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Application of improved iodine–azide procedure for the detection of thiouracils in blood serum and urine with planar chromatography

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Abstract

The application of iodine–azide reaction for the determination of thiouracils in thin-layer chromatography and high-performance thin-layer chromatography 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 s. The detection limits were established at pmol level. The factors depending on the detection limits were described. A comparison of iodine–azide tests reaction with other procedures is presented. The developed method was applied to detection of thiouracils in blood serum and urine. The possibility of detection of a thiouracils mixture was demonstrated.

Keywords: Iodine–azide reaction; Thiouracils

1. Introduction

Thiouracils derivatives have medical and biological properties. They inhibit nucleic acid metabolism [1,2]. Moreover, they also inhibit the formation of thyroid hormones and are used for the treatment of hyperthyroidism and Basedow's disease [3]. These thiols are applied to determinations of varied elements and in preparation of drugs and pesticides.

The use of the thyreostatic (i.e. thiouracils) drugs for promoting animal growth is prohibited in EU member states because not only could thyreostatic drug be harmful to human health but the meat derived from animals which were treated with such drug is lower quality. Because of the wide application of these compounds and control of application of growth promotors there was a need to develop selective and sensitive method of determination and/or detection of sulphur compounds.

There are numerous methods of detection of sulphur compounds, most being based on chromatography-related techniques. Most of the procedures cited in literature employ general rather than specific detection reagent for sulphur compounds. Considering this, the application of the iodine-azide reaction:

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

selectively induced by sulphur compounds, offers the opportunity for selective and sensitive detection technique. This method bases on visual observation of the plates after spraying of freshly prepared solution of sodium azide and iodine solution [4–6]. Sometimes the starch solution was applied [7,8]. As

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a continuation of this research, the improved procedure of detection of thiouracils in planar chromatography was presented. The proposed method can be applied to screening test of thiouracils in biological samples as well as in reaction mixtures where these thiols were used as reactant (synthesis of drugs and pesticides, in vulcanisation processes or photography). We focused on the methyl, propyl, and benzyl derivatives of 2-tiouracil and we elaborated the new detection methods in biological samples, since there are numbers of applications in medicine.

2. Experimental

2.1. Materials and apparatus

Thiouracils compounds were obtained from Aldrich or Fluka. Methylthiouracilum (Polfa-Kraków), Basdene (Laboratoires DOMS-ADRIAN), Propycil 50 (Kali-Chemie, Pharma, Hanower, Germany), Propyl-Thiouracil (Lederle AHP, Schweiz), Tyreostat II (DR. Herbrand KG) tablets were used.

The plates were developed in horizontal DS-Chamber (Chromades, Poland) and sprayed with a TLC-sprayer (Merck).

2.2. Solution and reagents

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

2.2.1. Solution for determination of induction coefficients

A 0.1 mol/l aqueous iodine solution containing 25 g/l of potassium iodide. A 2×10^{-2} mol/l aqueous sodium arsenite solution.

2.2.2. Reaction solution

The aqueous solution containing 20 g/l sodium azide buffered with hydrochloric acid to an appropriate pH.

2.2.3. Solution for planar chromatography

For the sodium azide solution, 20 ml 4% (w/v) aqueous sodium azide solution was added to 2 ml of 0.5% aqueous starch solution and adjusted to the appropriate pH with 0.1 *M* hydrochloric acid solution. A 0.1% (w/v) aqueous potassium permanganate solution was also prepared. Methanolic solutions of thiols were obtained by dissolving a specified amount of particular reagent in the suitable quantity of a solution of sodium hydroxide and adjusted with methanol. All solutions were prepared fresh daily and stored in a cold, dark place.

2.3. Determination of the inductive effect on the iodine–azide reaction

The efficiency of sulphur compounds as inductors in 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 was performed as in Refs. [9,10] or was adopted from Ref. [11]. 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 the inductor. To the stirred solution, 2 ml of 0.1 mol l^{-1} iodine solution was added and flask tightly locked with a stopper. From this moment, reaction time, 2 min was measured. The excess of iodine was then back titrated by means of 2×10^{-2} mol l^{-1} sodium arsenite solution in the presence of starch indicator. In the same way, the solution was titrated in the absence of inductor (blank).

2.4. 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 HPTLC RP-18 (5×5 cm, 0.2 mm thick layer) were used for all chromatographic experiments. The plates were spotted 1 cm (in TLC) or 0.5 cm (in HPTLC) from the edge of the plate (distances between spots was 1 cm) with an appropriate amount of compound (deposition area ≈0.2 cm^2) with 0.1–1 μl pipette (Brand) and developed using a horizontal DS-

Chamber (Chromdes, Poland) that was saturated with solvent vapour for a distance of 8 cm (for TLC) or 4 cm (for HPTLC) with methanol and then air dried with hair dryer. Spots were located by visualization with improved iodine–azide, iodine, UV_{254} detection, permanganate procedures. The detection limits were obtained in six experiments.

2.4.1. Detection of thiouracils by the improved iodine–azide procedure (I_2–N_3)

The developed plates were sprayed with a freshly prepared mixture of sodium azide and starch solution adjusted to a proper pH and were exposed to iodine vapour for 5 s. Due to the catalytic effect of the C–S or C=S bond, the spots became visible as white spots on a violet-grey background and they start to disappear after 20 min.

2.4.2. Detection of thiouracils by the non-improved iodine–azide procedure [4]

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.

2.4.3. Detection of thiouracils by the iodine procedure (I_2)

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

2.4.4. Detection thiouracils by the UV_{254} procedure (UV_{254})

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

2.4.5. Detection of thiouracils by the permanganate procedure ($KMnO_4$)

The developed plates were sprayed with a freshly prepared permanganate solution. The spots became yellow on a violet background.

2.5. Analytical application of developed procedure

Developed procedure was applied to detect thiouracils in blood serum, urine and drugs. The possibility of thiouracils detection in varied chromatographic system was also shown.

2.5.1. Detection in blood serum

2.5.1.1. Procedure I. A 1-ml aliquot of serum with appropriate amount of thiouracils was placed in the conical flask and fixed up to 5 ml with methanol. The precipitation was filtered. The filtrate was then evaporated to dryness. The residue was treated with 0.5 ml 12.5 *M* ammonium solution. The standards of thiouracils in 12.5 *M* ammonium solution and blank of serum were prepared. The 1-μl of the solutions were spotted on the HPTLC plates, which were developed for a distance of 4 cm with methanol.

2.5.1.2. Procedure II. A 1-ml aliquot of serum with appropriate amount of thiouracils was placed in the conical flask and fixed up to 5 ml with methanol. The precipitation was filtered. The 1-μl of the filtrate, standard and blank solution were spotted on the HPTLC plates.

2.5.2. Detection in urine

A 2-ml aliquot of urine with appropriate amount of thiouracils was placed in the conical flask and fixed up to 5 ml with methanol. If necessary the precipitation was filtered. The 1-μl of the filtrate, standard and blank solutions were spotted on the HPTLC plates.

2.5.3. Detection in drugs

Solution of 6-methyl-, or 6-propyl-, or 6-benzyl-2-thiouracil were spotted on the TLC or HPTLC RP-18 plates which were developed for a distance of 8 cm (for TLC) or 4 cm (for HPTLC RP-18) with methanol. The R_f value for thiouracils in drugs is in accord with the one for the thiol standards.

3. Results and discussion

3.1. Separation

The R_f data (TLC and HPTLC) are presented in Table 1. There was no need to fine new chromatography systems because several different ones had been found [12–14]. Slight differences in R_f value of

Table 1
HPTLC analysis of mixture of thiouracils (developed distance: 4 cm)

Mixture of compounds	Spotted amount [pmol/spot]		Solvent	R_f	
	I_2–N_3 [a]	UV_{254}		I_2–N_3 [a]	UV_{254}
2-Thiouracil	6	100	Methanol–chloroform	0.16	0.15
6-Methyl-2-thiouracil	1.5	120	(1:15, v/v)	0.21	0.20
6-Propyl-2-thiouracil	5	200		0.31	0.30
6-Benzyl-2-thiouracil	5	200		0.37	0.35

[a] Improved iodine–azide procedure.

thiouracils in biological samples result from different way of preparation of samples and pH of those samples.

The possibility of detection of a thiouracils mixture was also shown. The results are presented in Table 1.

3.2. Detection

The detection limits for thiouracils, using different detection systems are summarised in Table 2. In the proposed, improved iodine–azide methods, the detection limits are the lowest in comparison to applied common detection methods of the sulphur compounds.

Cysteine, cystine and glutathione are natural compounds of blood and other biological materials. Their detection limits are 4, 2.5 and 0.8 nmol/spot, respectively (chromatographic system: silica gel; methanol–12 *M* ammonium, 90:15, v/v). Values of R_f for cysteine, cystine and glutathione are nearly 0 when methanol was used as mobile phase. The thiols do not interfere in detection of the thiouracils in biological materials owing to low sensitivity of the iodine–azide procedure and different R_f. Applications of the iodine–azide procedure in detection of

Table 2
Detection limits of sulphur compounds in TLC and HPTLC

Compound	F_i [b] [pH]	Method	Detection limit [pmol/dot]				R_f
			I_2–N_3 [a]	I_2	UV_{254}	$KMnO_4$	
2-Thiouracil	1300	TLC	3	100	260	500	0.76
	[5.57]	HPTLC	2	70	100	300	0.77
6-Methyl-2-thiouracil	1600	TLC	2	300	500	1200	0.78
	[5.57]	HPTLC	0.8	100	120	600	0.72
5-Methyl-2-thiouracil	760	TLC	4	500	600	600	0.78
	[5.57]	HPTLC	3	100	120	300	0.80
5-Carboxy-2-thiouracil	520	TLC	3	200	400	1000	0.80
	[5.57]	HPTLC	2	80	200	400	0.80
6-Benzyl-2-thiouracil	1850	TLC	1	400	500	1500	0.79
	[5.57]	HPTLC	0.7	100	200	500	0.81
6-Propyl-2-thiouracil	1850	TLC	1	400	700	1000	0.79
	[5.57]	HPTLC	1	100	200	350	0.81
6-Amino-2-thiouracil	2360	TLC	0.9	80	250	1000	0.71
	[5.57]	HPTLC	0.9	60	100	200	0.71
5,6-Diamino-2-thiouracil	263	TLC	10	500	1000	3000	0.74
	[5.57]	HPTLC	10	200	650	2000	0.75
6-Amino-5-nitroso-2-thiouracil	370	TLC	10	250	760	1000	0.77
	[6.90]	HPTLC	7	100	500	760	0.80

[a] Improved iodine–azide procedure.
[b] Induction time $t = 2$ min.

Table 3
Detection limits (HPTLC) of 2-thiouracils in serum in improved iodine–azide procedure

Compound	Sample					
	Blood serum				Urine	
	Procedure I		Procedure II		Detection limit [nmol/1 ml sample]	R_f
	Detection limit [nmol/1 ml sample]	R_f	Detection limit [nmol/1 ml sample]	R_f		
6-methyl-2-thiouracil	0.75	0.82	7.5	0.82	3	0.90
6-propyl-2-thiouracil	2.5	0.87	27.5	0.85	3	0.87
6-benzyl-2-thiouracil	2.5	0.87	37.5	0.85	3	0.85

thiols in serum and urine were summarised in Table 3.

3.3. Influence of the varied factors on detection limit

3.3.1. Influence of iodine and iodide ions

Table 4 shows the comparison between the non-improved procedure and the new improved one. The detection limits obtained by the improved procedure are lower than the ones obtained by non-improved procedure. The excess of iodine makes the detection limits higher in the iodine–azide procedure and makes the white spots vanish. The phenomenon does not generally occur when others visualisation techniques of TLC plates are applied. The excess of visualisation solution does not make detection limit higher. It is important and difficult to spray the plates with very finely divided spray solution for optimum staining of the TLC plates [4–8]. It is overcome with applying iodine vapour in proposed procedure.

Table 4
Detection limit (nmol/spot) of thiouracils in TLC

Spray solution	Compound		
	6-Methyl-2-thiouracil	6-Propyl-2-thiouracil	6-Benzyl-2-thiouracil
0.5 *M* NaN_3, 0.5 *M* KI, 0.5 *M* I_2 [a]	2	2	2
4% NaN_3, 0.5 *M* KI, 0.5% starch solution	0.02	0.02	0.02
4% NaN_3, 0.5% starch solution[b]	0.002	0.001	0.001

[a] Non-improved procedure.
[b] Improved procedure.

The high concentration of KI in spraying solution (in non-improved procedure) depends on the detection limits to a low degree. When there are iodide ions in the same concentration in spraying solution (spraying solution with 0.5 or 1 *M* KI in improved procedure) the detection limits are not so high for 6-methyl-, 6-propyl- or 6-benzyl-2-thiouracil. Applying iodine vapour makes detection limits considerably lower.

Generally, the presence of iodide ions (in the range 1×10^{-3}–1×10^{-1} M concentration) makes the detection limits for all compounds (except 2-thiouracil) in spraying with iodine in potassium iodide solution procedure in TLC slightly higher. In the case of 6-amino-5-nitroso-2-thiouracil, the presence of iodide ions makes the detection limit in TLC lower. In the case of HPTLC, iodide ions do not influence detection limits of all compounds. The same phenomenon occurs in the case of 6-methyl-2-thiouracil in TLC. The addition of iodide ions to the reaction medium influences the induction coefficients of examined compounds [9]. The inductions coefficient and induction times increase with the increase of concentration of KI. That is why the induction time is so long in spectrophotometric determination [17]. In the case of chromatographic detection, iodine–azide reaction takes place with slower ratio in the presence of iodide ions and the white dots appear after slightly longer time, particularly in the case of higher concentration of KI.

The hampering effect of the iodide ions on iodine–azide reaction inducted with thiols or organothiophosphorus compounds is increased with increas-

ing iodide ions concentration [10]. The presence of iodide ions makes the detection limits higher for many compounds in spraying with iodine in potassium iodide solution procedure (Table 4). In this procedure, the spots corresponding to some organothiophosphorus compounds [4] became visible as brown ones on a yellow background. It is evident that, iodide ions hamper totally the iodide–azide reaction induced with these compounds. Applying the improved iodine–azide procedure for thiophosphorus compounds makes the detection limits greatly lower.

3.3.2. Induction coefficient

Induction coefficient shows how many times the consumption of iodine in the induced reaction is higher than in an iodometric one. From the analytical point of view, the value of induction coefficient is that it is the measure of sensitivity of the determination of a given sulphur compound in volumetric, coulometric, spectrometric and FIA determination [9,10,15–17]. Induction coefficient can be applied to prediction of detection limits in varied methods. Generally, it can be stated that the higher induction coefficient occurs the lower detection limit is. There is quite good correlation between the induction potency of thiouracils and their detectability in such analytical methods as coulometric, spectrometric and FIA determination and volumetric titration with starch as an indicator. There is no such correlation in the TLC method [4–6]. So varied factors influenced the course of the iodine–azide reaction and the detection limit in improved iodine–azide procedure were investigated. Fig. 1 illustrates good correlation between the induction potency (induction coefficient) of thiouracils and their detectability using the improved iodine–azide procedure.

3.3.3. pH of azide solution

The induction coefficient has been found to be dependent on pH for all thiouracils [9]. The pHs of spray solutions were summarized in Table 2. The influence of pH solutions on detection limits of 6-propyl- and 6-methyl-2-thiouracil are shown in Fig. 2. The use of solution whose pH is lower than 5.5 is not recommended because of the emission of

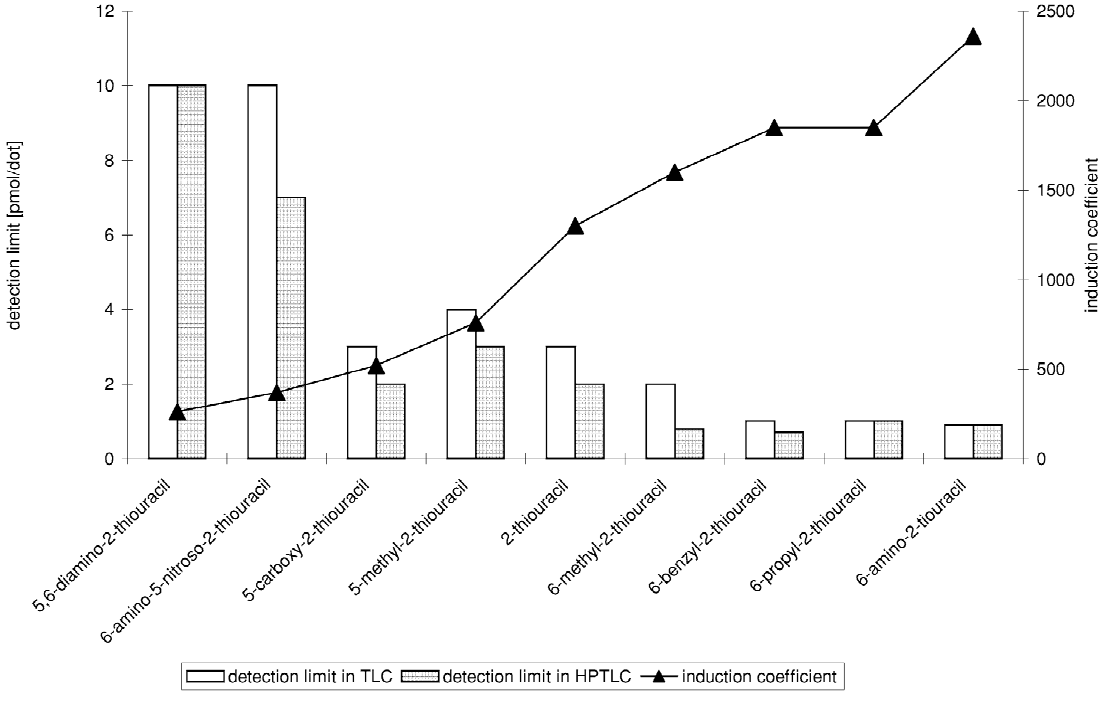

Fig. 1. The dependence of detection limits on induction coefficient.

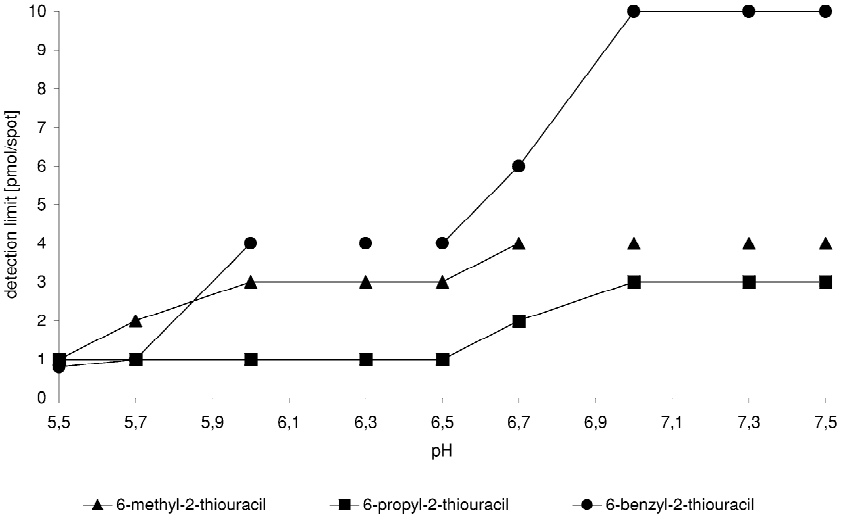

Fig. 2. The dependence of detection limits in HPTLC on pH of spraying solution.

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 iodine–azide reaction. In the case of thiouracils induction coefficient decreases with increase of the pH value (within range 5.5–8.0) [9,15–17]. Considering the influence of all parameters on the course of the iodine–azide reaction, pH 5.6 has been chosen as the most favourable pH for the measurement of the mixture of thiouracils (for details, see Table 2).

3.3.4. Concentration of azide ions

Increasing sodium azide concentration in 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, coulometric and FIA determination [9,10,15,16].

3.3.5. Induction time

The plates were 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. The exposure time is so short due to adsorption of iodine on the plate and vanishing of white dots. Appearance of white spots could take some time, due to the induction time conditioned by induction properties of the particular compound. It is time within which the induction reaction has finished. This is less than 2 min in each case. In the case of volumetric determination, the 2-min induction time is sufficient [9], in the case of spectrophotometric determination induction time is 20 min [17].

3.3.6. Chromatographic system

The detection limits obtained in HPTLC methods are similar to than those obtained in TLC using improved iodine–azide procedure. The differences in the obtained detection limits are greater that in the compared procedures.

4. Conclusion

In the light of the results presented here, only the improved iodine–azide detection system allows the selective and the most sensitive detection for thiouracils. The other applied detection systems, routinely used in the TLC (iodine, UV, permanganate 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. However, it cannot be applied in scanning densitometry since spots corresponding to the particular thiouracils are white and iodine gives

violet-grey background, which changes with time and disappears after 20 min.

References

[1] P.T. Cardeilhac, Proc. Soc. Exp. Biol. Med. 125 (1967) 692.
[2] J.A. Nelson, J.W. Carpenter, L.M. Rose, D.J. Adamson, Cancer Res. 35 (1975) 2875.
[3] D.J. Hurst, The Chemistry and Biochemistry of Pyrimidines, Purines and Pteridines, Wiley, New York, 1980.
[4] Z.H. Kudzin, A. Kotyński, P. Kiełbasiński, J. Chromatogr. A 588 (1991) 307.
[5] A. Kotyński, W. Ciesielski, Z.H. Kudzin, A. Okruszek, D. Krajewska, P. Lipka, J. Chromatogr. A 813 (1998) 135.
[6] Z.H. Kudzin, M. Saganiak, A. Kotyński, W. Ciesielski, J. Chromatogr. A 888 (2000) 335.
[7] E. Cherbuliez, B. Baehler, J. Rabinowitz, Helv. Chim. Acta 47 (1964) 1350.
[8] T. Cserháti, F. Ŏrsi, Period. Polytech. (Budapest) 26 (1982) 111.
[9] J. Kurzawa, Chem. Anal. (Warsaw) 32 (1987) 875.
[10] W. Ciesielski, Z.H. Kudzin, P. Kiebasiński, Talanta 41 (1994) 1493.
[11] Z. Kurzawa, Chem. Anal. (Warsaw) 5 (1969) 551.
[12] G. von Grissel, M. Schoal, Archiv für Lebensmittelhygiene 25 (1974) 8.
[13] L. Reio, J. Chromatogr. 68 (1972) 183.
[14] H.F. Brabander, R. Verbeke, J. Chromatogr. 108 (1975) 141.
[15] J. Kurzawa, Anal. Chim. Acta 173 (1985) 343.
[16] J. Kurzawa, Quim. Anal. 4 (1985) 117.
[17] W. Ciesielski, Acta Polon. Pharm. 44 (1987) 202.