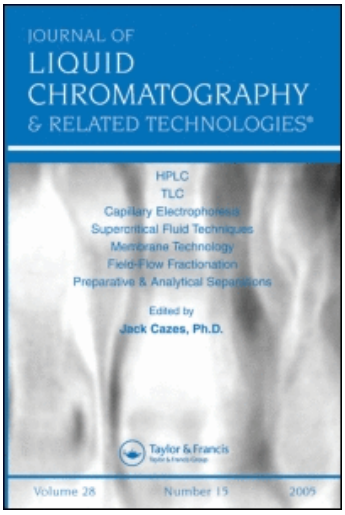

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Detection and Separation of Amino Acids as Butylthiocarbamyl Derivatives by Thin-Layer Chromatography with the Iodine-Azide Detection System

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Abstract: Protein amino acids were derivatized to butylthiocarbamyl derivatives by prechromatographic reaction at 40°C for 30 min with butyl isothiocyanate. The iodine-azide reaction was employed as the detection system for sulphur-containing derivatives. In practice, the plates were sprayed with a mixture of sodium azide and starch solution and then exposed to iodine vapour. The spots became visible as white spots on violet-grey background. The iodine-azide system enabled detection of quantities in the range of 2–90 pmol per spot. R_F values of BTC-derivatives in several solvent systems were established in order to carry out separation. The obtained data were applied to resolution of three 7-component mixtures of BTC-derivatives. Additionally, BTC-leucine and isoleucine were successfully distinguished.

Keywords: Amino acids, Butyl isothiocyanate, Iodine-azide reaction, Thin-layer chromatography (TLC)

INTRODUCTION

Considering the biological, medical, and pharmaceutical role of amino acids, their analysis has been a subject of importance for analytical scientists. One of the analytical methods that has its established position in amino acids studies is thin-layer chromatography.[1] TLC, a versatile, inexpensive, easy-to-carry-out technique, has been used for separation and determination of amino acids

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with a wide range of combinations of stationary and mobile phases. This method offers profound benefits in comparison with other chromatographic techniques: only a small amount of compounds needed for measurement, low purity requirements, and unsophisticated instrumentation. The main advantage of TLC, however, arises from the simplicity by which the chromatographic parameters can be obtained and the convenience with further data processing.

Owing to the necessity for derivatization of amino acids in order to improve the sensitivity, there are many different derivatization procedures available. Among the most frequently used is a group of isothiocyanates. Phenyl isothiocyanate seems to play a key role.[2] This reagent is a base for Edman degradation coupled with identification of phenyl thiohydantoin derivatives of amino acids (PTH) at each step.[3] However, identification of PTH derivatives is almost exclusively applied to studies on protein structures and is not suitable as an overall method for determination of amino acids. For this purpose, phenyl thiocarbamyl derivatives (PTC) are considered as the more efficient ones. Furthermore, PTC derivatives can be formed from all essential amino acids, both primary and secondary ones and offer pmol sensitivity range, stable final product, short analysis time, as well as no temperature requirements.

PITC as a derivatization agent has been the subject of our previous reports.[4–7] The present work, however, is focused on another isothiocynate, butyl isothiocynate (BITC), and the purpose of this study is to assess the possibility of BITC application towards the amino acids analysis. There were reports of BITC application in HPLC,[8,9] yet our aim is to use it as a labeling reagent for amino acids with regard to TLC and detection based on the iodine-azide reaction.

Employing the iodine-azide reaction for detection involves the presence of sulphur (II) compounds, which are inductors of this reaction. The reaction is depicted in the Scheme 1.

The derivatization process with BITC results in transforming amino acids into inductors of the iodine-azide reaction. Thus, it is an introduction of sulphur atoms into the given molecule. The detection is based on visual observation of the plate after spraying it with a freshly prepared solution of sodium azide and starch and exposure to iodine vapour. Due to induction properties of bivalent sulphur compounds, spots become visible as white spots on a violet-grey background.

The present work outlines the results of amino acids detection as their BTC-derivatives. The detection limits of BTC-amino acids were established.

$$I_2 + 2N_3^- \xrightarrow{\text{C=S inductor}} 2I^- + 3N_2$$

Scheme 1.

We have also made an effort to separate amino acids in mixture as BTC-derivatives.

EXPERIMENTAL

Solutions and Reagents

All amino acids, BITC, methanol, ethanol, 2-propanol, dioxan, toluene, tetrachloromethane, chloroform, acetone, tetrahydrofuran, triethylamine were purchased from Sigma-Aldrich (Steinheim, Germany) or LAB-SCAN Analytical Sciences (Dublin, Ireland).

Stock amino acid solutions: a specified amount of a particular reagent was dissolved in a suitable quantity of a 0.1 mol L^{-1} sodium hydroxide solution.

Standard amino acid solutions: a specified volume of stock amino acid solution was diluted in a mixture of 2-propanol and triethylamine, 15:2 (v/v).

Derivatization solution: 1 mL BITC was added to 13 mL of 2-propanol and 2 mL triethylamine.

Mobile phases: specified volumes of organic solvents were mixed (for details see proper Tables).

Spraying solution: 25 mL aqueous starch solution containing 0.250 g starch was added to 20 mL aqueous sodium azide containing 1.5 g sodium azide, and the mixture was adjusted to the appropriate pH (pH 5.5) with 0.1 mol L^{-1} hydrochloric acid solution and diluted to 50 mL with water to obtain 3% sodium azide solution and 0.5% starch solution. All solutions were prepared fresh daily.

Procedure for BITC Derivatization of Amino Acids

Procedure for Derivatization of Amino Acids in a Test Tube

Appropriate amounts of standard amino acids solutions and 1 mL of derivatizating reagent, 2-propanol-BITC-triethylamine (13:1:2), were placed in a stoppered tube on the magnetic stirrer. The magnetic stirrer was located in the thermostat at 40°C. The reaction time was 30 min. After the reaction was completed the sample was diluted to 5 mL with methanol. The plates were spotted with 1 μL of BTC-amino acid solution and developed with a mixture of suitable solvents.

Procedure for Derivatization of Amino Acids on the Chromatographic Plate (in situ)

The plates were spotted with 0.1–1 μL of amino acid solution and then dried. Derivatization solution was applied after sample application to the same

starting zone with a 1 μL pipette (Brand, Wertheim, Germany); as a result reagent solvent caused the sample to spread outward. The BITC procedure required placing the plate in a glass chamber in the thermostat at 40°C for 30 min, since under these conditions the BITC agent reacted with all of the protein amino acids. After that, the plate was developed with a mixture of suitable solvents.

Planar Chromatography

HPTLC silica gel 60 F_{254} aluminium sheets (Merck, Darmstadt, Germany; 5 × 5 cm, 0.2 mm thick layer) were used for the determination of detection limits of BTC-amino acids, and 10 × 10 cm HPTLC plates were used for separation of BTC-amino acids in mixtures. The plates were developed using a horizontal DS-Chamber (Chromdes, Poland), which was ready for use 30 min after the solvent had been poured into it. The developing distances were: 4 cm (for determination of detection limits on HPTLC) and 9 cm (for separation on HPTLC). Different solvents systems were used as mobile phases. The specification is indicated in Table 3.

Separation of Amino Acids Mixtures as Their BTC Derivatives

The amount of each amino acid in the separation mixture corresponded to a 3-fold excess of the established detection limit.

We selected three 7-component groups of protein amino acids to carry out the separation. The chosen options are presented in Table 1. The appropriate mixture of amino acids prepared in water solution was applied on the plate (HPTLC, 10 × 10 cm). Derivatization reagent, 2-propanol-BITC-triethylamine, 13:1:2 was spotted to the sample starting zone on the plate. In the next step, the plate was placed in a glass chamber in the thermostat at 40°C. The reaction time was 30 min and finally, the plate was developed

Table 1. R_F values of protein amino acids detected as BTC-derivatives obtained by one-dimensional normal-phase chromatography; mobile phase: ethanol-methanol-chloroform (1:1:2, v/v/v); derivatization procedure in situ; iodine-azide detection system for NP chromatography

Group I	Val	Lys	Trp	Cys	Arg	Thr	Tyr
R_f	0.64	0.03	0.51	0.22	0.07	0.31	0.45
Group II	Gly	Ile	Asn	Glu	Phe	Pro	Hyp
R_f	0.28	0.76	0.22	0.05	0.65	0.51	0.40
Group III	Ala	Leu	Ser	Asp	Gln	His	Met
R_f	0.49	0.71	0.33	0.03	0.24	0.08	0.54

in ethanol-methanol-chloroform (1:1:2, v/v/v). The iodine-azide procedure described below was used as the method of detection.

Detection of BTC-Amino Acids Derivatives

After drying, the developed plates were sprayed with a freshly prepared mixture of 3% sodium azide, and 0.5% starch solution adjusted to pH 5.5 and exposed to iodine vapour for 5 s. Due to the catalytic effect of the C═S bond, the spots became visible as white spots on a violet-grey background and they were stable for a couple of minutes.

RESULTS AND DISCUSSION

In this part of our article we would like to present the most relevant points of our study that we managed to establish.

Derivatization

The first aspect of our research into BTC-derivatives was a thorough examination of the reaction with amino acids. Following the pattern of in situ PITC derivatization,[5–7] we applied BITC reagent. Several experiments were done to establish optimal conditions considering BITC derivatization in situ on the normal phase TLC plates. In order to find the most sufficient composition of the derivatization solution, we examined the following organic solvents: 2-propanol, acetonitrile, 1,2-dioxane, methanol, and chloroform, as well as some buffer solutions: phosphate buffer (pH = 10–12), Britton-Robinson buffer (pH = 9–12), triethylamine and their different ratios. Consequently, we found the solution which consisted of: 2-propanol–triethylamine–BITC (13:2:1, v/v/v) the most advantageous for all tested amino acids listed in Table 2. Additionally, the time of derivatization was investigated within the range of 15 to 35 min. The optimum one occurred to be 30 min. Having examined the following range of derivatization temperatures: 20–50°C, we discovered that 40°C was the favourable one. These established conditions were applied to in tube derivatization and occurred to be advantageous as well.

Table 2 depicts the detection limits of BTC-amino acids obtained by the iodine-azide procedure as a method of detection on normal-phase plates. As it can be seen, the derivatization in tube is more successful for determination of BTC-derivatives than in situ. The detection limits of BTC-amino acids in situ are considerably higher than in the tube counterparts. This phenomenon seems to be due to the differences between these two derivatization procedures, which play a significant role, as they have an impact on the reactivity in the formation of the derivative. Both derivatization

Table 2. Detection limits of BTC-amino acids (pmol/spot) with the iodine-azide procedure as the detection method for NP chromatography; developing distance: 4 cm; derivatization in test tube and in situ

Amino acids	In tube	In situ
Glycine	4	100
Alanine	25	600
Aspartic acid	90	2,000
Arginine	10	600
Proline	2	60
Hydroxyproline	8	40
Lysine	3	50
Glutamic acid	28	600
Serine	13	500
Tryptophan	25	200
Valine	11	500
Phenylalanine	4	90
Isoleucine	10	800
Leucine	9	600
Asparagine	8	80
Methionine	10	60
Cysteine	50	600
Histidine	6	100
Threonine	5	90
Tyrosine	17	200
Glutamine	40	800

methods required derivatization time of 30 min and temperature of 40°C. As the time did not pose any problems, the temperature conditions obtained by placing the plate in the thermostat seemed to be insufficient. Moreover, the higher temperature makes the applied BITC reagent evaporate from the plate since it is a volatile compound. In consequence, the reaction progress is inhibited. This was confirmed by the detection of BTC-derivatives after derivatization in tube. The achieved lower detection limits indicate that the temperature requirements were met more effectively in the larger volume of the derivatization solution in tube than in situ. Thus, in tube derivatization method is beneficial considering forming BTC-derivatives of amino acids, and enables achieving pmol per spot level of detection limits.

Separation

Several solvent systems were examined in order to resolve BTC-amino acids mixtures. Table 3 presents the ones with the acceptable capability of resolving

Table 3. R_F values of protein amino acids detected as BTC-derivatives obtained in different solvent systems; normal-phase plates; iodine-azide reaction as a detection method for NP chromatography

BTC-amino acid	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV
Glycine	0.25	0.53	0.35	0.17	0.26	0.26	0.11	0.19	0.12	0.14	0.07	0.12	0.28	0.59	0.31
Alanine	0.31	0.62	0.39	0.27	0.43	0.54	0.17	0.39	0.20	0.30	0.11	0.27	0.49	0.69	0.37
Aspartic acid	0.00	0.11	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.03	0.04	0.00
Arginine	0.03	0.07	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.01	0.07	0.05	0.02
Proline	0.39	0.60	0.42	0.36	0.46	0.52	0.29	0.51	0.32	0.31	0.21	0.55	0.51	0.56	0.38
Hydroxyproline	0.20	0.44	0.25	0.13	0.18	0.24	0.13	0.45	0.18	0.15	0.15	0.28	0.40	0.43	0.22
Lysine	0.00	0.04	0.05	0.00	0.00	0.00	0.00	0.00	0.11	0.11	0.00	0.07	0.03	0.03	0.02
Glutamic acid	0.13	0.22	0.10	0.00	0.00	0.00	0.00	0.00	0.13	0.15	0.00	0.09	0.05	0.06	0.02
Serine	0.23	0.47	0.33	0.13	0.12	0.16	0.10	0.10	0.08	0.11	0.05	0.08	0.33	0.44	0.20
Tryptophan	0.30	0.65	0.06	0.28	0.35	0.46	0.62	0.85	0.26	0.27	0.03	0.29	0.51	0.65	0.37
Valine	0.52	0.75	0.55	0.65	0.71	0.73	0.85	0.88	0.30	0.61	0.30	0.56	0.67	0.76	0.65
Phenylalanine	0.21	0.48	0.58	0.54	0.57	0.62	0.75	0.89	0.44	0.53	0.37	0.57	0.65	0.72	0.57
Leucine	0.56	0.76	0.59	0.71	0.72	0.74	0.80^t	0.86	0.42^t	0.63	0.28^t	0.62	0.71	0.80	0.74
Isoleucine	0.59	0.77	0.63	0.79	0.76	0.83	0.84^t	0.85	0.55^t	0.73	0.44^t	0.74	0.76	0.83	0.79

(*continued*)

Table 3. Continued

BTC-amino acid	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV
Asparagine	0.17	0.29	0.17	0.06	0.05	0.04	0.05	0.04	0.03	0.07	0.03	0.03	0.18	0.22	0.13
Methionine	0.06	0.12	0.51	0.39	0.48	0.61	0.00	0.00	0.39	0.44	0.22	0.74	0.54	0.64	0.46
Cysteine	0.09	0.10	0.05	0.02	0.04	0.06	0.00	0.04	0.06	0.03	0.02	0.07	0.22	0.22	0.05
Histidine	0.10	0.12	0.08	0.03	0.03	0.04	0.00	0.00	0.04	0.04	0.03	0.03	0.08	0.08	0.05
Threonine	0.11	0.22	0.30	0.17	0.19	0.24	0.08	0.26	0.13	0.16	0.00	0.27	0.33	0.47	0.24
Tyrosine	0.06	0.32	0.40	0.35	0.47	0.61	0.00	0.00	0.33	0.37	0.09	0.67	0.45	0.66	0.42
Glutamine	0.18	0.39	0.22	0.06	0.08	0.08	0.06	0.08	0.04	0.07	0.04	0.05	0.22	0.24	0.17

[t]:Tailing.

Two-component solvent systems: I—methanol–chloroform (1:2), II—methanol–toluene (1:1), III—methanol–1,2–dichloroethane (1:1), IV—ethanol–chloroform (1:1), V—ethanol–chloroform (2:1), VI—ethanol–chloroform (3:1), VII—ethanol–1,2–dioxane (1:1), VIII—ethanol–1,2–dioxane (2:1), IX—ethanol–acetone (1:2), X—ethanol–toluene (2:1), XI—ethanol–tetrahydrofuran (2:1), XII—ethanol–tetrachloromethane (2:1).

Three-component solvent systems: XIII—ethanol–methanol–chloroform (1:1:2), XIV—ethanol–methanol–chloroform (4:1:2).

Four-component solvent systems: XV—ethanol–methanol–chloroform–toluene (2:1:2:1).

the chosen groups of derivatives and having no interfering effect of BITC spot on respective amino acids derivatives spots. Nevertheless, the most selective separation was predicted to be achieved in ethanol-methanol-chloroform (1:1:2). Therefore, this mobile phase was employed for the resolution. The final results are gathered in Table 1, as well as in Figure 1. According to this figure, good separation was obtained in the three seven-component mixtures of BTC-amino acids and no tailing effect was observed.

Another important point of separation worth stressing is the possibility of the entire resolution of leucine and isoleucine as BTC-derivatives. This issue has always been a difficult problem to address. In our previous attempt[6] to deal with it, we found PTC-derivatives of these two amino acids insufficient to obtain fully resolved spots, although a great number of solvent systems were investigated. However, our current experiments with BITC as a derivatization reagent solves this matter. We established that the application of the solvent system consisted of given solvents: ethanol-tetrachloromethane (2:1, v/v) distinguished, successfully, BTC-leucine and isoleucine. In Table 3, the appropriate R_F values are given. It should be added, that although there are some discrepancies between R_F of BTC-leucine and isoleucine in other mobile phases, the tailing effect makes it impossible to reach favourable separation.

Detection

We dealt with the optimum conditions of the improved method of detection using iodine-azide reaction for PTC-amino acid in the latest publi-

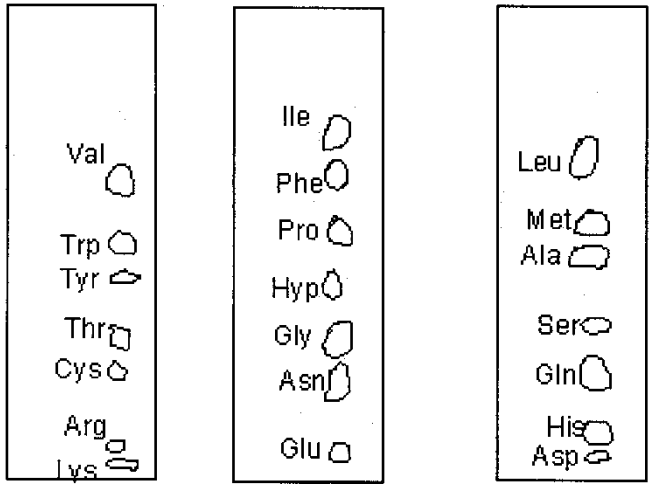

Figure 1. One-dimensional separation of amino acids as their BTC derivatives on normal phase silica gel plates.

cations.[5–7] In this report, we have utilized this method to the group of amino acids as their BTC-derivatives. The optimum conditions to conduct the iodine-azide detection for BTC-amino acids were found during experiments. It was observed that pH value (in the range of 5.5–8.0) of sodium azide solution did not have a huge impact on detection limits. This limited range of pH is determined by the fact that the use of solution whose pH is lower than 5.5 results in the emission of the poisonous hydrazic acid. Whereas, above pH 8.0, catalytic reaction does not occur as a consequence of forming hypoiodite, which is not a reagent in the iodine-azide reaction. However, the concentration of sodium azide solution influenced the results. In the range of 0.1–4.0% concentration of sodium azide, the most favourable outcome was achieved using 3%, and a further increase in concentration did not change results. Application of lower concentration solutions caused greater instability of spots and weaker colour of the background. As a result, the higher detection limits were obtained.

The iodine-azide procedure was compared with other commonly used detection techniques used in TLC, iodine vapour and UV, and proved to give the lowest detection limits (data not shown). Another advantage of this system over examined detection methods was the quality of obtained chromatograms. Spots of all the amino acids detected as BTC-derivatives were compact with sharp edges against a violet-grey background of the plate, and provided an accurate measurement of R_F values.

CONCLUSION

BITC was chosen as the prechromatographic derivatization reagent for analysis of amino acids in TLC with the application of the iodine-azide reaction in the detection system. This reagent reacted with all the protein amino acids at 40°C for 30 min to yield BTC-amino acids. The obtained detection limits in the tube procedure, together with iodine-azide detection system, were within the range of pmol. We established successful resolution of BTC-derivatives mixtures, as well as the distinction of leucine and isoleucine, two amino acids that are most difficult to separate. The presented system of detection of BTC-derivatives provides an efficient tool in amino acids analyses.

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