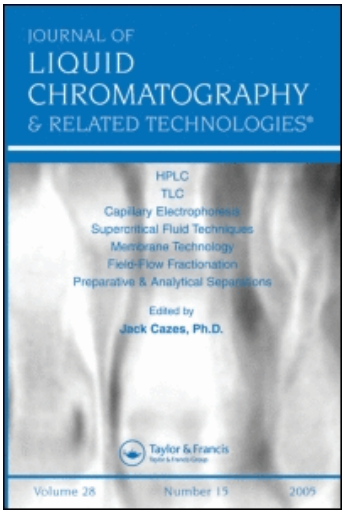

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APPLICATION OF THE IODINE–AZIDE PROCEDURE FOR THE DETECTION OF GLYCINE, ALANINE, AND ASPARTIC ACID IN PLANAR CHROMATOGRAPHY

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ABSTRACT

The application of iodine–azide reaction for the determination of non-sulphur amino acids (glycine, alanine, and aspartic acid) transformed into PTC-derivatives with phenyl isothiocyanate in TLC and HPTLC, is described. The developed plates were sprayed with a freshly prepared mixture of sodium azide, adjusted to a proper pH, and starch solution, and exposed to iodine vapour for 5 seconds. The detection limits were established at the pmole level. The factors depending on the detection limits were described. A comparison of iodine–azide test reactions with other procedures is presented. The developed method was applied to detection of aspartic acid in a drug. The possibility of detection of amino acid mixtures was demonstrated.

Key Words: Amino acids; PTC-derivatives; Iodine-azide reaction; TLC; HPTLC

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INTRODUCTION

The amino acids constitute an essentially significant group of organic compounds to living organisms. They are not only structural units of proteins, but they also occur in free state in numerous natural products. Additionally, most amino acids show various biological functions.

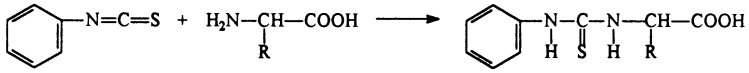
Glycine is considered to be a main component of porfyrine, purine, and glutathione in living organisms. Alanine and glycine can be found in proteins of blood serum. Alanine also builds bacterial cell walls. Aspartic acid exhibits strong biological activity in organic reactions such as: deaminations, transaminations, and synthesis of ammonia and urea.

Among the constantly improved analytical methods applied in amino acid studies, chromatographic techniques are most valuable, especially thin-layer chromatography, which enables separation and identification of amino acids and also the quantative analysis by densitometry.

Several spray reagents for the selective and non-selective detection of amino acids on thin layer chromatograms have been described, among which ninhydrin is most often used.

Derivatization is frequently used for sample preparation before analysis. Although this complicates the analytical procedure and is the cause of additional errors and lengthening of the analysis time, there are reasons for using derivatization in liquid chromatography. Improvement of detection, especially detection sensitivity, is the most important reason for derivatization. Among many reagents reacting with amino acids, the most frequently used are: phenyl isothiocyanate (PITC),[1] the chloride of 4-*N*,*N*-dimethylaminoazobenzene-4′-sulfonic acid (DABSYL-Cl),[2] 4-*N*,*N*-dimethylaminoazobenzene-4′-isothiocyanate (DABITC),[3] 5-methylaminonaphthalene-1-sulfonyl chloride (dansyl chloride),[4], and 1-fluoro-2,4-dinitrobenzene (FDNB).[5]

We applied phenyl isothiocyanate to transform amino acids into PTC-derivatives:



to detect glycine, alanine, and aspartic acid in TLC and HPTLC. The method of detection was the application of the iodine–azide reaction:[6]

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

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selectively induced by sulphur compounds (PTC-amino acid). It offers the opportunity for selective and sensitive detection. Previous application of the iodine–azide reaction in TLC was based on spraying a freshly prepared solution of sodium azide and iodine solution in potassium iodide.[7–10] Sometimes starch solution has been applied.[11,12] None of the authors investigated the influence of iodide ion and pH of the azide solution on the course of the iodine–azide reaction. In volumetric, spectrophotometric, or coulometric methods, iodide ions have an influence on the rate of induced iodide–azide reaction and the value of the induction coefficient.[13] As a continuation of this research, the improved iodide–azide procedure of detection of amino acids in planar chromatography was presented.

In this paper, we wish to present a new selective and sensitive method for the detection of non-sulphur amino acids. The detection limits of amino acids (as PTC-derivatives) were achieved at the pmol/spot levels; thus, they are lower than the well-known ninhydrine, iodine, and UV_{254} procedures.

EXPERIMENTAL

Materials

Amino acids (glycine, alanine, aspartic acid), phenyl isothiocyanate, pyridine, methanol, chloroform, and *n*-butanol were obtained from Aldrich or Lab-Scan Analytical Sciences. "ASPARGIN" (Farm. Sp. Pr. "FILOFARM" Bydgoszcz) tablets were used.

Solutions and Reagents

Aqueous amino acid solutions were obtained by dissolving a specified amount of a particular reagent in the suitable quantity of a solution of sodium hydroxide.

Solution for Determination of Induction Coefficients

A 0.1 M aqueous iodine solution containing $25\,g \times L^{-1}$ of potassium iodide. A 2×10^{-2} M aqueous sodium arsenite solution. Reaction solution: the aqueous solution containing $20\,g \times L^{-1}$ sodium azide buffered with hydrochloric acid to an appropriate pH.

Solution for Planar Chromatography

Sodium Azide Solution

Two mL of 0.5% aqueous starch solution was added to 20 mL 4% (w/v) aqueous sodium azide solution and adjusted to the appropriate pH with 0.1 M hydrochloric acid solution. Ninhydrine reagent: 0.15 g ninhydrine was dissolved in 1.5 mL acetic acid and diluted to 50 mL with *n*-butanol. All solutions were prepared fresh daily.

Synthesis of PTC-Derivatives

PTC-amino acids were obtained according to the Edman procedure.[1] Amino acids (0.01 mole), dissolved in 5 mL of 1 M NaOH was added to aqueous pyridine solution (1 : 1; 10 mL) in a stoppered tube. The solution was adjusted to pH 9.0 with 1 M HCl and placed in a water bath at 40°C. Phenyl isothiocyanate (2.4 mL) was added, with shaking, during a reaction time of about 4 hours. The mixture was extracted repeatedly with benzene (4×10 mL). HCl (1 mL 36%) was added to precipitate the PTC-derivatives. The resultant PTC-derivatives were used for determination of the inductive effect and the influence of pH solution and iodide ion on the course of the iodine–azide reaction.

Determination of the Inductive Effect on the Iodine–Azide Reaction

The efficiency of sulphur compounds as inductors in the iodine–azide reaction has been characterized on the basis of their induction coefficients, defined by the equation:

$$F_i = \frac{n_I}{n_s}$$

where n_I is moles of iodine consumed in the inducted reaction and n_S is moles of the inductor. The consumption of iodine in the induced iodine–azide reaction were adopted [14]. The reaction flask (25 mL conical flask equipped with a glass stopper and a magnetic stirrer) was charged with 10 mL of reaction solution, followed by addition of an appropriate amount of PTC-derivative. To the stirred solution, 0.5 mL of 0.1 M iodine solution was added and flask tightly locked with a stopper. From this moment, reaction time, 2 minutes, was measured. The excess of iodine was then back titrated by means of 2×10^{-2} M sodium arsenite solution in the presence of starch indicator. In the same way, the solution was titrated in the absence of inductor (blank). The results are given in Fig. 1.

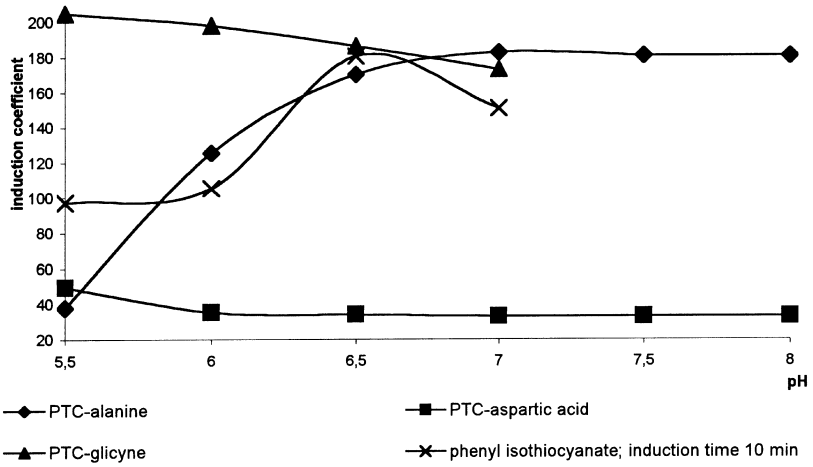

Figure 1. The dependence of the induction coefficient (F_i) of PTC-amino acids on the pH of the solution; 2% azide solution; induction time 2 min.

Determination of Influence on Iodide Ion on the Course of the Iodine–Azide Reaction

The influence of iodide ion on the course of the iodine–azide reaction was determined in the same way as the induction coefficient. The reaction solution contains, additionally, a proper concentration of iodide ions.[13] The results are given in Fig. 2.

Planar Chromatography

TLC silica gel 60 F_{254} aluminium sheets (10 × 5 cm, 0.2 mm thick layer) or HPTLC silica gel 60 F_{254} aluminium sheets (5 × 5 cm, 0.2 mm thick layer) or precoated cellulose DC plates (10 × 5 cm, 0.2 mm thick layer) were used for all chromatographic experiments. Proper amounts of amino acids, dissolved in 0.1 M NaOH, were added to aqeous pyridine (1 : 1; 1 mL) in a stoppered tube. The solution was adjusted to pH 9.0 with 1 M HCl and placed in a water bath at 40°C. Phenyl isothiocyanate (250 μL) was added, with shaking, during a reaction time of 30 min. The sample was diluted to 5 mL with methanol (solution 1). The plates were spotted with 1 μL of solution 1 or amino acid solution (deposition area *ca.* 0.2 cm^2), developed using a horizontal DS-Chamber (Chromdes, Poland) for a distance of 8 cm (for TLC) or 4 cm (for HPTLC) with proper solution mixtures and then air dried.

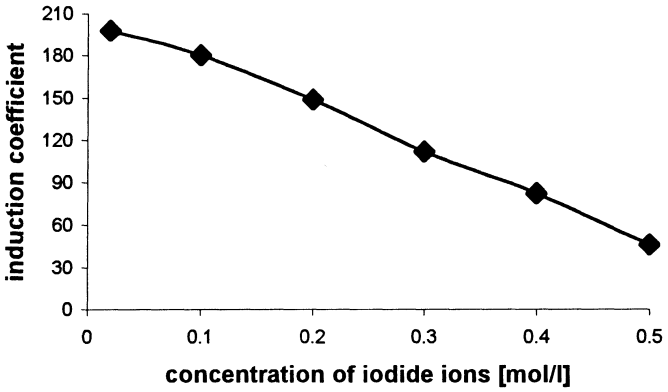

Figure 2. The dependence of the induction coefficient of PTC-glycine on iodide ion concentration; 2% sodium azide; pH 6.0, induction time 2 min.

Detection of Amino Acids (as PTC-Derivatives) by the Improved Iodine–Azide Procedure

The developed plates were sprayed with a freshly prepared mixture of 4% sodium azide adjusted to a proper pH with 0.5% starch solution (20 : 2) and were exposed to iodine vapour for 5 seconds. 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 20 min.

Detection of Amino Acids (as PTC-Derivatives) by the Non-Improved Iodine–Azide Procedure[8]

The developed plates were sprayed with a freshly prepared 1 : 1 (v/v) mixture of 1 M sodium azide and 1 M iodine solution (in 1 M potassium iodide solution). The spots became visible as white spots on a yellow background and they were stable for 30 min.

Detection of Amino Acids (as PTC-Derivatives) by the Iodine Procedure

The developed plates were exposed to iodine vapour for 10 min. The spots became visible as brown spots on a yellow background.

Detection Amino Acids (as PTC-Derivatives) by the UV_{254} Procedure

Substances were visualised under a UV-lamp (254 nm) by using TLC or HPLTC plates with fluorescent indicator.

Detection Amino Acids (Free or as PTC-Derivatives) by the Ninhydrine Procedure

The developed plates were sprayed with a freshly prepared ninhydrine solution and dried at 150°C for 5 min. The spots became yellow (PTC-glycine), pink (PTC-alanine), violet (PTC-aspartic acid), and blueviolet on a white or pinkish background when ammonia was added into mobile phase (free amino acids). The results are given in Tables 1 and 2.

Analytical Application of the Developed Procedure

The developed procedure was applied to detect aspartic acid in a drug ("ASPARGIN"; Farm. Sp. Pr. "FILOFARM" Bydgoszcz). The possibility of amino acid mixture detection was also shown.

Detection of Aspartic Acid in a Drug

To prepare solution (2), a quantity of the powdered tablets containing 30 mg aspartic acid was shaken with 10 mL 1 M NaOH and was adjusted to 100 mL with water. 1 mL of solution (2) was diluted to 5 mL with water (solution (3)). 300 μL (for TLC) or 100 μL (for HPTLC) was placed in a stoppered tube and the chromatographic procedure was performed in the same way as for the standard solution. The R_f value for PTC-aspartic acid in the drug is in accord with the one for the standard of PTC-amino acid.

Detection of Amino Acid Mixtures with the Iodine–Azide Procedure

Solutions containing proper amounts of amino acids were placed in a stoppered tube and the chromatographic procedure was performed in the same way as for standard solutions. The results and iodine–azide procedure detection conditions are given in Table 3.

Table 1. Detection Limits of Amino Acids Detected as PTC-Derivatives with Varied Methods; Silica Gel, Methanol : Chloroform (1 : 1)

	Detection Limit (pmol/spot)								pH		R_F	
	Improved Iodine–Azide		Iodine		UV		Ninhydrine					
	TLC	HPTLC	TLC	HPTLC	TLC	HPTLC	TLC	HPTLC	F_i		TLC	HPTLC
Glycine	13	7	50	30	100	50	1,000	500	5.5		0.66	0.66
									240			
Alanine	10	6	40	20	100	40	1,000	500	7.0		0.83	0.84
									183			
Aspartic acid	90	40	200	150	400	200	2,000	1,500	5.5		0.75	0.75
									51			

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Table 2. Detection Limits of Free Amino Acids in Varied Systems with Ninhydrine Detection Procedure

	Detection Limits (pmol/spot)							
	Silica Gel						Cellulose	
	Chloroform–Methanol (1 : 1)		Isopropanol–Ammonium (7 : 3)		Ethanol–Water (7 : 3)			
Amino Acid	TLC	HPTLC	TLC	HPTLC	TLC	HPTLC	Chloroform–Methanol–Ammonium (20 : 20 : 9)	Butanol–Acetic Acid–Water (4 : 1 : 5)
Glycine	300	100	2,300	1,200	900	100	600	300
Alanine	300	100	2,100	1,000	1,000	100	900	300
Aspartic acid	600	200	2,800	1,200	1,200	400	400	200

Table 3. HPTLC Analysis (Clean-up Procedure) of the Mixtures of Amino Acids as PTC-Derivatives; Silica Gel, Methanol : Chloroform (1 : 1)

	Iodine–Azide Procedure			
Mixtures	pH of Azide Solution	Amount [pmol/spot]	UV_{254} Procedure Amount [pmol/spot]	R_f
Glycine	6.7	8	30	0.65
Alanine		5	30	0.83
Glycine	5.5	3	30	0.64
Aspartic acid		10	150	0.77
Alanine	6.7	5	30	0.85
Aspartic acid		32	150	0.72
Glycine	6.7	8	30	0.63
Alanine		5	30	0.87
Aspartic acid		32	150	0.77

RESULTS AND DISCUSSION

Derivatization Procedure

The derivatization procedure was worked out according to the original work.[1] Increasing the temperature above 40°C or lengthening reaction time above 30 min does not cause an increase in efficiency of the derivatization procedure.

Separation

The R_f data (TLC and HPTLC) are presented in Table 1. We have not found any publications concerning separation of amino acids as PTC-derivatives in TLC or HPTLC. Use of methanol : chloroform (1 : 1) has been found extremely satisfactory for the discrimination between all reagents, as the difference in their R_f values was more than 0.1.

The possibility of detection of amino acids as PTC-derivative mixtures was also demonstrated. The results are shown in Table 3.

Detection

The detection limits for amino acids, using different detection systems, are summarised in Table 1. In the proposed improved iodine–azide methods, the

detection limits are lower in comparison to applied detection methods of PTC-derivatives or free amino acids (Table 2). Amino acids which do not contain a sulphur atom in the molecule exhibit no induction activity and are not detectable with the iodine–azide procedure. That is the reason why the investigated amino acids were transformed into PTC-derivatives.

Influence of the Varied Factors on Detection Limit

Stationary Phase

The detection limits obtained in HPTLC methods are lower than those obtained in TLC. High performance plates, produced from sorbents having narrow pore and particle size distributions are more efficient, thereby leading to tighter zones, a better resolution for smaller migration distances. It leads to the lower detection limit compared to TLC systems because molecular diffusion, which is the limiting factor, takes place to a smaller degree.

Induction Coefficient

Inducting properties of examined compounds were characterized by the induction coefficient. From the analytical point of view, the value of the induction coefficient is that it is the measure of sensitivity of the determination of a given PTC-derivative in volumetric, coulometric, and spectrophotometric determination. But, the induction coefficient is not the only factor which influences the detection limit in TLC and HPTLC. Figure 3 illustrates a good correlation between the induction potency (induction coefficient) of PTC-derivatives and their detectability using the improved iodine–azide procedure.

pH of Azide Solution

The induction coefficient has been found to be dependent on pH for all PTC-derivatives (Fig. 2). The use of a solution whose pH is lower than 5.5 is not recommended because of the emission of the poisonous, volatile hydrazoic acid. Over pH 8.0, catalytic reaction does not proceed since iodine forms iodite (I), which is not a reagent in the iodine–azide reaction. In the case of PTC-glycine, induction coefficient decreases with increase of the pH (within the range 5.5–8.0) while, in the case of PTC-alanine, the dependence is different. The induction coefficient of PTC-alanine increases with increase of the pH value (within the

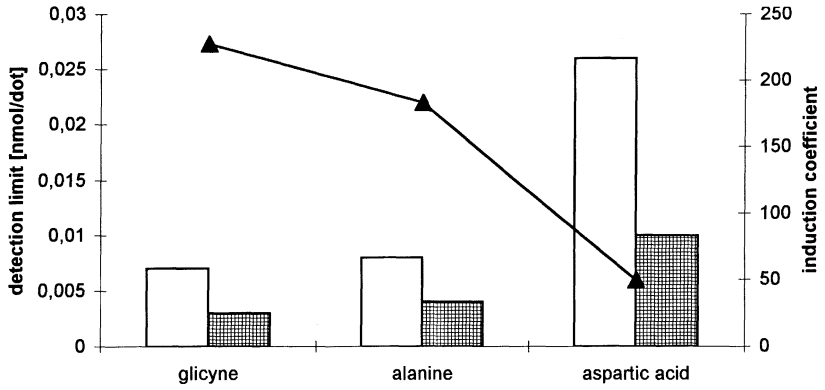

Figure 3. The dependence of the induction coefficient on detection limit; silica gel, chloroform–methanol (1 : 1), iodine–azide detection procedure.

range 5.5–7.0) and becomes independent (within range 7.0–8.0). In the case of aspartic acid, the induction coefficient decreases with an increase of the pH value (within the range 5.5–6.0) and remains constant (within the range 6.0–7.0). There is a peak on the curve of the dependence of the induction coefficient of phenyl isothiocyanate on pH of the solution (pH 6.5). It results in different pK values of the PTC-derivatives and phenyl isothiocyanate and different mechanisms of the iodine–azide reaction.

Phenylisothiocyanate does not interfere in the proposed methods because it has different R_f value than PTC-derivatives and the induction coefficient has a small value. Spots corresponding to the substance have small areas despite a great amount of it. The pH's of spray solutions are sumarized in Table 1. The influence of pH of solutions on detection limits is shown in Table 4.

Considering the influence of all parameters on the course of the iodine–azide reaction, pH 5.5 or 6.7 has been chosen as the most favourable pH range for the measurement for mixtures of amino acids (for details see Table 3).

Concentration of Reagents

Increasing sodium azide concentration in the spray reagent up to 4% causes a slight increase in detection limit. Application of more concentrated sodium azide solution, greater than 4%, does not increase the detection limit. Similar relationships occur in volumetric, spectrophotometric, and coulometric methods for other inductors.

Table 4. Influence of pH of Spray Solution on Detection Limits (pmol/spot) with Improved Iodine–Azide Procedure (with Clean-up Procedure)

	pH	TLC	HPTLC
Glycine	5.5	7	3
	6.7	15	8
Alanine	5.5	9	5
	6.7	8	4
Aspartic acid	5.5	26	10
	6.7	68	32

Clean-up of samples by solvent partitioning was used to improve detection limits. When the derivatization reaction was completed, the mixtures were extracted repeatedly with benzene to remove excess phenyl isothiocyanate and pyridine. The detection limits obtained with the clean-up procedure are slightly lower. Despite of this, we do not recommend the procedure because of the hazardous properties of benzene. The results are given in Table 5.

The effect of an excess of derivatization reagent was also studied. It was found that the three-fold excess of phenylisothiocyanate is enough to obtain sufficient detection limits with the studied amino acids.

Induction Time

The plates were exposed to iodine vapour for 5 seconds. Due to the catalytic effect of the C=S bond, the spots became visible as white spots on a violet-grey background. The exposure time is so short due to adsorption of iodine on the plate and vanishing of white spots. Appearance of white spots could take some time, due to the induction time conditioned by induction properties of the particular compound. It is the time within which the induction reaction has finished. This is less than 2 min in each case.

Influence of Iodine and Iodide Ion

Excess of iodine makes the detection limits higher in the iodine–azide procedure. It is important and difficult to spray the plates with very finely divided spray solution for optimum staining of the plates.[7–12] This is overcome, thanks to applying iodine vapour in the proposed improved procedure.

Table 5. Influence of Clean-up Procedure on Detection Limits (ppm) of Amino Acids Detected as PTC-Derivatives; Silica Gel, Methanol : Chloroform (1 : 1); Improved Iodine–Azide Detection

			Glycine	Alanine	Aspartic Acid
Improved iodine–azide	Without clean-up procedure	TLC	1	0.9	10
		HPTLC	0.6	0.7	3
	With clean-up procedure	TLC	0.6	0.7	3
		HPTLC	0.3	0.4	1
Iodine	Without clean-up procedure	TLC	4	3.5	50
		HPTLC	2	2	15
	With clean-up procedure	TLC	2	2	15
		HPTLC	1	2	7
UV	Without clean-up procedure	TLC	8	9.5	50
		HPTLC	4	3.5	30
	With clean-up procedure	TLC	4	3.5	30
		HPTLC	2	2	10
Ninhydrine	Without clean-up procedure	TLC	80	90	260
		HPTLC	30	20	130
	With clean-up procedure	TLC	30	20	130
		HPTLC	15	9.5	50

Table 6. Comparison of the Detection Limits (pmol/spot) for Glycine as PTC-Derivatives (with Clean-Up Procedure) Using Varied Iodine–Azide Procedures; Silica Gel, Methanol:Chloroform (1 : 1)

Spray Reagent	Improved Procedure	Non-Improved Procedure[8]	4% Sodium Azide, 0.01 mol · l^{-1} Potassium Iodide, 0.5% Starch Solution
TLC	7	600	40
HPTLC	3	200	20

We found that increasing the concentration of the iodide anions resulted in the decrease of the rate of the induced iodine–azide reaction and the value of the induction coefficient of PTC-glycine (Fig. 2). Presence of iodide ions makes the detection limits higher for all investigated compounds, when spraying with sodium azide and iodine in potassium iodine or sodium azide in potassium iodide solution procedure (Table 6).

In the light of the results presented here, only the iodine–azide detection system with derivative procedure allows the selective and the most sensitive detection for the amino acids. The other applied detection systems, routinely used in the TLC of free or PTC-amino acids (ninhydrine, iodine, UV procedure) gave a positive but less sensitive test. The iodine–azide procedure is characterised by a short analysis time, a simple procedure, and commonly available reagents.

REFERENCES

1. Edman, P. Preparation of Phenyl Thiohydantoins from Some Natural Amino Acids. Acta Chem. Scand. **1950**, *4*, 277–282.
2. Chang, J.Y.; Creaser, E.M. Separation of 4-*N*,*N*-dimethylaminoazobenzene-4′-sulfonyl Amino Acids on Polyamide Sheets. J. Chromatogr. **1976**, *116*, 215–217.
3. Chang, J.Y.; Creaser, E.M.; Beatly, K.W. 4-*N*,*N*-dimethylaminoazobenzene 4′-Isothiocyanate, a New Chromaphoric Reagent for Protein Sequence Analysis. Biochem. J. **1976**, *153*, 607–611.
4. Deyl, Z.; Rosmus, J. Thin-Layer Chromatography of Dansyl Amino Acids Derivatives. J. Chromatogr. **1965**, *20*, 514.
5. Rosmus, J.; Deyl, Z. Chromatographic Methods in the Analysis of Protein Stucture. The Methods for Identification of N-Terminal Amino Acids in Peptides and Proteins. J. Chromatogr. B **1972**, *70*, 221–339.
6. Raschig, F. Neue Reaktionen der Stickstoffwaserrstoffsäure. Chem. Ztg. **1908**, *32*, 1203.
7. Fischer, R.; Otterbeck, N. Zum Nachweis von Thiosphorsäureestern. Sci. Pharm. **1959**, *27*, 1–6.
8. Kudzin, Z.H.; Kotynski, A.; Kiel Basiński, P. Application of the Iodine–azide Reagent for Selective Detection of Thiophosphoryl Compounds in Thin-Layer Chromatography. J. Chromatogr. A **1991**, *588*, 307–313.
9. Kotyński, A.; Ciesielski, W.; Kudzin, Z.H.; Okruszek, A.; Krajewska, D.; Lipka, P.J. Iodine–azide Reagent for the Detection of Biologically Oriented Thiophosphoryl Compounds in Thin-Layer Chromatography Systems. J. Chromatogr. A **1998**, *813*, 135–143.

10. Kudzin, Z.H.; Saganiak, M.; Kotyński, A.; Ciesielski, W. Comparison of the Thin-layer Chromatographic Properties of Sulfur-Containing Amino Acids and their Aminophoshonic Analogues. J. Chromatogr. A **2000**, *888*, 335–339.
11. Cherbuliez, E.; Baehler, Br.; Rabinowitz, J. Etude de Structures Peptidiques à l'Aide du Phenylisothiocyanate. Helv. Chim. Acta **1964**, *5*, 1350–1353.
12. Cserhati, T.; Orsi, F. The Application of the Iodine–Azide Reaction in Thin-Layer Chromatography Studies of Pesticide Preparations. Periodica Polytechnica **1982**, *26*, 111–119.
13. Ciesielski, W.; Kudzin, Z.H.; Kiel Basiński, P. Organothiophosphorus Compounds as Inductor of the Iodine–Azide Reaction. Anal. Applic. Talanta **1994**, *41*, 1493–1498.
14. Kurzawa, Z. The Determination of Trace Amounts of Sulphides, Thiosulphates and Thiocyanides by the Reaction of Sodium Azide with Iodine. Chem. Anal. (Warsaw) **1959**, *4*, 649.

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