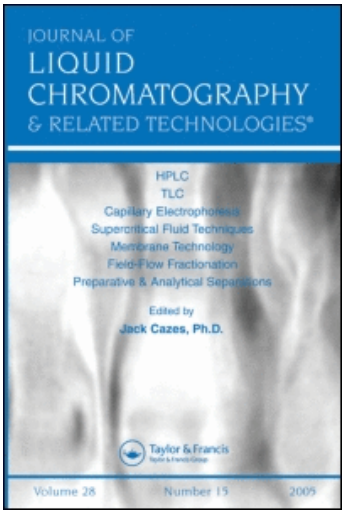

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Separation of Biologically Active Isomers of (E)-N-Meta- and Para-Nitroazastilbenes by the HPLC Technique

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Abstract: Results of investigations concerning the chromatography of bromides of (E)-N-m- and p-nitrobenzyl-4′-hydroxy-3′-methoxystilbazoles-4 are presented. Analyzed compounds, similarly to other azastilbene halogenides show antimicrobial activity. Optimal conditions of chromatographic separation and determination of two mentioned isomers have been elaborated. In the investigation three stationary phases (octadecyl, octyl, and naphthylpropyl), two mobile phases (acetonitrile, dichloromethane) and various flow were considered. The best selectivity and the highest separation factor, $\alpha = 1.74$, were obtained using a naphthylpropyl column and 100% acetonitrile, as the mobile phase. An application of an octadecyl phase, recommended by numerous analysts as standard, allowed only the observation of an existence of two compounds, but did not yield satisfactory results.

Keywords: (E)-Azastilbenes, Isomers, Aryl, Octyl, Octadecyl stationary phases

INTRODUCTION

(E)-Azastilbenes are nitrogen containing derivatives of stilbenes. They belong to a group of biologically active compounds with negative influence on microbes.[1–6] Cavallito and Grifantini showed their biological activity.[7–10]

Stilbenes are considered as derivatives of diphenylethylene. In plants they can be synthesized, e.g., from coumaric acid and cinnamic acid. Stilbenes resist the growth of fungi and destroy them, and also possess estrogenic activity. Some

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stilbenes belong to the group of synthetic estrogens (e.g., diethylstilbestrol) or are used directly to synthesize these compounds. Earlier, synthetic diethylstilbestrol was applied as an estrogen in menstruation disturbance. Now, because of the possibility of oncogenesis, induction is rarely used in medicine for humans. However, it was utilized in veterinary medicine as hormonal stimulators; therefore, its presence in meat products was found on frequent occasions. Depending on the concentration, it can act as an estrogenic or antiestrogenic.

Between 1940 and 1960, stilbestrol was used in abortion prophylaxis. However, this medicament later caused numerous cases of vaginal cancer. Careful investigation of stilbestrol therapy consequences proved, that it had caused an increase of formation defects in the sexual urinary system and an increase of embryo mortality. But, in some countries (e.g., in China) stilbestrol is still used in the health service. In Poland, its commercial confection Distilbene (coated 4 mg tablets) was withdrawn from circulation about four years ago.

Natural stilbenes exhibit different activity. They neither induce cancer, nor cause harmful side effects, typical of synthetic stilbenes. However, they are not recommended for 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 the 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.

Subject to investigations mentioned above, isomers were obtained according to a scheme (Figure 1, Table 1).[11] Analyzed compounds are (E)-azastilbene derivatives, they exhibit a negative influence on microorganisms (Table 2). In consideration of the biological activity of their bromides, the aim of this work-elaboration of optimal condition of separation and determination – is advisable. The optimization process concerns two isomers: (E)-N-m- and p-nitrobenzyl-4′-hydroxy-3′-methoxystilbazoles-4.

EXPERIMENTAL

HPLC Analysis of (E)-Azastilbenes

Samples of (E)-N-(m- or p-)nitrobenzyl-4′-hydroxy-3′-methoxystilbazoles-4 (Figure 1)[11] were dissolved in methanol (HPLC purity, Fluka AG, Buchs, Switzerland) to obtain a concentration at about 25 $\mu g \cdot mL^{-1}$. Analyses were

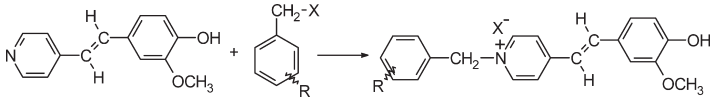

Figure 1. General scheme of preparation of analyzed isomers, where: X = Br, R = m-NO_2 or p-NO_2.[11]

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

Compound	Formula (mol. mass)	M.p. (°C)	IR (KBr) (cm^{-1})	UV λ_{max} (nm)	Analysis (calc./found) %C	%H	%N
1 (m-NO_2)	$C_{21}H_{19}BrN_2O_4$ 443.13	237–240	970	411.5	57.01 56.73	4.33 4.19	6.34 6.05
2 (p-NO_2)	$C_{21}H_{19}BrN_2O_4$ 443.13	146–149	960	412.5	57.01 56.75	4.33 4.39	6.34 6.09

performed at 412 nm and at temperature of 22°C. Three stationary phases were examined: octadecyl (S. Witko – J.T. Baker, Łódź, Poland), octyl (S. Witko – J.T. Baker, Łódź, Poland) and naphthylpropyl (RP Si–NAF, Figure 2).[12] Dimensions of the 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)-azastilbenes compounds were prepared by the method described in the literature (Figure 1).[11]

1 (m-NO_2): ^{1}H-NMR (DMSO-d_6) δ: 3.88 (s, 3H, OCH_3), 5.93 (s, 2H, CH_2-$^+$N), 6.90 (d, 1H), 7.22 (d, 1H), 7.42 (s, 1H), 7.42 (d, 1H, J = 16.2 Hz), 7.78 (t, 1H), 8.01 (d, 1H, J = 16.2 Hz), 8.07 (d, 1H), 8.23 (d, 2H), 8.29 (d, 1H), 8.55 (s, 1H), 9.11 (d, 2H), 9.89 (s, 1H, OH).

^{13}C-NMR (DMSO-d_6) δ: 55.83, 60.67, 110.87, 115.66, 119.73, 123.27, 123.45, 123.63, 124.03, 126.69, 130.70, 135.38, 135.43, 136.27, 142.27, 144.01, 147.96, 149.72, 153.99.

2 (p-NO_2): ^{1}H-NMR (DMSO-d_6) δ: 3.88 (s, 3H, OCH_3), 5.96 (s, 2H, CH_2-$^+$N), 6.90 (d, 1H), 7.22 (d, 1H), 7.42 (s, 1H), 7.44 (d, 1H, J = 16.1 Hz), 7.81

Table 2. Antimicrobial activity of isomers[11]

Compounds	Minimal inhibitory concentration (MIC) μg · mL^{-1} 1	2	3	4	5	6	7	8
m-NO_2	—	—	—	—	—	—	—	100
p-NO_2	—	100	—	—	—	—	—	250

1 - *Staphylococcus aureus* FDA209P, 2 - *Streptococcus faecalis* ATCC 8040, 3 - *Bacillus subtilis* ATCC 6633, 4 - *Escherichia coli* PZH 026B6, 5 - *Klebsiella pneumoniae* 231, 6 - *Pseudomonas aeruginosa* S 85/2, 7 - *Candida albicans* PCM 1499 PZH, 8 - *Microsporum gypseum* K1.

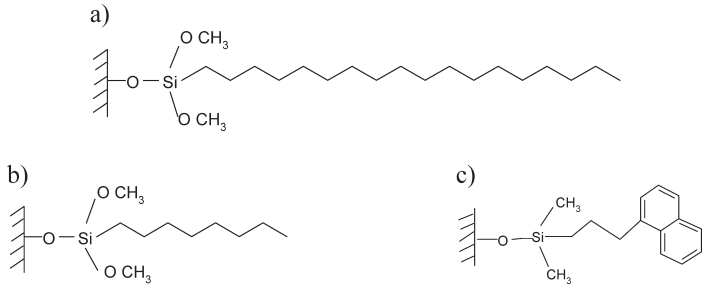

Figure 2. Scheme of chemically bonded stationary phases: a) octadecyl (RP Si–C_{18}), b) octyl (RP Si–C_8), and c) naphthylpropyl (RP Si–NAF).

(d, 2H), 8.03 (d, 1H, J = 16.1 Hz), 8.24 (d, 2H), 8.31 (d, 2H), 9.08 (d, 2H), 9.89 (s, 1H, OH).

^{13}C-NMR (DMSO-d_6) δ: 55.69, 60.73, 110.84, 115.68, 119.70, 123.34, 123.64, 124.03, 126.66, 129.74, 141.59, 142.34, 144.10, 147.61, 147.93, 149.72, 154.06.

Apparatus

Chromatographic measurements were performed on a liquid chromatograph SPD-6A (Shimadzu, Kyoto, Japan) equipped with a gradient pump LC-6A, UV/Vis detector, a sampling valve Rheodyne (Berkeley, CA, USA), model 7125, with a 20 μL sample loop, and a Shimadzu C-R6 A data recorder.

^{1}H NMR spectra were recorded on a Bruker-200 in DMSO-d_6, with HMDS as internal standard. The infrared (IR) spectra were recorded on a Nicollet Magna-IR 760 in potassium bromide. Chemical and physical data of (E)-azastilbenes compounds are described in Table 1. The antimicrobial activity of isomers is described in Table 2.

RESULTS AND DISCUSSION

Results obtained during the optimization process are collected in Table 4. Reported in this work are optimal data of chromatographic separation of two

Table 3. Characteristics of bonded phase

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

Table 4. Chosen dependence k′ for (E)-N-(m- or p-)nitrobenzyl-4′-hydroxy-3′-methoxystilbazoles-4 from on type of stationary and mobile phase. Chromatographic conditions: flow −1.0, 0.5 or 0.3 mL · min^{-1}, wavelength −412 nm, temperature −22°C

Type of stationary phase	[a]Mobile phase/flow	k'_1 (p-NO_2)	k'_2 (m-NO_2)	$\alpha = k'_2/k'_1$
RP Si–C_{18}	Acetonitrile/1.0	2.22	2.29	1.04
	Acetonitrile/0.5	5.77	5.82	1.01
	Acetonitrile/0.3	12.45	13.21	1.05
	Dichlomethane/1.0	2.27	2.31	1.02
	Dichlomethane/0.5	5.84	5.97	1.02
	Dichlomethane/0.3	12.67	13.98	1.10
RP Si–C_8	Acetonitrile/1.0	0.42	0.47	0.86
	Acetonitrile/0.5	1.83	1.84	1.00
	Acetonitrile/0.3	3.57	3.77	1.06
	Dichlomethane/1.0	0.44	0.50	1.14
	Dichlomethane/0.5	1.87	1.93	1.03
	Dichlomethane/0.3	3.75	3.91	1.04
RP Si–NAF	Acetonitrile/1.0	1.81	2.15	1.31
	Acetonitrile/0.5	3.11	3.77	1.18
	Acetonitrile/0.3	3.60	6.27	1.74
	Dichlomethane/1.0	1.92	2.28	1.19
	Dichlomethane/0.5	3.22	3.89	1.21
	Dichlomethane/0.3	3.75	6.34	1.69

[a]In the table are placed only optimal data of analyzed isomers separation.

synthesized isomers: (E)-N-m- and p-nitrobenzyl-4′-hydroxy-3′-methoxystilbazoles-4. During selection of the separation and determination conditions, two mobile phases (acetonitrile and dichloromethane) with various flow and three stationary phases (octadecyl, octyl, and naphthylpropyl) were tested. Octadecyl stationary phase is commonly considered as the standard for numerous determinations by means of high performance liquid chromatography; therefore, it was used for the experiments. Neither an application of various flow (Table 4) nor an addition of other solvents increased the separation. Different compositions of water containing solvent mixtures caused only an elongation of retention times (these results are not reported in the tables). Because octadecyl phase did not yield satisfactory results, octyl phase was used. It also did not help to separate isomers in the proper way; however, retention times were shorter by half. This failure led to a conclusion, that more and more frequently used so called dedicated stationary phases can solve this problem. Such phases are designed for determination of a special type of chemical structure, only.

Because analyzed compounds are contained in the structure aromatic rings, an application of aryl phase was reasonable. As a dedicated phase, naphthylpropyl chemically bonded stationary phase (RP Si–NAF) has been chosen. This type of phase is, first of all, designed to determine π electron

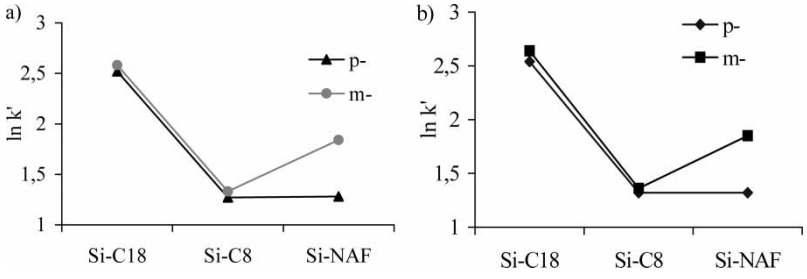

Figure 3. Effect of the separation of (E)-N-(m- or p-)nitrobenzyl-4′-hydroxy-3′-methoxystilbazoles-4 with the use of stationary phases RP Si–C_{18}, RP Si–C_8 and RP Si–NAF. Mobile phase: (a) acetonitrile (vol. 100%), flow rate: 0.3 mL · min^{-1}, (b) dichloromethane (vol. 100%), flow rate: 0.3 mL · min^{-1}, detection –412 nm (see Table 4).

containing compounds.[12–16] In the chromatographic process carried on with a participation of stationary phase and analyzed substances, interactions of $\pi - \pi$ are predominating. Owing to increased selectivity, in numerous determinations, a separation was improved and retention times became shorter.

Separation of investigated isomers was possible by the use of aryl stationary phase (RP Si-NAF) and acetonitrile (100%) or dichloromethane (100%) as the mobile phase. This effect is shown in Figures 3–5. Optimal conditions of the separation of two isomers: (E)-N-m- and p-nitrobenzyl-4′-hydroxy-3′-methoxystilbazoles-4 are shown in Table 2. It was established that naphthylpropyl chemically bonded stationary phase is characterized by the highest selectivity ($\alpha = 1.74$). The best separation was obtained using this stationary phase and acetonitrile as the mobile phase, whereas results obtained by means of RP C_{18} and C_8 only showed an existence of two compounds, but the separation was not satisfactory.

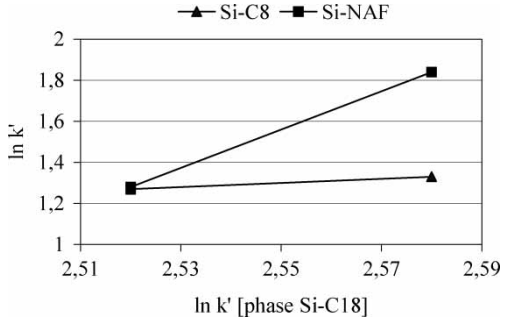

Figure 4. Dependence of ln k′ of the RP Si–C_8 and RP Si–NAF phases on ln k′ obtained for the octadecyl phase for (E)-N-(m- or p-)nitrobenzyl-4′-hydroxy-3′-methoxystilbazoles-4.

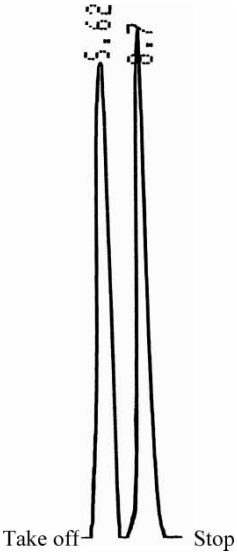

Figure 5. A chromatogram of separation of the (E)-N-(m- or p-)nitrobenzyl-4′-hydroxy-3′-methoxystilbazoles-4 on the stationary RP Si–NAF phase (m- 5.62 min and p- 8.7 min). Mobile phase: acetonitrile (100 vol.%); flow −0.3 mL · min^{-1}, wavelength −412 nm, temperature −22°C.

CONCLUSIONS

An application of octadecyl and octyl stationary phase did not enable desired separation of analyzed isomers: (E)-N-m- and p-nitrobenzyl-4′-hydroxy-3′-methoxystilbazoles-4. In order to obtain perfect separation of the isomers, an HPLC technique with UV/Vis detection required the use of aryl (naphthylpropyl) stationary phase.

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