Available online at www.sciencedirect.com
SCIENCE DIRECT®

JOURNAL OF CHROMATOGRAPHY A

Journal of Chromatography A, 1059 (2004) 171–174

www.elsevier.com/locate/chroma

Application of iodine-azide reaction for detection of amino acids in thin-layer chromatography

Dorota Kaźmierczak, Witold Ciesielski, Robert Zakrzewski*, Monika Żuber

Department of Instrumental Analysis, University of Łódź, Pomorska 163, 90-236 Łódź, Poland

Received 1 September 2004; received in revised form 27 September 2004; accepted 4 October 2004

Abstract

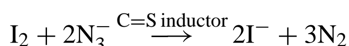
The iodine-azide reaction was employed to TLC detection of sulphur-containing derivatives of protein and some non-protein amino acids. The derivatization reaction with phenyl isothiocyanate (PITC) took place directly on the plate before the developing step. Subsequently, the plates were sprayed with a mixture of sodium azide and starch solution in NP-TLC and in the case of RP-TLC sodium azide solution with starch incorporated into mobile phase and then exposed to iodine vapour. The spots became visible as white spots on violet–grey background. The obtained detection limits of PTC-derivatives have been compared with other visualizing techniques commonly used in TLC practice (UV_{254} and iodine vapour). The iodine-azide system has been proved to be the most favourable and enabled to detect quantities per spot in the range of 1–60 pmol (HPTLC) and 3–100 pmol (TLC).
© 2004 Elsevier B.V. All rights reserved.

Keywords: Amino acids; Phenyl isothiocyanate; Iodine-azide reaction; Thin-layer chromatography

1. Introduction

A great variety of amino acids visualizing reagents was reported. Among them the most frequently applied was ninhydrin. Despite its common use, ninhydrin indicates decreased sensitivity towards amino acids for example proline and hydroxyproline [1]. Consequently, the prechromatographic derivatization of amino acids is widely recommended to enhance the sensitivity and obtain better results. A significant number of the suggested derivatization agents are isothiocyanates.

In this paper, we have focused on employing the iodine-azide reaction as the proposed method of detection of amino acids in order to improve the sensitivity. The reaction is depicted in the equation:



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

As it is clearly shown on the scheme above, only sulphur(II) compounds induce the reaction. This method bases on visual observation of the plate after spraying it with freshly prepared solution of sodium azide and starch and exposure to iodine vapour. Due to induction properties of bivalent sulphur compounds, the spots become visible as white spots on a violet–grey background. Application of this property to the detection of amino acids requires suitable derivatization. In our study, phenyl isothiocyanate as the prechromatographic reagent in determination of protein amino acids, as well as some of non-protein amino acids was chosen since it introduces bivalent sulphur into amino acid molecules to form proper PTC-derivatives. This derivatization agent is a relatively small molecule and reacts efficiently under basic conditions with all primary and secondary amino acids with a single derivatization step that is why it was chosen.

The present work outlines the results of the application of a new selective and sensitive method for detection of amino acids as PTC-derivatives. The obtained detection limits of PTC-derivatives have been compared with other visualizing techniques commonly used in a TLC laboratory (UV_{254} and

* Corresponding author. Tel.: +48 42 635 58 08; fax: +48 42 665 57 71.
E-mail address: robzak@chemul.uni.lodz.pl (R. Zakrzewski).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.10.009

iodine vapour). Detection of PTC-amino acids on silica gel and reversed bonded silica gel plates has been shown. Additionally, in the case of PTC-amino acid detection on reversed phase we have carried out derivatization procedure in a test tube.

2. Experimental

2.1. Solutions and reagents

All amino acids, PITC and all organic solvents 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 2 mL of 0.1 M sodium hydroxide solution and diluted to 10 mL with water to obtain 0.01 M concentration of amino acid solution. Standard amino acid solutions: specified volumes of stock amino acid solution (1000, 100 and 10 μL) were diluted to 10 mL with a mixture of 2-propanol and 0.0033 M phosphate buffer (pH 12), 2:1 (v/v).

Derivatization solution: 1 mL PITC was added to 7 mL of 2-propanol and 1 mL 0.005 M phosphate buffer (pH 12).

Mobile phases: (1) NP—specified volumes of methanol and chloroform (1:1, v/v) were mixed; (2) RP—specified volumes of acetonitrile and sodium azide solution (2:8, v/v) described below were mixed.

Sodium azide solution for the mobile phase in RP-TLC mode: 25 mL aqueous starch solution containing 1 g starch was added to 20 mL aqueous sodium azide containing 2 g of this compound. Then, the mixture was adjusted to the appropriate pH (pH 6.5) with 0.1 M hydrochloric acid solution and diluted to 50 mL with water to obtain 4% solution of sodium azide and 2% starch solution. All solutions were prepared fresh daily.

Spraying solution: 25 mL aqueous starch solution containing 0.250 g starch was added to 20 mL aqueous sodium azide containing 2 g and the mixture was adjusted to the appropriate pH (pH 5.5) with 0.1 M hydrochloric acid solution and diluted to 50 mL with water to obtain 4% sodium azide solution and 0.5% starch solution. All solutions were prepared fresh daily.

2.2. Procedure of derivatization of amino acids

2.2.1. Procedure of 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 (PITC) caused the sample to spread outward. The starting zone was covered with a glass strip for 15 min to complete the prechromatographic derivatization. After that, the plate was developed with a mixture of suitable solvents.

2.2.2. Procedure of derivatization of amino acids in a test tube

The appropriate amount of standard amino acids solution and 1 mL of derivatizating reagent [2-propanol-PITC-0.005 M phosphate buffer (pH 12) (7:1:1)] were placed in a stoppered tube on the magnetic stirrer. The reaction times were 15 min. After the reaction was completed, the sample was diluted to 5 mL with methanol. The plates were spotted with 1 μL of PTC-amino acid solution and developed with a mixture of suitable solvents.

2.3. Planar chromatography

TLC silica gel 60 F_{254} aluminium sheets (Merck, Darmstadt, Germany; 10 cm × 5 cm, 0.2 mm thick layer), HPTLC silica gel 60 F_{254} aluminium sheets (Merck, Darmstadt, Germany; 5 cm × 5 cm, 0.2 mm thick layer) were used for the determination of detection limits of PTC-amino acids. We also applied 10 cm × 5 cm TLC RP-18_{254s} aluminium sheets (Merck, Darmstadt, Germany; 0.2 mm thick layer) for the determination of detection limits of PTC-amino acids. The plates were developed using a horizontal DS-Chamber (Chromdes, Poland), which was ready for use 30 min after the solvent has been poured into it. The developing distances were: 8 cm (for TLC) and 4 cm (for HPTLC). The solvent systems used as mobile phases are indicated in Tables 1 and 2.

2.4. Detection of amino acids derivatives

2.4.1. Detection of PTC-amino acids in normal-phase chromatography

- The iodine-azide procedure:
  After drying, the developed plates were sprayed with a freshly prepared mixture of 4% 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 several minutes.
- The iodine procedure:
  After drying, the developed plates were exposed to iodine vapour for 3 min. The spots became visible as brown spots on a yellow background.
- The UV_{254} procedure:
  After drying substances were visualized under a UV lamp (254 nm) using TLC or HPTLC plates with a fluorescent indicator.

2.4.2. Detection of PTC-amino acids in reversed-phase chromatography

The developed plates were exposed to iodine vapour for 5 s without being dried. Since the mobile phase consisted of sodium azide solution (4%, pH 6.5, 2% starch), the catalytic effect of the C=S bond occurred and the spots were visible as white spots on a violet–grey background and they were stable for several minutes.

Table 1
Detection limits of protein amino acids detected as PTC-derivatives (pmol/spot) in the various methods of detection and stationary phases

Amino acids derivatives	NP; methanol–chloroform (1:1, v/v)								RP; acetonitrile–sodium azide solution (4 %, 2 % starch, pH = 6.5) (2:8, v/v)		
	R_F		In situ						R_F	In situ	In tube
	TLC	HPTLC	Iodine-azide procedure		Iodine		UV_{254}			Iodine-azide procedure	
			TLC	HPTLC	TLC	HPTLC	TLC	HPTLC	TLC		
Glycine	0.75	0.72	20	10	100	50	1000	260	0.54	1000	25
Alanine	0.83	0.80	50	25	250	100	500	250	0.43	10000	83
Aspartic acid	0.78	0.77	75	25	500	250	740	500	0.83	10000	49
Arginine	0.21	0.21	7	3	40	25	70	30	0.39	200	7
Proline	0.66	0.64	3	1	35	20	85	45	0.43	200	31
Lysine	0.14	0.12	8	3	90	70	100	90	0.95	200	25
Hydroxyproline	0.86	0.85	25	10	120	60	280	80	0.69	1000	25
Glutamic acid	0.76	0.77	90	50	250	100	500	250	0.79	10000	83
Serine	0.83	0.81	75	45	260	150	260	150	0.59	1000	69
Tryptophan	0.83	0.79	100	50	150	75	110	60	0.09	10000	83
Valine	0.84	0.83	80	40	750	300	1160	800	0.24	1000	83
Phenylalanine	0.88	0.86	25	17	510	200	500	200	0.07	10000	14
Isoleucine	0.89	0.87	20	14	170	50	1700	1000	0.13	10000	25
Leucine	0.80	0.77	20	10	150	80	1700	1000	0.13	10000	31
Asparagine	0.72	0.71	12	10	85	50	250	100	0.61	10000	49
Methionine	0.80	0.78	70	30	300	100	480	100	0.13	10000	83
Cysteine	0.80	0.79	90	60	380	200	440	200	0.12	10000	250
Histidine	0.20	0.19	30	20	340	200	1400	1000	0.41	2000	58
Threonine	0.72	0.69	70	30	200	100	200	100	0.47	2000	69
Tyrosine	0.88	0.86	20	10	210	100	210	100	0.25	1000	14
Glutamine	0.76	0.73	20	10	120	70	190	100	0.55	1000	25

Table 2
Detection limits of non-protein amino acids detected as PTC-derivatives (pmol/spot) in the various methods of detection; mobile phase: methanol–chloroform (1:1)

PTC-amino acid	R_F		Iodine-azide procedure		Iodine		UV_{254}	
	TLC	HPTLC	TLC	HPTLC	TLC	HPTLC	TLC	HPTLC
β-Alanine	0.74	0.72	90	45	180	90	270	135
Sarcosine	0.80	0.78	13	8	130	52	260	130
Norleucine	0.76	0.74	20	11	250	100	1900	1200
α-Aminobutyric acid	0.69	0.68	50	20	700	400	1100	600
β-Aminobutyric acid	0.70	0.68	35	19	550	200	1000	400
γ-Aminobutyric acid	0.76	0.74	21	10	600	300	1000	500
Ornithine	0.06	0.05	10	4	150	100	200	160
Citrulline	0.53	0.53	60	40	350	200	500	300
3,5-Di-iodotyrosine	0.75	0.75	40	20	450	250	450	250
3-Iodotyrosine	0.65	0.64	30	20	400	280	400	280
Taurine	0.77	0.78	200	70	1000	500	2000	1000

3. Results and discussion

In this study, we attempted to broaden the application of the previous elaborated method of detection [2] on all protein, as well as on some non-protein amino acids using NP-TLC, NP-HPTLC and RP-TLC modes.

3.1. Derivatization

We managed to significantly simplify the method in comparison with our initial efforts to detect amino acids as PTC-derivatives [3]. By applying in situ derivatization, the number of the stages, in the entire process, was reduced and a less time-consuming method was established. The general conditions for derivatization of three amino acids (lysine, proline and arginine) using PITC were discussed in our previous report [2] and were exploited in this work to carry out the reaction with all protein and some non-protein amino acids.

According to results summarized in Table 1, the detection limits established on RP plates are significantly higher than the ones on NP plates during derivatization in situ. It was presumed that different interactions between the long carbonyl chains from RP and the molecules taking part in derivatization interfere with and inhibit the reaction. The assumption seems to be proved since the detection limits of PTC-amino acids obtained during the derivatization in tube and determined on

RP are lower than the ones in situ. Furthermore, they reached a comparable level in relation to PTC-derivatives on NP in situ. Additionally, it was established that such influence of the stationary phase on the derivatization did not occur in the case of NP because the methods of derivatization in tube and in situ enabled to obtain the same range of detection limits (data not shown). The interfering effect of this particular RP phase was also observed and the sequence in which the spots were placed on the plates played an important role. Spotting the amino acids solutions followed by the application of derivatization reagent required longer time to react with each other even up to 90 min (data not shown). Consequently, the obtained detection limits were higher than in the case of applying the opposite order of spotting. Hence, all the presented results were established using the latter order.

3.2. Detection

The optimum conditions of the improved method of detection using iodine-azide reaction for three PTC-amino acid derivatives (lysine, proline and arginine) were presented in the previous report [2]. In this paper, we have expanded the application of the method to the group of protein and some non-protein amino acids as their PTC-derivatives using iodine-azide procedure. All studied amino acids listed in Tables 1 and 2 were checked considering the optimum conditions for the detection method of PTC-derivatives and they remained the same as the previous ones [2].

It was established that using the mobile phases listed in Tables 1 and 2, in combination with the respective stationary phases, was satisfactory in providing the detection limits since the derivatization agents zones did not interfere with amino acids derivatives spots.

The comparison of the detection limits of PTC-amino acids achieved by using different detection systems (iodine-azide procedure, iodine chamber and UV) and silica gel stationary phase is listed in Tables 1 and 2. The presented outcome indicates that the iodine-azide method is the most favourable one. In general, the detection limits were established at pmol/spot for almost all of the analyzed amino acids PTC-derivatives using iodine-azide detection system.

Another advantage of iodine-azide procedure over other examined methods of detection was the quality of obtained chromatograms. Spots of all of the amino acids detected as PTC-derivatives with the proposed method were compact with sharp edges against the violet–grey background of the plate and provided an accurate measurement of R_F values.

Considering the RP-mode, the procedure of visualizing chromatograms was modified. Due to the decreased wettability of this phase, the step involving spraying the plates with azide solution was excluded. However, the requirement of the presence of azide ions was met by incorporating the sodium azide solution into the mobile phase. The optimum conditions of this phase were established during experimental work (acetonitrile–sodium azide solution (4%, 2% starch, pH 6.5), 2:8 (v/v)). It should be stressed that not only did the modification bring about no changes to the quality of the detection process in comparison with NP-mode, but it simplified the procedure, too. The obtained detection limits are summarized in Table 1.

4. Conclusion

The discussed results confirm the abilities and the beneficial effects of phenyl isothiocyanate in the TLC combined with iodine-azide procedure of detection. The proposed derivatization methods and detection allow sensitive examination of amino acids. Moreover, it is less complicated, with inexpensive, readily available chemicals and with shorter time analysis compared with Edman procedure [3,4]. This method could be regarded as the excellent support for amino acids analysis with possible application for separation.

Acknowledgement

This work was supported by Grant No. 505/678 from the University of Łódź, Poland.

References

[1] S. Blackburn, Amino Acids Determination: Methods and Techniques, Marc Dekker, New York, 1978.
[2] R. Zakrzewski, W. Ciesielski, D. Kaźmierczak, J. Sep. Sci. 26 (2003) 1063.
[3] R. Zakrzewski, W. Ciesielski, D. Kaźmierczak, J. Liq. Chromatogr. Related Technol. 25 (10/11) (2002) 1599.
[4] P. Edman, Acta Chem. Scand. 4 (1950) 33.