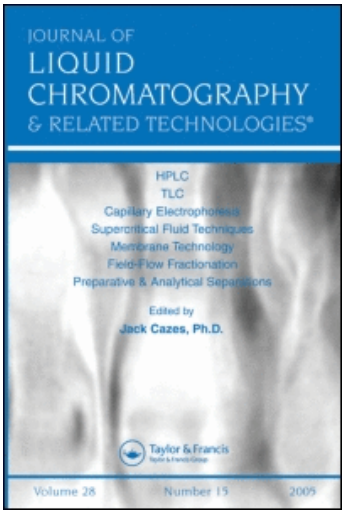

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Application of the Iodine-Azide Procedure for Detection of Biogenic Amines in TLC

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Application of the Iodine-Azide Procedure for Detection of Biogenic Amines in TLC

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Abstract: The iodine-azide reaction was employed for TLC detection of sulphur containing derivatives of biogenic amines. 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 was incorporated into the mobile phase and then exposed to iodine vapour. The spots became visible as white spots on violet grey background. The results of the detection limits proved to be advantageous to other commonly used detection techniques (UV and iodine chamber). The attempt to separate the mixtures of phenyl thiocarbamyl biogenic amines was also made.

Keywords: Biogenic amines, Phenyl isothiocyanate, Iodine-azide reaction, Thin-layer chromatography (TLC)

INTRODUCTION

Several biological studies on biogenic amines have proven their crucial role for all living organisms of different evolution levels. These organic bases are formed in plants, animals, and microorganisms as well as in food during storage.[1] Apart from their essential importance for biological cycles, biogenic amines pose a toxicological threat when ingested in large amounts.[2,3] Since the concentration of these compounds in tissues, with few exceptions, is rather low there is a necessity for the sensitive

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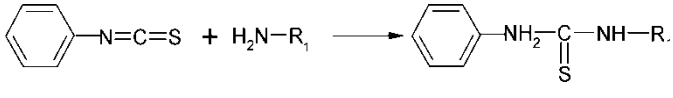
determination. Among the numerous techniques recommended for biogenic amines analyses, TLC[4–6] and HPLC[7–9] have been the most frequently chosen. Although HPLC preferred status is indicated, TLC is still a competitive method, owing to recent developments. Simplicity, convenience, and cost effectiveness makes TLC an efficient analytical tool for biogenic amines determination.

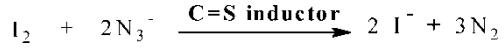
Due to their high polarity, biogenic amines tend to streak during the chromatographic development. To overcome this obstacle, a conversion to derivatives is usually applied. As a result, amines acquire properties that facilitate the chromatographic analysis. There is a wide range of derivatization reagents for biogenic amines determination.[10] Ninhydrine[11–13] and fluorescamine[14,15] are frequently used as post chromatographic reagents. Among prechromatographic derivatization agents, dansyl chloride[16–18] is the one most commonly used. However, the application of ninhydrine and fluorescamine is restricted to primary amines (a ninhydrine reaction with secondary amines gives the yellowish product with decreased sensitivity). In the case of dansyl chloride, the formation of side products during the procedure causes some difficulties. There is also a group of isothiocyanates that form both coloured[19,20] and fluorescent derivatives.[10] The aim of this paper is to present the application of phenyl isothiocyanate (PITC) as a potential derivatization reagent for biogenic amines. This agent reacts rapidly with primary and secondary amines under basic conditions to give thiourea derivatives. The PITC compound was extensively used in Edman degradation of proteins, coupled with identification of phenyl thiohydantoin (PTH) derivatives of amino acids.[21] However, PTH derivatives identification is almost exclusively applied to studies on protein structures and is not suitable as a general method for determination of amino acids. For this purpose, phenyl thiocarbamyl derivatives (PTC) are considered as the more effective ones. The derivatization reaction proceeds as follows in Scheme 1.

Since the PTC- as well as PTH-amino acid derivatives enable UV detection, these compounds were in an analytical range of interest. However, our purpose of using PITC derivatization was to apply the iodine-azide detection system. This system is based on the iodine-azide reaction. The reaction is induced by sulphur compounds as it is depicted in Scheme 2.

Its analytical application involves 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 the induction properties of bivalent sulphur compounds, the spots become visible as white spots on a violet-grey background. PITC derivatization results in obtaining inductors of the



Scheme 1.



Scheme 2.

presented reaction within a short analysis time, and stable final products of both primary and secondary amines. The procedure involving this derivatization together with the iodine-azide detection proved to be beneficial for the amino acids analyses.[22,23] Thus, it was interesting to extend the research to biogenic amines. Moreover, up to this point there have been no reports considering in situ PITC derivatization of biogenic amines in TLC.

The main objective of our work is to present results of introducing the iodine-azide reaction into the biogenic amine analysis using TLC. The chemical structures of the studied compounds are listed in Figure 1. It is also important to indicate its favourable detection limits in comparison with other visualizing techniques commonly used in TLC laboratory practice (UV_{254} and iodine chamber).

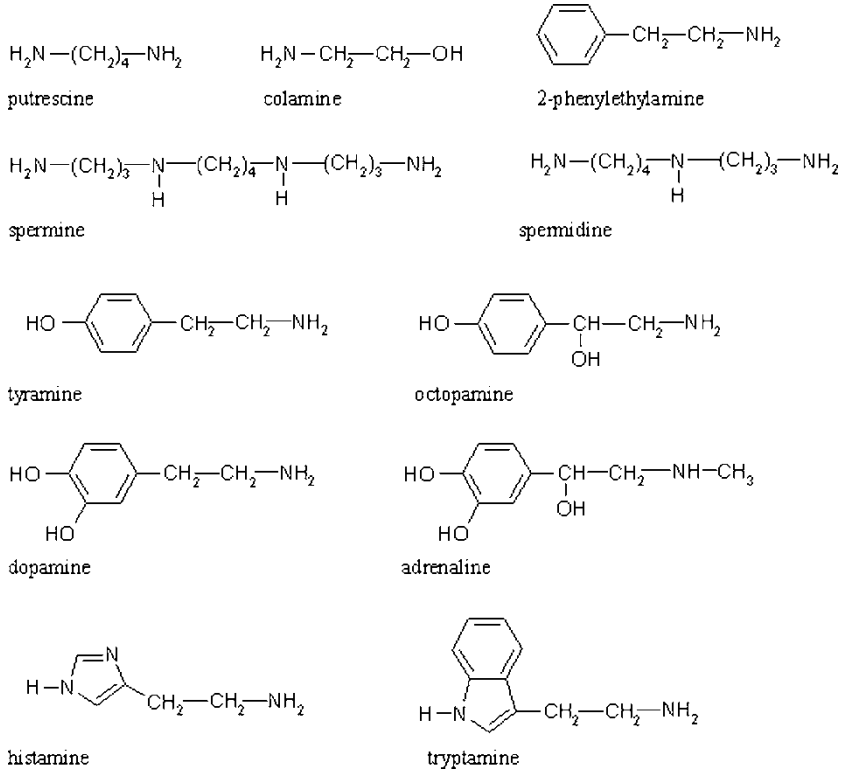


Figure 1. Molecular structures of the studied biogenic amines.

EXPERIMENTAL

Solutions and Reagents

All biogenic amines, PITC, sodium azide, methanol, ethanol, 2-propanol, dioxan, toluene, dichloromethane, acetone, hexane, and acetonitrile were purchased from Sigma-Aldrich (Steinheim, Germany) or LAB-SCAN Analytical Sciences (Dublin, Ireland).

Stock biogenic amines solutions: a specified amount of a particular reagent was dissolved in water to obtain the final concentration of 0.1 mol L^{-1}. In the case of adrenaline, a suitable quantity was dissolved in 2 mL of 0.1 mol L^{-1} sodium hydroxide solution and then diluted to the volume of 10 mL. Considering a tryptamine solution, a specified amount was dissolved in 2 mL of 0.1 mol L^{-1} hydrochloric acid and then diluted to the volume of 10 mL. Standard biogenic amines solutions: a specified volume of stock biogenic amines solution (1000 μL, 100 μL, 10 μL) was diluted to 10 mL with water.

Derivatization solution: PITC of 1 mL 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 organic solvents were mixed (for details see Table 1), 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: aqueous starch solution (25 mL) containing 1 g starch was added to 20 mL aqueous sodium azide containing 2 g of this compound. Then, the mixture was adjusted to 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 sodium azide, and the mixture was adjusted to pH = 6.0 with 0.1 mol L^{-1} 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.

Procedure of Derivatization of Biogenic Amines

Procedure for Biogenic Amines Derivatization on the Chromatographic Plate (in situ)

The plates were spotted with 0.1–1.0 μL of biogenic amine solution and air-dried. The derivatization solution was applied to the same starting zone with a 1 μL pipette (Brand, Wertheim, Germany). As a result, the reagent solvent (PITC) caused the sample to spread outward. The starting zone was covered with a glass strip for an appropriate time (for details see Table 1)

Table 1. Optimum conditions of PTC-biogenic amines determination

PTC-biogenic amine	Derivatization time (min)	Mobile phase (NP)	R_F (NP)	
			TLC	HPTLC
2-Phenylethylamine	20	Hexane–dioxane (1 : 1)	0.72	0.74
Tyramine	20	Hexane–dioxane (1 : 1)	0.50	0.48
Octopamine	20	Hexane–dioxane (1 : 2)	0.73	0.74
Dopamine	20	Hexane–dioxane (1 : 1)	0.13	0.11
Adrenaline	40	Hexane–dioxane-toluene (1 : 1 : 1)	0.57	0.56
Histamine	20	Hexane–ethanol (1 : 1)	0.13	0.14
Tryptamine	40	Hexane–dioxane (2 : 3)	0.76	0.78
Putrescine	20	Hexane–ethanol (1 : 1)	0.13	0.14
Spermidine	20	Methanol–dioxane (2 : 1)	0.03	0.02
Spermine	20	Methanol–dioxane (2 : 1)	0.03	0.03
Colamine	20	Hexane–ethanol (1 : 1)	0.78	0.77

to complete the prechromatographic derivatization. Subsequently, the plate was developed with a mixture of suitable solvents (for details see Table 1).

Procedure of Derivatization of Biogenic Amines in a Test Tube

The appropriate amount of standard biogenic amine solution together with 1 mL of derivatizing reagent [2-propanol-PITC-0.005 M phosphate buffer (pH 12) (7 : 1 : 1)] were placed in a stoppered tube on the magnetic stirrer for the derivatization time (for details see Table 1). After the reaction was completed, the sample was diluted to 5 mL with methanol. The plates were spotted with 1 μL of PTC-biogenic amine solution and developed with a mixture of acetonitrile and sodium azide solution (4%, pH = 6.5; 2% starch) (2 : 8, v/v).

Planar Chromatography

TLC silica gel 60 F_{254} aluminium sheets (Merck, Darmstadt, Germany; 10 × 5 cm, 0.2 mm thick layer), 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 the detection limits of PTC-biogenic amines. TLC RP-18_{254s} aluminium sheets (Merck, Darmstadt, Germany; 10 × 5 cm, 0.2 mm thick layer) were used for the determination of the detection limits of PTC-biogenic amines. A horizontal DS-Chamber (Chromdes, Poland) was applied for the developing process. The chamber was ready for use 30 min after the solvent had 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 Table 1.

Separation

The amount of each biogenic amine in the separation mixtures corresponded to a 3-fold excess of the established detection limit.

Normal-Phase Chromatography of Biogenic Amines Detected as PTC-Derivatives in Mixture

We selected two 4-component groups of biogenic amines: 1) colamine, tryptamine, histamine, spermine, and 2) colamine, tryptamine, putrescine, spermidine, to carry out the separation. The appropriate mixture of biogenic amines prepared in a water solution was applied on the plate (HPTLC) in 1 μL volume. Derivatization reagent: 2-propanol-PITC-0.005 M phosphate buffer (pH 12, 7 : 1 : 1) was spotted to the sample starting zone on the plate in 1 μL volume. In the next step, the plate was placed in a glass chamber. The reaction time was 40 min and, subsequently, the plates with mixture 1 and mixture 2 were developed in ethanol-dichloromethane (2 : 1, v/v) and ethanol-hexane (1 : 1), respectively. The iodine-azide procedure described below was used as the method of detection.

Reversed-Phase Chromatography of Biogenic Amines Detected as PTC-Derivatives in Mixture

In this case, the derivatization in the tube was applied. The appropriate amount of each biogenic amine (2-phenylethylamine, histamine, tyramine, octopamine) was taken to prepare the solution, and 500 μL of derivatization reagent (2-propanol-PITC-0.005 M phosphate buffer (pH 12), 7 : 1 : 1) was added. The stoppered tube was placed on the magnetic stirrer. The reaction time was 40 min. After the derivatization was completed, 1 μL of the solution was spotted on the plate (RP-TLC), as well as the standard solutions of each PTC-biogenic amine. The development was performed in acetonitrile-sodium azide solution (4%, pH = 6.5; 2% starch) (2 : 8, v/v). The iodine-azide procedure described below for reversed-phase chromatography was used as the method of detection.

Detection of PTC-Biogenic Amines

Detection of PTC-Biogenic Amines in Normal-Phase TLC

The iodine-azide procedure follows. The air dried developed plates were sprayed with a freshly prepared mixture of 4% sodium azide and 0.5% starch solution adjusted to pH = 6.0 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. Their stability lasted for several minutes.

The Iodine Procedure

The air dried 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

The air dried developed plates were visualized under a UV lamp (254 nm) using TLC or HPTLC plates with a fluorescent indicator.

Detection of PTC-Biogenic Amines in Reversed-Phase Chromatography

After the development completion the 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. Their stability lasted for several minutes.

RESULTS AND DISCUSSION

Derivatization

To perform a successful analysis of chosen PTC-biogenic amines it was essential to thoroughly examine the conditions of the derivatization process. Following the pattern of in situ PITC amino acid derivatization,[23] the research into the biogenic amines was completed. The main goal was to establish optimal conditions considering PITC derivatization in situ on the normal phase TLC plates. The first aspect of this experiment was to find the most advantageous composition of the derivatization solution. Methanol, ethanol, 2-propanol, acetonitrile, and 1,2-dioxane were taken into consideration as organic solvents. In order to find the most sufficient buffer solution, phosphate buffer (pH = 10–12), Britton-Robinson buffer (pH = 9–12), and triethylamine were examined. As a consequence, the final composition was

chosen: PITC - 2-propanol - 0.005 M phosphate buffer (pH 12) (1 : 7 : 1). The next point to consider was the derivatization time. Having examined the following range of derivatization times: 15–50 min, it was discovered that for almost all of the examined amines 20 min occurred to be enough to complete the reaction. The exceptions were histamine and putrescine for which it was necessary to lengthen the time to 40 min. The results are listed in Table 1. The elaborated conditions of the reaction were applied to in tube derivatization in RP-TLC and seemed to be favourable as well. The reason for a different (in tube) procedure in the case of RP-TLC was found during the experimental work. It was observed that the detection limits established on RP plates were significantly higher than the ones on NP plates during derivatization in situ (data not shown). The different interactions between the long carbonyl chains from RP and the molecules taking part in derivatization were supposed to interfere and inhibit the reaction. The assumption was supported by the results, since the detection limits of PTC-biogenic amines obtained during the derivatization in tube and determined on RP are lower than the ones in situ, and reached a comparable level in relation to PTC-derivatives on NP in situ. The data are presented in Table 2.

Detection

In order to perform a successful determination of detection limits of PTC-biogenic amines it was necessary to find a suitable mobile phase. Several

Table 2. Detection limits of biogenic amines detected as PTC-derivatives (pmol/spot) in the various methods of detection and stationary phases

	NP (In situ)				RP (In tube)	
Biogenic amines derivatives	Iodine-azide procedure		Iodine	UV_{254}	Iodine-azide procedure	
	TLC	HPTLC	HPTLC	HPTLC	(TLC)	R_F
2-Phenylethylamine	100	70	700	700	140	0.06
Tyramine	100	70	700	700	80	0.68
Octopamine	40	20	300	300	40	0.83
Dopamine	400	100	800	900	450	0.87
Adrenaline	200	110	600	600	260	0.29
Histamine	40	10	500	500	30	0.24
Tryptamine	100	85	750	750	100	0.25
Putrescine	200	100	800	1000	250	0.96
Spermidine	200	70	600	600	220	0.96
Spermine	100	60	400	400	120	0.96
Colamine	100	40	600	600	80	0.24

solvent systems were examined, including methanol, ethanol, dioxane, hexane, toluene, acetonitrile, dichloromethane, and their different mixtures. The main criteria applied during the search was that there is no tailing effect of PTC-amines as well as no interfering effect of PITC spot on respective biogenic amines derivatives spots. The results are presented in Table 1.

Although these mobile phases were optimum for determination of detection limits for each amine alone, it was not possible to choose one of them as a solvent system to separate PTC-biogenic amines mixtures. However, a close analysis of R_F values of PTC-derivatives in other examined mobile phases enabled us to perform the separation for the chosen amines. It was possible to resolve three four-component mixtures using NP- and RP-modes. The obtained resolution is depicted in Figure 2 and Figure 3.

Having established the derivatization procedure together with the favourable mobile phase the iodine-azide detection system was employed. Considering the application of this method, experiments were done to find the optimum conditions. The pH value (in the range of 5.5–7.0) of sodium azide solution was checked. 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. As to the outcome, the chosen value was 6.0 in NP and 6.5 in RP. Additionally, it was observed that there was no dramatic impact of the concentration of sodium azide solution within the range of 1–4% on detection limits. Consequently, the 4% sodium azide solution was applied.

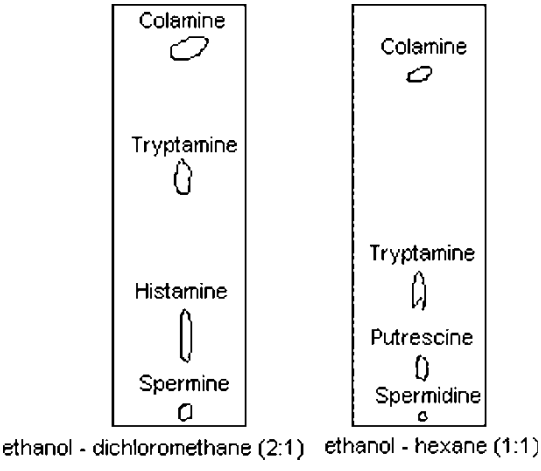


Figure 2. Separation of PTC-biogenic amines in NP-TLC.

Figure 3. Separation of PTC-biogenic amines in RP-TLC; acetonitrile-sodium azide (4%, pH = 6.5, 2% starch) (2 : 8).

The comparison of the detection limits of PTC-biogenic amines obtained by using different detection systems (iodine-azide procedure, iodine chamber, and UV) is listed in Table 2. The presented outcome gives the iodine-azide method the most advantageous position with the lowest detection limits. It is also important to stress that the widely used ninhydrine procedure resulted in the nmol range of detection limits (11–13) and proved to be less sensitive than the iodine-azide system with PITC derivatization (pmol detection level).

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

The careful study of the results in Table 2 confirmed the well known fact that high performance layers are slightly thinner than conventional layers (0.2 mm instead of 0.25 mm) and, thus, need smaller samples to show the same measuring result. Therefore, all of the detection limits determined on HPTLC plates are lower than the ones on TLC plates.

When the RP-mode is taken into account, the modification of detection must be stressed. The step involving spraying the plates with azide solution was omitted. The reason for this action was the decreased wettability of this phase. 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 pointed out 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 as well. The obtained detection limits at pmol level are summarized in Table 2.

CONCLUSION

Since the derivatization process of biogenic amines is crucial for their successful TLC analysis it is important to apply an effective procedure. The phenyl isothiocyanate reaction combined with the iodine-azide detection proved to be beneficial. It enabled selective and sensitive examination of biogenic amines on the pmol level. Moreover, it is less time consuming with simple analytical steps, during which the derivatization is performed directly on the plate (NP-TLC) or in tube with the simplified detection method (RP-TLC). Cost effectiveness should also be stressed, because the procedure relies on inexpensive equipment, as well as on readily available chemicals. The presented system provides an efficient tool in biogenic amines analysis and can be applied in daily routine laboratory practice.

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