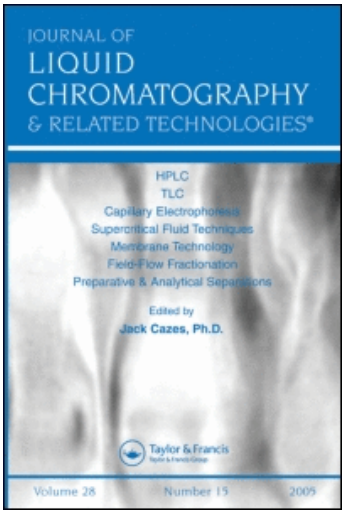

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Chromatography of Biologically Active Chlorides of (*E*)-N-*o*-(*m*- or *p*-)chlorobenzyl-γ-azastilbenols-2′(3′ or 4′)

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Abstract: results of investigations concerning optimization of chromatographic separation and determination of (*E*)-N-*o*-(*m*- or *p*-)chlorobenzyl-γ-azastilbenols-2′(3′ or 4′) are presented. Analyzed isomers show antimicrobial activity. In the investigation, were considered three stationary phases (octadecyl, octyl, and naphthylpropyl), two mobile phases (acetonitrile, dichloromethane), and various intensities of flow. The best selectivity ($\alpha_1 = 1.34$, $\alpha_2 = 1.47$) was obtained using naphthylpropyl column and 100% acetonitrile, as the mobile phase. An application of octadecyl phase, recommended by numerous analysts as standard, did not yield satisfactory results.

Keywords: (*E*)-Azastilbenoles, Isomers, Stationary phases: Aryl, Octyl and Octadecyl, HPLC

INTRODUCTION

(*E*)-Azastilbenoles, similarly as previously described azastilbenes, are derivatives of stilbene.[1–4] (*E*)-Azastilbenoles is the group of biologically active compounds, with a negative influence on microorganisms. Among these microorganisms, 9 species can be distinguished: Gram-positive cocci

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(*Staphylococcus aureus* 209P FDA, *Streptococcus faecalis* ATCC 8040), aerobic bacilli (*Bacillus subtilis* ATCC 1633), Gram-negative rods (*Escherichia coli* PZH 026B6, *Klebsiella pneumoniae* 231, *Pseudomonas aeruginosa* SR1), yeasts (*Candida albicans* PCM 1409 PZH), dermatophytes (*Microsporum gypseum* K_1), and moulds (*Aspergillus fumigatus* C_1). The biological activity of this group was already proven a few dozen years ago.[5–8]

Starting compounds, stilbenes, necessary to obtain derivatives of (*E*)-azastilbenoles, have structures of diphenylethylene. They can be synthesized in plants from coumaric acid and cinnamic acid. Stilbenes show properties of growth resistance of fungi and can destroy them. Some stilbenes belong to the group of synthetic estrogens (e.g., diethylstilbestrol) and often were used in medicines. Formerly, synthetic diethylstilbestrol was applied as an estrogen in menstruation disturbance. Now, because of the possibility of oncogenesis, induction is rarely used in human medicine.

In the 1960s, diethylstilbestrol was used in abortion prophylaxis.[9,10] However, its negative influence appeared several years later, because this medicament caused numerous cases of vaginal cancer. Careful investigation of diethylstilbestrol therapy consequences proved that it had caused an increase of formation defects in sexual-urinary systems and an increase of embryo mortality. Currently, in Poland, diethylstilbestrol is not used. The highest consumption of this drug is reported in China.[2]

However, it is often utilized in veterinary medicine as a hormonal stimulator; therefore its presence in meat products was found on frequent occasions. Depending upon the concentration, it can act estrogenically or anti-estrogenically.[6,7]

Natural stilbenes exhibit different activity. They neither induce cancer, nor cause harmful side effects, typical for synthetic stilbene. However, they are not recommended to healthy men, because they moderate the activity of testosterone and can distemper male attributes. On the other hand, stilbenes are prescribed to patients with an overgrowth of prostate, because they block the disadvantageous influence on the gland epithelium. Natural stilbenes, as estrogenic agents, can be used as a prophylaxis against the effects of menopause. Furthermore, they improve the state of the hair and nails.[7]

Three above mentioned isomers, considered in the investigation were obtained according to the scheme (Figure 1). Their most important

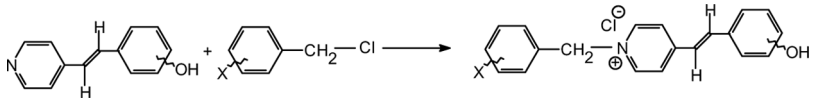

Figure 1. General scheme of reaction of analyzed compounds synthesis, where: X = *m*-Cl, *p*-Cl, *o*-Cl.[11]

Table 1. Chemical and physical data of compounds[11]

Compound	Yield (%)	M.p. °C	IR (KBr) (cm^{-1}) δ $_{CH=CH}$	UV_{max} (nm)	^{1}HNMR δ (ppm) $-CH_{2-}{}^{+}N$
o-Cl	87.7	233–236	955	402	5.93 s
m-Cl	78.3	227–230	970	402	5.81 s
p-Cl	67.0	240–243	985	402	5.81 s

properties are reported in Table 1. Analyzed isomers, belonging to the group of (*E*)-azastilbenols are derivatives of stilbenes. Their negative influence on the microorganisms is shown in Table 2. Verified biological activities of the analyzed compounds justify a need of elaboration of the conditions of the chromatographic separation and determination, i.e., the aim of this work. To the process of optimization were subjected three synthesized isomers: (*E*)-N-*o*-(*m*- or *p*-)chlorobenzyl-γ-azastilbenols-2′(3′ or 4′).

EXPERIMENTAL

HPLC Analysis of (*E*)-Azastilbenoles

Samples of chlorides of (*E*)-N-*o*-(*m*- or *p*-)chlorobenzyl-γ-azastilbenols-2′(3′ or 4′) (Figure 1)[11] were dissolved in DMSO (HPLC purity, Fluka AG, Buchs, Switzerland); to obtain a concentration at about $20\,\mu g \cdot mL^{-1}$. Analyses were performed at 402 nm and at temperature 20°C. Three stationary phases were examined: octadecyl (S. Witko – J.T. Baker, Łódź, Poland), octyl (S. Witko – J.T. Baker, Łódź, Poland),

Table 2. Antimicrobial activity of isomers. Minimal inhibitory concentration (MIC $\mu g \cdot mL^{-1}$)[11]

Compounds	Minimal inhibitory concentration (MIC) $\mu g \cdot mL^{-1}$								
	1	2	3	4	5	6	7	8	9
o-Cl	100	500	500	1000	1000	1000	>500	>500	>500
m-Cl	100	500	500	500	1000	1000	>500	>500	>500
p-Cl	100	100	500	100	500	1000	>500	>500	>500

1-*Staphylococcus aureus* 209P FDA, 2-*Streptococcus faecalis* ATCC 8040, 3-*Bacillus subtilis* ATCC 1633, 4-*Escherichia coli* PZHO 26B6, 5-*Klebsiella pneumoniae* 231, 6-*Pseudomonas aeruginosa* 5 R1, 7-*Candida albicans* PCM 1409 PZH, 8-*Microsporum gypseum* K_1, 9-*Aspergillus fumigatus* C1.

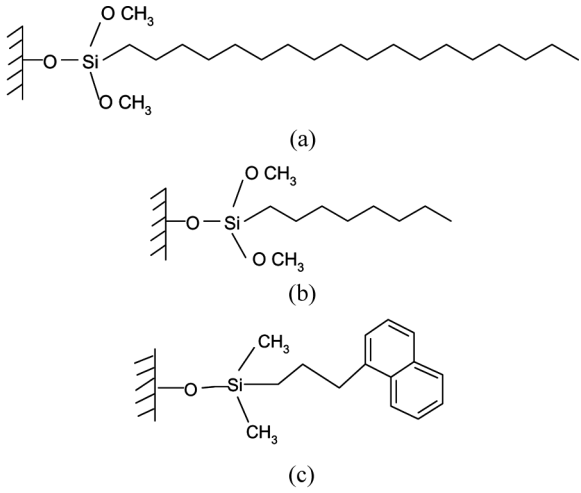

Figure 2. Scheme of chemically bonded stationary phases: (a) octadecyl (RP Si–C_{18}), (b) octyl (RP Si–C_8) and c) naphthylpropyl (RP Si–NAF).

and naphthylpropyl (RP Si–NAF, Figure 2).[12] Dimensions of steel columns were: for RP Si–C_{18} –250×4.6 mm, for RP Si–C_8 and RP Si–NAF–125×4.6 mm (Table 3). Two anhydrous systems of mobile phase were applied: acetonitrile and dichloromethane.

The (*E*)-N-*o*-(*m*- or *p*-)chlorobenzyl-γ-azastilbenols-2′(3′ or 4′) compounds were prepared by the method described in the literature.[11]

(*E*)-N-*o*-chlorobenzyl-γ-azastilbenols-2′(3′ or 4′): 13C NMR ($DMSOd_6$) δ (ppm) = 59.71, 115.95, 119.28, 123.06, 125.90, 127.87, 129.79, 130.16, 130.97, 131.64, 132.85, 142.03, 144.04, 154.00, 160.24. ^{1}H NMR, (DMSO-d_6) δ (ppm).[11] UV (DMSO): λ_{max} (Table 1).[11]

(*E*)-N *m*-chlorobenzyl-γ-azastilbenols-2′(3′ or 4′): 13C NMR ($DMSOd_6$) δ (ppm) = 60.86, 115.97, 119.33, 123.28, 125.94, 127.25, 128.51, 128.95, 130.15, 130.84, 133.41, 136.74, 141.82, 143.81, 153.79, 160.18. ^{1}H NMR, (DMSO-d_6) δ (ppm).[11] UV (DMSO): λ_{max} (Table 1).[11]

Table 3. Characteristics of bonded phase

Stationary phases	Type of packing	Carbon content (vol.%)	Length of column (mm)	Manufacturer of column
Octadecyl	RP Si–C_{18}	18.09	250×4.6	S. Witko – J.T. Baker
Octyl	RP Si–C_8	13.49	125×4.6	Home made
Naphthylpropyl	RP Si–NAF	16.10	125×4.6	Home made

(*E*)-N-*p*-chlorobenzyl-γ-azastilbenols-2′(3′ or 4′): 13C NMR ($DMSOd_6$) δ (ppm) = 60.79, 115.91, 119.23, 123.17, 125.86, 128.84, 130.05, 130.42, 133.36, 133.70, 141.75, 143.68, 153.72, 160.15. ^{1}H NMR, (DMSO-d_6) δ (ppm).[11] UV (DMSO): λ_{max} (Table 1).[11]

Apparatus

Chromatographic measurements were performed on a liquid chromatograph SPD-6A (Shimadzu, Kyoto, Japan) equipped with a gradient pump LC-6A, UV detector, a sampling valve Rheodyne (Berkeley, CA, USA), model 7125, with a 20 μL sample loop, and a Shimadzu C-R6 A data recorder. IR spectra were recorded on a Perkin-Elmer M180 spectrophotometer in KBr pellets. ^{1}H NMR spectra were determined on a Varian EM-360 (60 MHz) in DMSO-d_6 solution with TMS as internal standard. The UV/Vis spectra were recorded on a spectrophotometer DU-68 (Beckman, USA).

RESULTS AND DISCUSSION

Optimum results obtained during the chromatographic process are collected in Table 4. This paper contains the optimum of chromatographic separation and determination of three synthesized isomers: (*E*)-N *o*-(*m*- or *p*-)chlorobenzyl-γ-azastilbenols-2′(3′ or 4′). During selection of separation and determination conditions, two mobile phases (acetonitrile and dichloromethane) with various intensity of flow and three stationary phases (octadecyl – reference phase, octyl and naphthylpropyl) were tested. The octadecyl stationary phase is commonly considered as the standard phase in numerous determinations carried out by means of the high performance liquid chromatography method. At the beginning of the investigation, the octadecyl phase was applied in order to separate the mentioned isomers. Various intensities of a flow were used, but this phase was incapable of achieving satisfactory results (Table 4).

Different compositions of water containing solvent mixtures caused only an elongation of retention times (these results are not reported in the tables).

As a consequence of the failure connected with the octadecyl phase, the next step was an application of octyl phase. However, good separation of the isomers was not obtained. As a result, retention times were slightly shorter. At present, so called dedicated stationary phases are often used. They are aimed at the determination of a specific group of compounds, only. Because analyzed compounds contain aromatic rings in the structure, an application of aryl phase was reasonable. Consequently, stationary phase (RP Si–NAF) was used. Repeatedly it was

Table 4. Chosen dependence k′ for (*E*)-N *o*-(*m*- or *p*-)chlorobenzyl-γ-azastilbenols-2′(3′ or 4′) from on type of stationary and mobile phase. Chromatographic conditions: flow–1.0, 0.5 or 0.3 mL · min^{-1}, wavelength – 402 nm, temperature –20 °C

Type of stationary Phase	[a]Mobile phase/flow	k'_1 (*p*-Cl)	k'_2 (*m*-Cl)	k'_2 (*o*-Cl)	$\alpha_1 = k'_2/k'_1$	$\alpha_2 = k'_3/k'_2$
RP Si–C_{18}	Acetonitrile/1.0	2.50	2.51	2.52	1.00	1.00
	Acetonitrile/0.5	5.94	5.99	5.95	1.01	0.99
	Acetonitrile/0.3	10.52	10.65	10.77	1.01	1.01
	Dichlomethane	2.53	2.53	2.54	1.00	1.00
	Dichlomethane/0.5	5.97	6.01	6.02	1.01	1.00
	Dichlomethane/0.3	10.54	10.58	10.66	1.00	1.01
RP Si–C_8	Acetonitrile/1.0	0.62	0.62	0.56	1.00	0.90
	Acetonitrile/0.5	2.83	2.83	2.87	1.00	1.02
	Acetonitrile/0.3	3.94	3.94	4.16	1.00	1.06
	Dichlomethane	0.58	0.60	0.61	1.04	1.02
	Dichlomethane/0.5	2.81	2.82	2.83	1.00	1.00
	Dichlomethane/0.3	3.95	3.96	4.01	1.00	1.01
RP Si–NAF	Acetonitrile/1.0	0.36	0.19	0.38	0.53	2.00
	Acetonitrile/0.5	1.41	2.08	2.67	1.48	1.28
	Acetonitrile/0.3	2.04	2.98	3.45	1.46	1.16
	Dichlomethane	0.31	0.34	0.37	1.10	1.09
	Dichlomethane/0.5	1.39	1.86	2.73	1.34	1.47
	Dichlomethane/0.3	2.12	2.87	3.41	1.35	1.19

[a] In the Table are reported only optimum data of analyzed isomers separation.

shown that this type of phase is, first of all, designed for determination of π electron containing compounds.[12–16] In the chromatographic process carried out with a participation of such stationary phase and analyzed substances, interactions of π – π type predominate. Owing to increased selectivity in numerous determinations, separation was improved and retention times shortened.

Separation of investigated isomers was possible by the use of aryl stationary phases and acetonitrile (100%) or dichloromethane (100%) as the mobile phase. This effect is clearly shown in Figures 3–5. Optimum conditions of separation of analyzed isomers: (*E*)-N-(*o*-, *m*- and *p*-)nitrobenzyl-4′-hydroxy-3′-methoxystilbazole-4 are shown in Table 4. It was established that naphthylpropyl chemically bonded stationary phase is characterized by the highest selectivity ($\alpha_1 = 1.34$, $\alpha_2 = 1.47$). The best separation was obtained using this stationary phase, acetonitrile (100%), as the mobile phase. Results earlier obtained by means of octadecyl (reference) and octyl phase only showed an existence of isomers. Decreasing the flow to 0.1 mL · min^{-1}in these stationary phases

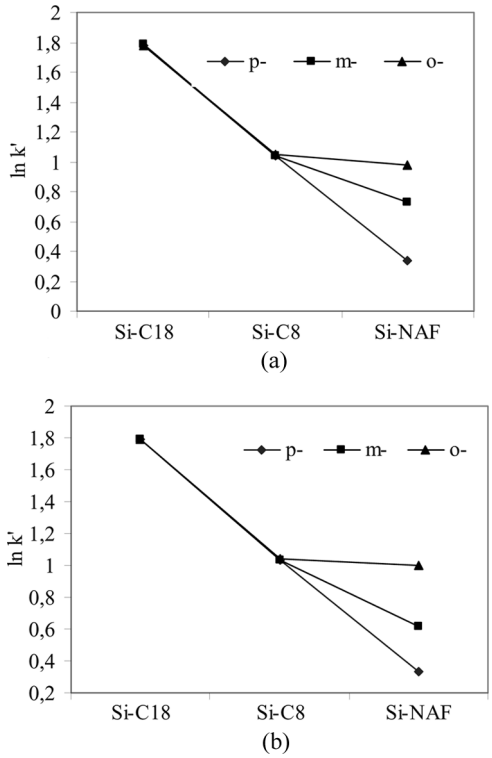

Figure 3. Effect of the separation of (*E*)-N-*o*-(*m*- or *p*-)chlorobenzyl-γ-azastilbenols-2′(3′ or 4′) with the use of stationary phases RP Si–C_{18}, RP Si–C_8 and RP Si–NAF. Mobile phase: (a) acetonitrile (vol. 100%), flow rate: 0.5 mL · min^{-1}, (b) dichloromethane (vol. 100%), flow rate: 0.5 mL · min^{-1}, detection –402 nm (see Table 4).

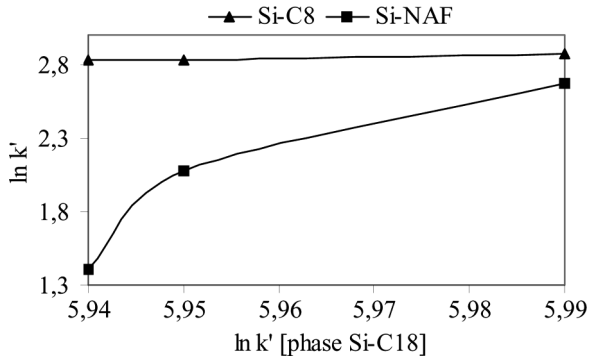

Figure 4. Dependence of ln *k*′ of the RP′ Si–C_8 and RP Si–NAF phases on ln *k*′ obtained for the octadecyl phase for (*E*)-N-*o*-(*m*- or *p*-)chlorobenzyl-γ-azastilbenols-2′(3′ or 4′).

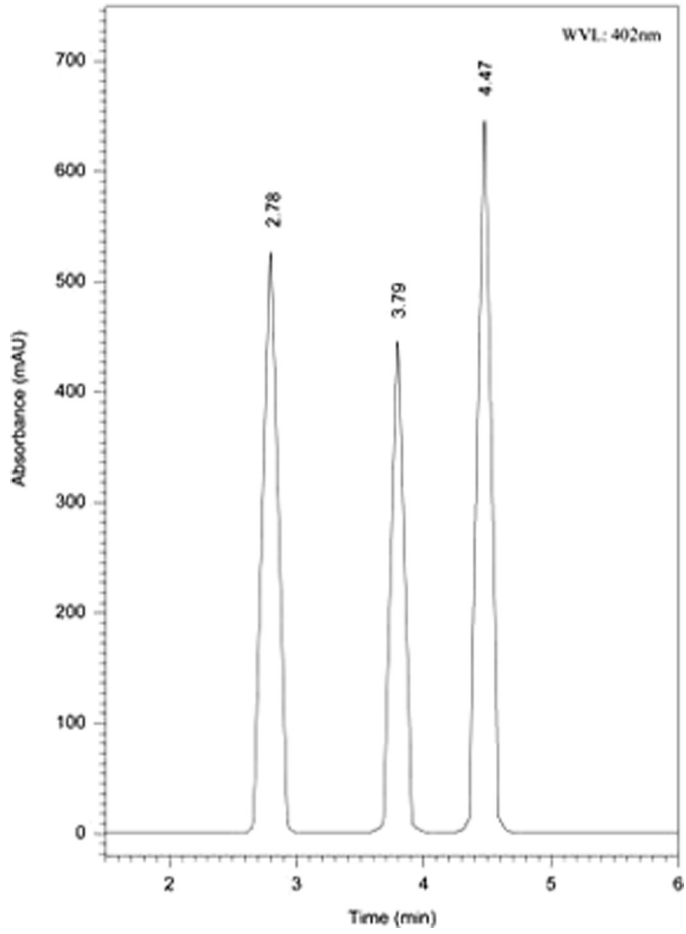

Figure 5. A chromatogram of separation of the chlorides of (*E*)-N-*o*-(*m*- or *p*-) chlorobenzyl-γ-azastilbenols-2′(3′ or 4′) on the stationary RP Si–NAF phase (*p*- 2.78 min., *m*-3.79 min., *o*- 4.47 min.). Mobile phase: acetonitrile (100 vol.%); flow –0.5 mL · min^{-1}, wavelength –402 nm, temperature –20°C.

caused only elongation of retention time over 25 min. Therefore the use of alkyl phases did not permit obtaining satisfactory results.

CONCLUSIONS

An application of alkyl stationary phases: octadecyl and octyl did not enable the desired separation of analyzed isomers: (*E*)-N-(*o*-, *m*- and *p*-)chlorobenzyl-γ-azastilbenols-2′-(3′ or 4′). In order to obtain perfect

separation of these isomers, by HPLC, the use of aryl (naphthylpropyl) stationary phase was necessary.

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