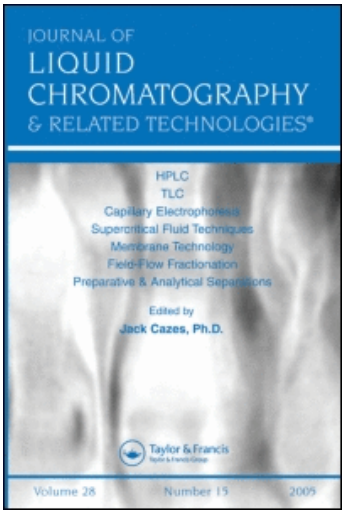

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Iodine-Azide Detection System for Dipeptides in Thin-Layer Chromatography

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Abstract: Dipeptides were derivatized with phenyl thiocarbamyl (PITC) to sulfur-containing compounds. The reaction was performed directly on the chromatographic plate in normal phase chromatography. The detection system was based on an iodine-azide reaction that was induced by sulfur compounds. The procedure involved spraying the developed plate with sodium azide and starch solution and, subsequently, exposing the plate to iodine vapor. Due to the catalytic effect of C═S bonds, white spots appeared on the violet-gray background. The method was found to be competitive with the other detection techniques and its sensitivity range was as low as pmol per spot.

Keywords: Iodine-azide reaction, Phenyl isothiocyanate, Dipeptides, Detection, Separation, Thin-layer chromatography

INTRODUCTION

Dipeptides are a group of biologically active compounds that exhibit numerous functional properties. They are known to serve as surfactants, antioxidants, hormones, as well as antimicrobial substances. In addition, there are some widely recognized physiological attributes of dipeptides such as buffering capacity, neurotransmitter activity, or enzyme modulation. They seem to contribute to biomedical research, therapeutic applications, and proteomic studies. Moreover, dipeptides are technologically important and may affect some

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physiochemical characteristics of different products and foodstuffs.[1] The essential role of these biomolecules in several fields is, to a great degree, accepted. As a result, there is a large need for fast, reliable, and sensitive methodologies capable of detecting dipeptides. A variety of different analytical procedures have been developed for this purpose and most of them are related to derivatization reagents that are applied to improve the overall performance. Often these chemicals are common for amino acids, peptides, proteins, or amino compounds. Reports from literature contain a great number of examples: o-phthalaldehyde,[2] related naphthalenedialdehyde,[3] fluorescein isothiocyanate,[4] fluorescamine,[5] dansyl chloride,[6] 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate,[7] 3-(4-carboxybenzoyl)-2-quinolinecarboxyaldehyde,[8] reagents selective for arginine-[9] and tyrosine containing peptides,[10] 9-fluorenmethoxycarbonyl chloride,[11] 4-(5,6-dimethoxy-2-phatalimidinyl)-2-methoxyphenylsulfonyl chlorides,[12] N-hydroxysuccinimidyl-α-(9-acridine)-acetate,[13] benzoyl chloride,[14] and nitrobenzoyl chloride.[15] The aforementioned peptide reagents have been employed mainly to thin-layer chromatography as a separation technique coupled with a ninhydrin detection system.[16–28] The above listed reagents are mainly fluorogenic and their application is restricted due to their incomplete reactions, highly fluorescent hydrolysates, their toxity, or limitations to primary amino groups. Although some of these methods reach a favorable level of sensitivity and selectivity, they involve a complex sample manipulation and expensive instrumentalization that often prove to be disadvantageous.

The intention of this contribution is to indicate a novel application area of iodine-azide reaction as a detection system for dipeptides using phenyl thiocarbamyl (PITC) derivatization in normal phase thin-layer chromatography. The procedure has already been successfully used in protein and non-protein amino acids,[29] biogenic amines,[30] and amphetamines.[31] It has proven to be considerably sensitive and reliable, as well as inexpensive and easy to follow. The phenomenon of the iodine-azide reaction induced by sulfur compounds has been described previously.[32] The current report deals with new experimental conditions for derivatization and detection of dipeptides in TLC. Additionally, it makes a comparison between detection limits obtained with different visualizing techniques that are common for TLC laboratory practice.

EXPERIMENTAL

Solutions and Reagents

Chemicals

All dipeptides, phenyl iothiocyanate (PITC), sodium azide, ninhydrin, methanol, ethanol, 2-propanol, dichloromethane, acetonitrile, dioxan, were

purchased from Sigma-Aldrich (Steinheim, Germany) or LAB-SCAN Analytical Sciences (Dublin, Ireland).

Dipeptide Solutions

Each dipeptide stock solution was prepared at a concentration of 0.1 mol L^{-1} in water. The working solutions were prepared by diluting specified volumes of stock dipeptide solutions (1000 μL, 100 μL, 10 μL) to 10 mL with water.

Derivatization Solution

PITC, 1 mL, was added to 7 mL of 2-propanol and 2 mL 0.05 M phosphate buffer (pH 12).

Solvents Systems

Specified volumes of organic solvents were mixed (for details see Table 1).

Spraying Solution

Aqueous starch solution, 25 mL, containing 2.5 g starch was added to 20 mL aqueous sodium azide containing 2 g sodium azide. The mixture was adjusted to pH = 5.5 with 0.1 mol L^{-1} hydrochloric acid solution and diluted to 50 mL with water to obtain a 4% and 5% solution for sodium azide and starch, respectively. All solutions were prepared fresh daily.

Ninhydrin Solution

Ninhydrin, 0.2 g, was dissolved in 100 mL of ethanol.

Derivatization

Dipeptide solutions were spotted on the plate with a micropipette of the 0.1–1.0 μL range (Brand, Wertheim, Germany) and air dried. Subsequently, dipeptide dots were spotted with a derivatization solution by means of a 1 μL micropipette (Brand, Wertheim, Germany). The reagent solvent caused the sample to spread outward. The plate was placed in a chromatographic chamber for 30 minutes in order to reach the prechromatographic derivatization completion. Afterwards, the development was initiated with a mixture of suitable solvents.

Table 1. Mobile phases and respective R_F values for PTC-dipeptides determination in NP chromatography

	Mobile phase		R_F	
PTC-dipeptides	Iodine-azide detection	Ninhydrine detection	Iodine-azide detection	Ninhydrine[a] detection
Gly-Gly	Ethanol-dichloro-methane (2:1)		0.42	0.42
Ala-Gln	Methanol-dichloro-methane (1:1)		0.48	0.47
Pro-Leu	Ethanol-dichloro-methane (2:1)		0.40	0.60
Pro-Asp	Methanol-dichloro-methane (2:1)		0.65	0.32
Pro-Gly	Methanol-dichloro-methane (1:1)	Ethanol–water (7:3)	0.35	0.30
Leu-Pro	Ethanol-dichloro-methane (2:1)		0.28	0.56
Ala-Pro	Methanol-dichloro-methane (1:1)		0.68	0.45
Phe-Pro	Ethanol-dichloro-methane (2:1)		0.33	0.55
Val-Pro	Ethanol-dichloro-methane (2:1)		0.25	0.55

[a]Underivatized dipeptides detected.

Planar Chromatography

Chromatography was performed at room temperature on TLC silica gel 60 F_{254} aluminum sheets (Merck, Darmstadt, Germany; 10×5 cm, 0.2 mm thick layer) and HPTLC silica gel 60 F_{254} aluminum sheets (Merck, Darmstadt, Germany; 5×5 cm, 0.2 mm thick layer). The horizontal DS-Chamber (Chromdes, Poland) equilibrated for 20 min was applied to the process. The developing distances were: 8 cm (for TLC) and 4 cm (for HPTLC) with a starting line positioned at 1 cm (for TLC) and 0.5 cm (for HPTLC) from the edge of the plate by the use of the solvent systems indicated in Table 1.

Detection

The Iodine-Azide Procedure

The TLC-Sprayer (Merck) was utilized to spread the mist of the homogeneous sodium azide and starch mixture on the air dried plate. After, the plate was

exposed to iodine vapor for 5 s. Due to the catalytic effect of the C═S bond, white, stable for several minutes, spots on a violet gray background appeared.

The Iodine Procedure

The air dried developed plates were exposed to iodine vapor for 3 min. The spots became visible as brown spots on a yellow background.

The UV_{254} Procedure

The air dried developed plates with a fluorescent indicator were examined under a UV light (254 nm). The compounds extinguished the fluorescence of the plate background.

The Ninhydrin Procedure

In this case, the underivatized dipeptides were analyzed. The air dried developed plates were sprayed with ninhydrin solution with the TLC-Sprayer (Merck) and dried at a temperature of 110°C. As a result, the purple red spots on a slight pink background were observed.

RESULTS AND DISCUSSION

The objective of this study was to perform sensitive dipeptides detection methods as PTC-derivatives, using an iodine-azide reaction in normal phase TLC. The series of experiments were divided into two parts. The first one involved establishing optimal conditions for the derivatization process and the second one was connected with optimal conditions for detection itself. As a result, different parameters were taken under consideration that have an impact on both processes. The starting point for each part was the results obtained for amino acid analysis[29] and, if necessary, they were modified to increase the sensitivity.

Derivatization

A simple and rapid derivatization performed directly on the chromatographic plate was applied. The method involved spotting the derivatization reagent and dipeptide solution on one another, waiting for some time, and starting the development. There was no need for any special conditions (temperature, pressure, light exposure) to complete the reaction.

When it comes to the derivatization step, a certain group of parameters was examined (data not shown). First of all, the time necessary to complete the reaction on the plate was checked in the range of 10–40 min. As a

result, 30 min seemed to be sufficient for all dipeptides. The effect of an excess of derivatization reagent in the reaction mixture was also examined. It was found that a three-fold excess of PITC is enough to obtain sufficient detection limits with the studied compounds. Then, the composition of the derivatization reagent was taken into consideration. At this point we dealt with:

1. The kind of buffer – phosphate or Britton-Robinson – the choice was limited by the range of pH to examine (7–12).
2. pH value of the buffer – the range of 7–12 – in lower pH the reaction does not proceed and over the value of 12 the PITC hydrolyses.
3. The concentration of the buffer – the range of 10^{-3}M–10^{-2}M – the criterium of the homogeneity of the solution was considered.
4. The organic solvent – methanol, ethanol, propan-2-ol, dioxan, dichloromethane – the criterium of the homogeneity of the solution was considered.

Having analyzed the above mentioned factors, the optimum composition of the derivatization reagent was established (described in the experimental part) to ensure the maximum of sensitivity.

Detection

The detection step in the proposed procedure is dependent on several factors as well. To find the optimum conditions, these parameters were carefully checked.

The first thing to consider was the spraying solution. A number of experiments made to establish the optimal spraying solution included (data not shown):

1. pH of the sodium azide solution in the range of 5.5–8.0: Safety standards excluded values below 5.5 due to the emission of poisonous, volatile hydrazoic acid. Whereas, above 8.0 value the catalytic reaction was hampered as a result of hypoiodite formation that in turn was not the reagent of the iodine-azide reaction.
2. The concentration of sodium azide solution in the range of 0.1–5%: It was observed that below this concentration the iodine-azide reaction hardly proceeded. While, above 4% value no further changes were detected in detection limits.
3. The concentration of iodide ions in the range of $0–5 \cdot 10^{-3}$ M: The increase in iodide ions in spraying solution resulted in slightly increased detection limits of PTC-dipeptides. It is explained by the slower rate of the iodine-azide reaction in the presence of iodide ions.

4. The concentration of starch solution in the range of 0.5–6%: The improvement in the contrast between white spots and violet-gray background was observed with the increase in the starch solution concentration up to 5%. Above this value no further changes were detected.

In each case, the choice of the best parameter was determined by the lowest detection limit obtained as a result. Consequently, the optimal spraying solution was found (described in the experimental part).

As it is indicated in Table 1, different solvent systems were chosen to perform detection limit analyses of each PTC-dipeptide. They were selected through experimental work to ensure spots without tailing effects and with easy R_F determination, as well as to exclude any interference with the derivatization reagent spot. The developments of the plates in the chosen mobile phase were repeated at intervals of 24 hours for five consecutive days.

Table 2 presents detection limits established with different visualization techniques. The best results are obtained with the iodine-azide reaction detection system on pmol level per spot. The differences in values of detection limits favor the proposed procedure over other commonly used ones and make it very sensitive not only for TLC standards. Additionally, the interpretation of chromatograms with the iodine-azide detection is accurate and exact due to the sharp white spots on the violet gray background.

In the case of Pro-Asp, the relatively high detection limit was obtained (at hundred pmol per spot level; Table 2). The molecule of the dipeptide consists of two carboxylic group, which lowered the derivatization solution. Applying the new derivatization solution with sodium hydroxide solution of

Table 2. Detection limits of dipeptides detected as PTC-derivatives (pmol/spot)

	Iodine-azide procedure		Iodine	UV_{254}	Ninhydrine[a]
PTC-dipeptides	TLC	HPTLC	HPTLC	HPTLC	HPTLC
Gly-Gly	200	70	2000	2000	1000
Ala-Gln	200	100	1000	1000	100
Pro-Leu	4	2	20	20	500
Pro-Asp	100	80	1000	1000	200
	20[b]	10[b]	500[b]	500[b]	
Pro-Gly	20	10	100	100	300
Leu-Pro	20	10	600	1000	40
Ala-Pro	2	1	200	400	100
Phe-Pro	20	8	300	300	100
Val-Pro	20	8	1000	1000	100

[a]Underivatized dipeptides detected.

[b]*Derivatization solution:* 1 mL PITC was added to 7 mL of 2-propanol and 1 mL 0.1 M sodium hydroxide solution (pH 13) and 1 mL water.

pH = 13 (1 mL PITC was added to 7 mL of 2-propanol and 1 mL 0.1 M sodium hydroxide solution and 1 mL water), lowers the detection limits at several pmol/per spot level.

It is also important to stress, that the widely used ninhydrin procedure resulted in the nmol range of detection limits for dipeptide, containing proline and proved to be less sensitive than the iodine-azide system with PITC derivatization. The same relationship was observed for free proline.[33]

Interferences

In general, applying the iodine-azide detection system makes only sulfur (II) compounds visible in chromatograms. However, three groups of additional spots may be found due to certain compounds: 1) iodine-azide reaction inductors (e.g., cysteine or cystine), 2) compounds which react with iodine under experimental conditions (e.g., ascorbic acid), 3) compounds that react with PITC to obtain PTC derivatives (e.g., amino acids).

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

CONCLUSION

The procedure based on the PITC derivatization and the iodine-azide detection system that was successful for amino acids, biogenic amines, and amphetamines proved to be applicable to dipeptides as well. The established detection limits indicate great sensitivity of the method. The ability of the iodine-azide procedure to detect as little as pmol quantities per spot of PTC-dipeptides makes it very competitive with other TLC methods. The usual level of detection in TLC is nmol per spot. In some cases, it could be lowered by fluorogenic reagents application, however, the procedures themselves are complicated and require sophisticated sample treatment. In contrast, the proposed procedure is simple, inexpensive, and short, with easy to follow analytical steps. It was applied to a certain group of dipeptides to show its ability as an efficient tool in analysis.

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