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Short communication

Development of a high-performance liquid chromatography-fluorescence detection method for analyzing nonylphenol/dinonylphenol-polyethoxylate-based phosphate esters

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ABSTRACT

A novel reversed phase HPLC method for the simultaneous analysis of surfactants containing nonylphenol/dinonylphenol-polyethoxylates and their *o*-phosphate esters is reported, in which eluting substances are detected fluorescently. Their chemical structures were elucidated by direct infusion electrospray-mass spectrometry in positive mode. The limits of quantification and range of the method were determined to be 0.1 mg and 0.1–100 mg surfactant L^{-1}, respectively, with a reproducibility (RSD) at a concentration of 38 mg surfactant L^{-1} of 5.6%. The accuracy was determined by spiking selected process water samples with known amounts of surfactant, and recoveries were typically in the 82–102% range.

1. Introduction

Nonylphenol/dinonylphenol-polyethoxylate-based phosphate esters (NPEO/DNPEO-PEs) are anionic surfactants that are stable in strongly alkaline solutions and have industrial applications, mainly as lubricants or surfactants. The phosphate esters are produced from mixtures of nonylphenol (NP, mostly 4-NP, with minor amounts of 2-NP) and dinonylphenol (DNP, predominantly 2,4-DNP, with minor amounts of 2,6-DNP), which are ethoxylated then phosphorylated. The commercially available phosphate esters often include various amounts of free alcohols (NPEOs and DNPEOs) and minor amounts of free *o*-phosphoric acid. The esters are mainly mono- and di-esters, although minor amounts of tri-esters can also occur [1,2], and phosphate esters originating from polyphosphoric and metaphosphoric acids may be present as by-products. Chemical structures of mono-, di- and tri-NPEO and DNPEO-PEs are shown in Fig. 1. The average ethoxy chain lengths (n) of NPEOs/DNPEOs and the corresponding phosphate esters in the surfactants investigated here should be 10, according to the manufacturer's specification, although their oligomer distribution is broad (3–19).

NPEOs/DNPEOs are of considerable environmental concern since they bioaccumulate and the NPEO degradation products 4-nonylphenol (4-NP), 4-nonylphenol-monoethoxylate (4-NP1EO) and 4-nonylphenol-diethoxylate (4-NP2EO) are estrogenic and toxic to aquatic organisms, causing endocrine disrupting effects both *in vitro* and *in vivo* [3–8].

Thus, there is a need for a convenient method to determine their concentrations in industrial process streams. In the presented study an analytical method was developed that is capable of quantifying NPEO/DNPEO-PEs, NPEOs/DNPEOs in a simultaneous chromatographic run. Chromatographic separation of NP- and DNP-containing substances was desired, due to the differences in the strength of their environmental effects.

The samples used in the analysis originated from a protein separation media manufacturing factory (GE Healthcare) in Uppsala, Sweden, where surfactants are removed from the effluent stream by a filtration procedure. The samples were obtained from the effluent stream before and after this treatment. The purpose of the developed method is to monitor the efficiency of this procedure.

Reversed-phase (RP)-HPLC was deemed to be the most convenient HPLC mode since it allows water-containing samples to be directly injected in a mobile phase comprising water miscible solvents. RP-chromatography with C_8- and C_{18}-packings allows the resolution of most alkylphenol polyethoxylates (APEOs) according to alkyl chain length. Cheng and Ding presented a method for determining levels of commercial NPEOs in household detergents, in the

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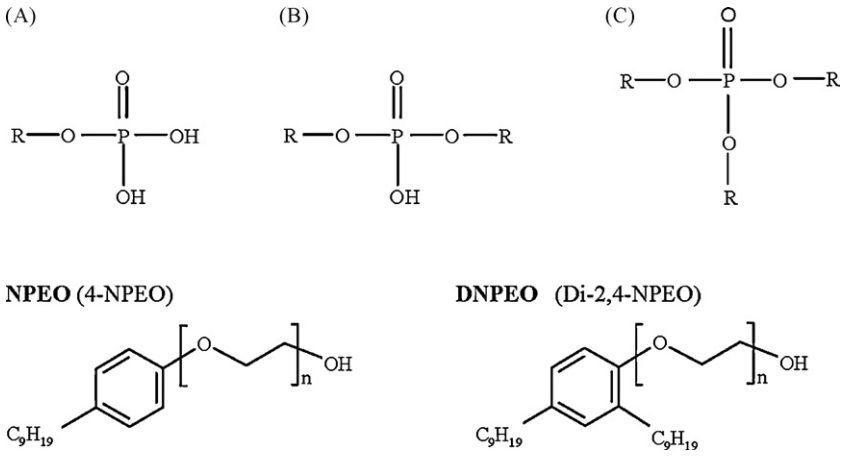

Fig. 1. Chemical structures of (A) mono-*o*-phosphate esters, (B) di-*o*-phosphate esters, (C) trip-*o*-phosphate esters. NPEOs and DNPEOs contain structure and chain-isomers. R = NPEO or DNPEO, n = 3–19.

concentration range 0.1–100 mg L^{-1}, using a C_8 reversed phase column and fluorescence detection with an excitation wavelength of 228 nm and an emission wavelength of 305 nm [9]. Furthermore, Tsuda et al. showed that baseline-separation of NP, NP1EO and NP2EO could be obtained using the phenylic RP stationary phase Inertsil PH [10].

LC–MS systems are often used for both quantitative and qualitative analysis of NP and NPEOs. The most common technique for ionizing NP and NPEOs is electrospray ionization (ESI), using either ion trap (LC–IT-ESI-MS) or quadropole (LC–Q-ESI-MS) instruments [11–16]. MS-based quantification of NPEOs is troublesome since the response of the MS detector rapidly decreases with reductions in the number of ethoxy groups [17], hence the oligomer distributions in the calibration standard and the sample need to be very similar. Therefore, in the present study, RP-HPLC with fluorescence detection was used for quantification purposes (following optimization to maximize the separation of the analytes) and LC–IT-ESI-MS was used to confirm the identities of the separated peaks.

2. Experimental

2.1. Chemicals

4-Nonylphenol-polyethylene glycol with an average of 10 EO units (NP10EO) was purchased from Fluka, Switzerland. Surfonic DNP-40, including regio and chain-isomers of DNP4EO, was supplied by Huntsman Petrochemical Corporation (USA). Rhodafac RM 510 including NPEO/DNPEO-PEs (mono- and diphosphate esters) and Igepal DM-430 containing branched NPEOs and DNPEOs were supplied from Novecare, Rhodia Inc. (USA). In addition to the phosphate esters, RM 510 also contains non-ionic constituents (polyethoxylates), but the main constituents are phosphate esters.

Process water samples: BF-water and AF-water (aqueous effluent streams from a protein separation media manufacturing factory) (GE Healthcare) in Uppsala, before and after the filtration treatment, respectively.

HPLC chemicals: acetonitrile (gradient grade for LC) and trifluoroacetic acid (TFA) for spectroscopy (99.8%) were both obtained from Merck (Germany) and Milli-Q^{TM} water from Millipore Corporation (USA). TFA oxidizes with age which may lead to ghost peaks [18]. Therefore, TFA was used within six months following its purchase.

2.2. Instrumentation

HPLC-FLD analyses were performed using a Shimadzu-Prominence system including a SIL-20AC autosampler, a CTO-20AC column oven, a CBM-20A system controller, a DGU-20A5 degassing unit, a LC-20AD solvent delivery unit and a RF-10AXL fluorescence detector with a 12 μL flow-cell. The HPLC-column used was an ACE 3 Phenyl column (3 mm × 150 mm, 3 μm particles) from Advanced Chromatography Technologies (ACT, USA), except in some optimization tests, described in Section 3.

Confirmatory MS-analyses were performed using an Agilent 1100 Series LC/MSD ion trap instrument from Agilent Technologies (USA), which has an orthogonal electrospray ionization (ESI) source.

2.3. Methods

2.3.1. HPLC-FLD analyses

The surfactant Rhodafac RM 510 was used as a calibration standard for quantification purposes since its composition resembles the chemical profile of the BF-water. According to the supplier, the surfactant contains at most 36% non-ionic ethoxylates and DNP-containing substances predominate since the initial DNP/NP blending ratio is 65/35 (w/w).

Standard calibrations were performed using two concentration ranges including different sensitivity settings to avoid FLD-signal saturation and to increase the sensitivity at low surfactant concentration levels: 0.05–2 and 2–20 mg surfactant L^{-1} (the former with the high sensitivity setting of the fluorescence detector, and the latter with the medium sensitivity setting). The calibration solutions are generally stored for one week in a refrigerator between measurements.

Prior to HPLC-FLD analysis (under conditions found to be optimal in the optimization trials reported below), 10 mL samples were centrifuged in polypropylene centrifuge tubes for 5 min at 5000 rpm using a Hettich, Universal 320 centrifuge, then 15 μL portions of the supernatant were injected with the mobile phase flow rate set to 0.6 mL min^{-1}. The column oven temperature was 50 °C, and the mobile phase consisted of a linear gradient of 40–64% B from t = 0 to t = 4.8 min, followed by a linear rise to 97% B at t = 18.0 min, held isocratically until t = 23.0 min, before returning to 40% B, at 23.1 min, which was maintained to re-equilibrate the column until t = 28 min. The HPLC-mobile phases were A: 0.1% (v/v) TFA in Milli-Q water, B: 0.1% (v/v) TFA in acetonitrile. The fluorescence detection was most sensitive with an excitation wavelength of 230 nm and emission wavelength of 310 nm.

2.3.2. ESI-MS-analyses

15 μL of a 1000 mg L^{-1} sample of the RM 510 surfactant was injected into the HPLC system and eight fractions, each of ca. 300 μL,

Table 1
Electrospray operation conditions.

Dry temp (set)	325 °C
Nebulizer (set)	20.00 psi
Dry gas (set)	5.00 L min^{-1}
Trap drive	85.6
Octopole RF amplitude	200.0 Vpp
Capillary exit	181.0 V
Skimmer	40.0 V
Oct 1 DC	12.00 V
Oct 2 DC	3.11 V
Scan begin	300 *m/z*
Scan end (1)	1200 *m/z*
Scan end (2)	1600 *m/z*
Averages	50 spectra
Max. accu time	20,000 μs
ICC target	300,000
Charge control	0

containing the main chemical constituents at concentrations in the 1–3 mg L^{-1} range, were manually collected. A 100 μL portion of each fraction was continuously pumped (directly infused) to the electrospray interface (operating under conditions shown in Table 1) using a Hamilton syringe mounted in a KD Scientific syringe pump at a flow rate of 3 μL min^{-1}. The positive ion detection mode was used for all fractions. The 1600 *m/z* scan end was used for the NPEO/DNPEO-di-PE-analyses.

3. Results and discussion

3.1. HPLC-FLD analyses

Since the samples containing NPEO/DNPEO-PEs and their free alcohols contained solid residues, it was necessary to establish a clean-up procedure to remove these substances prior to HPLC injection. The analytes readily adsorb to solid glass-containing surfaces, so the initial use of glassfilters was rejected. However, a simple centrifugation procedure was sufficient for clean-up purposes (see Section 2.3.1).

The HPLC-FLD method was optimized to resolve the chromatographic peaks for both quantification and identification purposes, by varying the stationary phase, mobile phase and gradient profile. In these experiments the performance of three columns – an ACE 3 phenyl-propyl (150 mm × 3 mm, 3 μm particles), an ACE 3 octyl (C8; 150 mm × 3 mm, 3 μm particles) and a Luna phenyl-hexyl (150 mm × 3 mm, 3 μm particles, Phenomenex, USA) column – as stationary phases was investigated. In addition, the FLD excitation and emission wavelengths were optimized with respect to signal/noise ratios and ghost peak occurrences [19]. The emulsifier components were best resolved using the ACE 3 phenyl-propyl stationary phase. The optimal resolution was achieved with the TFA in Milli-Q water and acetonitrile gradient described in Section 2.3.1. The optimized HPLC-FLD conditions yielded eight peaks that were adequately resolved, as shown in Fig. 2, and the total surfactant concentrations in the samples were quantified by summarizing all integrated peaks.

From spiking experiments, it was concluded that peak 2 corresponds to 4-NPEO, while peaks 4 and 6 correspond to different constituents of technical DNPEO, probably the regio-isomers 2,4- and 2,6-DNPEO [20] (see details in Supplementary Material). Thus, the other peaks presumably correspond to NPEO-PEs and DNPEO-PEs. The chemical structures of the substances represented in all peaks were determined by direct infusion ESI-MS analysis of manually collected fractions of each peak (see details in Supplementary Material). The chemical structure elucidation revealed that the most polar compounds (NPEO-mono-PEs) eluted first from the HPLC system and the most lipophilic compounds (DNPEO-di-PEs) last (fractions 1 and 8 in Fig. 2). Typical chromatograms for AF- and BF-water samples are shown in Figs. 3 and 4. The relative increase of NPEOs in the BF-water sample (peak 2) in Fig. 3 may be explained by a less tendency to adsorb onto solid surfaces.

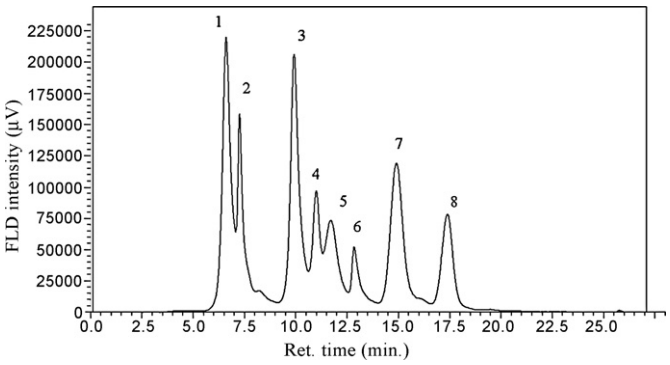

Fig. 2. HPLC-FLD chromatogram of RM 510 (20 mg L^{-1}) showing designated numbers of collected fractions. 1 = NPEO-mono-PEs, 2 = NPEOs, 3 = DNPEO-mono-PEs, 4 = DNPEOs, 5 = NPEO-di-PEs, 6 = DNPEOs, 7 = NPEO/DNPEO-di-PEs, 8 = DNPEO-di-PEs. For chromatographic conditions, see Section 3.1.

3.2. Validation procedure

The distribution of the detected components in wastewater samples is affected by the pH and both the types and amounts of adsorbents present in the streams. Orthophosphoric acid has three ionizable groups, and thus three pKa-values: $pKa_1 = 2.1$, $pKa_2 = 7.2$ and $pKa_3 = 12.3$. When the pH in an investigated sample falls close to pKa_1, the PEs become unionized, hence their aqueous solubility declines and their tendency to adsorb to hydrophobic surfaces increases. In the process streams examined here (before and after filtration), the pH is maintained at 4.5. Thus, both mono- and di-PEs are ionized to high degrees. In the BF-water, the relative proportions of the analytes are similar to those illustrated in Fig. 2. In the AF-water, trace amounts (<0.1 mg surfactant L^{-1}) of NPEO-mono-PEs, NPEO/DNPEO-di-PEs and DNPEO-di-PEs are generally present, but the other components are completely removed (or at least the amounts that are not removed are sub-detectable). See Figs. 3 and 4.

The fluorescence detector emission signal is proportional to the content of aromatics in the surfactant. Therefore, quantification conditions are optimal when the relative amounts of compounds in the samples are similar to those in the calibration standard (surfactant RM 510). For AF-water samples this is not the case, but the

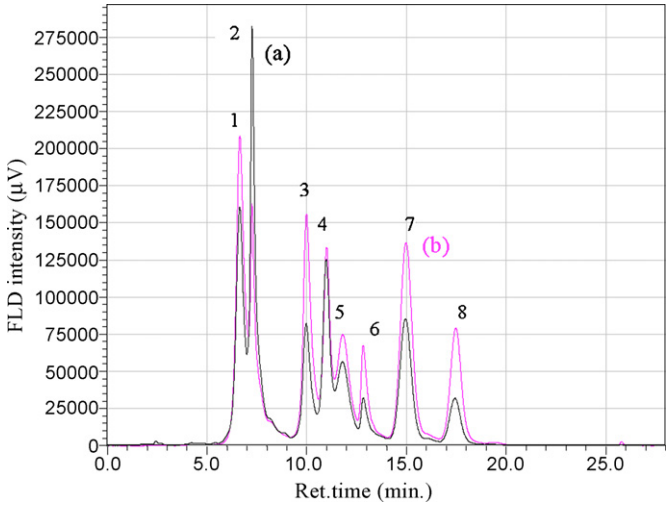

Fig. 3. Typical HPLC-FLD chromatogram of BF-water containing 17 mg surfactant L^{-1} (a) and reference chromatogram of RM 510 (20 mg L^{-1} (b)) using medium FLD-sensitivity setting.

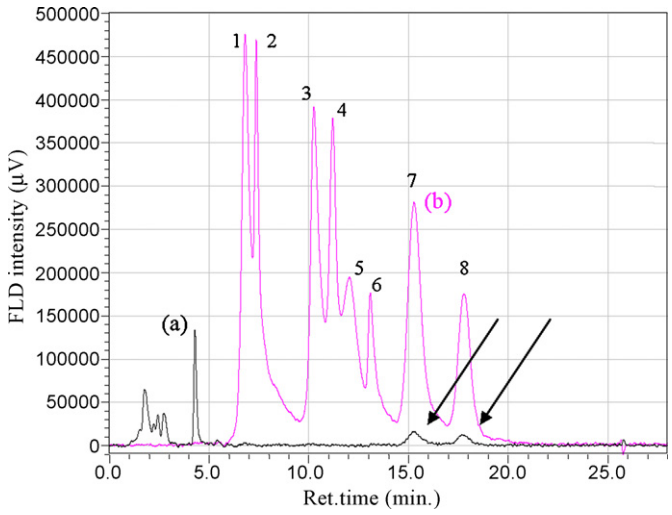

Fig. 4. Typical HPLC-FLD chromatogram of AF-water (a) and reference chromatogram of RM 510 (1 mg L^{-1} (b)) using high FLD-sensitivity setting. The AF-water contains <0.1 mg L^{-1} surfactants (arrows). The early eluting polar substances are fluorescent contaminants.

analytical results are still expressed as mg surfactant L^{-1} here, since no NPEO-mono-PE standards are currently available. The fluorescence detector responds more strongly to the NP10EO standard than to the RM 510 surfactant, but the difference in response between NPEO-mono-PEs and the RM 510 surfactant is probably smaller, since most of the constituents in RM 510 are present as phosphate esters. The validation procedure was performed using RM 510 as a test substance since it is the main surfactant in the process streams examined here. The quantification was performed by integrating all the peaks in the HPLC-FLD chromatograms and calculating the concentrations from RM 510 calibration curves.

3.2.1. Range and linearity

Since concentrations of the surfactants could rise to 100 mg L^{-1}, in some cases, while concentrations lower than 0.1 mg L^{-1} could be safely released, the method should ideally be able to quantify the surfactants within the 0.1–100 mg L^{-1} range. However, for concentrations exceeding 20 mg L^{-1}, the samples were diluted fivefold to avoid HPLC memory effects that could occur when higher surfactant concentrations are injected. Two calibration intervals were established, 0.1–2 and 2–20 mg L^{-1}, for calibrating measurements of AF-water samples (low) and BF-water samples (high). Correlation coefficients (r) exceeding 0.9979 were obtained for both concentration ranges.

3.2.2. Precision and accuracy determinations

The within- and between-day repeatability of the measurements was determined by performing triplicate analyses on different days of three samples of BF-water and one AF-water sample, with concentrations in the low and high ranges (BF-water samples) and in the low range (AF-water sample). The accuracy of the method was determined by spiking BF-water samples ($n = 3$) with the surfactant RM 510. Since the surfactant concentration of the chosen AF-water sample was very close to the LOQ (0.1 mg surfactant L^{-1}), it was not possible to assess the accuracy of measurements of this sample. Generally, the concentrations of surfactants in AF-water samples are below this level. The spikings were performed in such a manner that the spiked samples contained 50% more of the surfactant than the unspiked samples (see *Validation results* in Supplementary Material).

The reproducibility of the method was calculated by analyzing a BF-water sample on three different occasions by three different workers in triplicate. The mean surfactant concentration of the sample was found to be 38 mg surfactant L^{-1} with an RSD of 5.6%.

3.2.3. Limit of quantification (LOQ)

Calibration solutions in the 0.01–2.0 mg surfactant L^{-1} concentration range were analyzed in triplicate on three different days to determine the lowest concentration at which the precision could be maintained at a sufficiently high level (see *Validation results* in Supplementary Material).

The results clearly showed that good repeatability could be maintained even when concentrations of the surfactants were very low, and the LOQ was determined to be 0.1 mg surfactant L^{-1} (5% RSD is acceptable). However, the measurements of the BF-water samples were less precise than those of the calibration standards, presumably because surfactants adsorbed to residual substances in the samples. As noted above, it is necessary to remove these materials by centrifugation prior to analysis, and it should be pointed out that the surfactants adsorb readily to most solid surfaces, including glass and plastics, so it is essential to perform the analyses in a manner that minimizes this risk.

4. Conclusions

A method was developed for the quantitative analysis of NPEO/DNPEOs and their phosphate esters in selected process waters. The chromatographic separation of eight constituents present in a commercial surfactant mixture was accomplished using a reversed phase ACE 3 phenyl stationary phase and a mobile phase system consisting of a gradient of TFA-acidified Milli-Q water and acetonitrile. The use of fluorescence detection enables highly sensitive analyses with an LOQ of 0.1 mg surfactant L^{-1}.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2009.08.057.

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