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Determination of amino acids in the foods by reversed-phase high-performance liquid chromatography with a new precolumn derivative, butylthiocarbamyl amino acid, compared to the conventional phenylthiocarbamyl derivatives and ion-exchange chromatography

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Abstract

A new derivatizing reagent, butylisothiocyanate (BITC), reacts quantitatively with the 22 standard amino acids and the amino acids in the acid hydrolysate of food and the protein standard, bovine serum albumin (BSA), at 40°C for 30 min to yield butylthiocarbamyl (BTC) amino acids. The sensitivity of BTC-amino acid was similar to phenylthiocarbamyl (PTC) amino acid. The detection limit in both derivatives was about 3.9 pmol at 0.05 AUFS that showed a stable baseline for the quantitative determination. Analysis of the results obtained with BSA and food samples as BTC derivatives showed good agreement with those determined as PTC derivatives, ion-exchange chromatography and data presented in the literatures, except for a few amino acids. Especially the values compared to those of ion-exchange chromatography were very close, except for histidine. The advantage of the BITC reagent over the phenylisothiocyanate was that it had high volatility, so the excess reagent and by-products were removed in about 10 min, compared to about 1 h in the PITC reagent, with a common aspirator, In the BTC derivatives, cystine and cysteine were determined separately but in the PTC derivatives they were resolved into a single peak.

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

1. Introduction

Phenylthiocarbamyl (PTC) derivatives have been widely used with a precolumn derivatization method for amino acid analysis by RP-HPLC and UV detection [1–8]. Analysis by RP-HPLC and UV detection following precolumn derivatization is popular because of the great versatility of the instrument. PTC derivatization is an excellent method for this purpose and especially for the derivatization of secondary amino acids like proline and hydroxyproline, but this method requires a high vacuum system and a long time for removing the excess reagent and by-products produced during derivatization.

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In a previous report [9] we found a new pre-column derivatizing reagent, butylisothiocyanate (BITC), an aliphatic compound, on the protein amino acids for RP-HPLC and UV detection. All of the 22 protein amino acids were successfully derivatized to butylthiocarbamyl (BTC) derivatives and resolved on the C_{18} reversed-phase column. The advantages of this reagent were high volatility and the ability of the separate derivatization on the cysteine and cystine, which had not been found with phenylisothiocyanate (PITC) for PTC derivatives. The high volatility of this reagent substantially reduced the analysis time because the excess reagent and by-products produced during the derivatizing reaction could be easily removed. Moreover, BTC derivatives of the secondary amino acids like proline and hydroxyproline were also detected with high peak responses. But this reagent was only used on the standard amino acids, so there is a need for analysis of food samples and protein standard.

In this study we attempted to analyze the amino acids by BTC derivatives on food samples, whole egg and soybean, and on the protein standard, bovine serum albumin (BSA), and compared the results to those obtained with PTC derivatives and ion-exchange chromatography.

2. Experimental

2.1. Materials

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

2.2. Standard amino acid solution

A mixture solution of standard amino acids, except glutamine, cysteine and cystine, was prepared at a concentration of 2.5 μmol/ml 0.01 *M* HCl. Standard solutions of glutamine and cysteine were prepared with water because of the conversion to pyroglutamic acid and cystine, respectively, on prolonged storage of these amino acids in HCl solution [10]. Cystine was prepared at a concentration of 0.5 μmol/ml 0.01 *M* HCl because of the solubility.

2.3. Derivatization

Derivatization to the BTC derivatives of standard amino acids and hydrolyzed amino acids of a sample was achieved by employing the method described in a previous report [9]. Aliquots (25 μl) of standard amino acid solution and hydrolyzed sample solution were placed into 2-ml conical vials and the solvent was completely removed by a stream of dry nitrogen gas at 50°C. An appropriate amount of acetonitrile was added and the sample was dried again. The residue was dissolved in about 50 μl of coupling buffer [acetonitrile–methanol–triethylamine (10:5:2)] and 3 μl BITC was added. After tightly capping the vial was treated with ultrasound for 1 min and stood at 40°C for 30 min for derivatization. After derivatization the contents were completely dried with nitrogen gas at room temperature (ca. 10 min), and then 100 μl of acetonitrile was added and the sample was dried again (ca. 5 min). The residue was dissolved in 1 ml 0.02 *M* ammonium acetate solution and filtered through a 0.25 μm membrane filter. A 10-μl volume of filtrate was injected onto the HPLC system.

For the PTC-amino acids, the derivatization was carried out in a 2-ml conical vial with an open-hole stopper with a septum. The reaction condition was 30 min at room temperature. The procedure for the derivatization of the PTC derivatives was the same as for BTC derivatives except the drying process after derivatization. After derivatization, two stainless-steel injection needles were pierced through the septum into the vial. One needle was connected to nitrogen supply and the other to a vacuum pump. Nitrogen was infused into the vial and simultaneously evacuated with a vacuum pump to complete dryness (ca. 40 min), and then 100 μl of acetonitrile was injected into the vial with a microinjection syringe and the contents were dried again (ca. 20 min).

2.4. Sample preparation

The food samples used for analysis were soybean and whole egg purchased from a commercial market and the protein standard was BSA.

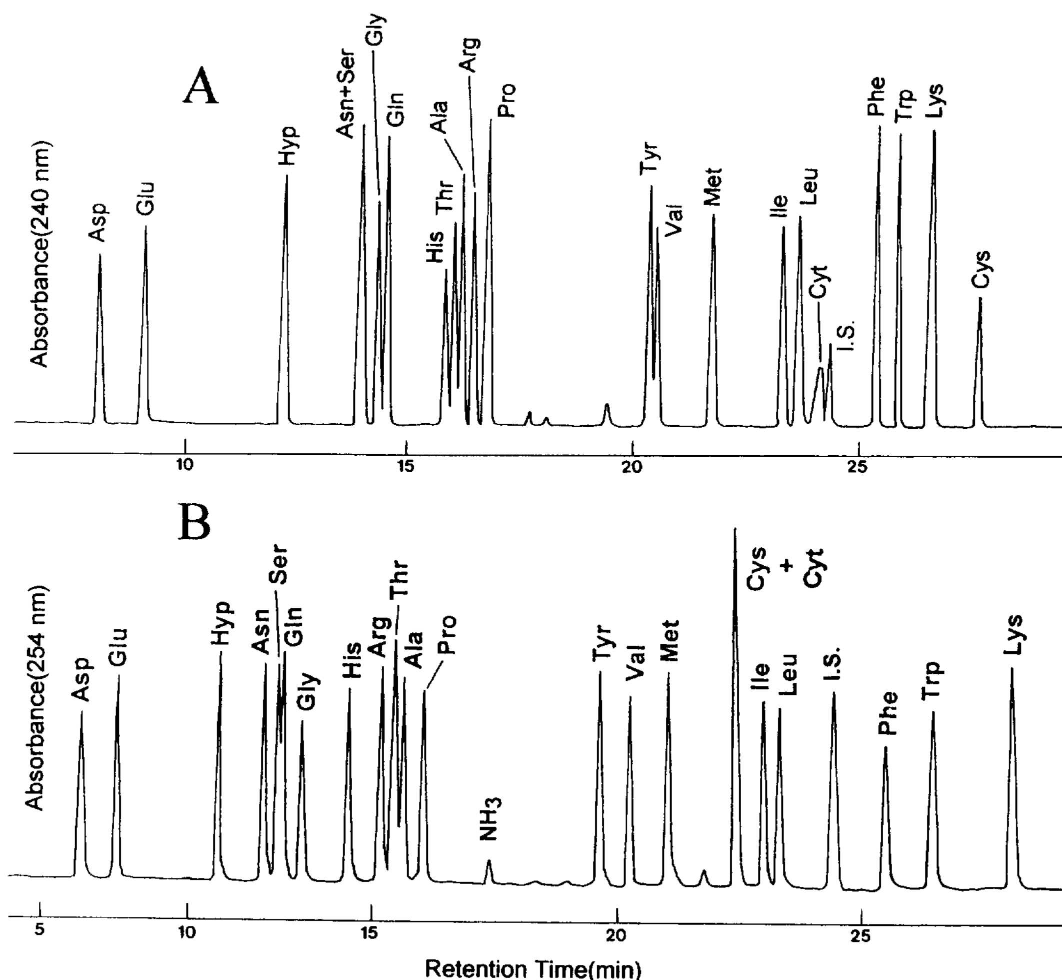

Fig. 1. Chromatogram of standard protein amino acid derivatives resolved on a Nova-Pak (30 cm×3.9 mm) C_{18} column. I.S.=norleucine, injected amount 0.625 nmol. (A) BTC-amino acid; (B) PTC-amino acid. Cyt=cystine; Cys=cysteine.

The procedures of sample hydrolysis and clean-up with a cation-exchange column were carried out with the method described in a previous report [10].

For the analysis with ion-exchange chromatography and ninhydrin derivatization method the clean-up sample solutions were dried in rotary evaporator and dissolved in 0.2 *M* sodium citrate buffer (pH 2.2), and injected into the automatic amino acid analyzer (LKB 4150 Alpha, Ultrapac-11 cation-exchange column, particle size 11±0.5 μm).

2.5. Chromatography

The HPLC used for analysis was a Spectra-Physics 8800 ternary solvent-delivery system and a Spectra 200 programmable-wavelength UV detector. The column temperature was maintained at 40°C with an Eppendorf CH-30 column heater. The column was Nova-Pak C_{18} (300×3.9 mm I.D.; 4 μm dimethyloctadecylsilyl-bonded amorphous silica, Waters). A solvent stabilization and degassing system with a blanket of helium (Spectra-Physics) was employed on the solvents for separation. The solvent system for the BTC derivatives consisted of three eluents; (A) 0.05 *M* ammonium acetate (pH 6.7, adjusted with phosphoric acid); (B) 0.02 *M* sodium phosphate dibasic solution containing 5% methanol and 1.5% tetrahydrofuran–acetonitrile (50:50); (C) acetonitrile–water (70:30). The flow-rate was 1.0 ml/min. The solvent gradient was as follows; 0 min,

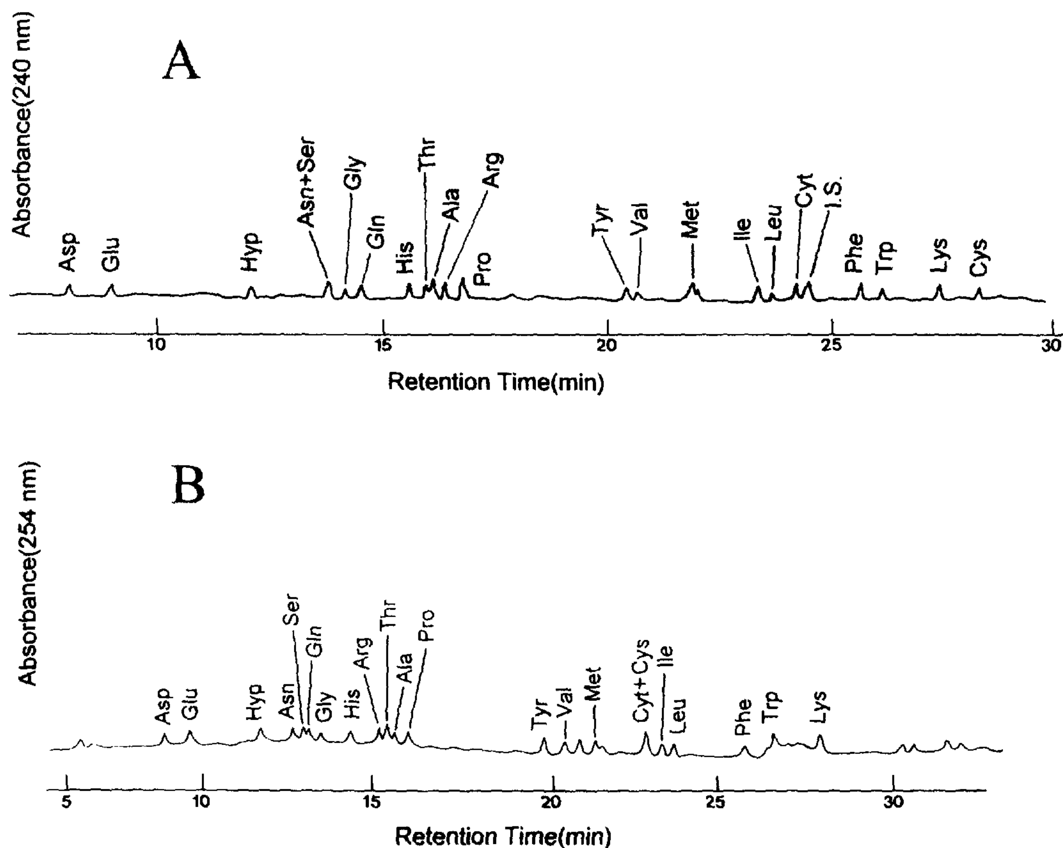

Fig. 2. Chromatogram of standard protein amino acid derivatives showing the sensitivity. Injected amount 3.9 pmol. Range 0.05 AUFS. (A) BTC-amino acid; (B) PTC-amino acid. Cyt=cystine; Cys=cysteine.

100% A; 8.0 min, 85% A–15% B; 14.0 min, 70% A–20% B–10% C: 20.0 min, 60% A–20% B–20% C; 25.0 min, 30% A–20% B–50% C; 30.0 min, 10% A–20% B–70% C. The solvent system for the PTC derivatives also consisted of three eluents; (A) 0.02 *M* sodium phosphate dibasic solution containing 5% methanol and 1.5% tetrahydrofuran (pH 6.8, adjusted with phosphoric acid); (B) solvent A–acetonitrile (50:50); (C) acetonitrile–water (70:30). The flow-rate was 1.2 ml/min. The solvent gradient was as follow; 0 min, 100% A; 10.0 min, 80% A–20% B; 15.0 min, 76% A–20% B–4% C; 20.0 min, 70% A–20% B–10% C; 30.0 min 50% A–30% B–20% C; 40.0 min, 30% A–35% B–35% C. After gradient program a washing step about 20 min with solvent C substantially extended the column life. The wave-lengths for detection were at 240 nm for the BTC derivatives and at 254 nm for the PTC derivatives.

3. Results and discussion

3.1. Chromatography of the standard amino acids

The chromatographic elution of BTC and PTC derivatives of standard amino acids separated on the Nova-Pak C_{18} column is shown in Fig. 1. As in the previous report on the BTC derivatives [9], asparagine and serine were not separated. BTC-cystine and cysteine were individually eluted but the cystine peak was resolved with a tailing following Leu, different from the previous report [9] in which was eluted just at behind proline peak. In case that large amount of cysteine was derivatized to the BTC derivatives, BTC-cysteine was resolved into two peaks with the second peak eluting at the position of cystine with a small tailing peak, which could be assumed that cysteine would be partially converted

into cystine during derivatization, due to the fact that cysteine can be oxidized [11]. The BTC derivatives of the secondary amino acids, proline and hydroxyproline, were detected with high peak responses, as were the corresponding PTC derivatives.

In the PTC derivatives, serine and glutamine were not completely separated. Cystine and cysteine were resolved at the same position, which could also lead one to assume that cysteine might be completely converted into cystine during derivatization. Some articles also reported that PTC-cysteine and cystine were eluted at the same position [2,4,7].

3.2. Sensitivity of BTC- and PTC-amino acids

The chromatograms of the BTC- and PTC-amino acids and their detection limits are shown in Fig. 2. The detection limits in the both derivatives were estimated to be about 3.9 pmol in the detector at 0.05 AUFS, which was the limit of the stable baseline for

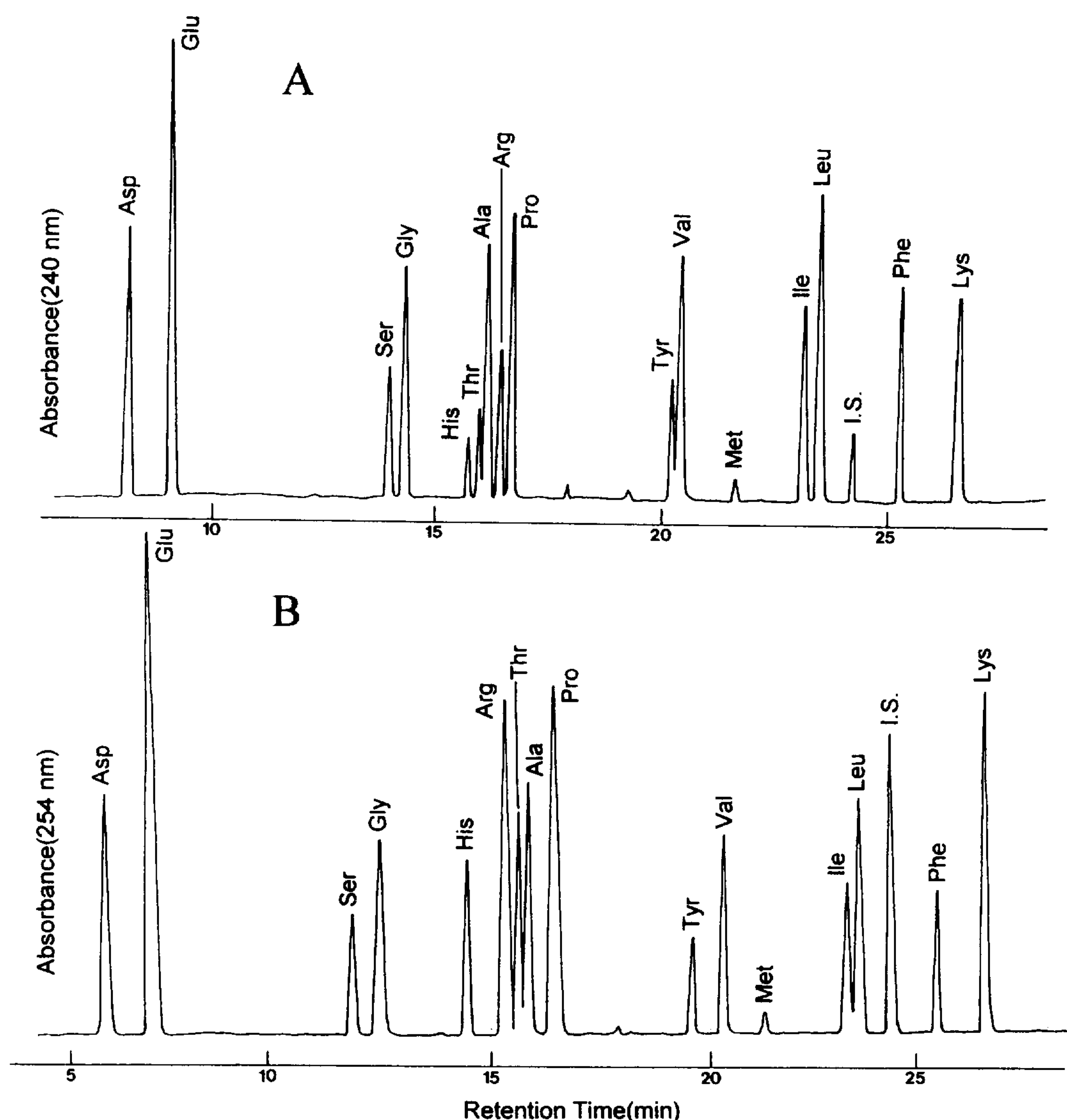

Fig. 3. Chromatogram of amino acids in soybean hydrolysate. (A) BTC derivatives; (B) PTC derivatives.

the practical quantitative analysis in this study. At lower levels than 3.9 pmol and at 0.05 AUFS, contamination of amino acids and other compounds from the solvents, instruments and reagents was serious.

3.3. Amino acid compositions of soybean and whole egg

Chromatograms of the amino acid composition of soybean and whole egg resolved with BTC and PTC derivatives are shown in Fig. 3 and Fig. 4. In both derivatives, we could detect the same amino acids, 16 in soybean and 17 in whole egg. As is shown in the chromatograms of food samples, assuming that some kinds of contaminants were contained, there were few ghost peaks. This phenomenon indicates that BITC and PITC are very good reagents having superior selective reactivity with amino acids.

The amino acid composition of both samples as determined with BTC derivatives compared to PTC derivatives and ion-exchange chromatography is shown in Table 1 and Table 2. In the soybean sample, a comparison between the contents of the BTC derivatives and ion-exchange chromatography showed a deviation of less than 5%, except for

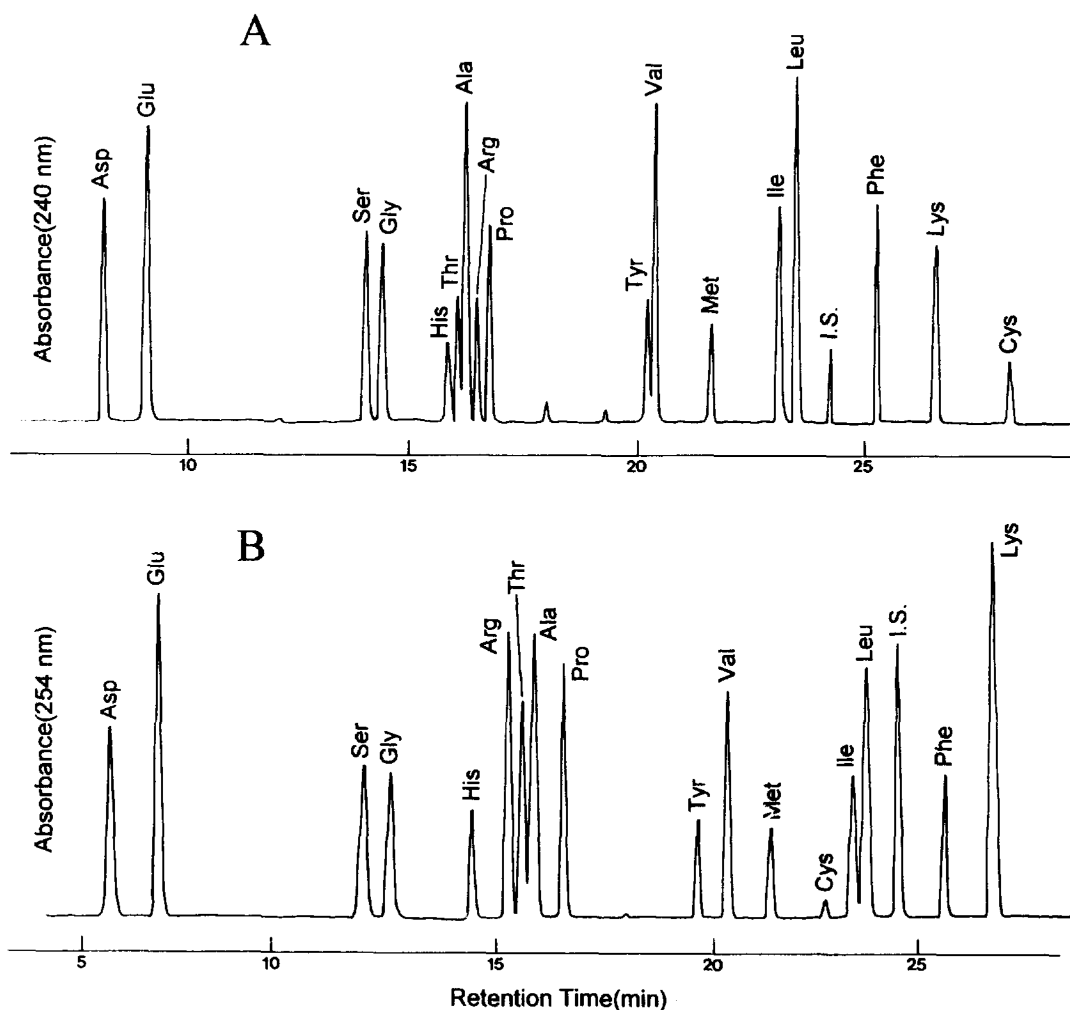

Fig. 4. Chromatogram of amino acids in whole egg hydrolysate. (A) BTC derivatives; (B) PTC derivatives. Cys=cysteine.

Table 1
Amino acid composition of soybean determined by the BTC derivatives method compared to the use of PTC derivatives and ion-exchange chromatography

Amino acid	BTC derivatives	PTC derivatives	Ion-exchange chromatography (g/100 g dry matter)
Asp	4.17±0.24	4.64±0.28	4.25±0.32
Glu	6.96±0.42	7.25±0.34	7.19±0.12
Ser	1.61±0.12	1.59±0.02	1.65±0.01
Gly	1.59±0.07	1.77±0.10	1.62±0.05
His	1.50±0.06	1.52±0.04	1.32±0.05
Arg	3.38±0.17	2.28±0.08	3.50±0.09
Thr	1.19±0.04	1.79±0.17	1.24±0.10
Ala	1.72±0.10	1.89±0.35	1.80±0.12
Pro	2.15±0.12	2.06±0.08	2.20±0.06
Tyr	1.20±0.04	1.29±0.08	1.15±0.05
Val	2.88±0.24	2.47±0.10	3.01±0.14
Met	0.42±0.08	0.54±0.06	0.40±0.03
Ile	2.46±0.14	2.21±0.06	2.25±0.18
Leu	3.38±0.18	3.03±0.31	3.57±0.21
Phe	2.27±0.16	2.10±0.10	2.19±0.14
Lys	3.03±0.13	3.15±0.16	2.86±0.19

All values are mean±S.D., n=4.

histidine (13%) and isoleucine (9%). But in the PTC derivatives, most of amino acids showed a deviation of more than 5% (Table 1). In the whole egg sample, the values for histidine and tyrosine in BTC derivatives showed deviations of more than 5% compared to those obtained by ion-exchange chromatography (Table 2). A common feature in the both derivatives was the fact that histidine was determined with

Table 2
Amino acid composition of whole egg determined by the BTC derivatives method compared to the use of PTC derivatives and ion-exchange chromatography

Amino acid	BTC derivatives	PTC derivatives	Ion-exchange chromatography (g/100 g dry matter)
Asp	4.20±0.09	4.74±0.07	4.23±0.05
Glu	5.48±0.05	5.85±0.59	5.76±0.07
Ser	3.50±0.09	2.61±0.42	3.54±0.10
Gly	1.56±0.09	1.54±0.31	1.53±0.02
His	1.90±0.14	1.91±0.15	1.79±0.06
Thr	1.75±0.13	2.15±0.11	1.82±0.15
Ala	2.71±0.16	3.29±0.19	2.70±0.11
Arg	3.55±0.10	3.43±0.48	3.61±0.16
Pro	1.95±0.15	1.87±0.24	1.98±0.22
Tyr	2.36±0.62	1.96±0.20	2.23±0.30
Val	3.64±0.20	3.83±0.36	3.59±0.32
Met	1.68±0.11	1.58±0.18	1.63±0.06
Ile	3.42±0.16	2.92±0.28	3.26±0.21
Leu	4.63±0.21	4.32±0.42	4.35±0.09
Phe	2.94±0.14	2.65±0.22	2.88±0.15
Lys	4.16±0.20	4.30±0.33	4.18±0.30
Cys	1.35±0.16	0.40±0.10	1.41±0.23

All values are mean±S.D., n=4. Cys=cysteine.

relatively high value in both samples compared to the values obtained with ion-exchange chromatography.

3.4. Amino acid composition of bovine serum albumin

Chromatograms of the amino acid composition of BSA obtained with BTC- and PTC-derivatives is shown in Fig. 5. There are also few ghost peaks, of the same kind as in the food samples, which illustrates that the ghost peaks were not from sample contaminants but from by-products produced during the acid hydrolysis or derivatization.

The amino acid composition of BSA was recalculated on the basis of a total of 581 residues, excluding two Trp residues that were completely destroyed during acid hydrolysis, is shown in Table 3. In the BTC derivatives, the cysteine content was low, with a deviation of more than 23%, and the histidine content was high, with a deviation of more than 30%, compared to any value reported in the literature. In comparison to ion-exchange chromatography, the amino acids with a deviation of more than 5% were histidine (27.2%), threonine (5.3%), pro-

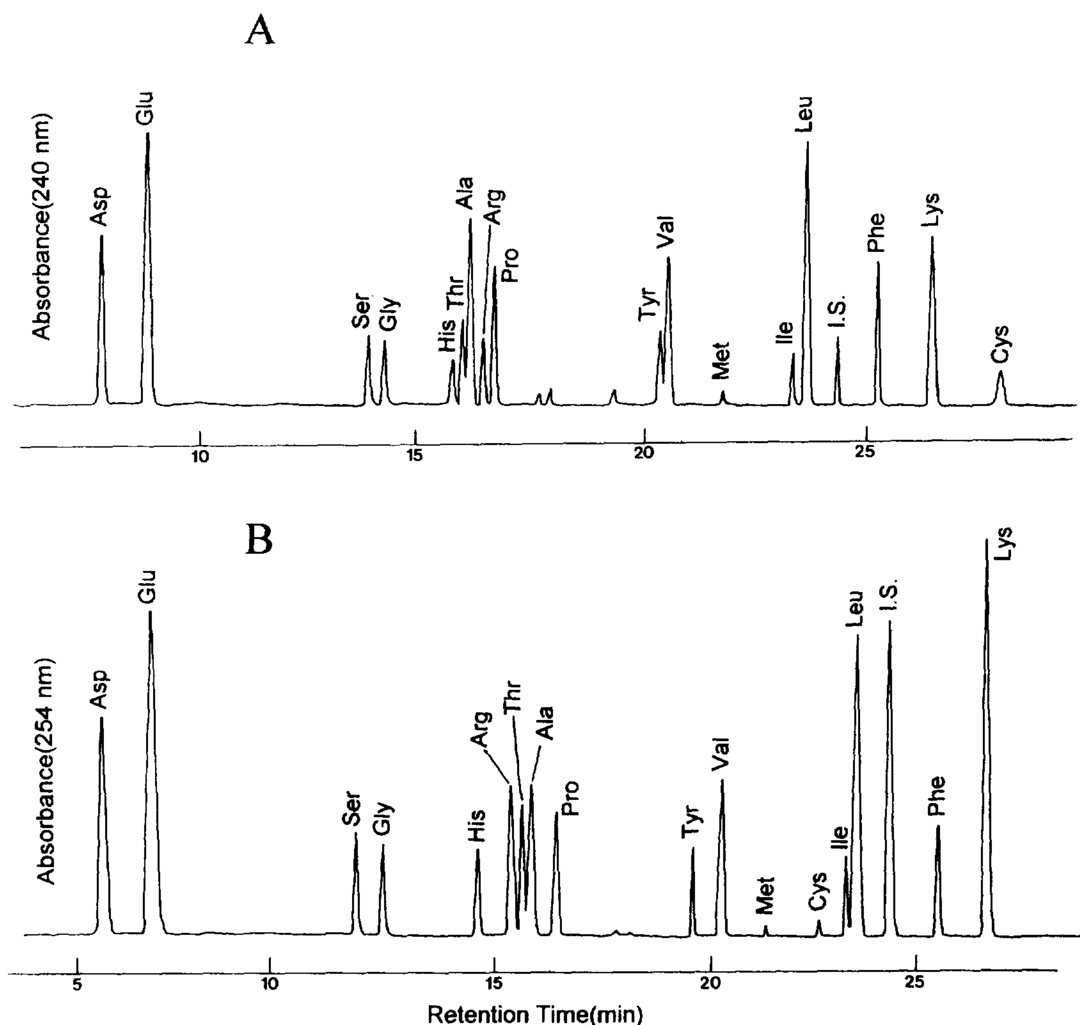

Fig. 5. Chromatogram of amino acids in bovine serum albumin hydrolysate. (A) BTC derivatives; (B) PTC derivatives. Cys=cysteine.

Table 3
Amino acid composition of BSA determined by BTC derivatives method compared to the use of PTC derivatives, ion-exchange chromatography and the analytical results reported in the literature

Amino acid	BTC derivatives[a]	PTC derivatives[a]	Ion-exchange chromatography[a]	Starch chromatography[c]	Automatic analyzer[d]	Sequence[e]	Sequence[f]
Asp	54.85±3.01[b]	53.78±4.75[b]	54.49±3.51[b]	55.4	55.0	53	41
Glu	73.52±5.04	72.61±0.94	75.99±4.14	75.8	82.0	78	59
Ser	24.60±0.45	26.22±4.38	25.84±0.15	27.3	26.9	28	28
Gly	16.68±0.28	14.96±0.50	16.31±0.37	16.4	15.3	15	16
His	22.39±0.49	20.85±2.40	17.60±0.87	17.4	17.0	17	18
Arg	21.94±0.22	31.06±1.51	22.98±0.17	22.9	23.6	23	25
Thr	30.35±0.82	31.16±0.76	32.05±0.94	33.1	33.0	34	34
Ala	46.07±0.94	38.17±0.27	46.06±1.33	47.4	45.2	46	46
Pro	26.85±1.13	29.27±0.78	28.60±2.11	27.9	28.5	28	29
Tyr	20.47±0.55	20.72±0.60	20.22±1.22	18.9	19.9	19	20
Val	38.61±0.85	36.87±0.84	37.28±0.96	34.2	35.4	36	36
Met	4.61±0.28	6.97±0.48	4.43±0.11	3.6	3.8	4	4
Ile	15.25±0.29	18.67±0.55	15.65±0.32	13.5	14.1	14	14
Leu	61.77±0.26	60.88±0.65	61.13±0.70	63.3	59.2	61	60
Phe	29.20±1.07	29.26±0.65	27.27±0.80	27.0	26.1	26	26
Lys	58.75±1.97	54.58±4.65	59.42±3.65	59.3	59.1	59	58
Trp	–	–	–	1.9	2.1	2	2
Cys	26.82±3.60	9.32±0.59	28.66±5.05	36.7	36.0	35	35
Gln	–	–	–	–	–	–	20
Asn		–	–	–	–	–	12
Total	572.73	555.35	573.98	582.0	582.2	578	583

Cys=cysteine.
[a] $n=3$.
[b] The values were recalculated on the basis of a total of 583 residues considering the fact that two Trp residues were completely destroyed and cysteine residues were substantially destroyed during acid hydrolysis.
[c] Data from Ref. [12].
[d] Data from Ref. [13].
[e] Data from Ref. [14].
[f] Data from Ref. [16].

line (6.1%) and phenylalanine (7.1%). Histidine also had a high deviation as well as the results of the food samples, which could be caused by inappropriate reaction conditions for histidine. In the PTC derivatives, the alanine and cysteine content was low, and the methionine, isoleucine, arginine and phenylalanine content was high, with a deviation of more than 10% compared to ion-exchange chromatography and any value in the literature.

The difference in cysteine content between both derivatives might be due to the fact that cystine and cysteine could be determined separately in the BTC derivatives. The low value of cysteine in the both derivatives as compared to the results in the literature could be due to the fact that a substantial amount of cysteine was destroyed during hydrolysis with 6 *M* HCl without conversion of cysteine to cysteic acid [10,15].

4. Conclusion

BITC was chosen with a new precolumn derivatization reagent for analysis of amino acids in the HCl hydrolysate of food samples and protein standard. The standard amino acids and HCl-hydrolyzed amino acids were successfully derivatized to the BTC derivatives and resolved on the Nova-Pak C_{18} column by RP-HPLC and UV detection. The results compared to PTC derivatives and ion-exchange chromatography on the soybean and whole egg samples were similar, except for a few amino acids.

For BSA, a protein standard, the results in the BTC derivatives were substantially close and accurate compared to the ion-exchange chromatography and the results in the literature, except for histidine and cysteine.

Acknowledgments

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