Preparation of standard amino acid solution

The concentration of mixture solution of standard amino acids except glutamine, cysteine and cystine was 2.5 μmol/ml 0.01 *M* HCl. Standard solutions of glutamine+cysteine and cystine were made individually just before derivatization because of their destruction by long-time storage in HCl solution. The concentration of cystine was only 1.0 μmol/ml of 0.01 *M* HCl because of its limited solubility.

2.4. Sample preparation

BSA (4 mg) was placed in a 5-ml test tube with an open-hole screw-cap with a septum and 0.5 ml 6 *M* HCl containing 0.1% phenol was added. Soybean meal (0.2 g) was placed into a 20-ml test-tube with the same type of cap and 15 ml 6 *M* HCl containing 0.1% phenol was added. After tightly capping, the septum was pierced with two stainless-steel injection needles. One needle was connected to the vacuum pump and the other to a nitrogen supply. The test-tubes were evacuated with a vacuum pump for 5 min and then flushed with nitrogen. This step was repeated twice. Hydrolysis was carried out at 145°C for 4 h. The soybean meal hydrolysate was filtered and dried in a rotary evaporator at 50°C. The residue was dissolved in 0.01 *M* HCl and the volume was adjusted to 50 ml. A 5-ml sample was passed

through a 100×13 mm I.D. cation-exchange column (Dowex 5X8) and the retained amino acids were eluted with 4 *M* ammonia solution. The eluted solution was dried in a rotary evaporator at 50°C, dissolved in 0.01 *M* HCl, diluted to 50 ml and used for derivatization. The BSA hydrolysate was dried with nitrogen at 50°C and dissolved in 5 ml 0.01 *M* HCl to use for derivatization.

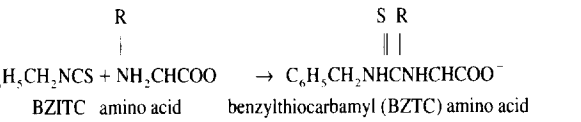
2.5. Derivatization

A mixture solution of standard amino acids (20 μl; standard solution of cystine, 50 μl) and sample solutions (soybean, 500 μl; BSA, 100 μl) were each placed in 2-ml conical vials and completely dried with nitrogen at 50°C. About 30 μl acetonitrile was added and dried again. The residues were dissolved in 50 μl coupling buffer [acetonitrile–methanol–triethylamine (10:5:2)] containing L-norleucine (2.5 μmol/ml) with internal standard and then 3 μl of BZITC or PITC was added. After tightly capping the vial with an open-hole screw-cap with a septum, derivatization of BZTC-amino acids was carried out at various reaction temperatures and times as indicated in the legends to the respective figures. PTC derivatization was accomplished at room temperature for 20 min [12]. After derivatization, two stainless-steel injection needles were pierced through the septum into the vial. One needle was connected with a nitrogen supply and the other with a vacuum pump. Nitrogen was infused into the vial and simultaneously evacuated with the pump to complete dryness at room temperature (ca. 25 min). After that 50 μl of acetonitrile was injected into the vial with a microinjection syringe and dried again as above. The residue was dissolved in 1 ml of solvent A and filtered through a 0.20-μm membrane filter. A 10-μl aliquot of the filtrate was injected onto the HPLC system.

3. Results and discussion

3.1. UV spectrum of BZTC–amino acid derivative

The derivatization reaction between amino acids and BZITC proceeds as follows:



The UV spectra of the BZTC–amino acid mixture and the PTC–amino acid mixture dissolved in 0.02 *M* NaH_2PO_4 are shown in Fig. 1. The wavelengths giving strong absorbance were 220 nm and 238 nm in the BZTC derivative and 215 nm and 270 nm in the PTC derivative, but the most efficient wavelengths were 246 nm in the BZTC derivative and 254 nm in the PTC derivative, which avoided interference by the absorption spectra of the impurities and the electrolyte, and showed a stable baseline.

3.2. Chromatography

Chromatograms of BZTC-amino acids derivatized at 50°C for 30 min and PTC-amino acids are shown in Fig. 2. In the BZTC derivatives, all 22 protein amino acids and norleucine (internal standard) were successfully resolved in 35 min. Cystine and cysteine

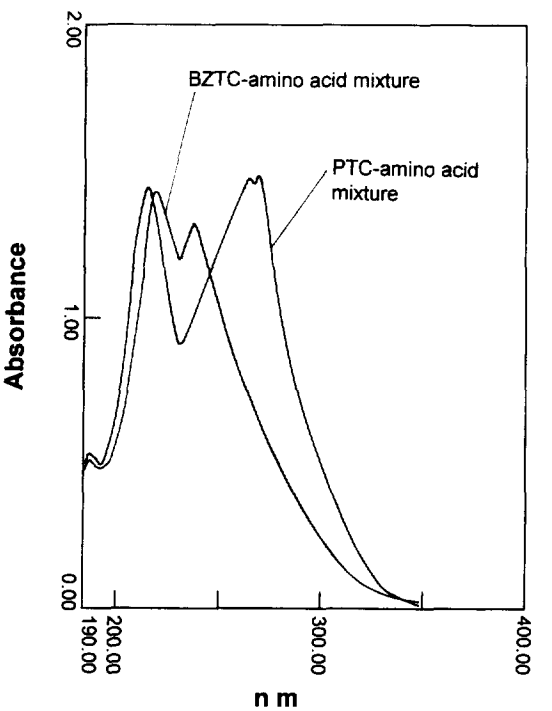


Fig. 1. UV spectra of the BZTC–amino acid mixture and the PTC–amino acid mixture. Solvent, 0.05 *M* NaH_2PO_4. Absorbance of solvent at 254 nm = 0.

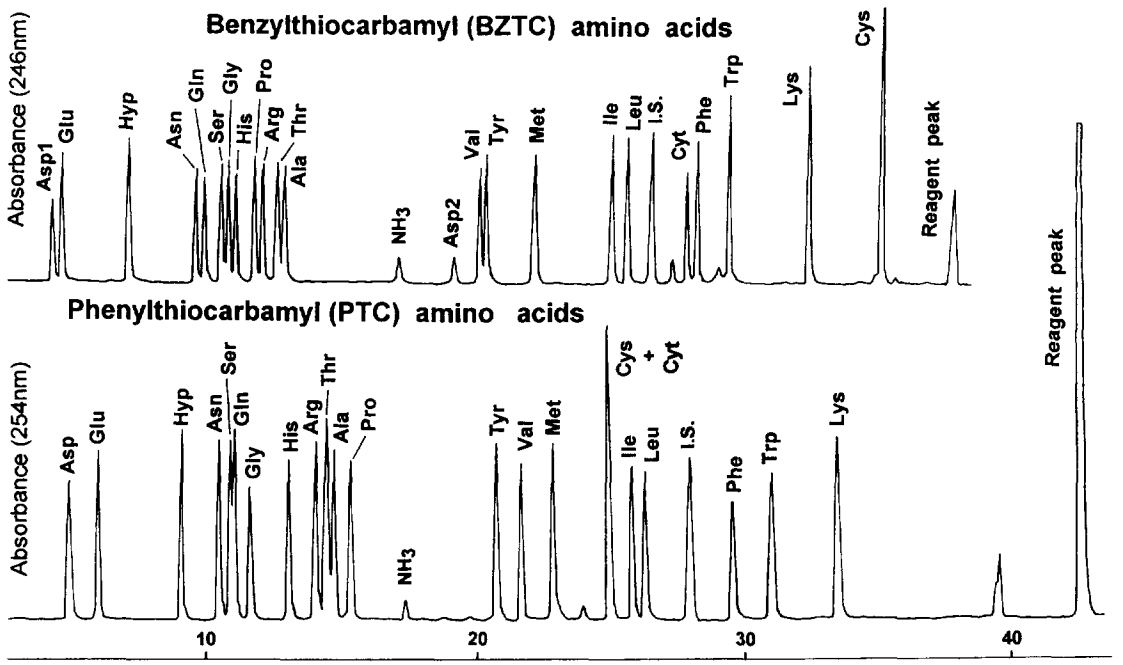


Fig. 2. Chromatogram of standard protein amino acid derivatives resolved on a Nova-Pak (30 cm×3.9 mm) C_{18} column. Injected amount = 0.625 nmol. I.S. = norleucine; Cyt = cystine; Cys = cysteine.

were individually separated and especially secondary amino acids like proline and hydroxyproline showed peak responses similar to their PTC derivatives. Aspartic acid had two peaks. Increasing of the reaction temperature and time, the peak response of the aspartic acid peak 2 was increased compared to that of the aspartic acid peak 1, but we could not explain the reason for the two aspartic acid peaks. In the PTC derivatives, serine and glutamine were separated poorly. Cystine and cysteine were eluted as same peak; we assumed that they were derivatized into the same type of derivative compound, but we could not illustrate the type of compound. In some reports, we found that whenever the analyses on the PTC derivatives of cystine and cysteine were carried out, those peaks were eluted at same position [12,15–17]. The only example where cystine and cysteine were determined separately by RP-HPLC and UV detection would be in the BTC derivatives [14], except for this study. In the elution order of peaks, some differences between BZTC and PTC derivatives were observed in serine, glutamine, proline, valine, cystine and cysteine peaks. In the BZTC derivatives, the -SH moiety of cysteine would be converted to the thiocyanate derivative by BZITC. To find out whether 1 mol of cysteine reacts with 2 mol of BZITC, absorbances of the BZTC derivative on the mole fraction of BZITC in the solution for derivatization between BZITC and cysteine were determined (Fig. 3). The determined wavelength was 238 nm, which gave maximum absorption for the BZTC derivatives. Maximum absorbance appeared at

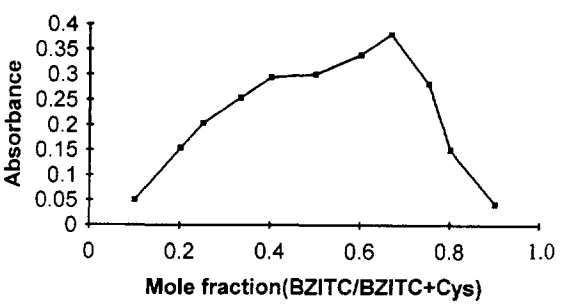


Fig. 3. Absorbance plot of BZTC-cysteine derivative at 238 nm showing maximum absorption of the mole fraction of BZITC in the derivatizing solution for the reaction between benzylisothiocyanate and cysteine.

0.667 of the mole fraction, which meant that the mole ratio of the reaction between cysteine and BZITC was 1:2. So we could draw the conclusion that the -SH group, as well as the amino group of cysteine was derivatized. Interference due to the fact that -SH moiety of cysteine was readily converted to the thiocyanate derivative with 2-nitro-5-thiocyanobenzoic acid [18] could support this conclusion. BZTC-cysteine might be eluted last because of the long less polar side chain by the derivatization of -SH group.

3.3. Formation of BZTC–amino acid derivatives

The peak-area responses of BZTC derivatives on various reaction times at 50°C and various reaction temperatures for 30 min are shown in Fig. 4 and Fig. 5. In most derivatives, the highest peak responses appeared at reaction times of 15 and 30 min when the reaction temperature was fixed at 50°C. For reaction times longer than 30 min, the peak responses decreased gradually in most cases, except for aspartic acid, which increased with the increment of the reaction time, and the secondary amino acids proline and hydroxyproline, which showed almost constant peak responses for all reaction times (Fig. 4). BZITC, as well as PITC is undoubtedly an excellent reagent for precolumn derivatization of the secondary amino acids. When the reaction time was fixed at 30 min, the peak responses on the reaction temperatures in most derivatives were almost constant over all of the measured temperatures, except for aspartic acid which showed the highest value at 50°C; we concluded that it was very difficult amino acid for the BZTC derivatization. The optimum condition of the formation of BZTC derivatives was determined with a reaction time of 30 min at 50°C because of the lowest values of the relative standard deviation (R.S.D.) on the relative molar response (RMR) at this condition.

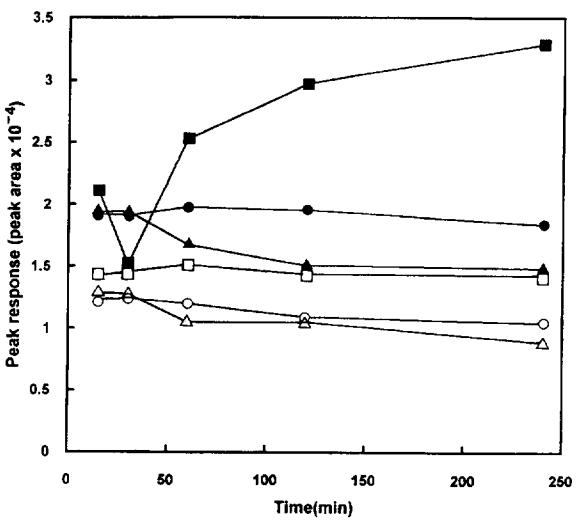


Fig. 4. Effect of reaction time on the formation of BZTC derivatives of aspartic acid (■), hydroxyproline (●), tyrosine (▲), threonine (○), proline (□) and alanine (△) (reaction temperature 50°C). Each point is the mean value of three measurements.

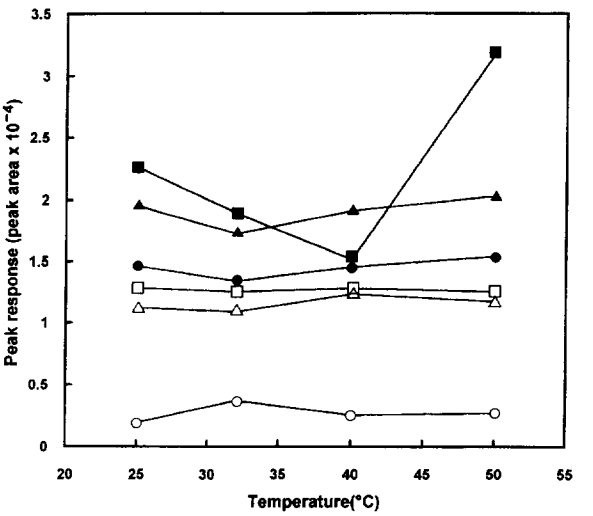


Fig. 5. Effect of reaction temperature on the formation of BZTC derivatives of aspartic acid (■), cysteine (○), hydroxyproline (▲), serine (△), glycine (□) and proline (●) (reaction time 30 min). Each point is the mean value of three measurements.

3.4. Relative molar response and calibration graph

The RMR values of the individual amino acid derivatives with respect to the norleucine derivative by the reaction for 30 min at 50°C are shown in Table 1. In the BZTC derivatives, the R.S.D. values of all derivatives except cysteine (7.22%) were less than 5%. However, for the PTC derivatives of glutamine, proline, alanine and leucine the R.S.D. exceeded 5%, and for threonine, cystine+cysteine and lysine the R.S.D. exceeded 10%. In the PTC

Table 1
RMR of BZTC and PTC derivatives of standard amino acids derivatized at 50°C for 30 min

Amino acid	BZTC derivatives		PTC derivatives	
	RMR	R.S.D.	RMR	R.S.D.
Asp	1.89[a]	3.82	0.89[a]	2.48
Glu	1.21	0.83	0.84	1.83
Hyp	1.61	4.50	0.93	1.62
Asn	1.12	4.48	0.91	2.37
Gln	1.07	2.80	1.03	5.89
Ser	1.05	1.46	0.76	2.02
Gly	0.96	2.63	0.65	1.68
His	0.84	2.49	0.83	0.87
Pro	1.17	3.42	0.85	7.37
Arg	1.00	1.15	0.91	3.02
Thr	0.92	1.09	1.01	16.6
Ala	0.88	1.73	0.90	6.64
Val	0.95	1.08	0.83	4.68
Tyr	1.39	2.20	0.92	2.64
Ile	1.05	0.95	0.85	3.76
Leu	0.84	1.38	0.84	6.86
Cyt	0.79	3.64	–	10.5
Phe	1.52	1.32	0.76	2.46
Trp	2.04	0.98	0.92	1.64
Lys	1.27	0.91	1.24	10.8
Cys	3.63	7.22	1.62[b]	–

R.S.D., relative standard deviation ($n=3$).
[a] Values are relative to norleucine.
[b] Cyt+Cys. Cyt=Cystine, Cys=Cysteine.

derivatization, the PITC reagent must be stored at −20°C under the inert gas to prevent breakdown [17] and a high vacuum system (50–100 mTorr; 1 Torr=133.322 Pa) is needed to remove the excess reagent and by-products that could interfere with the main peaks [13]. In this study, we did not use the high vacuum system, so this seems to be one of the reasons why the R.S.D. vaules exceeded 5%. We concluded that BZTC derivatization was less fastidious compared to PTC derivatization.

Calibration graphs for all of the BZTC derivatives with reaction at 50°C for 30 min showed good linearity in the measured range (0.125–5 nmol). The correlation coefficients (r) of the calibration graphs for all of the BZTC derivatives were highly significant ($P<0.001$) and exceeded 0.99, except for cysteine (0.952) and glutamine (0.976). In the PTC derivatives, cystine+cysteine had the lowest r value (0.967). There are some reports that the linearities of the calibration graphs for BTC-cystine and PTC-cystine were the poorest compared to those of other derivatives, but they could be used for quantitative determination [12,14]. However, there is a report that the linearity for PTC-cystine was so poor that it could not be used for quantitative analysis [19]. Cystine and cysteine seem to cause problems for the

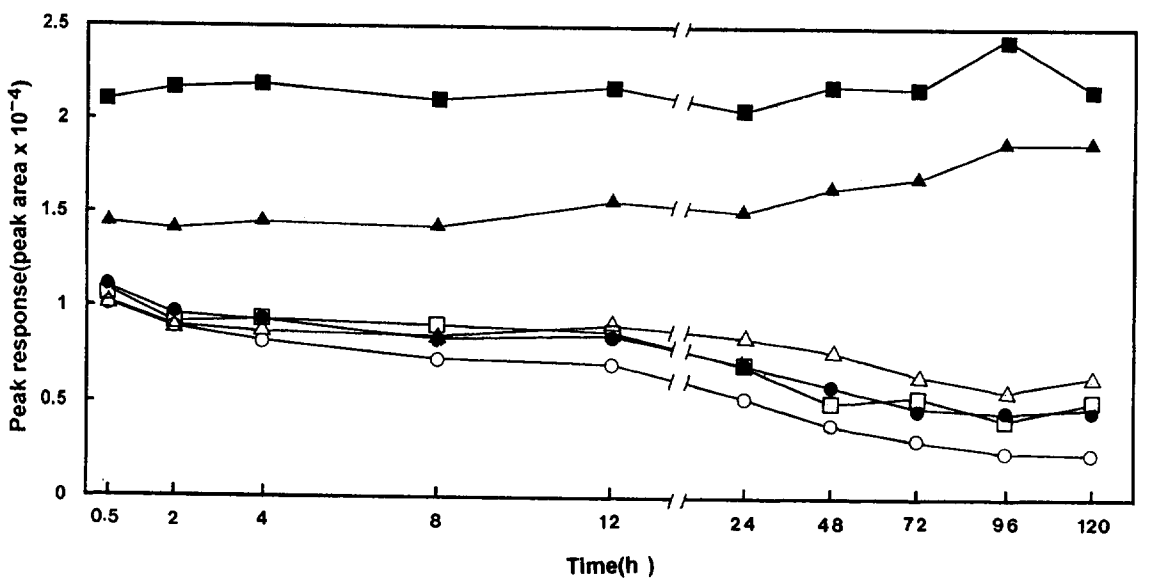


Fig. 6. Stability of BZTC derivatives of hydroxyproline (■), proline (▲), serine (●), cystine (□), alanine (△) and threonine (○). Each point is the mean value of three measurements.

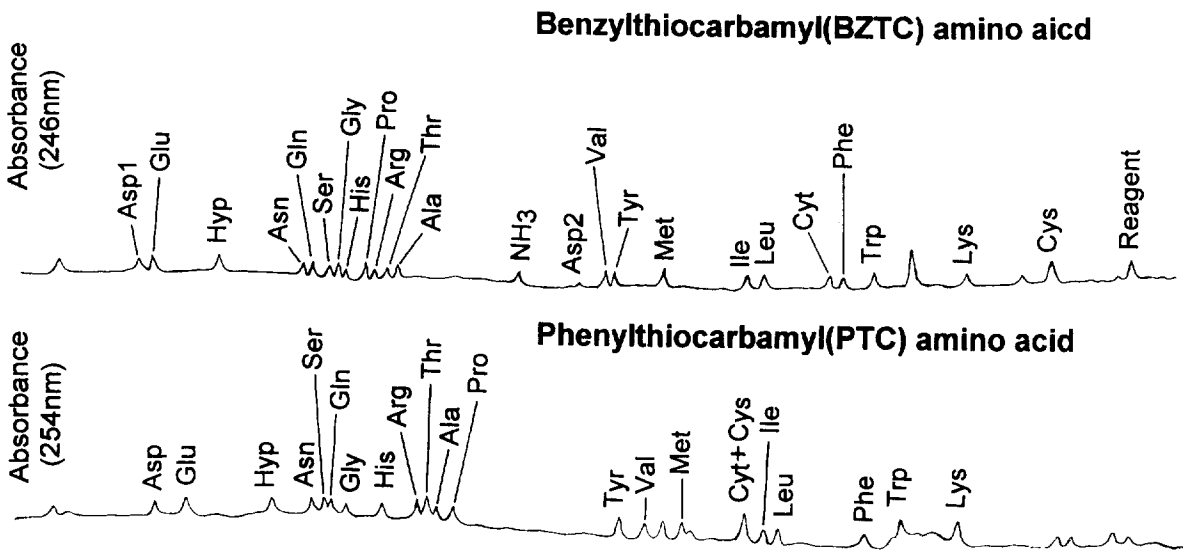


Fig. 7. Chromatograms of standard amino acid derivatives showed the sensitivity. Injected amount 3.9 pmol; range, 0.05 AUFS. Cyt = cystine; Cys = cysteine.

quantitative derivatization with PITC, BITC and BZITC reagents.

3.5. Stability of the BZTC derivatives

The stability measured with the variation of the peak-area responses on the storage time at room temperature is shown in Fig. 6. In most derivatives, the peak-area responses were decreased to less than 5% up to 120 h storage, but threonine, alanine, cystine and serine were decreased to 18.9, 13.7, 15.1 and 15.6% after 4 h. With regard to the stability, we knew that the optimum pH of the solvent dissolving the derivatives was very important. For the PTC derivatives the optimum pH of the solvent was 7.5;

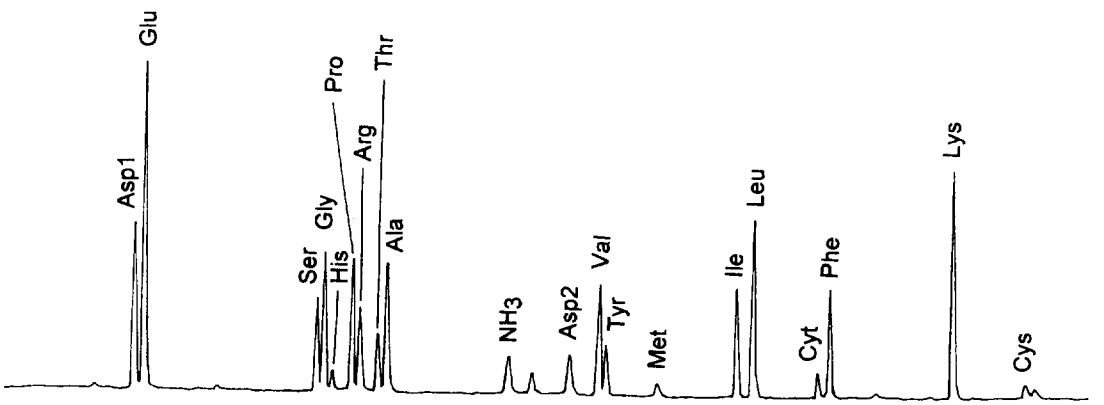


Fig. 8. Chromatogram of BZTC derivatives of amino acids in soybean hydrolysate. Cyt = cystine, Cys = cysteine.

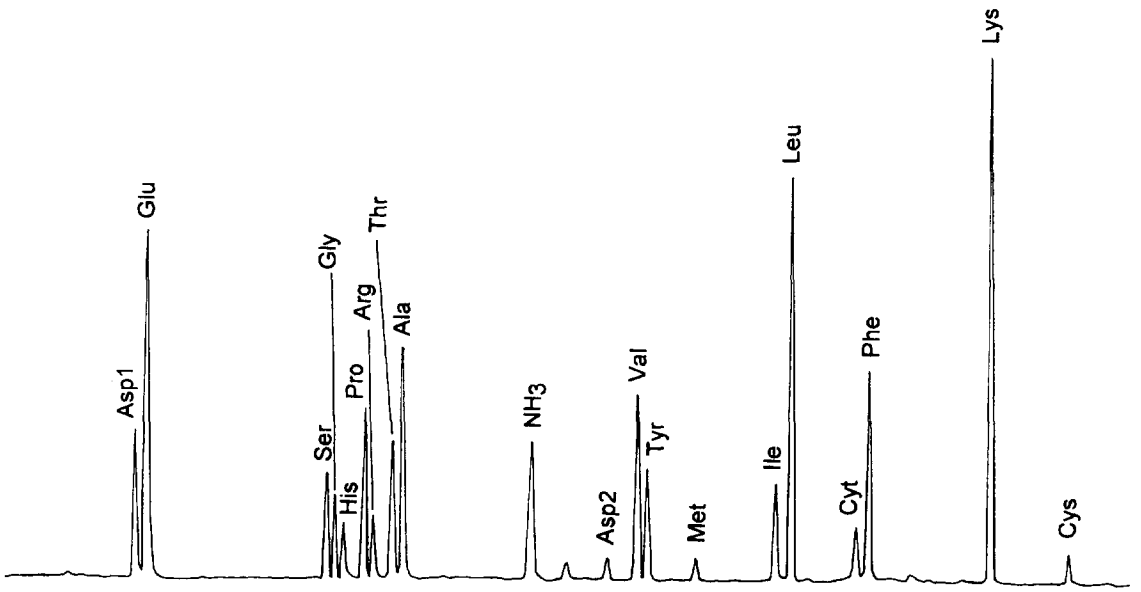


Fig. 9. Chromatogram of BZTC derivatives of amino acids in BSA hydrolysate. Cyt = cystine, Cys = cysteine.

the loss of derivatives at this pH was in the range 0–10% after 10 h storage at room temperature [17]. We assumed that the stability of the BZTC derivatives was superior to that of the PTC derivatives [17] or BTC derivatives [14], except for threonine, alanine, cystine and serine.

Table 2
Amino acid composition of soybean determined by the BZTC derivative method compared to the PTC derivative method and the analytical results reported in the literature (g/100 g dry matter)

Amino acid	BZTC derivatives	PTC derivatives	Food composition table[a]	INFIC[b]
Asp	4.45±0.25[c]	4.72±0.63[c]	4.92	4.37
Glu	7.41±0.14	7.76±0.84	8.00	7.30
Ser	2.00±0.16	2.03±0.43	2.09	1.98
Gly	1.56±0.03	2.09±0.13	1.75	1.64
His	1.06±0.08	1.15±0.04	1.13	1.04
Pro	1.96±0.20	2.38±0.18	2.24	1.71
Arg	3.61±0.13	2.65±0.36	2.89	3.21
Thr	1.78±0.11	1.79±0.05	1.67	1.77
Ala	2.00±0.12	1.89±0.35	1.88	1.51
Val	1.97±0.10	2.12±0.10	1.97	1.61
Tyr	1.20±0.05	1.26±0.11	1.11	1.04
Met	0.73±0.01	0.50±0.25	0.56	0.51
Ile	1.70±0.05	1.83±0.16	1.96	1.66
Leu	2.95±0.18	3.10±0.24	3.34	2.85
Cyt	0.49±0.12	–	–	0.52
Phe	2.06±0.15	2.63±0.18	2.30	1.82
Lys	2.72±0.10	3.73±0.25	2.64	2.56
Cys	0.17±0.04	0.11±0.01[d]	–	1.66[d]

[a] Data from Ref. [22].
[b] Data from Ref. [23].
[c] Mean±S.D., $n=5$.
[d] Cys+Cyt. Cyt = Cystine, Cys = Cysteine.

Table 3
Amino acid composition of BSA obtained with the BZTC derivative method compared to the PTC derivative method and the analytical results reported in the literature

Amino acid	BZTC derivatives[a]	PTC derivatives[a]	Automatic analyzer[b]	Sequence[c]	Sequence[d]
Asp	57.5±4.76[e]	52.2±0.42[f]	55.0	53	41
Glu	79.8±5.41	78.5±2.92	82.0	78	59
Ser	24.4±1.60	22.3±1.92	26.9	28	28
Gly	13.8±1.42	16.3±2.03	15.3	15	16
His	16.4±3.22	18.4±0.70	17.0	17	18
Pro	28.3±2.65	32.7±0.35	28.5	28	29
Arg	23.0±2.55	23.9±1.80	23.6	23	25
Thr	32.4±1.52	32.6±0.80	33.0	34	34
Ala	47.6±2.24	40.0±0.28	45.2	46	46
Val	38.2±1.50	40.2±2.42	35.4	36	36
Tyr	22.9±1.56	20.3±2.12	19.9	19	20
Met	4.2±0.25	4.6±0.56	3.8	4	4
Ile	15.9±1.09	16.9±0.25	14.1	14	14
Leu	66.6±2.02	63.3±3.68	59.2	61	60
Cyt	12.5±1.63	–	–	–	–
Phe	28.7±2.44	30.3±1.70	26.1	26	26
Lys	58.1±2.73	64.4±2.42	59.1	59	58
Trp	–	–	2.1	2	2
Cys	12.4±1.60	25.8±5.30[g]	36.0[g]	35[g]	35
Gln	–	–	–	–	20
Asn	–	–	–	–	12
Total	582.7	582.7	582.2	578	583

[a] The values are recalculated based on a total of 583 residues.
[b] Data from Ref. [24].
[c] Data from Ref. [25].
[d] Data from Ref. [27].
[e] $n=12$.
[f] $n=4$.
[g] Cyt+Cys. Cyt=Cystine, Cys=Cysteine.

3.6. Sensitivity

Chromatograms of the BZTC and PTC derivatives (injected amount 3.9 pmol) at 0.05 AUFS, which was the limit that gave a stable, baseline are shown in Fig. 7. At levels lower than 3.9 pmol, several amino acids were not detected. The peak responses were similar in both derivatives, so we could conclude that the sensitivity of BZTC derivatives was as good as that of the PTC derivatives. There is a report that the sensitivity of the PTC derivatives was 1 pmol in the detector at 0.005 AUFS (signal-to-noise ratio 5:1). But at this level, because the contamination of amino acids by the reagents, instruments and solvents was serious, the adaptation of the practical analysis was impossible [12]. At the level used in our study, there were no ghost peaks interfering in the analysis of the blank test.

3.7. Amino acid composition of soybean and BSA

Fig. 8 and Fig. 9 show chromatograms giving the amino acid composition of soybean and BSA resolved with BZTC derivatives. We could detect 18 amino acids in both samples with BZTC derivatives, but with PTC derivatives 17 amino acids were detected because of the coelution of cystine and cysteine.

A comparison of the amino acid composition of soybean and BSA determined with BZTC and PTC derivatives to other analytical results reported in the literature is shown in Table 2 and Table 3. It is known, by sequence analysis, that BSA has 17 disulfide bonds (cystine) and one free cysteine [26]. In this study the number of cystine and cysteine residues was 12.5 and 12.4, respectively. The amino acid composition in samples analyzed with both

derivatives, except for cysteine, was similar with results reported in the literature. It is known that a substantial amount of cysteine and cystine was destroyed during hydrolysis of protein with 6 *M* HCl when the hydrolysis was carried out without conversion of these amino acids to cysteic acid [20,21]. During the hydrolysis with 6 *M* HCl a substantial amount of disulfide bonds seems to be destroyed. Cysteine and cystine determination seems to give unavoidable error, unless they are modified to other compounds, i.e., pyridylethyl-cysteine or cysteic acid, prior to quantification [18,21].

4. Conclusions

A new derivatizing agent, BZITC, reacted quantitatively with all 22 protein amino acids at 50°C for 30 min to yield BZTC derivatives and the derivatives were successfully separated on the Nova-Pak C_{18} column (300×3.9 mm I.D.; 4 μm) at 246 nm with a UV detector. However, by the conventional PTC derivatization method cystine and cysteine were not separately derivatized. The calibration graphs of the BZTC-amino acids showed good linearity and R.S.D. values of the relative molar response were less than 5%, except for cysteine (7.22%). The sensitivity that could be used for practical analysis was 3.9 pmol in the detector range 0.05 AUFS, which was similar to the level in the PTC and BZTC derivatives. The amino acid composition of soybean and BSA determined with BZTC derivatives showed good agreement with the PTC derivatization method and the other reports in the literature, except for cysteine.

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