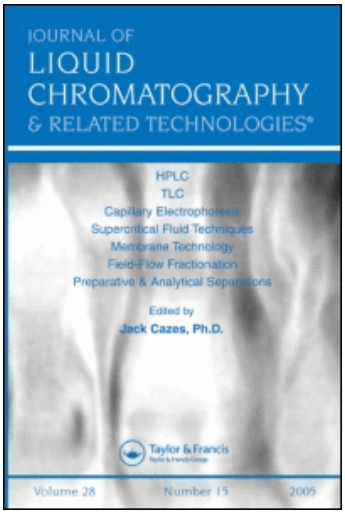

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Prukała, Wiesław , Prukała, Dorota , Pypowski, Krzysztof , Chrząścik, Ireneusz and Kluska, Mariusz(2008) 'Separation of Biologically Active Isomers of Nitroazastilbenes by the HPLC Technique', Journal of Liquid Chromatography & Related Technologies, 31: 18, 2784 — 2793
To link to this Article: DOI: 10.1080/10826070802388391
URL: http://dx.doi.org/10.1080/10826070802388391

Journal of Liquid Chromatography & Related Technologies®, 31: 2784–2793, 2008

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802388391

Separation of Biologically Active Isomers of Nitroazastilbenes by the HPLC Technique

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Abstract: Optimum conditions for chromatographic separation of bromides of (E)-N o-, m-, and p-nitrobenzyl-4′-hydroxy-3′-methoxystilbazole-4 are presented. This work was a continuation of an earlier investigation, which was necessary because of difficulties when standard (octadecyl) phases had been applied. Analyzed isomers show antimicrobial activity. In the investigation were considered three aryl stationary phases (phenylbutyl, naphthylpropyl, and Hypercarb), two mobile phases (acetonitrile, dichloromethane), and various intensities of flow. The best selectivity ($\alpha_1 = 1.74$, $\alpha_2 = 1.22$) was obtained using the naphthylpropyl column and 100% acetonitrile as the mobile phase. An application of the octadecyl phase, recommended by numerous analysts as the standard, allowed only to observe an existence of two compounds, but did not yield satisfactory results.

Keywords: Aryl stationary phases, Azastilbenes, Isomers, HPLC

INTRODUCTION

Cavallito and Grifantini supplied documentary evidence for biological activity of stilbenes and their derivatives decades ago.[1,2] Stilbenes are considered as derivatives of diphenylethylene. They exist as natural or synthetic compounds. Natural stilbenes can be synthesized in plants, e.g., from coumaric acid and cinnamic acid. Stilbenes resist growth of

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fungi and destroy them, also they possess estrogenic activity. Some stilbenes belong to the group of synthetic estrogens (e.g., diethylstilbestrol) or are used directly for the syntheses of these compounds.[3]

Earlier, in the 1950s synthetic diethylstilbestrol was applied as estrogen in menstruation disturbance. Now, because of a possibility of oncogenesis, induction is rarely used in human medicine. However, it was utilized in veterinary medicine as a hormonal stimulator; therefore, its presence in meat products was found on frequent occasions. Dependent of the concentration, it can act an estrogenic or antiestrogenic.[3,4]

In the 1960s, 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.[3] But, in some countries (e.g. in China), stilbestrol is still used in the health service.

Natural stilbenes exhibit different activity. They neither induce cancer, nor cause harmful side effects, typical for 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 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.[5,7]

Azastilbenes are an important group of nitrogen containing stilbenes derivatives. They also exhibit biological activity; they have a negative influence on microbes (Table 1).[8–10] The most important physicochemical data of analyzed isomers are shown in Table 2.[11] Because of the biological activity of bromides of (E)-azastilbenes, it was advisable to elaborate optimum conditions of their separation and determination. Continuation of the investigation concerning this task was the aim of our work. Optimization included chromatography of three (orto-, meta-, and para-) isomers of (E)-N-(o-, m-, or p-)nitrobenzyl-4′-hydroxy-3′-methoxystilbazole-4.

EXPERIMENTAL

HPLC Analysis of (E)-Azastilbenes

Samples of (E)-N-(**o**-, **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 of about $20\,\mu g \cdot mL^{-1}$. Analyses were performed at 412 nm and at temperature

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
(m-NO_2)	C_{21} H_{19} BrN_2 O_4 443.13	237–240	970	411.5	57.01 56.73	4.33 4.19	6.34 6.05
(o-NO_2)	C_{21} H_{19} BrN_2 O_4 443.13	142–145	960	412.0	63.30 63.01	4.81 4.82	7.03 6.79
(p-NO_2)	C_{21} H_{19} BrN_2 O_4 443.13	146–149	960	412.5	57.01 56.75	4.33 4.39	6.34 6.09

Table 2. Antimicrobial activity of isomers[11]

	Minimal inhibitory concentration (MIC) $\mu g \cdot mL^{-1}$							
Compounds	1	2	3	4	5	6	7	8
m-NO_2	–	–	–	–	–	–	–	100
o-NO_2	–	–	–	–	–	–	–	250
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.

19°C. Three aryl stationary phases were studied: a commercial column Hypercarb, packed with porous graphitized coal (PGC, Thermo Electron Corporation UK – Figure 2a), phenylbutyl (RP Si – PB, Figure 2b),[12] and naphthylpropyl (RP Si NAF, Figure 2c).[12] Dimensions of steel columns were: for PGC–100×4.6 mm, for RP Si–PB 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]

(**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.

(**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 (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).

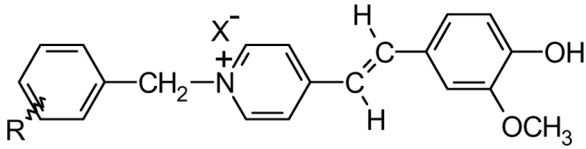

Figure 1. General structures of analyzed isomers, where: X = Br, R = **o**-NO_2, **m**-NO_2 or **p**-NO_2.

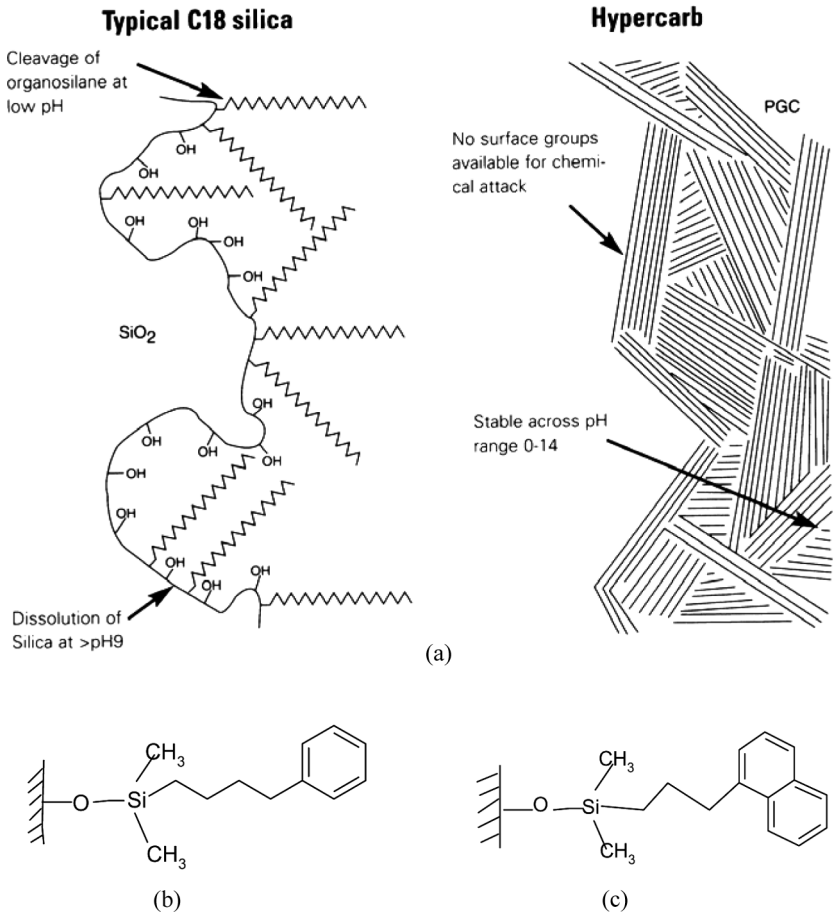

Figure 2. Scheme of chemically bonded stationary phases: (a) typical C_{18} silica and Hypercarb (PGC) (b) phenylbutyl (RP Si–PB) (c) naphthylpropyl (RP Si–NAF).

Table 3. Characteristics of aryl bonded phase

Phase code	Carbon (%)	Manufacturer of column	Column dimensions (mm)
RP Si–PB	14.9	home made	125×4.6
RP Si–NAF	16.1	home made	125×4.6
PGC	100	Thermo Electron Corporation	100×4.6

^{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.

(**o**-NO_2): ^{1}H-NMR (DMSO-d_6) δ: 3.87 (s, 3H, OCH_3), 6.17 (s, 2H, CH_2 -$^+$N), 6.98 (d, 1H), 7.22 (d, 2H), 7.42 (s, 1H), 7.46 (d, 1H, J = 16.4 Hz), 7.74 (t, 1H), 7.83 (t, 1H), 8.04 (d, 1H, J = 16.4 Hz), 8.25–8.28 (m, 3H), 8.94 (d, 2H), 10.12 (s, 1H, OH).

^{13}C-NMR (DMSO-d_6) δ: 55.68, 59.21, 110.96, 115.74, 119.73, 123.19, 123.63, 125.47, 126.59, 129.64, 130.02, 130.21, 134.82, 142.46, 144.47, 147.35, 147.97, 149.93, 154.21.

Apparatus

Chromatographic measurements were performed on a liquid chromatograph SPD-6A (Shimadzu, Kyoto, Japan) equipped with a gradient

Table 4. Chosen dependence k′ for (E)-N-(**o-**, **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 –19°C

Type of stationary Phase	[a]Mobile phase/	k′$_1$ (**p**-NO_2)	k′$_2$ (**m**-NO_2)	k′$_3$ (**o**-NO_2)	α_1 = k′$_2$/k′$_1$	α_2 = k′$_3$/k′$_2$
RP Si–PB	Acetonitrile/1.0	0.65	0.89	1.02	1.37	1.15
	Acetonitrile/0.5	2.27	2.25	2.27	0.99	1.01
	Acetonitrile/0.3	2.85	3.45	4.06	1.21	1.18
	Dichlomethane/1.0	0.68	0.94	1.28	1.38	1.36
	Dichlomethane/0.5	2.31	2.43	3.17	1.05	1.30
	Dichlomethane/0.3	2.93	3.51	4.12	1.20	1.17
PGC	Acetonitrile/1.0	−0.42	−0.43	−0.18	1.02	0.42
	Acetonitrile/0.5	0.20	0.19	0.36	0.95	1.89
	Acetonitrile/0.3	1.35	1.47	1.72	1.09	1.17
	Dichlomethane/1.0	−0.44	−0.40	−0.23	0.91	0.58
	Dichlomethane/0.5	0.21	0.24	0.39	1.14	1.63
	Dichlomethane/0.3	1.37	1.52	1.78	1.11	1.17
RP Si–NAF	Acetonitrile/1.0	1.81	2.15	3.50	1.19	1.63
	Acetonitrile/0.5	3.11	3.77	4.59	1.21	1.22
	Acetonitrile/0.3	3.60	6.27	7.68	1.74	1.22
	Dichlomethane/1.0	1.92	2.28	3.67	1.19	1.61
	Dichlomethane/0.5	3.22	3.89	4.65	1.21	1.20
	Dichlomethane/0.3	3.75	6.34	7.13	1.69	1.12

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

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 $CDCl_3$, 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 is described in Table 1. However, antimicrobial activity of isomers described in Table 2.

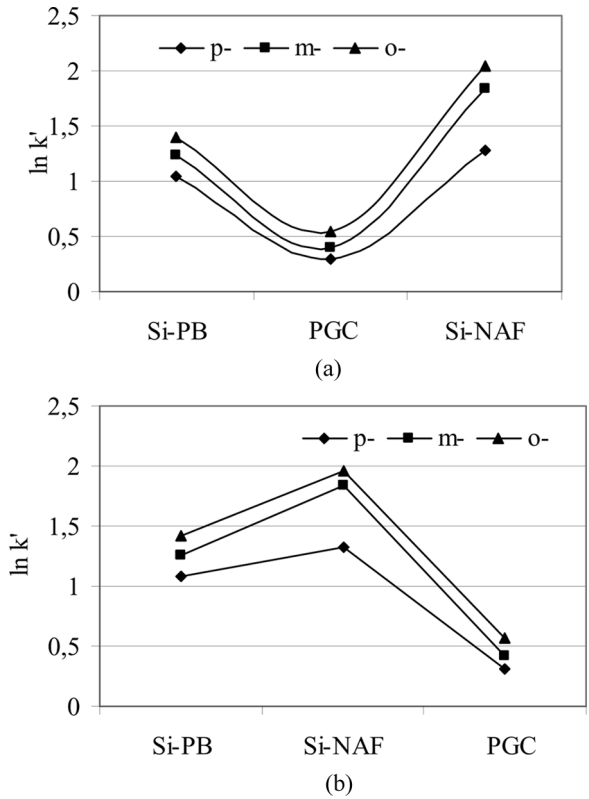

Figure 3. Effect of the separation of (E)-N-(**o-**, **m-** or **p-**)nitrobenzyl-4′-hydroxy-3′-methoxystilbazoles-4 with the use of stationary phases PGC, RP Si–NAF and RP Si–PB. Mobile phase: (a) acetonitrile (vol. 100%), flow rate: 0.3–mL min^{-1}, (b) dichloromethane (vol. 100%), flow rate: 0.3 mL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

RESULTS AND DISCUSSION

This work is a continuation of the investigation concerning optimization of the chromatographic process of separation and determination of isomers, which belong to the group of nitroazastilbenes. This continuation was caused by unsatisfactory results obtained by the use of standard columns.[13] The obtained optimum results are collected in Table 4. Optimization of chromatographic separation concerned three synthesized isomers: (E)-N-(**o-**, **m-**, and **p**−) nitrobenzyl-4′-hydroxy-3′-methoxystilbazole-4.

During selection of the separation and determination conditions, two mobile phases (acetonitrile and dichloromethane) with various intensities of flow and three stationary phases (phenylbutyl, naphthylpropyl, and Hypercarb) were tested. Different compositions of water containing solvent mixtures caused only an elongation of retention times (these results are not reported in the tables). Because analyzed compounds contain aromatic rings in their structures, an application of aryl phase was reasonable. Consequently, three aforementioned stationary phases were used. These types of phases is, first of all, designed to determine π electron containing compounds.[14–17] In the chromatographic process carried out with a participation of such stationary phases and analyzed substances, interactions of π–π types are predominating. Owing to increased selectivity, in numerous determinations separation was improved and retention times were shorter. The same effect appeared in our case.

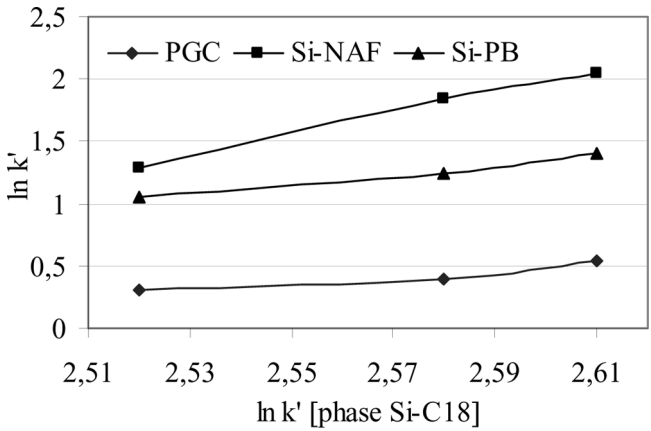

Figure 4. Dependence of ln k′ of the PGC, RP Si–PB and RP Si–NAF phases on ln k′ obtained for the octadecyl phase for (E)-N-(**o-**, **m-** or **p-**) nitrobenzyl-4′-hydroxy-3′-methoxystilbazoles-4.

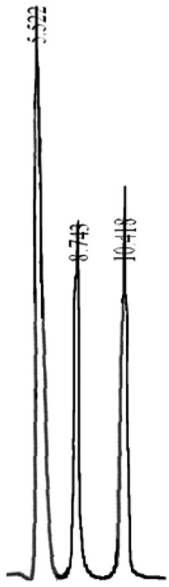

Figure 5. A chromatogram of separation of the (E)-N-(**o-**, **m-**, or **p-**)nitrobenzyl-4′-hydroxy-3′-methoxystilbazoles-4 on the stationary RP Si–NAF phase (**p**-5.522 min., **m-**8.723 min., **o-**10.418 min.). Mobile phase: acetonitrile (100 vol.%); flow–0.3 mL · min^{-1}, wavelength−412 nm, temperature −19°C.

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 shown in Figures 3–5. Satisfactory results were obtained with all three aryl phases. 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 the naphthylpropyl chemically bonded stationary phase is characterized by the highest selectivity ($\alpha_1 = 1.74$, $\alpha_2 = 1.22$). The best separation was obtained using this stationary phase, acetonitrile as the mobile phase, and flow 0.3 mL · min^{-1}.

CONCLUSIONS

An application of aryl stationary phases: phenylbutyl, naphthylpropyl, and Hypercarb enabled the desired separation of analyzed isomers: (E)-N-(o-, m-, and p-)nitrobenzyl-4′-hydroxy-3′-methoxystilbazole-4. In order to obtain perfect separation of the isomers, the HPLC technique with UV/V is detection and the use of aryl stationary phase was

necessary. Standard phases (octadecyl and octyl) were incapable of achieving satisfactory results, which was shown in a preceding paper.

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Received September 12, 2007
Accepted October 18, 2007
Manuscript 6325