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High-performance liquid chromatography of polyethylene glycols as their α,ω-bis(1-naphthylurethane) derivatives and signal monitoring by fluorescence detection

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Abstract

Polyethylene glycols (PEGs) of average molecular mass (M_r) 600, 1000 and 3000 were converted to their α,ω-bis(1-naphthylurethane) derivatives with 1-naphthylisocyanate and subjected to high-performance liquid chromatography on a Si 80 bare silica gel stationary phase. Signal monitoring was done by fluorescence detection at wavelengths of 232 nm for excitation and 358 nm for emission. A binary acetonitrile–water gradient effected good separation of PEG 600 and PEG 1000 into a wide variety of individual oligomers and almost baseline separation was achieved for PEG 600 and PEG 1000. In contrast, PEG 3000 requires a ternary acetonitrile–water–tetrahydrofuran gradient for both efficient elution and acceptable satisfactory signal resolution. Although separation of PEG 3000 into individual oligomers was substantially lower than that observed for PEG 600 and PEG 1000, the chromatograms impressively reveal the oligomeric composition of the polyether sample. Using serial dilutions of the α,ω-bis(1-naphthylurethane) derivatives, the detection limits for PEG 600, PEG 1000 and PEG 3000 are approximately 0.1 ppm, respectively. Using 50 μl of sample dissolved in either water or physiological saline, a detection limit of 1 μg/ml was achieved corresponding to an absolute amount injected of 10 ng. Taken together both, separation efficiency and detection sensitivity, this new technique, termed as a ''pseudo-reversed-phase separation process'', may therefore be applicable to investigations of intestinal permeability and resorption in the living organism. Furthermore, with PEG 3000 as model compound, incorporation of two 1-naphthyl substituents to the corresponding α,ω-bis(1-naphthylurethane) derivative was confirmed by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Keywords: Derivatization, LC; Mass spectrometry; Polyethylene glycols; 1-Naphthylurethane derivatives

1. Introduction

Polyethylene glycols (PEGs) are increasingly used as probes for intestinal adsorption and resorption in man [1–5], whereas determination of the concentration as well as elucidation of the metabolic fate of their alkyl(arylalkyl) derivatives, belonging to the substance group of non-ionic surfactants, plays an important role in investigations of environmental protection [6–12]. In order to simulate the transport and/or diffusion of appropriate target compounds across biological membranes exhibiting a distinct molecular mass (M_r) cut-off, as typically encountered in the living organism, PEGs widely differing

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in M_r values, such as, e.g., PEG 600, PEG 1000 and PEG 3000 were chosen as appropriate targets. Due to the fact that PEGs compose of a large number of homologues, they are excellently suited for this type of investigation. Therefore, efficient separation of the individual oligomers is a fundamental prerequisite for method development and the PEGs should then be identified on the basis of their ''chromatographic fingerprint''. Furthermore, it is assumed that the ability to cross biological membranes is a M_r-dependent process. The PEG oligomers being able to cross this barrier are then to be determined in highly diluted fluids, such as, e.g., dialysates. However, as a consequence of the effect of dilution, extremely low concentrations of PEGs are usually found in these samples, which lie only in the lower ppm range and thus, a highly sensitive method had to be developed.

For permeability and resorption tests performed up to the present, signal monitoring was preferably done by measurement of the refractive index (RI) and UV responses [1–4]. However, only low-M_r PEG samples can be measured by means of RI detection, because for PEGs of higher M_r the gradient technique is required for either satisfactory signal resolution or complete elution from the column matrix. Unfortunately, this alternative cannot be applied in this case and thus, RI detection is restricted to isocratic high-performance liquid chromatography (HPLC). A further drawback of the isocratic alternative is given by the observation that peaks for late-eluting oligomers are often very broad and more and more merge with the baseline, by which the method's sensitivity in this elution range is either markedly decreased or recognition of distinct polyether samples on the base of the ''chromatographic fingerprint'' substantially impaired. Another disadvantage is, that either RI or UV responses are insufficient for measurement of PEGs in the lower ppm range, and for this reason, much more sensitive assays are required. Detection sensitivity is crucial for this substance class because PEGs are composed of a multitude of oligomers, which is associated with a more or less pronounced lack to determine the sample constituents of low frequency. This problem could yet be solved by merging the signals from the individual oligomers into one peak by the choice of appropriate chromatographic conditions. However, in the latter case no information will be available with respect to the preferential M_r-dependent permeation or resorption of PEG oligomers across biological barriers as well as selective removal of them during their biotransformation in, e.g., sewage treatment plants. As a logical consequence, efficient separation methods are needed, still allowing sufficient insight into the oligomeric distribution of PEGs. Recently a very sensitive method for the determination of PEG 400 in plasma and urine based on sample enrichment by solid-phase extraction (SPE) and gas chromatography–mass spectroscopy (GC–MS) was published [5]. However, this alternative cannot be applied for PEGs exceeding the mass range of M_r 500 as a consequence of insufficient volatility.

Due to the lack of intrinsic chromophors or fluorophors, PEGs had to be reacted with appropriate reagents. At present, only introduction of fluorophors into PEG fulfils the requirement for their determination down to the lower ppm level. A variety of fluorogenic reagents is available for selective derivatization of hydroxy endgroups of polyethers, among them 4-dimethylamino-1-naphtoylnitrile [13], 1-anthroylnitrile [14], 9-anthroylnitrile [15,16], 7-methoxycoumarin-3-carbonyl azide [17], 3,4-dihydro- 6, 7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonylazide [18], 1-naphtylisocyanate [12,19], 1-naphtoylchloride [12], 9-fluorenyl-methyloxycarbonylchloride [20] and carbazol-9-carbonylchloride [21] being the most attractive examples. All these reagents afford the corresponding derivatives at both gentle reaction conditions and high yield. It should be emphasised however, that a lot of them are not commercially available and thus have to be synthesised in tedious and time-consuming multi-step procedures.

In contrast to their monoalkyl(aryl) ethers (esters), native PEGs constitute a relatively polar substance group, which for this reason, affords some difficulties for appropriate separation into individual oligomers on reversed-phase materials, such as, e.g., C_{18}, C_8, C_4 and C_{Phenyl} stationary phases [22]. Furthermore, after derivatization with 3,5-dinitrobenzoylchloride, separation efficiency almost completely disappears on reversed-phase materials [22]. However, as shown in a previous paper [23], much better signal resolution (R_s), at least for underivatized (native) PEGs, is achieved on a bare silica gel column by use with of binary acetonitrile-water as

well as acetone–water gradients, by which the problems associated with too strong peak broadening, as observed in isocratic HPLC, are extensively eliminated. Therefore, we applied this unusual ''normal-phase sorbent–reversed-phase solvent'' HPLC technique to the chromatography of the three PEG samples after prior derivatization with 1-naphthylisocyanate and measurement of the fluorescence responses at λ=232 nm for excitation and λ=358 for emission. In the case of sufficient separation into individual oligomers, selective changes in the oligomer distribution should then be easily recognisable with these members of the PEG family in either permeability and resorption studies in the living organism or subjection to environmental degradation processes.

In this paper we report on the development of an HPLC-based method for either highly efficient separation of PEG oligomers or sensitive detection of the corresponding α,ω-bis(1-naphthylurethane) derivatives by means of fluorescence detection.

2. Experimental

2.1. Materials

PEG samples PEG 600[1], PEG 1000 and PEG 3000 (''pract.'' quality) were purchased from Fluka (Buchs, Switzerland). The derivatization reagent 1-naphthylisocyanate was obtained from Carbolabs (Bethany, CT, USA). Acetonitrile, methanol, tetrahydrofuran (THF), diethylether (all of HPLC quality) and sodium chloride (puriss. analytical-reagent grade) were from Fluka. Water for the use in HPLC was purified with a Milli-Q reagent water system from Millipore-Waters (Milford, MA, USA). For HPLC a Spherisorb Si 80 bare silica gel stationary phase (125×4.6 mm I.D., 5 μm particle size, 80 Å pore diameter) from Stagroma (Wallisellen, Switzerland) was used. For comparative purpose, a SpheriGrom Si 80 (125×4.6 mm I.D., 5 μm particle size, 80 Å pore diameter), a GromSil Si 80 (125×4.6 mm I.D., 5 μm particle size, 80 Å pore diameter) and a Lichrospher Si 60 (125×4.6 mm I.D., 5 μm particle size, 60 Å pore diameter) stationary phase, all obtained from Stagroma, and a Nucleosil 100-5 column (125×4.6 mm I.D., 5 μm particle size, 100 Å pore diameter) from Macherey-Nagel (Oensingen, Switzerland) were tested.

[1]The numbers indicate the average molecular mass M_r as specified by the manufacturer.

2.2. Analytical equipment

The HPLC apparatus consisted of a P 4000 quaternary HPLC pump, a vacuum degassing unit, an AS 3000 autosampler equipped with a 100-μl sample loop, a UV 2000 UV detector, a FL 2000 fluorescence detector and a PC 1000 data acquisition unit, all obtained from Thermo Separation Products (Freemont, CA, USA).

2.3. Derivatization and sample preparation

The procedure originally used by Lemr et al. [19] was modified in our laboratory. In brief, the PEG 600, PEG 1000 and PEG 3000 standards were dissolved in acetonitrile (10 mg/ml). From these solutions 100 μl (absolute amount≡1 mg) of each sample were separately transferred into a 1.5-ml glass vial and the solvent evaporated at 60°C by a gentle stream of nitrogen. To the individual residues was added 20 μl of 1-naphthylisocyanate. After 2 h at 60°C, the reaction mixtures were dissolved in 100 μl methanol for conversion of excessive reagent to the 1-naphthylurethane. Each sample was then transferred into a calibrated 10-ml flask and filled up to volume with THF yielding a concentration of 100 μg/ml (100 ppm). This solution was further diluted with either THF or water–THF (i.e., at final water–THF ratios of 95:5 and 90:10, respectively), yielding concentrations of 10 μg/ml (10 ppm), 1 μg/ml (1 ppm) and 0.1 μg/ml (0.1 ppm), respectively.

For matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) investigations 25 mg PEG 3000 was reacted with 200 μl 1-naphthylisocyanate (60°C, 2 h) and the excess of reagent converted to its urethane derivative with 200 μl methanol. After addition of 3 ml water, the formed colourless suspension was treated with 1 ml diethylether on a Vortex mixer for about 15 s for removal of excessive derivatization reagent and the aqueous layer evaporated to dryness at 60°C using a

gentle stream of nitrogen. The remaining colourless pasty residue was used for MALDI-TOF-MS experiments without further purification.

In order to simulate low concentrations of PEG 3000 encountered in real life samples, three concentrations of PEG 3000 (i.e., 50 μg/ml=sample 1; 5 μg/ml=sample 2 and 1 μg/ml=sample 3) all dissolved in either water or physiological sodium chloride (0.9%), were used for derivatization with 1-naphthylisocyanate. From each sample 50 μl corresponding to absolute amounts of 2.5 μg, 0.25 μg and 0.05 μg, respectively, were withdrawn and subjected to derivatization with 20 μl of reagent after prior evaporation of the solvent. In the case of sample 1 the residue was dissolved in 1 ml methanol, 100 μl withdrawn and 900 μl water subsequently added slowly under Vortex mixing. To the immediately formed colourless suspension was given 500 μl of diethylether and the mixture rapidly shaken on a Vortex mixer for about 10 s. The aqueous phase was removed and 100 μl injected. The residue of sample 2 was dissolved in 100 μl methanol followed by addition of 900 μl of water. The formed suspension was extracted with 500 μl of diethylether as described before and 100 μl of the aqueous phase injected. To the residue of sample 3, dissolved in 50 μl methanol was added 450 μl water and the resulting suspension extracted with 250 μl diethylether, processed as described for sample 1 and 100 μl of the aqueous phase injected. In order to estimate the influence of sample extraction on either ''chromatographic fingerprint'' or signal intensities, identical concentrations of an α,ω-bis(1-naphthylurethane) PEG 3000 standard (1 ppm) were injected directly or after treatment with water and following extraction with diethylether.

2.4. Chromatography and detection

Separation on the Si 80 column matrix was performed at a flow-rate of 1.5 ml/min at ambient temperature (approximately 22°C) applying the solvent gradient technique. Gradient programmes I (Table 1), II (Table 2) and III (Table 3) were used for the separation of the α,ω-bis(1-naphthylurethane) derivatives of PEG 600, PEG 1000 and PEG 3000, respectively. For method optimisation relatively concentrated samples dissolved in 10 μl THF were injected onto the column. Signal responses were measured by fluorescence detection (FD) at wavelengths set at 232 nm for excitation and 358 nm for emission. The photomultiplier tube (PMT) current was adjusted to 800 V yielding an optimum signal-to-noise ratio (S/N). In order to prevent PMT overload due to the high concentration of 1-naphthylurethane formed from excessive derivatization agent with methanol, wavelengths for excitation and emission were set at 600 nm and 750 nm, respectively, for the first 10 min of chromatographic separation. During this time period no signals attributable to PEG oligomers were measured. For rapid determination of the elution region of the PEG oligomers, the UV responses of sufficiently high concentrations were measured at 230 nm prior to signal monitoring by FD.

Table 1
Gradient programme I for the separation of the α,ω-bis(1-naphthylurethane) derivatives of PEG 600 on a Spherisorb Si 80 column

Time (min)	Acetonitrile (%)	Water (%)
0	10	90
40	60	40
41	10	90
55	10	90

Table 2
Gradient programme II for the separation of the α,ω-bis(1-naphthylurethane) derivatives of PEG 1000 on a Spherisorb Si 80 column

Time (min)	Acetonitrile (%)	Water (%)
0	20	80
40	60	40
41	20	80
55	20	80

Table 3
Gradient programme III for the separation of the α,ω-bis(1-naphthylurethane) derivative of PEG 3000 on a Spherisorb Si 80 column

Time (min)	Acetonitrile (%)	Water (%)	THF (%)
0	20	80	0
40	80	0	20
41	20	80	0
55	20	80	0

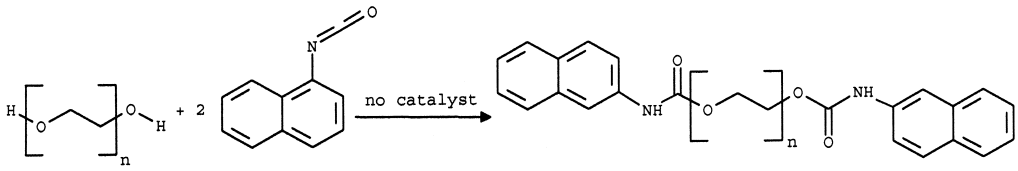

Fig. 1. Schedule of the derivatization recation between PEGs and 1-naphthylisocyanate.

2.5. MALDI-TOF-MS investigations

All MS spectra were obtained using a LDI 1700 instrument (Linear Scientific, Reno, NV, USA) with a pulsed nitrogen laser (337 nm) and an acceleration voltage of +30 kV for the positive ion mode. The vacuum inside of the 1.7 m flight tube was always kept between 10^{-7} and 10^{-6} Torr (1 Torr=133.322 Pa). For the MALDI mass measurements 3 ml of an aqueous solution of the PEG samples was mixed with the same amount of a 100 m*M* solution of 2,6-dihydroxy acetophenon as MALDI matrix [24] in a water–2-propanol (1:1) mixture. After applying of 0.3 ml onto the probe tip, the solution was dried by vacuum. Then the sample was ready for introduction into the mass spectrometer.

3. Results

In general, the use of 10 μl 1-naphthylisocyanate is sufficient for complete derivatization of all sample constituents (see Fig. 1), because no further increase in signal intensity was observed when the samples are reacted with either 20 μl or 50 μl of reagent. As a compromise, we used a volume of 20 μl of derivatization reagent, which takes into consideration an additional marked consumption of reagent by the hydroxy-, amino-, carboxyl- and thiol-functionalities encountered in various samples of environmental as well as biological origin. It is noteworthy that excessive reagent did not impair chromatographic performance and, under the chosen conditions, was eluted as 1-naphthylurethane near the column's void volume.

From different bare silica gel stationary phases (i.e., Spherisorb, Nucleosil, SpheriGrom, GromSil and Lichrospher) tested during the investigations, the Spherisorb Si 80 column revealed optimum signal resolution of the three polyethers. Only in the case of PEG 3000, the SpheriGrom Si 80 column matrix exhibited a nearly identical chromatographic pattern. The separation efficiency of the three PEGs on all tested columns increased in the range Lichrosorb Si 60<Nucleosil 100<GromSil Si 80<SpheriGrom Si 80<Spherisorb Si 80. Therefore, the Spherisorb matrix was used in all further separations.

Approximately baseline separation of the α,ω-bis(1-naphthylurethane) derivatives of PEG 600 and PEG 1000 oligomers was achieved using a binary solvent gradient of acetonitrile and water (Tables 1 and 2). The chromatograms obtained from the injection of 100 ppm PEG 600 and PEG 1000 dissolved in 10 μl THF are shown in Fig. 2a and b[2].

Under the conditions used for chromatography of PEG 600 and PEG 1000, the PEG 3000 derivative was extensively retained on the column matrix. Nevertheless, when using a binary acetonitrile–water gradient, the PEG 3000 derivative could be eluted by a column change to a less polar CN matrix, but unfortunately, no separation into individual oligomers was achieved. When acetonitrile was replaced by THF in the binary solvent mixture, PEG 3000 could also be released from a silica gel column matrix, but as already observed in previous investigations using typical reversed-phase matrices (e.g., C_8, C_{18} columns), no separation into individual oligomers was achievable and the whole amount of oligomers merged into a single unresolved signal envelope. Although the elution system composed of

[2]Note that in almost all chromatograms the baseline drops to negative mV values after the change of wavelength (see Section 2.4). Although this effect could yet be overcome by a set of more-fold wavelength changes at distinct time intervals and ''zero'' adjustment at each change, peak area calculations are not impaired. For this reason, no further attempt was made in order to correct for the drop in baseline.

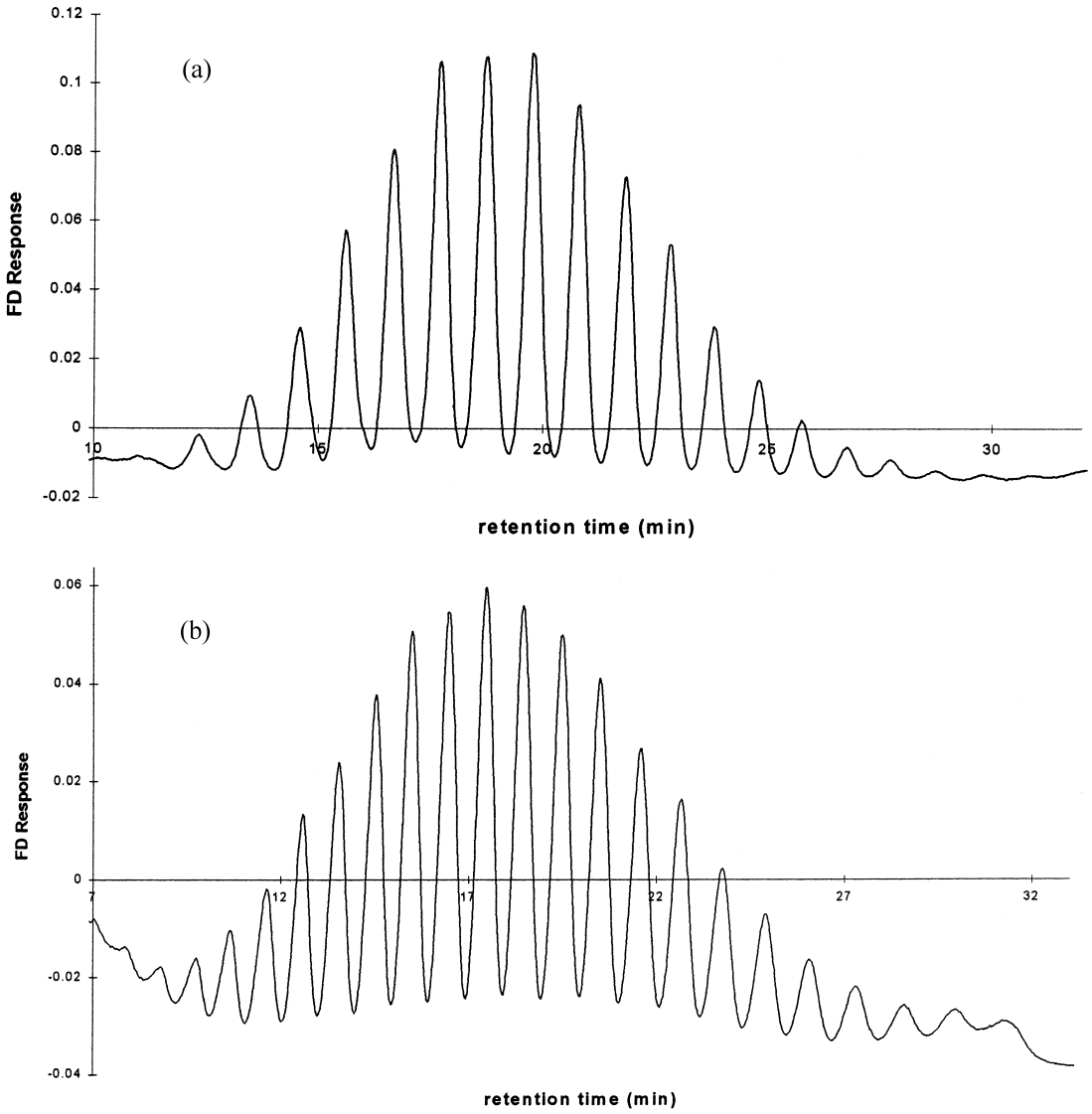

Fig. 2. Chromatograms of 100 ppm of the α,ω-bis(1-naphthylurethane) derivatives of each PEG 600 (a) and PEG 1000 (b) dissolved in 10 μl THF on a Spherisorb Si 80 (125×4.6 mm I.D., 5 μm particle size) column with gradient programmes I and II (Tables 1 and 2).

a binary gradient of acetone and water, successfully used for PEG 1000 [23], provides acceptable separation of native (underivatized) PEG 3000 (chromatographic conditions see Ref. [23]), as shown in Fig. 3, it could not be used due to complete quenching of FD responses. Nevertheless, similar separation of PEG 3000 was achieved with a ternary gradient system of acetonitrile, water and THF (Table 3), as depicted in Fig. 4. Application of these elution conditions to the corresponding α,ω-bis(1-naphthylurethane) derivatives, as revealed in Fig. 5 for an injection of 100 ppm PEG 3000 dissolved in 10 μl THF, yielded markedly better signal resolution than that observed for the corresponding underivatized sample.

Fig. 2a and b provide evidence that no too pronounced peak broadening of late-eluting oligomers with respect to their early-eluting homologues

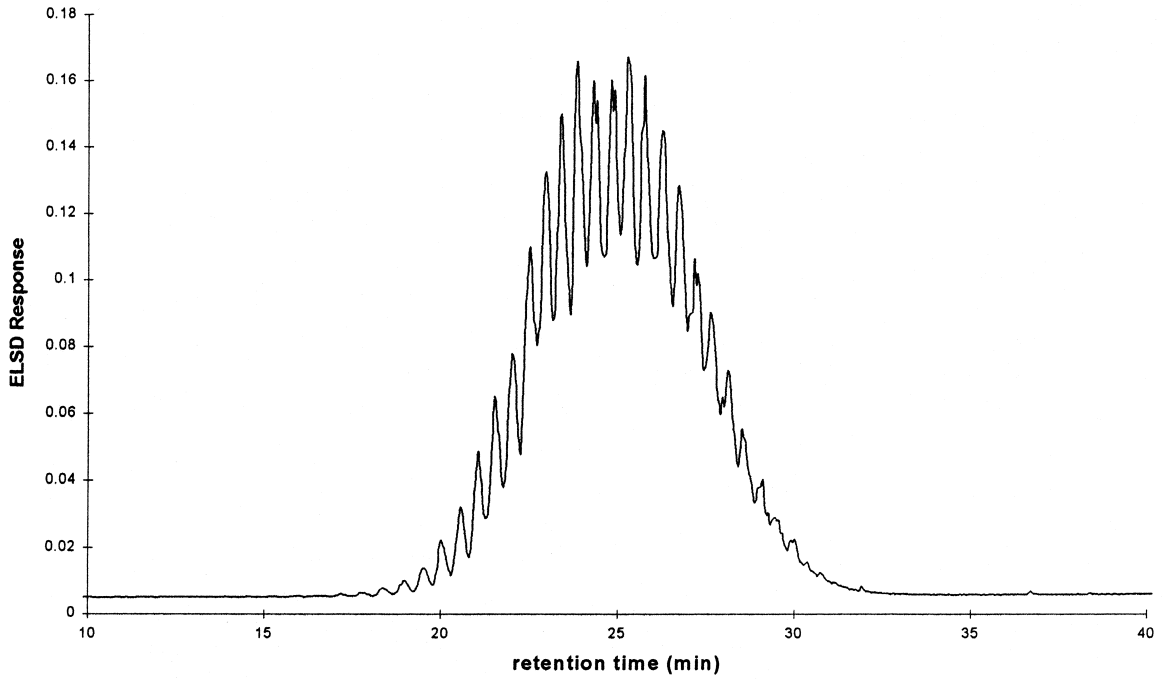

Fig. 3. Chromatogram of underivatized (native) PEG 3000 dissolved in 10 μl THF on a Spherisorb Si 80 (125×4.6 mm I.D., 5 μm particle size) column with a binary gradient of acetone and water (chromatographic conditions see Ref. [22]); detection mode: ELSD.

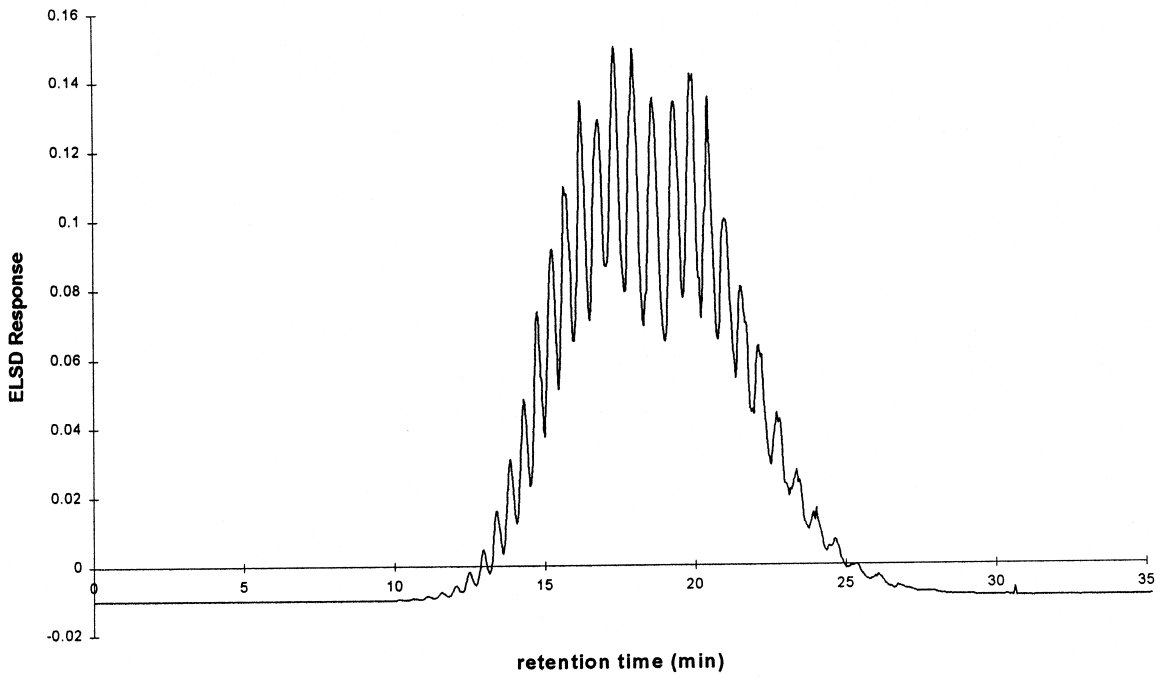

Fig. 4. Chromatogram of underivatized (native) PEG 3000 dissolved in 10 μl THF on a Spherisorb Si 80 (125×4.6 mm I.D., 5 μm particle size) column with a ternary gradient of acetonitrile, water and THF (Table 3); detection mode: ELSD.

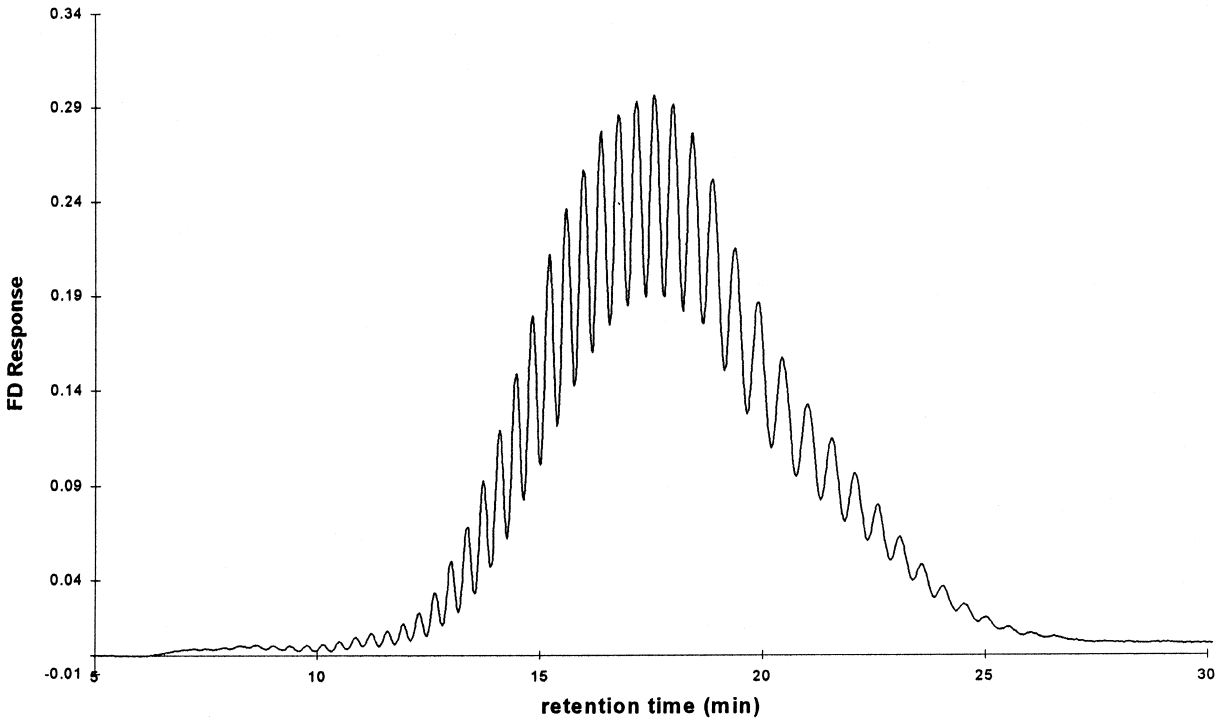

Fig. 5. Chromatogram obtained from 100 ppm of the α,ω-bis(1-naphthylurethane) derivative of PEG 3000 dissolved in 10 μl THF on a Spherisorb Si 80 (125×4.6 mm I.D., 5 μm particle size) column with a ternary gradient of acetonitrile, water and THF with gradient programme III (Table 3).

was observed for PEG 600 and PEG 1000. Only in the case of PEG 3000 (Fig. 5) peak broadening proves to be substantially higher for the stronger retained later-eluted oligomers. Nevertheless, these signals show sufficiently small peak widths in order to obtain acceptable satisfactory detection sensitivity.

As reasonably expected, separation of the corresponding α,ω-bis(1-naphthylurethane) derivative of PEG 3000 was not as good as that observed with the corresponding lower M_r samples, but the chromatogram still impressively reveals its oligomeric composition (Fig. 5). Among the three investigated PEGs, signal resolution R_s decreases in the range PEG 600≥PEG 1000>PEG 3000. This general phenomenon can be satisfactorily explained by the higher relative mass difference of individual oligomers of the lower M_r members of PEG, such as, e.g., PEG 600 and PEG 1000 versus PEG 3000. This means that the lower the M_r of the PEGs the more is the relative mass difference between individual oligomers, which consequently, is associated with corresponding higher differences in solute–matrix interactions and thus better R_s.

MALDI-TOF-MS spectra of the α,ω-bis(1-naphthylurethane) derivative of PEG 3000 revealed, that our new HPLC system was indeed capable of separating the whole entity of PEG oligomers. No additional signals other than those obtained by the chromatographic procedure could be observed in the spectrum. Nevertheless, as shown in Fig. 6a, substantial deviations from the manufacturers specification were seen and, for this reason, the M_r value of 2294 (n=44, M_r=1954 for native PEG 3000) for the oligomer of highest abundance fits more with PEG 2000 than PEG 3000. Compared with the native PEG 3000 sample, endgroup determination of the α,ω-bis(1-naphthylurethane) derivative yields an increase in M_r for each oligomer of 338.4 u, corresponding to incorporation of two naphthylurethane moieties, as shown in Fig. 6b Fig. 6c, respectively.

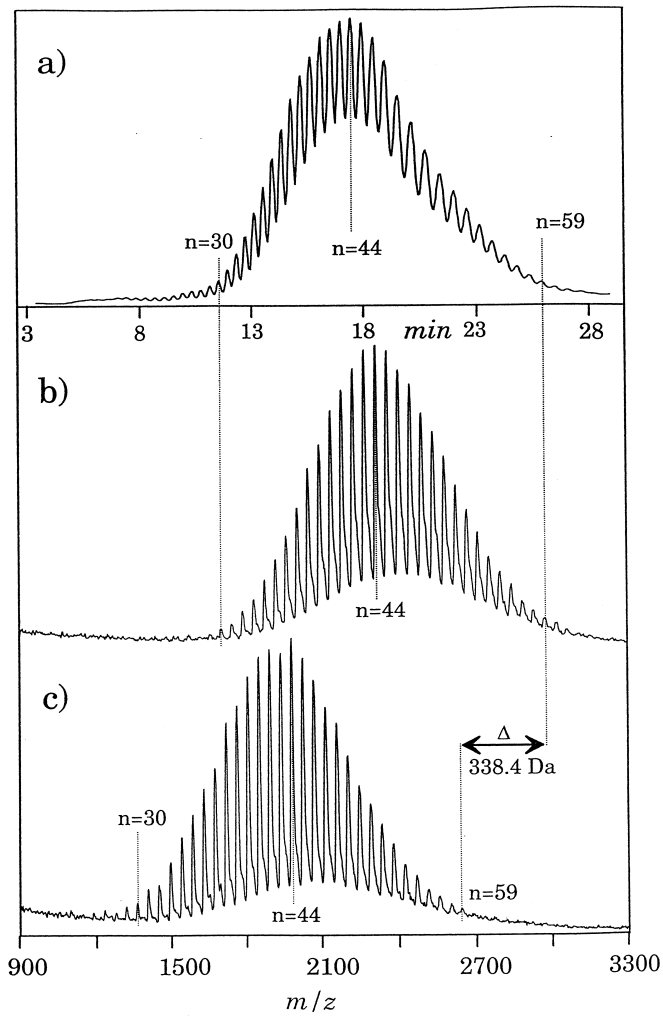

Fig. 6. HPLC chromatogram of the α,ω-bis(1-naphthylurethane) derivative of PEG 3000 (a), MALDI-TOF mass spectrum of the α,ω-bis(1-naphtylurethane) derivative of PEG 3000 (b), MALDI-TOF mass spectrum of native PEG 3000 (c).

In contrast to sample dissolution in THF, where the injection volume has to be kept between 5 and 10 μl, the sample volume can be markedly increased, by use of conditions, effecting concentration of the sample in a small band at the column head, i.e., at high concentrations of water. This is impressively shown in Fig. 7, which shows the superposition of the HPLC traces obtained from injection of 10 μl, 20 μl, 50 μl and 100 μl of the derivatives obtained from PEG 1000 (5 ppm) in a mixture of water–THF (95:5). Similar results were obtained for water–THF (90:10). A comparison of the chromatographic patterns as well as the peak heights of identical concentrations of the α,ω-bis(1-naphthylurethane) derivative of PEG 3000 either injected directly or after extraction with diethylether revealed no visible differences (results not shown).

The limits of detection (LODs) for PEG 600, PEG 1000 and PEG 3000 are approximately 0.1 ppm, corresponding to absolute amounts of 10 ng of each sample injected on-column in a volume of 100 μl based on ''chromatographic fingerprint'' recognition. This value is in a similar order of magnitude as those obtained by measurement of the intrinsic fluores-

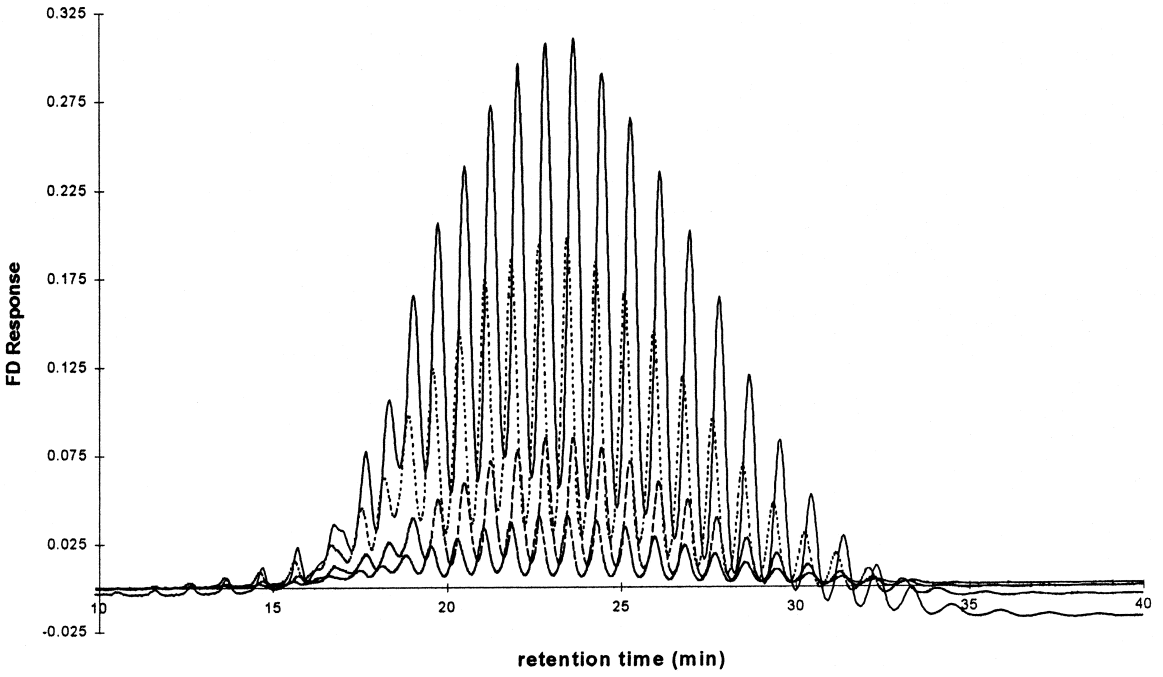

Fig. 7. Dependence of signal resolution of the α,ω-bis(1-naphthylurethane) derivative of PEG 1000 (5 ppm) dissolved in water–THF (95:5) on the injection volume: solid line (lower trace): 10 μl, dashed line (lower middle trace): 20 μl, dotted line (upper middle trace 2): 50 μl, solid line (upper trace): 100 μl.

cence responses of alkylphenolethoxylates or alkylethoxylates having been reacted with the same fluorophor (see Section 4.3). The chromatograms obtained from injections of 1 ppm and 0.1 ppm, respectively, of the α,ω-bis(1-naphthylurethane) derivative of PEG 3000, which represents the most important sample under investigation, dissolved in 100 μl water–THF (90:10) are shown in Fig. 8a and b.

When PEG 600, PEG 1000 and PEG 3000 dissolved in physiological saline are subjected to the derivatization procedure, no changes in terms of ''chromatographic fingerprint'' and relative peak height of the different oligomers are recognisable in the chromatograms compared with those obtained from the samples dissolved only in organic solvent (results not shown). The chromatograms obtained from PEG 3000 dissolved in 0.9% sodium chloride (50 μg/ml, 5 μg/ml and 1 μg/ml), from which 50 μl corresponding to absolute amounts of 2.5 μg, 0.25 μg and 0.05 μg were used for derivatization, are shown in Fig. 9a–c. This fact impressively demonstrates that even small sample volumes containing low concentrations of PEG 3000 can be measured with sufficient sensitivity. Although the sample containing 1 μg/ml of PEG 3000, corresponding to an absolute amount injected of 10 ng, still shares a ''chromatographic fingerprint'' (Fig. 9c) comparable with that of a sample of substantially higher concentration, it may be regarded as the limit of detection because oligomers of low frequency abundance merge with the baseline noise (signal-to-noise ratio$<$2:1).

Due to the fact that an ''unprotected'' silica gel stationary phase was required for sufficient signal resolution, the column lifetime is substantially lower compared with the more generally used reversed-phase matrices. However, the column still shows satisfactory performance after about 11 000 column volumes corresponding to about 200 individual separations each lasting 55 min including column re-equilibration.

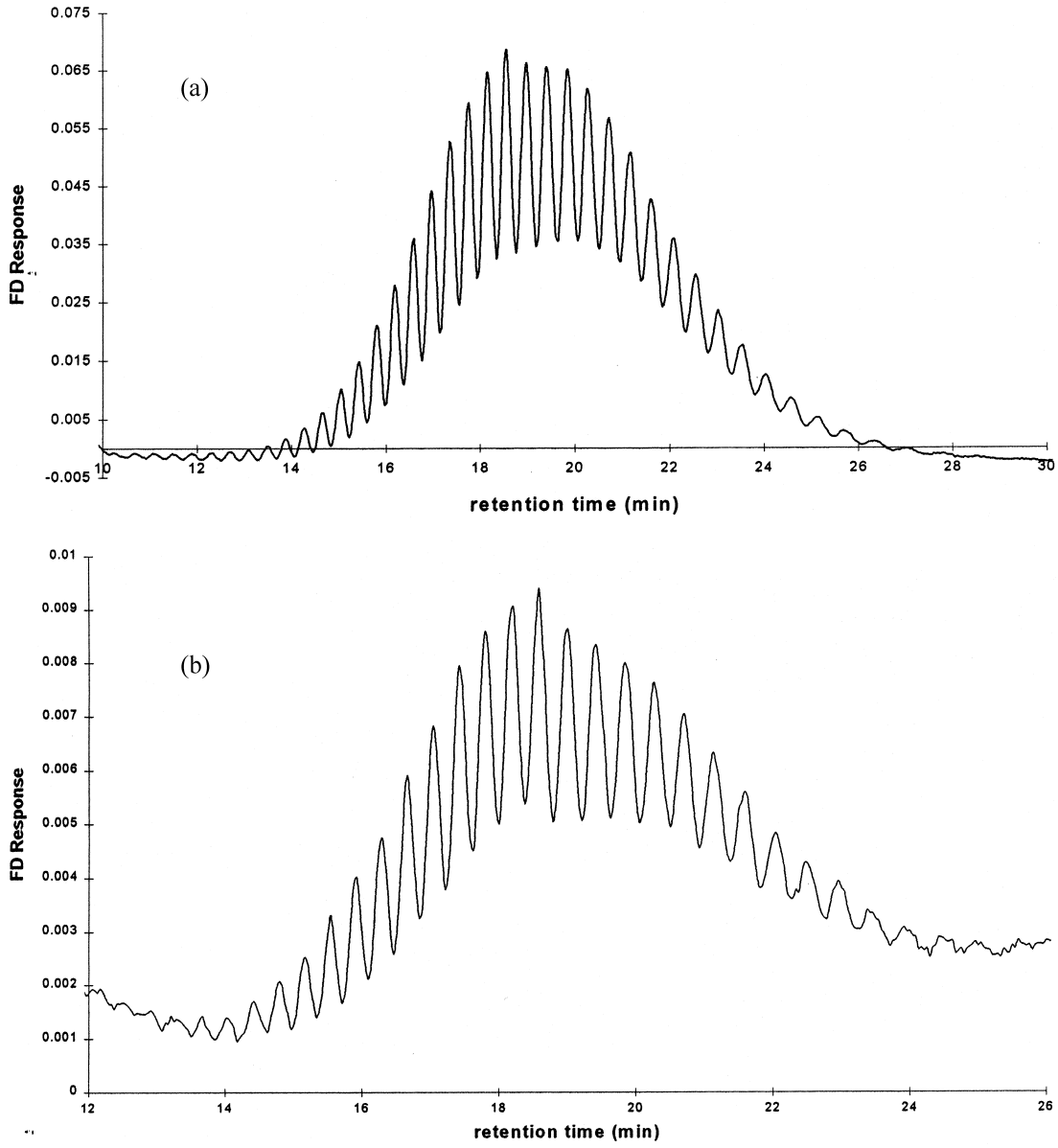

Fig. 8. Chromatograms of the α,ω-bis(1-naphthylurethane) derivatives of 1 ppm PEG 3000 (a) and 0.1 ppm (b) dissolved in 100 μl of water–THF (90:10) on a Spherisorb Si 80 (125×4.6 mm I.D., 5 μm particle size) column with gradient programme III (Table 3).

4. Discussion

4.1. Derivatization and sample preparation

Derivatization with 1-naphthylisocyanate offers some strong advantages over competing procedures, such as, e.g., reaction with 1-naphtoylchloride, 9-anthroylnitrile, 9-fluorenyl-methyloxycarbonylchloride and carbazol-9-carbonylchloride. This primarily concerns the simplicity of reaction performance, requiring neither solvent nor base catalyst and thus being directly applicable to the solid sample residue.

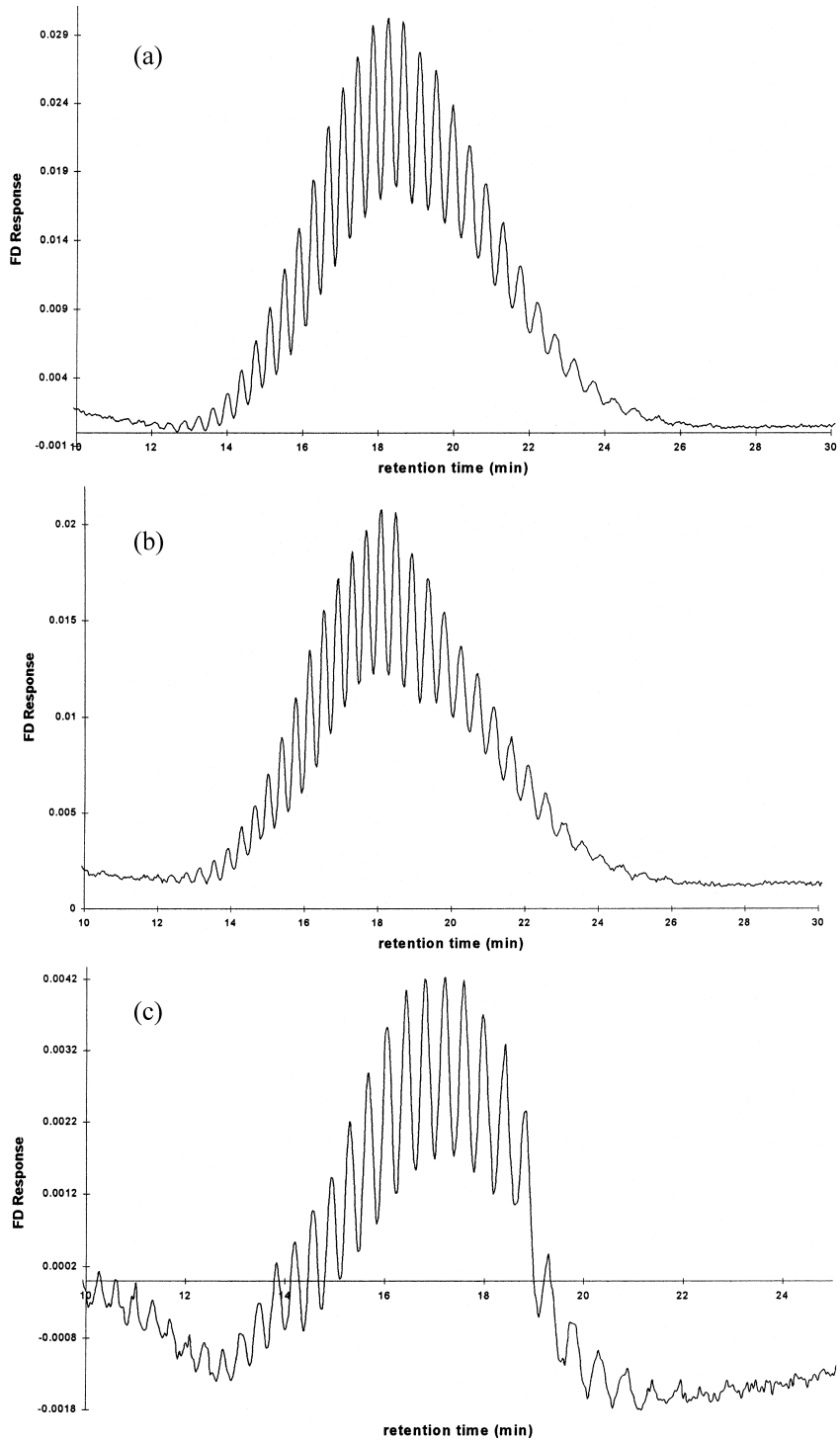

Fig. 9. Chromatograms of the α,ω-bis(1-naphthylurethane) derivatives obtained from 50 μl of ''artificial PEG 3000 samples'' dissolved in 0.9% sodium chloride: 50 μg/ml (a), 5 μg/ml (b), 1 μg/ml (c) on a Spherisorb Si 80 (125×4.6 mm I.D., 5 μm particle size) column with gradient programme III (Table 3).

Furthermore, excessive reagent can be easily converted to the corresponding 1-naphthylurethane and naphthylurea derivatives by addition of methanol or aqueous ammonia, which both elute near the column's void volume and therefore, do not interfere with separation of the PEG derivatives. As an additional advantage, it should be noted that among the five derivatives prepared during the course of the investigations, only the 1-naphthylurethane derivatives of the investigated PEGs show either sufficient detection sensitivity or chromatographic separation (results not shown). For this reason, 1-naphthylisocyanate was chosen in all further derivatizations of PEG samples. Due to the fact that no substantial increase in signal intensity was observed by increasing the amount of derivatization reagent from 10 μl to 50 μl, we assume a nearly quantitative conversion of the selected PEG samples, although no determination of individual derivatization yields was done. With PEG 600, PEG 1000 and PEG 3000 originally dissolved in acetonitrile as well as water and physiological saline, no influence of the sample matrix on derivatization yields of the α,ω-bis(1-naphthylurethanes) was observed and relative peak heights of the individual oligomers are almost identical. Unlike determination of the LODs of the PEG α,ω-bis(1-naphthylurethane) derivatives, where sequential dilution of the samples is necessary, the real samples containing the target component at the μg/ml level, require rather concentration than dilution. Therefore, dilution of the derivatized sample reconstituted in methanol with water, which is necessary to achieve optimum signal resolution, should be kept as low as possible (see below). However, precipitation of excessive derivatizing agent occurs at these conditions. The first approach using simple filtration of the suspension in order to remove the reagent was unsuccessful, presumably due to co-precipitation of the small amounts of the α,ω-bis(1-naphthylurethane) derivatives. As a consequence, we decided to removed the excessive reagent by extraction into an extensively water-immiscible solvent. From three tested solvents, i.e., cyclohexane, chloroform and diethylether, only the latter proved to be superior in terms of reproducibility. When compared with a ''non-extracted'' sample of identical concentration, extensive trapping of the analyte in the aqueous layer was achieved yielding about 80% recovery. For this reason, despite the two hydrophobic endgroups, only minor amounts of the derivative are extracted into the sparingly water-soluble organic solvent, which can be ascribed to the pronounced hydrophilicity of the PEG backbone.

At the first sight sample preparation seems to be very tedious and time-consuming and thus may not be suited for large sample numbers. Nevertheless, it should be emphasised that all steps prior to and following derivatization, which still takes place ''off-line'', can be performed by means special sample preparation devices, available from a wide variety of suppliers of chromatographic systems. As a consequence, despite a fully ''off-line'' procedure was chosen at this stage of method development, most operations can be automated and thus save time and money.

4.2. Chromatography

Signal resolution of the α,ω-bis(1-naphthylurethane) derivatives of PEG 600 and PEG 1000 on the Spherisorb Si 80 column is comparable to that observed for the corresponding underivatized PEG 600 and PEG 1000 samples as well as their α,ω-diacetates on the same stationary phase and by evaporative light scattering detection (ELSD) [23].

However, in contrast to Zanette et al. [12], who reported excellent separation of the 1-naphthylurethane derivative of PEG 600 on a Lichrosorb C_{18} matrix, we did neither achieve a corresponding signal resolution on the same stationary phase nor on a variety of other reversed-phase materials from different manufacturers (results not shown) and thus the authors' results remain questionable. Only in some cases, the signal attributable to the derivative of PEG 600 became more or less broad, but resolution into distinct oligomers was not observed.

Whereas separation of both the α,ω-bis(1-naphthylurethane) derivatives of PEG 600 and PEG 1000 (Fig. 2a and 2b) has been achieved with a binary gradient system of acetonitrile and water, the corresponding PEG 3000 sample was almost completely retained on the column matrix under these conditions. It is worthy to note that a recently published paper [25] describes investigations targeted to separate synthetic polyesters into a maximum number of oligomers for their assignment on the base of the

''chromatographic fingerprint''. Herein, THF was successfully applied as a ternary co-eluent due to its excellent solvation properties for components of higher M_r. For this reason, an attempt was started to apply this novel technique to the separation of underivatized PEG 3000 oligomers. Indeed, superposition of a shallow THF gradient up to a final concentration of 20% for 40 min (Table 3) effected separation into a wide variety of oligomers being sufficient for recognition of PEG 3000 on the base of its characteristic ''chromatographic fingerprint'' (Fig. 4). As expected, application of the same elution conditions provided also acceptable satisfactory signal resolution of the corresponding α,ω-bis(1-naphthylurethane) derivative, as demonstrated in Fig. 5. Admixture of THF as the ''co-solvent'' is essential, because its absence causes almost complete retention of both underivatized PEG 3000 and its α,ω-bis(1-naphthylurethane) derivative on the strongly polar silica gel stationary phase. A further gain in R_s may yet be achievable by modifying the gradient profile, but in this case signals, in particular those attributable to late-eluting oligomers, will become substantially broader and, as a consequence, detection sensitivity decreases.

The complete lack of separation of the α,ω-bis(1-naphthylurethane) derivatives of all investigated PEGs into individual oligomers on a C_{18} column matrix (results not shown) is indicative that highly polar stationary phases are an ultimate prerequisite for optimum solute–matrix interactions. Due to the fact that neither native PEG 3000 nor its α,ω-bis(1-naphthylurethane) derivative could be eluted from a bare silica gel column by use of a binary acetonitrile–water gradient, a stationary phase of substantially lower polarity compared with silica gel, such as a CN column was initially chosen for separation of PEG 3000. Unfortunately, no signal resolution into individual homologues was achieved, but unlike chromatography on a C_{18} matrix yielding only a single signal of small peak width, a more or less broad peak envelope resulted on the CN material. This observation can be satisfactorily explained that in contrast to the C_{18} stationary phase showing no discrimination differentiation of homologues, the CN column exhibits substantially better individual interactions with the oligomers of PEG 3000. Nevertheless, polarity of the CN matrix still proves too low for sufficient signal resolution, which unfortunately could not be improved by replacement of acetonitrile as the organic modifier by methanol, i.e., a solvent being capable of hydrogen bond formation (results not shown).

It is very important to note that the residue of a PEG 3000 sample after reaction with 1-naphthylisocyanate cannot be dissolved in a small volume of THF and then injected onto the stationary phase due to the huge excess of reagent, which strongly interferes with FD and thus has to be removed before injection. For this reason, the derivatized hydrophilic sample has to be trapped in the aqueous phase and the reagent eliminated by extraction into a sparingly water-soluble solvent. Nevertheless, in order to compensate for this inevitable dilution step, rather high analyte volumes can be injected.

Due to the excellent separation properties of PEG derived non-ionic surfactants on bare silica gel stationary phases compared with classical RP-HPLC, this technique becomes increasingly important. Desmaizières et al. [26] evaluated separation conditions for alkyl(arylalkyl) polyethoxylates on silica gel matrices under typical ion-exchange conditions by use of different cations and anions in the mobile phase for complexation of the oxyethylene moieties and also discussed the influence of temperature on separation efficiency. The authors achieved excellent separation of oligomers up to $n=60$ using a binary eluent system of acetonitrile and water containing 5 m*M* of sodium acetate under isocratic conditions and temperature programming. Ibrahim and Wheals [27] also applied binary mixtures of acetonitrile and 14 m*M* phosphate buffer of pH 3 and 7, respectively, for either isocratic or gradient chromatography of alkylphenol ethoxylate surfactants on a Spherisorb silica gel column and obtained satisfactory separation of up to 20 oligomers. The authors termed chromatography performed in this way as a ''pseudo-reversed-phase process''. Kibbey et al. [28] effected baseline separation of both alkylethoxylates and alkylphenol ethoxylates on different silica gel stationary phases by use of a binary gradient built up from acetonitrile and water. Last but not least, it is worthy to note that at the end of the present investigations Sun et al. [29] described separation of PEGs with average M_r 400 to 2000 on an aminopropyl (NH_2) column by means of a binary gradient

composed of acetonitrile and water. The promising technique was tested in our laboratory for its ability to separate the α,ω-bis(1-naphthylurethane) derivative of PEG 3000 into a maximum number of oligomers using the experimental conditions reported by the authors and either native (underivatized) PEG 3000 or its α,ω-bis(1-naphthylurethane) derivative were investigated. We found that native PEG 3000 exhibits slightly lower separation efficiency on the NH_2 column matrix compared with bare silica gel, whereas comparable resolution of the individual oligomers was obtained with the corresponding α,ω-bis(1-naphthylurethane) derivative (results not shown). Although the late-eluting oligomers exhibit markedly higher peak widths compared with the silica gel matrix and thus show lower sensitivity, both methods work well and offer similar application ranges. Therefore, the mutual advantages and drawbacks should only be verified in long-term investigations. However, the most conspicuous feature was that the α,ω-bis(1-naphthylurethane) derivative of PEG 3000 was much less retained on the NH_2 stationary phase compared with the underivatized sample, which can be interpreted that the hydrophobic endgroups significantly influence solute–matrix interactions on this adsorbent. This is in contrast to the situation on a bare silica gel column, on which either native PEG 3000 or its α,ω-bis(1-naphthylurethane) derivative show similar retention.

Despite its excellent separation properties in the case of the investigated PEGs, bare silica gel is a relatively critical stationary phase for chromatography in aqueous organic media and thus will tend to be destructed when marked amounts of water are used in the mobile phases. A further drawback lies in the fact that in contrast to typical reversed-phase materials, more or less marked batch-to-batch variations have to be considered. Fortunately, when using different batches of adsorbent, only a more or less marked shift of the individual PEG oligomers to either lower or higher retention times takes place without substantial change of the characteristic ''chromatographic fingerprint''. Furthermore, signal resolution can slightly change, but no marked changes between different column batches are observed. For this reason, slight modifications of both the starting conditions and the profile of the gradient may sometimes be necessary in order to correct for changes in retention time. As a remedy to improve column lifetime, Kibbey et al. [28] recommend the use of a ''silica saturation column'' prior to the injection system. However, as experienced in numerous injections in our laboratory, it should be taken into account, that an upcoming column deterioration cannot be predicted on the basis of, e.g., continuously proceeding more or less marked changes on signal resolution. Surprisingly, it is a suddenly occurring process, which is characterized by an almost complete loss of oligomer separation within only a few injections. Eventually an NH_2 material offers the advantage of higher stability against hydrolytic dissociation of the aminopropyl substituents compared with its bare silica gel counterpart and, for this reason, higher column lifetimes may be expected.

4.3. Detection

Besides satisfactory separation of PEGs into a maximum number of individual oligomers, highly sensitive detection techniques are required for application to low-level determinations of this substance group in investigations of both intestinal permeability and environmental degradation processes.

Despite the fact that both hydroxy groups of the PEGs are reacted and thus the fluorescence response is doubled, LODs of the well-separated homologues will be of at least one-order of magnitude lower compared with those obtained when the whole amount of homologues is lumped into one signal envelope, as experienced by the so-called ''liquid chromatography under critical conditions'' (LCCC) [30–33]. Unlike real LCCC, where separation is only effected with respect to the chemistry of endgroups (i.e., the 1-naphthylurethane substituents) and not of repeating oxyethylene units by a simple change in solvent composition, merging of individual signals of PEG oligomers is also accomplished by a change from bare silica gel to C_{18} stationary phases. Despite yielding a gain in detection sensitivity, this alternative cannot be applied to the identification of ''selectively removed'' PEG oligomers, which is often required in analyses of these components in biological fluids as well as effluents from sewage treatment plants. Therefore, only methods providing an optimum signal resolution have to be used.

Unfortunately, most authors did not mention in

their reports whether LODs are related to individual oligomers or the whole entity of homologues. Calculation of LODs based on S/N ratios of approximately 2–3 as done with low M_r analytes is difficult because the concentrations of each oligomer should be known, which includes baseline separation of all oligomer peaks. However, this goal is only achievable in limited applications, in particular when oligomeric mixtures of low M_r are investigated. Therefore, in this study in accordance with Ref. [22], LODs are defined as the minimum sample concentration, which is necessary for an unambiguous identification based on the ''chromatographic fingerprint''. In earlier investigations Kudoh et al. [34] reported detection limits of about 0.2 ppm for alkylphenolethoxylates after separation on a silica gel column by normal-phase HPLC exploiting the intrinsic fluorescence of the alkylphenol group and 0.05 ppm (≡50 ppb) and for alkylethoxylates by RP-HPLC after prior derivatization with 1-anthroylnitrile. Holt et al. [35] obtained detection limits of 0.2 ppm for individual oligomers of octylphenolethoxylates after normal-phase HPLC, which means that the detection limits related to the whole entity of oligomers will be at least one-order of magnitude higher. Detection limits of about 5–10 ng of both alkylethoxylates and PEGs derivatized with 1-naphthylisocyanate ($S/N \approx 10$) as well as 50 ng for the corresponding alkylphenoxy derivatives, respectively, both injected on-column, were calculated by Zanette et al. [12] and Scullion et al. [36]. Marcomini and co-workers [37,38] observed detection limits of nonylphenolethoxylates in the lower ppb region. However, the RP-HPLC technique used by the latter authors did not discriminate between the different oligomers and, as a consequence, the excellently low limits of detection are ascribed to the merging of individual homologues into one peak.

When compared with the closely related family of alkylphenol- or alkylpolyethoxylates [33–35], our observed detection limits for the α,ω-bis(1-naphthylurethane) derivatives of PEGs lie within the same order of magnitude or are even better. In particular, it is of interest that, despite the different findings in chromatographic separation between Zanette et al. [12] and our report, the determined limits of detection are in a comparable range.

It is also of importance to mention a rather new method, termed ''condensation nucleation light-scattering detection'' (CNLSD), published by Koropchak et al. [39], which can be regarded as a further development of ELSD. In this technique the dry particles obtained after ''stripping off'' the solvent shell of the aerosol droplets taking place in classical ELSD are mixed with a second flow of gas, which is saturated with a condensible fluid, such as, e.g., butanol. The mixture of dry particles and saturated vapor is then rapidly cooled causing ''supersaturation'' of the mixture, which effects condensation and growth of the particles from the nm range, as observed in typical ELSD, to the μm range. As a logical consequence, the larger particles are much more effective in light scattering and detection sensitivity increases about 1000-fold compared with ELSD. Average detection limits of 15 ng/ml for PEGs ranging from M_r 1000–45 000 were determined. However, the chromatographic system used by Koropchak et al. [39] did not effect separation of the PEGs into individual oligomers, which raises the question if this method provides sufficient sensitivity for its application to underivatized (native) PEGs after satisfactory separation into individual oligomers and thus making derivatization superfluous. As published recently by Szostek et al. [40], a further gain in sensitivity is achieved when the aerosol is formed using the electrospray (ES) technique instead of pneumatic nebulization. By coupling ES-CNLSD to capillary zone electrophoresis (CZE), underivatized peptides and amino acids could be detected in the sub μg/ml range corresponding to absolute amounts injected in the sub pg range.

5. Conclusions

The aim of this study was to develop a HPLC assay providing both highly efficient separation and sensitive detection of the α,ω-bis(1-naphthylurethane) derivatives of PEG 600, PEG 1000 and PEG 3000 for future investigations of the permeability of these components across biological membranes. Optimum separation of PEG derivatives was only achievable on bare silica gel stationary phases with a binary solvent gradient of acetonitrile and water for PEG 600 and PEG 1000 and a ternary system consisting of acetonitrile, water and THF for PEG

3000. The hydrophilic backbone of the PEGs permits sample dissolution in aqueous organic solvents lean in organic modifier. Therefore, the sample is trapped in a small band on the column head allowing injection of relatively large volumes without substantial loss in peak resolution. The new technique can be termed as a ''pseudo-reversed-phase separation process'' as proposed by Ibrahim and Wheals [27]. Determination of the samples down to the lower ppm or even upper ppb range was achieved by measurement of the fluorescence responses at $\lambda_{ex}=232$ nm and $\lambda_{em}=358$ nm. For this reason, these detection limits should be sufficient for the determination of PEGs of different M_r in samples of environmental and biological origin, such as those obtained from dialysates or sewage treatment plants, providing satisfactory information of selectively ''removed'' or ''permeated'' oligomers. In order to exploit the whole gain in sensitivity given by the method, it is advisable to insert a prior sample clean-up or preconcentration step, such as, e.g., SPE [35]. In contrast, alternative sample preparation techniques, such as, e.g., ''exhaustive steam distillation'' [41] and ''gaseous stripping'' into ethyl acetate [42] will be the methods of choice for enrichment of PEGs in environmental samples.

To our knowledge, this is the first report describing highly efficient separation of PEGs covering a wide range of oligomeric distribution combined with detection limits in the lower ppm region based on the ''chromatographic fingerprint''. In particular, more than 30 signals attributable to individual oligomers of PEG 3000 were observed and MALDI-TOF-MS confirmed that the whole entity of oligomers in the sample had been separated chromatographically. Considering the fact that these high M_r homologues differ only in 44 u, the degree of mass resolution is adequate. Taking into account the fact, that the observed excellent signal resolution provides a detection limit of approximately 0.1 ppm for the whole entity of oligomers, the ''true'' detection limits for the individual oligomers will be at least one-order of magnitude lower, i.e., in the lower ppb range.

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