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Determination of protein amino acids as butylthiocarbamyl derivatives by reversed-phase high-performance liquid chromatography with precolumn derivatization and UV detection

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Abstract

Twenty-two protein amino acids were derivatized to butylthiocarbamyl derivatives by precolumn reaction at 40°C for 30 min with butyl isothiocyanate. All of the derivatives were quantitatively resolved in 27 min by RP-HPLC on a Nova-Pak C_{18} column and detected at 250 nm with a UV detector. Asparagine and serine were resolved in a single peak. The relative standard deviations of the relative molar responses with respect to the methionine peak were less than 5% for all of the derivatives except cysteine. The calibration graphs showed good linearity in the range 0.5–2.5 nmol. Correlation coefficients for all of the calibration graphs were highly significant ($p < 0.001$). The stability of the derivatives at room temperature was about 8 h.

1. Introduction

The determination of amino acids by high-performance liquid chromatography (HPLC) has developed substantially in recent years owing to its sensitivity and speed of analysis compared with specialized amino acid analysers. Especially analysis with a reversed-phase (RP) column and UV detection following precolumn derivatization is popular owing to the greater versatility of the instrument.

The formation of *o*-phthalaldehyde (OPA) [1,2], dansyl [3,4], dabsyl [5,6], phenylthiocarbamyl (PTC) [7–9] and phenylthiohydantoin (PTH) derivatives of amino acids has been widely adapted used in RP-HPLC. However, these derivatives have several disadvantages. With the OPA derivatives, secondary amino acids, proline and hydroxyproline, were not detected because OPA does not react with secondary amines in the absence of oxidizing agents. Moreover, as OPA derivatives are unstable, complete automation of the precolumn reaction with accurate control of the reaction time is essential for acceptable reproducibility [10]. For higher sensitivity, fluorescence instead of UV detection is essential.

Dansyl derivatives are formed under optimum conditions at pH 9–9.5 and room temperature within 30–35 min in the dark. Dansylamino acids are unstable towards prolonged reaction times, solvents and exposure to light and interfering peaks arise due to the by-products during the derivatization process [4].

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It was reported that the sulphonamide bond of dabsyl derivatives was very stable [6]. The limitation of the dabsyl derivative method is that the presence of an excess amount of urea, salt, phosphate or ammonium hydrogencarbonate will change the pH of the reaction buffer and interfere with derivatization [5,6]. The standard of dabsyl amino acids must be prepared by hydrolysis of an amino acid standard mixture and derivatized in parallel with unknown samples under the same conditions [10].

Derivatization of amino acids with phenylisothiocyanate (PITC), which has been used for several decades in the Edman degradation, is an excellent precolumn derivatization method especially for secondary amino acids, proline and hydroxyproline [8,9,11,12]. Excess reagent and by-products produced during derivatization of PTC-amino acids must be completely removed with a high vacuum system in order to avoid interfering peaks.

Identification of PTH-amino acids is almost exclusively used in the elucidation of the structures of protein and this derivatization is unsuitable in a general method for the determination of amino acids.

There is a need for a simple, sensitive and stable precolumn derivatization reagent for RP-HPLC with UV detection. In this work, we chose butyl isothiocyanate for this purpose and applied it to the determination of 22 standard protein amino acids.

2. Experimental

2.1. Materials

Butyl isothiocyanate (BITC) was obtained from Aldrich (Milwaukee, WI, USA), standard amino acids from Sigma (St. Louis, MO, USA) and HPLC-grade acetonitrile from Merck (Darmstadt, Germany). All other reagents and solvents were of analytical-reagent grade.

2.2. Derivatization

A mixture of standard amino acids (60 μl of solution containing 1.25 μmol/ml of 0.01 *M* HCl) was placed in a 1-ml conical vial and the solvent was completely evaporated with dry nitrogen at 50°C. About 50 μl of acetonitrile were added and the mixture was evaporated with dry nitrogen. The residue was dissolved in 50 μl of coupling buffer [acetonitrile–methanol–triethylamine (10:5:2)] with ultrasonic treatment for 1 min and 3 μl of BITC were added. After tightly capping the vial with an open-hole screw-cap with a septum, the contents were derivatized using various temperatures and times as indicated later. 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 vacuum pump until the contents were completely dried (*ca.* 15 min), and then 100 μl of acetonitrile were injected into the vial with a microinjection syringe and the contents were continuously dried with nitrogen. After completely drying, residue was dissolved in 500 μl of 0.2 *M* ammonium acetate solution and filtered through a 0.20-μm membrane filter. A 10-μl volume of the filtrate was injected into the HPLC system.

2.3. Chromatography

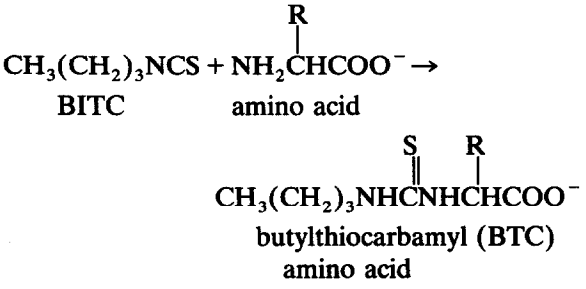
The HPLC system consisted of a Spectra-Physics 8800 ternary solvent-delivery system and a Spectra 200 programmable wavelength UV detector. The column temperature was maintained at 40°C with an Eppendorf CH-30 column heater. The column was Nova-Pak C_{18} (300 × 3.9 mm I.D.; 4-μm dimethyloctadecylsilyl-bonded amorphous silica) (Waters). 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) 0.05 *M* ammonium acetate (pH 6.7, adjusted with phosphoric acid); (B) solvent A–acetonitrile (50:50); and (C) acetonitrile–water (70:30). The flow-rate was 1.0 ml/min. The gradient was as follows: 0 min, 100% A; 8.0 min, 85% A–15% B; 14.0 min, 70% A–20% B–10% C; 20 min, 60% A–20% B–20% C; 25 min, 20% A–80% C; 30 min, 100% C.

After this a washing step was programmed with 100% C for 5 min.

3. Results and discussion

3.1. UV spectrum of BTC amino acid derivative

We assumed that the derivatization reaction between amino acids and BITC proceeds as follows:



The UV spectra of the BTC-amino acid mixture and BITC, the coupling reagent, are shown in Fig. 1. The λ_{max} of BTC-amino acids was about 234 nm, but the most efficient wavelength was 250 nm, which avoided the absorption spectra of the impurities and the electrolyte (ammonium acetate) in the solvent.

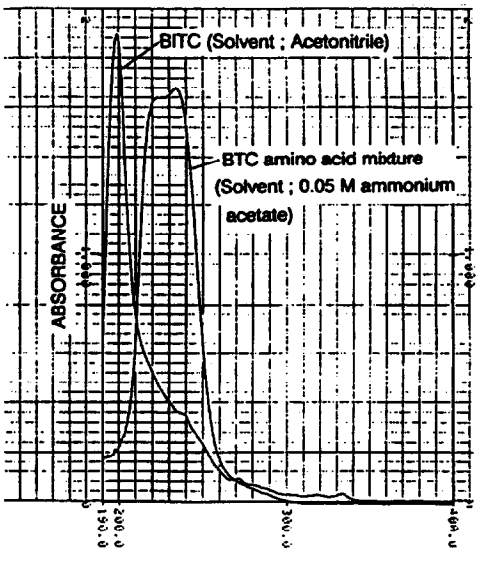


Fig. 1. UV spectra (x-axis in nm) of BTC-amino acid mixture and the coupling reagent BITC. Absorbance of solvents at 250 nm = 0.

3.2. Chromatography

The chromatographic elution of BTC-amino acids derivatized by reaction at 40°C for 30 min is shown in Fig. 2. All 22 protein amino acids were resolved in 27 min. Especially secondary amino acids, hydroxyproline and proline, were completely detected with high peak responses as PTC-amino acids [7–9]. Glutamine and asparagine were also separated from their acids, but asparagine and serine could not be resolved.

Incompletely resolved derivatives were threonine, alanine and arginine. The separation of tyrosine and valine is not as good as for the standard PTC derivatives [8]. All of the BTC derivatives were resolved into single peaks. However, glutamine stored in 0.01 *M* HCl for a long period showed three peaks. This phenomenon seems to be due to the partial conversion of glutamine into ammonia and glutamic acid on prolonged storage of the standard in acidic solution (Fig. 3). It has been reported that glutamine is converted into pyroglutamic acid on prolonged storage at low pH [13–15]. However, no pyroglutamic acid peak appeared on the chromatogram and also we could not derivatize standard L-pyroglutamic acid to the BTC derivative although we consider that this acid is produced in the standard solution on prolonged storage. The ammonia peak in Fig. 3 seemed to be produced by derivatization of non-volatile ammonium chloride formed between the amino group deaminated from glutamine and HCl on prolonged storage, because it was not observed with an aqueous ammonia solution (Fig. 3) but it

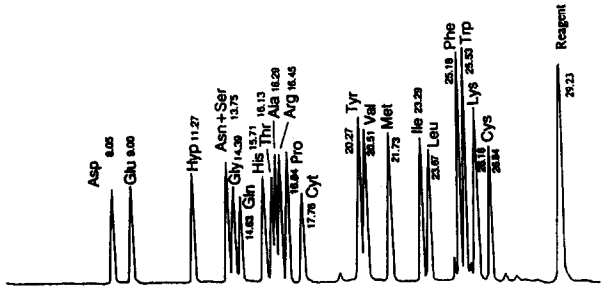


Fig. 2. Chromatogram of BTC-amino acids resolved on a Nova-Pak C_{18} column (300 × 3.9 mm I.D.). Amount injected, 1.5 nmol. Numbers at peaks indicate retention times in min.

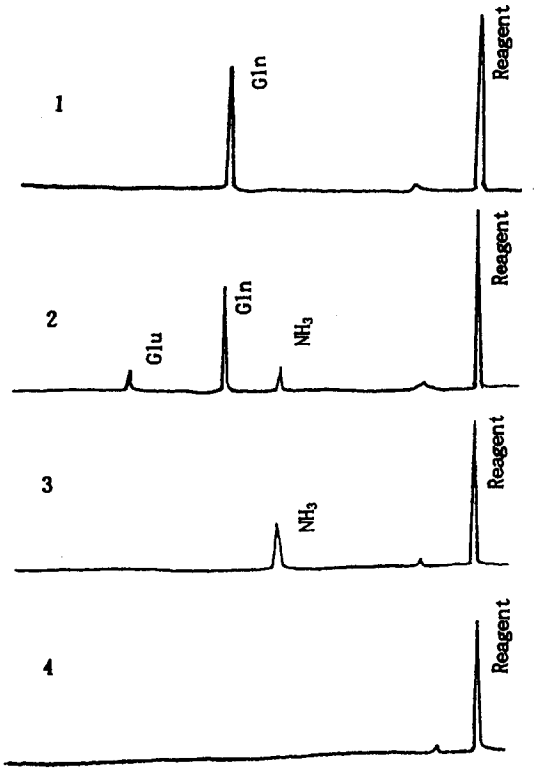


Fig. 3. Chromatogram of BTC-glutamine. (1) Chromatogram of glutamine derivatized immediately after preparation of standard solution with 0.01 *M* HCl; (2) chromatogram of glutamine derivatized after prolonged storage of standard solution; (3) chromatogram of NH_3 with derivatization with NH_3–0.01 *M* HCl solution; (4) chromatogram of NH_3 with derivatization with NH_3 solution.

was observed with an aqueous solution of ammonium chloride.

The order of resolution of BTC-amino acids in this study was similar to that of PTC-amino acids on a Pico-Tag column except for cystine (Cyt), cysteine (Cys) and arginine [11,16].

Although it is a methodological problem, there is a report that when the guard column is regularly changed after 120 analyses to avoid the damage of the column that can arise as a result of incomplete removal of the excess reagent, the separation remains satisfactory for at least 700 analyses with OPA, 800 analyses with fluorenylmethyl chloroformate, 150 analyses with PITC and 500 analyses with dansyl chloride [17]. With the BTC derivatives in this study, the resolution and peak response remained satisfactory for more than 700 samples without the need for a guard column.

The reagent peak appeared at 29 min after all of the amino acid derivatives had been resolved, so there is no risk of interfering peaks due to incomplete removing of excess reagent. The boiling point of BITC (70–71°C/35 mmHg; 1 mmHg = 133.322 Pa) is much lower than that of PITC (117.1°C/33 mmHg), so the excess BITC reagent will be more easily removed than PITC.

3.3. Relative molar response (RMR), peak response and calibration graphs

The RMRs with respect to the methionine derivative for reaction times of 10, 20, 30, 60, 120 and 180 min at 40°C and for reaction temperatures of 25, 40, 50, 60 and 70°C for 30 min were determined. The highest precision was obtained with reaction at 40°C for 30 min, although the RMRs and the peak area responses were higher for the reaction at 40°C for 120 min (Table 1, Fig. 5).

The amount of each amino acid injected was 1.5 nmol. The derivative that showed the highest relative standard deviation (R.S.D.) on reaction for 30 min at 40°C was cysteine (5.24%); all other R.S.D.s were less than 5%.

The peak-area responses on using various reaction temperatures for 30 min and various reaction times at 40°C are shown in Figs. 4 and 5. The peak-area responses on various reaction temperatures were the highest at 40°C for 30 min for almost all derivatives. The highest peak-area responses appeared at a reaction of 120 min at 40°C, but the R.S.D.s were higher than those for reaction for 30 min, although they were not so high to prevent these conditions being used for quantitative purposes (Table 1). With the PTC derivatives the derivatization reaction was completed in 10–20 min at room temperature [12], but with the BTC derivatives 120 min were required at 40°C. The peak response of cystine derivatives on reaction for 120 min compared with that for reaction for 30 min showed the greatest difference of 4.2-fold and the second was arginine with 1.6-fold. The differences for

Table 1
Relative molar responses of BTC-amino acids with reaction at 40°C for 30 and 120 min

Amino acid[a]	30 min			120 min		
	RMR[b]	S.D.[c]	R.S.D. (%)[c]	RMR[b]	S.D.[c]	R.S.D. (%)[c]
Asp	0.50	0.007	1.40	0.54	0.014	2.59
Glu	0.48	0.006	1.25	0.57	0.011	1.93
Hyp	0.67	0.011	1.64	0.63	0.027	4.29
Asn + Ser	0.66	0.003	0.45	0.66	0.026	3.94
Gly	0.52	0.005	0.96	0.58	0.014	2.41
Gln	0.71	0.009	1.27	0.75	0.025	3.33
His	0.62	0.007	1.13	0.65	0.011	1.69
Thr	0.29	0.005	1.72	0.33	0.013	3.94
Ala	0.75	0.019	2.53	0.74	0.028	3.78
Arg	0.44	0.003	0.68	0.69	0.012	1.74
Pro	0.71	0.011	1.55	0.67	0.017	2.54
Cyt	0.49	0.021	4.29	1.69	0.068	4.02
Tyr	0.83	0.011	1.33	0.92	0.010	1.09
Val	0.88	0.034	3.86	1.03	0.035	3.40
Ile	0.84	0.007	0.83	0.80	0.039	4.88
Leu	0.86	0.019	2.21	0.73	0.044	6.03
Phe	1.01	0.026	2.57	0.91	0.039	4.29
Trp	0.93	0.036	3.87	0.99	0.043	4.34
Lys	1.10	0.036	3.27	1.25	0.032	2.56
Cys	0.63	0.033	5.24	0.47	0.030	6.38

[a] Cyt = Cystine; Cys = cysteine.
[b] Values relative to methionine = 1.
[c] $n = 3$.

the other amino acid derivatives were *ca.* 1.2-fold. If we consider only the precision and economy of analytical time rather than higher sensitivity, however, the optimum conditions for determination are 30 min at 40°C.

Calibration graphs for all of the amino acid derivatives with reaction 40°C for 30 min showed good linearity in the range 0.5–2.5 nmol. The correlation coefficients (r) of the calibration graphs for all the derivatives were highly signifi-

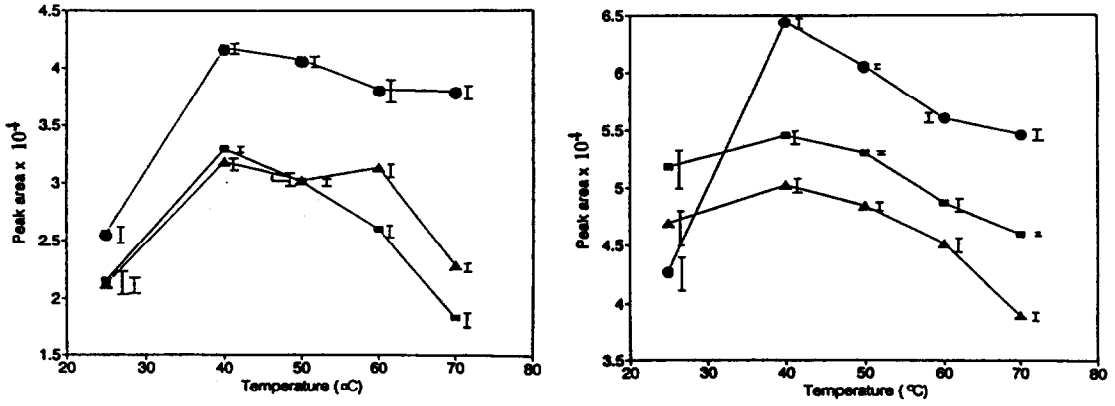


Fig. 4. Effect of reaction temperature on the formation of BTC-amino acids. Reaction time, 30 min. Left: ■ = Asp; ● = Hyp; ▲ = Glu. Right: ■ = Ile; ● = Phe; ▲ = Leu.

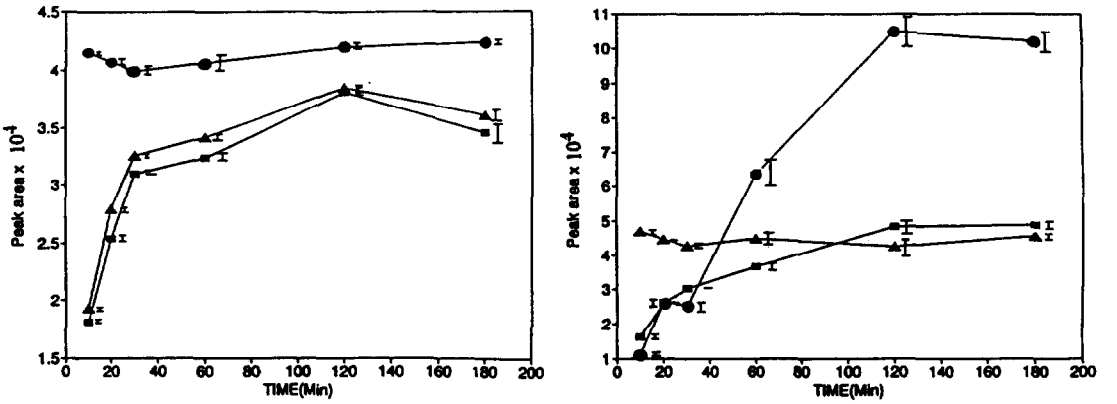


Fig. 5. Effect of reaction time on the formation of BTC-amino acids. Reaction temperature, 40°C. Left: ■ = Asp; ● = Hyp; ▲ = Glu. Right: ■ = Arg; ● = Cyt; ▲ = Pro.

cant ($p < 0.001$) and the lowest value was $r = 0.926$ for the cystine derivative. It has been reported that the linearity of the calibration graph for PTC-cystine was the poorest compared with those of other derivatives, but the correlation coefficient exceeded 0.999 [8]. However, there is a report that the linearity for PTC-cystine was so poor that it could not be used for quantitative purposes [17]. In this study, BTC-cystine showed good linearity for quantitative analysis. We also carried out PTC-cystine derivatization with the method used in this study. The linearity for PTC-cystine ($r = 0.997$) was as good as that for BTC-cystine.

3.4. Stability of BTC derivatives

The variation in the peak-area responses of BTC derivatives (with reaction at 40°C for 30 min) with storage time at room temperature is shown in Fig. 6. Up to 2 h of storage the peak-area responses increased for most of the derivatives, which indicated that the derivatization reaction was continuing at room temperature. After 8 h of storage the derivative that showed the greatest decrease was cysteine (17.3%); the loss of the other derivatives after 8 h was in the range 0–6.9%. The derivatives that decreased to less than 5% after 14 h were glutamic acid, asparagine + serine, glutamine, threonine, tyrosine, proline, lysine and tryptophan. With the PTC derivatives, the optimum pH of the solvent used to dissolve the derivatives was 7.5 and in solution, at this optimum pH, the loss of derivatives was in the range 0–10% after 10 h at room temperature [12]. We assumed that the stability of BTC-amino acids was similar to that of the PTC derivatives.

4. Conclusions

The derivatizing agent BITC reacts quantitatively with all of the protein amino acids at 40°C for 30 min to yield BTC-amino acids. All 22

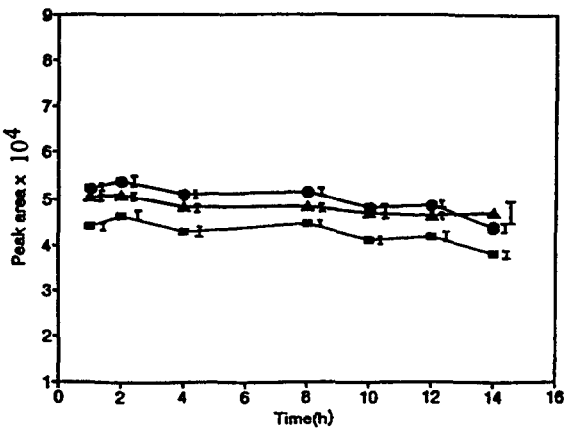


Fig. 6. Stability of BTC-amino acids at room temperature. ■ = Glu; ● = Ile; ▲ = Leu.

BTC-amino acids were separated on the Nova-Pak C_{18} column (300 × 3.9 mm I.D.; 4 μm), except asparagine and serine, which were resolved as a single peak. The calibration graphs showed good linearity and the R.S.D.s of the relative molar response were less than 5% except for cysteine (5.24%). The stability at room temperature was estimated to be *ca.* 8 h. This method could be useful for the determination of protein amino acids.

5. References

[1] M. Roth, *Anal. Chem.*, 43 (1971) 880.
[2] K. Yaegaki, J. Tonzetich and A.S.K. Ng, *J. Chromatogr.*, 356 (1986) 163.
[3] Y. Tapuhi, D.E. Schmidt, W. Lindner and B.L. Karger, *Anal. Biochem.*, 115 (1981) 123.
[4] C. De Jong, G.J. Hughes, E. Van Wieringen and K.J. Wilson, *J. Chromatogr.*, 241 (1982) 345.
[5] J.K. Lin and J.Y. Chang, *Anal. Chem.*, 47 (1975) 1634.
[6] R. Knecht and J.Y. Chang, *Anal. Chem.*, 58 (1986) 2375.
[7] D.R. Koop, E.T. Morgan, G.E. Tarr and M.J. Coon, *J. Biol. Chem.*, 257 (1982) 8472.
[8] B.A. Bidlingmeyer, S.A. Cohen and T.L. Tarvin, *J. Chromatogr.*, 336 (1984) 93.
[9] R.L. Heinrikson and S.C. Meredith, *Anal. Biochem.*, 136 (1984) 65.
[10] J.A. White and R.J. Hart, in L.M.L. Nollet (Editor), *Food Analysis by HPLC*, Marcel Dekker, New York, 1992, p. 53.
[11] J.A. White, R.J. Hart and J.C. Fry, *J. Autom. Chem.*, 8 (1986) 170.
[12] S.A. Cohen and D.J. Sytrydom, *Anal. Biochem.*, 174 (1988) 1.
[13] H. Wilson and R.K. Cannan, *J. Biol. Chem.*, 119 (1937) 309.
[14] J.P. Greenstein and M. Winitz, *Chemistry of Amino acids*, Vol. 3, Wiley, New York, 1961, p. 1934.
[15] G. Fortier, D. Tenaschuk and S.L. MacKenzie, *J. Chromatogr.*, 361 (1986) 253.
[16] R.F. Ebert, *Anal. Biochem.*, 154 (1986) 431.
[17] P. Fürst, L. Pollack, T.A. Graser, H. Godel and P. Stehle, *J. Chromatogr.*, 499 (1990) 557.