SCIENTIFIC PAPERS
OF THE UNIVERSITY OF PARDUBICE
Series A
Faculty of Chemical Technology
**17** (2011)

# ANALYSIS OF ETHOXYLATED NONYLPHENOLS BY COMBINATION OF HPLC AND CZE

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Received September 30, 2011

*The separation of samples of ethoxylated nonylphenol type surfactants by two-dimensional method combining liquid chromatography with capillary zone electrophoresis has been studied. In the first part of the work, separation conditions using reversed-phase liquid chromatography with isocratic elution were optimized. Thus six different stationary phases were compared to provide highest resolution of ethoxylated nonylphenol surfactants with various degrees of ethoxylation in water/methanol and water/acetonitrile mobile phases. The separation of nonylphenol branch isomers was carried out using capillary zone electrophoresis in non-coated and poly(acryl amide) coated capillaries employing solvofobic interaction approach in background electrolyte containing sodium dodecyl sulfate and acetonitrile. Finally, nonionic surfactants were analyzed using combination of optimized methods of liquid chromatography and capillary zone electrophoresis in off-line automated two-dimensional system.*

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## Introduction

Nonionic surfactants namely nonylphenol ethoxylates (NPEs) were recently used both in industry and household. Their household use is restricted now due to endocrinal toxicity of short chain ethoxylates (nonylphenols with 1-3 oxyethylene units) and their precursor (and final degradation product) nonylphenol. The industrial application of NPEs consisted mainly in the cleaning agents and detergents in the production of wool, textiles, wood, paper, preparation of coatings and resins [1]. Nonylphenol used for production of NPEs is usually prepared by alkylation of phenol using nonene, resulting in a mixture of C9 branch isomers with very close chromatographic behaviour [2]. Many methods were developed and published for the separation of NPEs by high-performance liquid chromatography (HPLC) [3-15] or by capillary electrophoresis based on number of oxyethylene (EO) units [16-23].

Development of analytical methods for separation of NPEs is complicated by the solubility of homologues with long hydrophobic chains, complexity of the samples and lack of analytical standards available in required purity. Separation of NPEs by HPLC usually utilizes normal-phase (NP) or reversed-phase (RP) separation systems. The NP-HPLC is suitable to easily separate NPE oligomers, while in RP systems, separation of alkylphenolic surfactants with different length of alkyls can be also achieved (octyl-, nonyl-, decyl-) [3]. In NP-HPLC, silica gel [4,5], aminopropyl silica gel or nitrile columns [6] have been reported as suitable to separate NPE oligomers. Ferguson *et al*. [7] utilizes mixed-mode HPLC separation of NPEs on a column packed with cross-linked poly(vinyl alcohol) stationary phase, which exhibits size-exclusion based retention for NPEs with molar masses between 200 and 1500 and adsorptive retention at certain concentrations of methanol in mobile phase. Another possibility for separation of sulfated and non-sulfated NPEs in NP system represents nitrile and amino chemically bonded stationary phases with mobile phases containing tetraalkylammonium additives [5].

The separation of NPEs in reversed-phase systems is generally based on hydrophobic interactions with analytes and it is more suitable for separation of alkyl phenol ethoxylates according to the length of alkyl chains [3] and usually utilizes octyl- or octadecyl- stationary phases [8-10]. The method for determination of NPEs in emulsions and textile lubricants using amino propyl silica gel column and acetonitrile/water mobile phase has been developed by Sun *et al*. [11]. Retention mechanism of sulfated and non-sulfated NPE type surfactants using ion-pair liquid chromatography on C18 column can be described by simple theoretical model [12]. Retention of both types of surfactants decreased with increasing concentration of ion-pairing reagent cetyltrimethylammonium bromide and with increasing concentration of propane-2-ol in aqueous-organic mobile phases.

In capillary zone electrophoresis (CZE), non-ionic NPEs have to be

separated in background electrolytes, which contain additives capable of creating charged complexes with NPEs. Micellar electrokinetic chromatography mode, where the neutral analytes are separated according to the partitioning equilibria in micelles, is not suitable for the separation of NPEs, due to the low selectivity for oligomers of NPEs differing in EO units. The CZE separations are usually based on the slightly modified MEKC conditions, where the formation of micelles is suppressed using high concentration of organic solvents. Analyzed NPEs can however interact with the molecules of surfactants by solvofobic interactions and the separation is based on the differences in stability of NPE-surfactant associates [16]. The most frequently used surfactant is sodium dodecyl sulfate, usually applied in combination with acetonitrile [17,18]. Another possibility for separation of NPEs by capillary electrophoresis can be provided using non-aqueous separation conditions in background electrolyte composed of organic solvents [19] or by using capillary electrochromatography [20].

In this work, the automated off-line two-dimensional LC-CZE method previously developed in our laboratory [25] was applied to the analysis of NPE samples with the aim to separate them also according to the nonyl- chain isomers. In the first RP-HPLC dimension the different columns were tested to obtain the best separation of oligomers of NPEs. In the second CZE dimension both uncoated and coated capillaries, different buffer composition and addition of different types of cyclodextrins as potential isomer selectors were investigated to resolve the possible branch isomers of nonylphenol group. An autosampler of CE instrument was employed as the interface between the LC and the CZE separations. The experimental setup is based on automated fraction collection before the reanalysis of the collected LC fractions in the second CZE dimension which is less limited in the second dimension separation time in comparison to the on-line system. The automated manipulation with the collected fractions further improves reliability of the 2D system.

## Experimental

### Samples and Chemicals

Acetonitrile, methanol (both HPLC grade), acryl amide (99 %, CE grade), sodium dihydrogen-phosphate, sodium hydrogenphosphate, sodium tetraborate (all analytical grade), sodium dodecylsulphate (98 %) and methyl-β-cyclodextrin were obtained from Sigma-Aldrich (Steinheim, Germany). β-Cyclodextrin, (2-hydroxypropyl)-β-cyclodextrin, tetramethylethylenediamine (CE grade), boric acid (analytical grade) and 3-(trimethoxysilyl)propylmethacrylate (99 %) were obtained from Fluka (Buchs, Switzerland). Potassium persulfate (analytical grade) was from Laborchemie (Apolda, Germany). Phosphoric acid (85 %), nitric acid (65 %) and

sodium hydroxide (all analytical grade) were obtained from Lach-Ner (Neratovice, CR). Water purified using SG Ultra Clean apparatus (SG, Hamburg, Germany) was used.

Analyzed surfactants with different degree of ethoxylation Serdox NNP *n* (*n* = 5-20 EO units per nonylphenol molecule, see Fig. 1) were obtained from Servo, Delden, Netherlands.

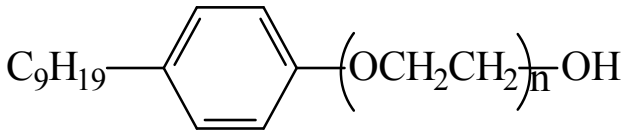


Fig. 1 Structure of nonylphenol and ethoxylated nonylphenol samples (*n* is average degree of ethoxylation)

## Equipment and Methods

LC analyses were carried out using a chromatograph consisting of Beta LC pump, six port injecting valve type D, column oven LCO 101 and UV detector Saphire 600 (all Ecom, Prague, The Czech Republic). Six columns in total packed with different stationary phases were tested: nonpolar type C18, C8 and C4, pentafluorophenyl-, cyanopropyl- and polyethyleneglycol- type. The columns used are listed in Table I. Water/acetonitrile and water/methanol mixtures were used as the mobile phases. Experiments were carried out at 40 °C and detector was set at 254 nm.

Table I List of tested columns

| Column | Dimensions | Manufacturer |
|---|---|---|
| Biospher PSI 100 C18, 7 mm | 150×4.6 mm I.D. | Labio, Praha, The Czech Republic |
| Zorbax Eclipse XDB-C18, 5 mm | 150×4.6 mm I.D. | Agilent, Palo Alto, USA |
| Zorbax Eclipse XDB-C8, 3.5 mm | 150×4.6 mm I.D. | Agilent, Palo Alto, USA |
| Ace 3 C4, 3 mm | 50×4.6 mm I.D. | Advanced Chromatography Technologies, Aberdeen, UK |
| Discovery HS F5, 5 mm | 150×4.6mm I.D. | Supelco Belfonte, USA |
| Discovery HS PEG, 3 mm | 50×2.1 mm I.D. | Supelco Belfonte, USA |
| Separon SGX CN, 5 mm | 150×3 mm I.D. | Tessek, Praha, The Czech Republic |

For CZE separations, Agilent $^{3D}$CE electrophoretic instrument (Agilent, Palo Alto, USA) was used with uncoated (50 mm I.D.) and polyacryl amide coated (75 mm I.D.) capillaries (both 48/40 cm long). The capillary was coated in our laboratory using modified Hjerten's method [26]. As the background electrolyte for analyses in the uncoated capillary, a mixture of 25 mmol $l^{-1}$ borate buffer (pH = 8.9)/acetonitrile 80:20 (v/v) with addition of SDS was used. A mixture of 10 mmol $l^{-1}$ phosphate buffer (pH = 6.8)/acetonitrile (60:40) with addition of SDS was used for separations in the coated capillary.

An autosampler of CZE instrument was used as the interface between the LC and CZE dimension for automated off-line two-dimensional LC-CZE analyses (Fig. 2). The maximum number of the collected and reanalyzed fractions was given by the capacity of the autosampler carrousel. Six-port switching valve was used to cut-off non-collected/reanalyzed portions of LC effluent. After collection of appropriate number of the fractions from LC dimension, each fraction was separated in CZE dimension with the advantage of repeated analyses, if necessary.

## Results and Discussion

### Optimization of LC Separation of Nonylphenol Ethoxylates

Samples with higher dimensionality containing two or more different chemical properties (e.g., different number of repeated structural units or positional isomers) can be potentially separated by liquid chromatography according to the number of oxyethylene groups using the two-dimensional system with orthogonal separation selectivities. For ethoxylated nonylphenols, degree of ethoxylation affects the polarity of surfactant and wetting properties, hence its distribution is important for characterization of these samples. Moreover, different configuration of nonyl-chains influences toxicity of the degradation products of NPE surfactants [2]. To achieve the best separation according to these chemical properties, separation in two-dimensional system was optimized employing LC separation in the first dimension combined with the CZE separation in the second dimension. Both methods were optimized separately to achieve the highest resolution of NPE isomers.

In the first dimension LC separation step, reversed-phase separation conditions were applied and columns with different chemically bonded silica gel stationary phases were tested (Table I). Thus columns with alkyl silica bonded stationary phases with three different lengths of carbon chains (C4, C8 and C18), cyanopropyl-, pentafluorophenyl- and polyethyleneglycol-silica phases were used in combination with aqueous-organic mobile phases consisting of acetonitrile and methanol with different volume ratios. In reversed-phase separation systems, NPEs are generally separated according to degree of ethoxylation, while lesspolar

ethoxylates with lower number of EO units in the molecules are more strongly retained in the stationary phase. Retention and selectivity of separation strongly depends on the type and polarity of chemically bonded stationary phase. For alkyl silica bonded stationary phases, the retention of NPEs increases with increasing length of alkyl chain in order C4 < C8 < C18, while the separation selectivity is best for the C8 column. Selectivity of separation of NPEs also depends on the brand of column with alkyl silica bonded stationary phase, as the resolution of NPE oligomers is higher on Zorbax Eclipse XDB-C18 column than on Biospher PSI 100 C18 column. Using polar stationary phases (cyanopropyl-, pentafluorophenyl- and polyethyleneglycol-silica), there is no visible separation of NPEs oligomers, so these columns are not suitable for application in two-dimensional LC-CZE system. Comparing acetonitrile/water and mixtures methanol/water, quality of separation was slightly better for the former one, probably due to the different type of interactions in mobile phase corresponding to the structural differences of the number of oxyethylene groups (prevailing dipole-dipole interactions for acetonitrile in comparison to proton-acceptor interactions of methanol).

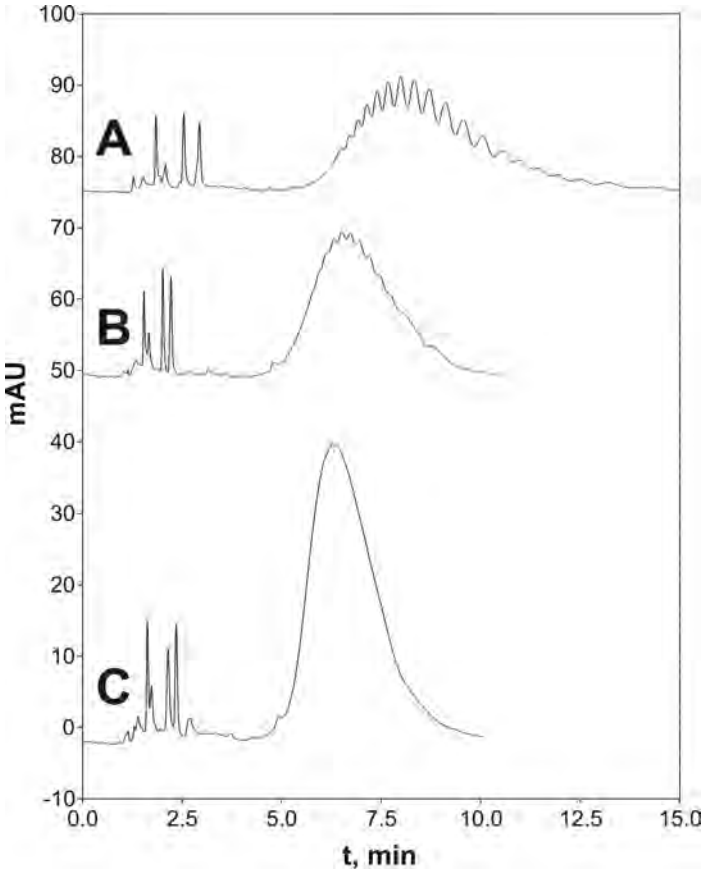


Fig. 2 Separation of ethoxylated nonylphenol sample with 12 oxyethylene units using different LC columns. (A) – Zorbax Eclipse XDB C8 3.5 mm, (B) Zorbax Eclipse XDB C18 5 mm, (C) Biospher PSI 100 C18 7 mm (dimensions of all columns 150×4.6 mm). Mobile phase 60 % acetonitrile in water, flow rate 1 ml $min^{-1}$, 40 °C, detection UV at 254 nm

The highest selectivity for separation of NPEs with different numbers of EO units achieved using combination of Zorbax Eclipse XDB-C8 column and mobile phase consisting of mixture of water and acetonitrile 40:60 (v/v) can be seen in Fig. 2. These separation conditions were further applied for separation of NPEs as the first dimension separation in two-dimensional system in combination with capillary zone electrophoresis. Using column with octyl silica stationary phase, all tested samples of ethoxylated nonylphenols were analyzed and comparison of separation of NPEs with different degree of ethoxylation is shown in Fig. 3. In this system, the best resolution of each oligomer of NPEs in the samples decreases with increasing degree of ethoxylation.

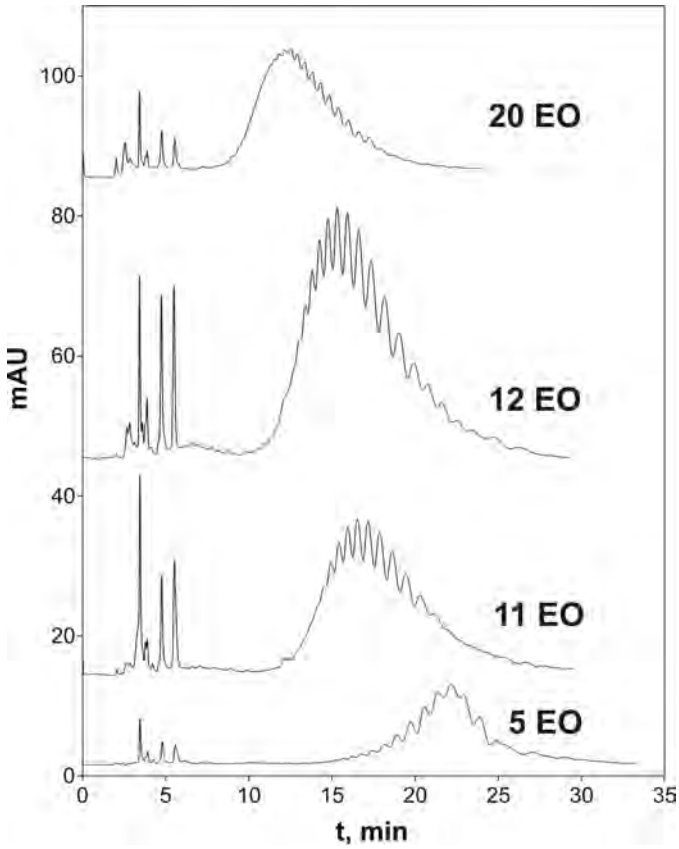


Fig. 3 Separation of ethoxylated nonylphenol samples with different degree of ethoxylation using Eclipse XDB C8 column (3.5 mm, 150×4.6 mm). Mobile phase 60 % acetonitrile in water, flow rate 0.5 ml $min^{-1}$, 40 °C, detection UV at 254 nm

## Optimization of CZE Separation Conditions

In the CZE step, separation conditions based on solvofobic interactions of analyzed NPEs with sodium dodecyl sulfate were used [16]. Separations were tested in both, non-coated and polyacryl amide coated capillaries. We have tested two phosphate buffers (10 mmol $l^{-1}$ pH 2.5 and 6.8) and 25 mmol $l^{-1}$ borate buffer as background

electrolytes (BGE) for separation in non-coated capillaries. Buffers were mixed with 20, 30 and 40 % (v/v) acetonitrile, and 40, 50 and 60 mmol $l^{-1}$ sodium dodecyl sulfate was added. Analyses were carried out at 25 °C. In phosphate buffer, the separation of NPE oligomers was achieved in buffer with pH 6.8 with addition of 40 % acetonitrile and 50 mmol $l^{-1}$ sodium dodecyl sulfate, however, the electroosmotic velocity was close to the migration velocity of SDS-NPE oligomer associates and the separation time was long. Therefore, BGE was changed to 25 mmol $l^{-1}$ borate buffer pH = 8.9 to obtain electroosmotic flow velocity significantly higher than the electrophoretic velocity of NPEs-SDS complex and to increase speed of the analysis. The best separation of oligomers of NPEs was achieved using borate buffer BGE mixed with 20 % of acetonitrile (Fig. 4). The addition of 60 mmol $l^{-1}$ sodium dodecyl sulfate provided the highest resolution of NPE oligomers, but the higher conductivity of the electrolyte led to the unstable current and even to the interruption of electric circuit and depreciation of analyses. Therefore, BGE with lower concentration of SDS 50 mmol $l^{-1}$ was considered best for separation of NPE oligomers in non-coated fused silica capillaries.

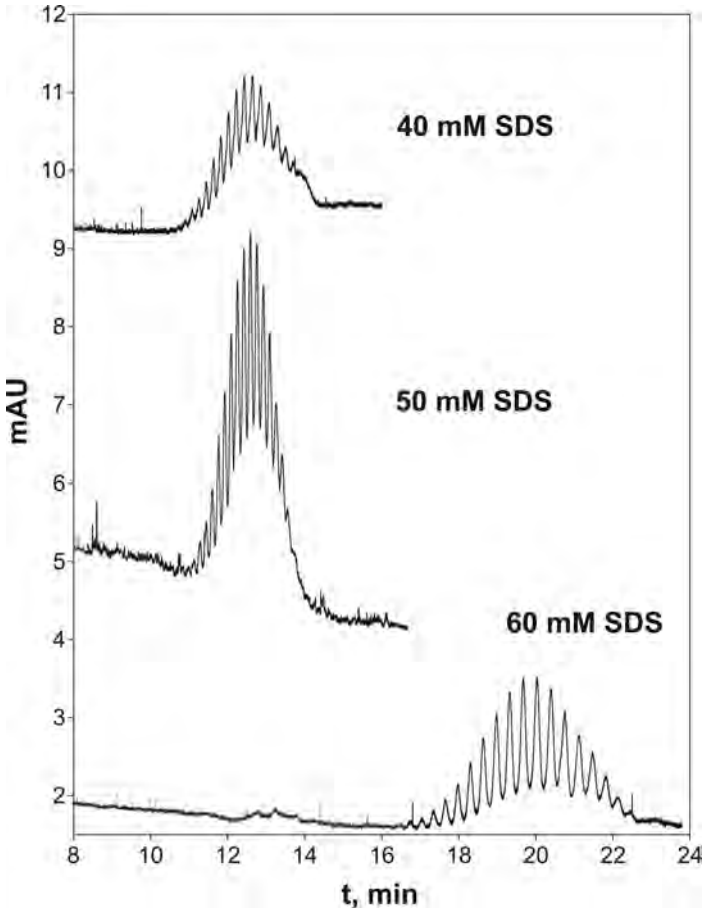


Fig. 4 Separation of ethoxylated nonylphenol sample with 12 oxyethylene units by capillary zone electrophoresis with different concentration of sodium dodecyl sulphate. Non-coated fused silica capillary, 50 mm×48 cm total length (40 cm to the detector), 25 mmol $l^{-1}$ borate background electrolyte pH 8.9, 20 % acetonitrile with addition of SDS. Applied voltage 25 kV, 25 °C, detection UV at 200 nm

In polyacryl amide coated capillaries, BGE was changed to 10 mmol $l^{-1}$ phosphate buffer pH 6.8 which provide better stability of the polymeric inner wall coating in comparison to higher pH [27]. The applied voltage was in polyacryl amide coated capillary reversed with respect to the non-coated fused silica capillary (anode was at the detector end and cathode at the inlet end of the capillary). Sodium dodecyl sulfate was added to both BGE, which were mixed with acetonitrile in appropriate ratios to inhibit formation of micelles. Mixtures of phosphate BGE with 20, 30 and 40 % acetonitrile were tested with addition of 40, 60 and 70 mmol $l^{-1}$ sodium dodecyl sulfate. The best separation in polyacryl amide coated capillary was achieved using 10 mmol $l^{-1}$ phosphate buffer pH 6.8 in mixture with 40 % (v/v) acetonitrile and with addition of 60 mmol $l^{-1}$ SDS.

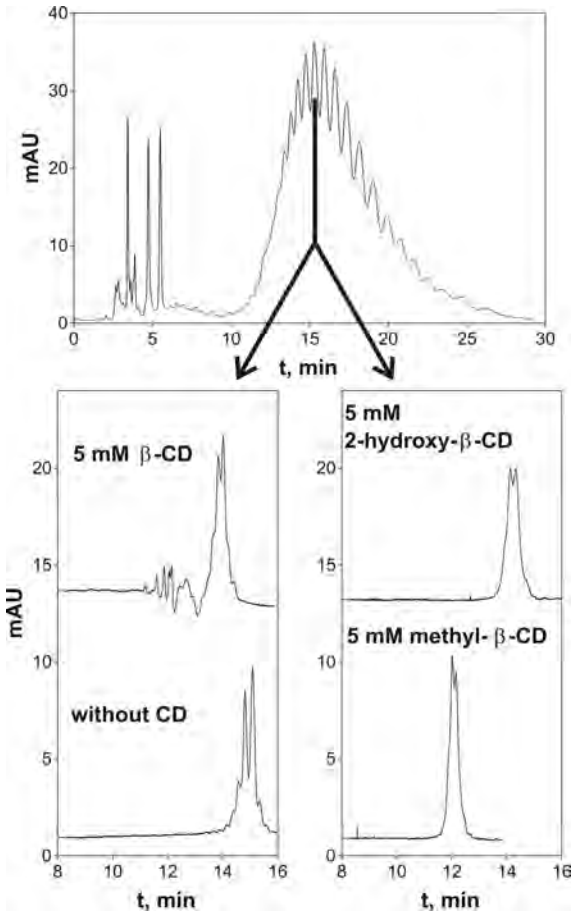


Fig. 5 Reanalysis of selected fraction of ethoxylated nonylphenol sample with 12 oxyethylene units collected from LC step by CZE in electrolytes with different cyclodextrins. LC conditions as in Fig. 3, CZE conditions as in Fig. 4, BGE with addition of 50 mmol $l^{-1}$ SDS

Samples of NPEs with different number of EO units were analyzed in both types of capillaries with similar results; however, the polyacryl amide coating was not durable under the separation conditions (10 mmol $l^{-1}$ phosphate buffer (pH = 6.8)/acetonitrile (60:40) with addition of SDS) and the quality of separation

decreased within several analyses. The quality and fastness of the separation depend both on the buffer/acetonitrile ratio and on the concentration of SDS in the background electrolyte. As the optimum BGE for two-dimensional separation was found 25 mmol $l^{-1}$ borate buffer (pH = 8.9)/acetonitrile 80:20 (v/v) with addition of 40-50 mmol $l^{-1}$ SDS in non-coated fused silica capillary (Fig. 4).

Prior to the automated 2D separation, one selected fraction from LC was analyzed by CZE (Fig. 6) with various cyclodextrins added to the BGE with the aim to separate the potential different positional branch isomers of nonyl group in surfactant molecule. Besides being excellent chiral selectors, cyclodextrins showed significant selectivity for separation of positional [28] or stereoisomeric forms [27]. We have investigated influences of non-substituted β-cyclodextrin and substituted 2-hydroxypropyl-β-cyclodextrin and methyl-β-cyclodextrin on separation of NPE oligomer with 12 EO units (Fig. 5). The addition of cyclodextrins did not significantly improve the separation of NPE oligomer with respect to the electrolyte without cyclodextrins. This may possibly be caused by the fact that the used cyclodextrins are not suitable position isomer selectors or in the tested samples of Serdox NNP only one branch isomer is dominant. On the other hand, the separation achieved in BGE without cyclodextrins can be probably attributed to the oligomers with one EO unit less and/or one EO unit more than collected NPE oligomer with 12 EO units, which may get to the sample by low resolution in the first LC dimension. Therefore, the application of CZE separation method in the second dimension in combination with LC separation can further improve the resolution of oligomers in NPE samples.

## Separation of Nnonylphenol Ethoxylates in Two-dimensional System

Finally, the optimized conditions for LC and CZE separation of NPE oligomers were used in two-dimensional system combining these two methods. Automated off-line approach for two-dimensional LC-CZE separation previously reported for separation of flavones related compounds and phenolic acids [25] was applied to the analysis of the Serdox NNP 12 sample. Thus 40 fractions with volume of 0.5 ml each were collected in the CE autosampler with fraction collection time 1 min, starting in the $12^{th}$ minute of the LC analysis. After the collection of the samples, the fractions were reanalyzed by CZE in 25 mmol $l^{-1}$ borate background electrolyte pH 8.9 in a mixture with 20 % (v/v) acetonitrile and with addition of 50 mmol $l^{-1}$ SDS. Resulting record of analysis is shown in Fig. 6. The total analysis time in the two-dimensional method is approximately 17 hours, however, this disadvantage is partly compensated by the automatic analysis, which can be run unattended overnight. The two-dimensional separation record represents a complex characterization of sample of NPE oligomers which might be further used as fingerprint identification of specific brand of surfactants and/or for quantitative analysis of each separated NPE oligomers.

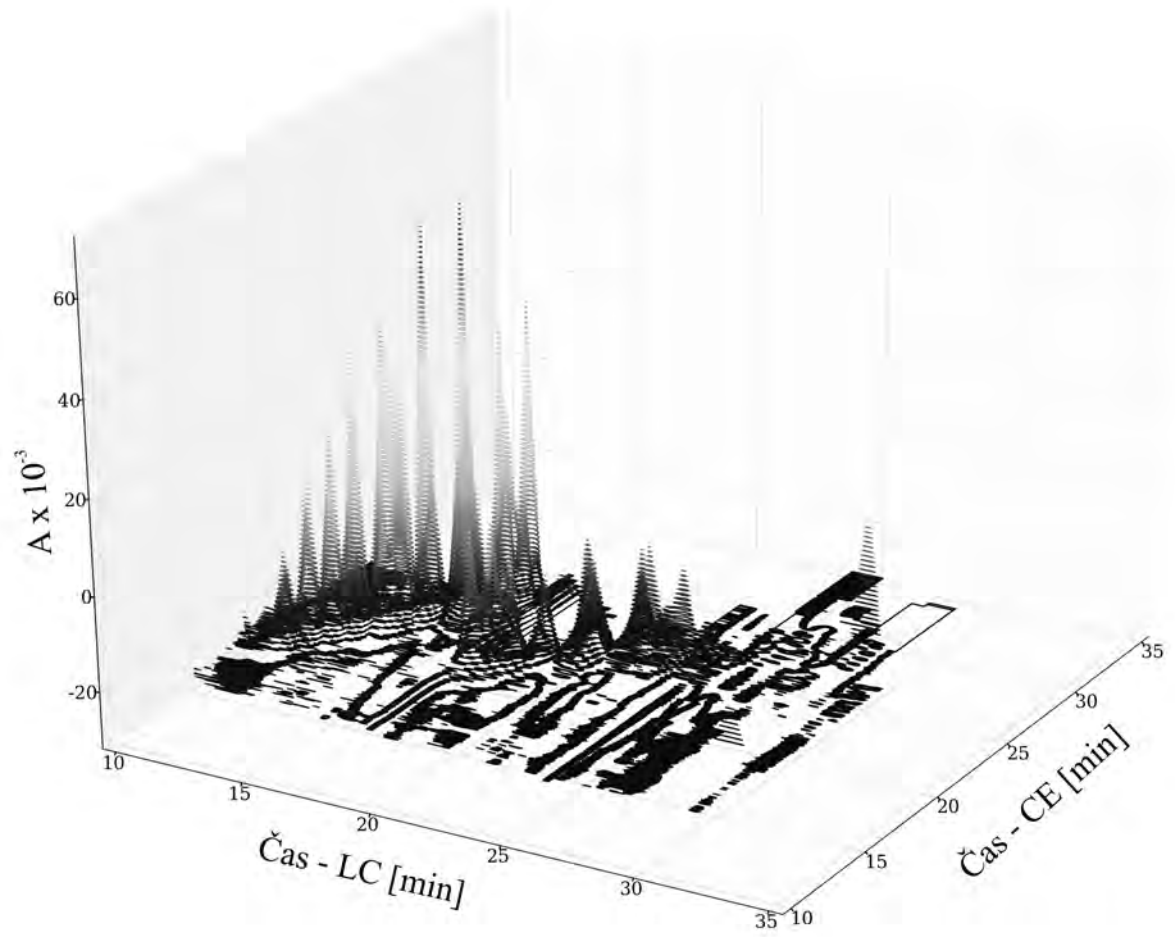


Fig. 6 Automated off-line two-dimensional LC-CZE separation of ethoxylated nonylphenol with different degree of ethoxylation. LC separation as in Fig. 3, CZE conditions as in Fig. 5 without cyclodextrin. 40 samples of 0.5 ml each collected starting in the 12th minute of LC and reanalyzed by CZE

## Conclusion

Nonionic nonylphenol ethoxylate surfactants Serdox NNP were successfully separated by the automated off-line two-dimensional LC-CZE method. Both the LC and CZE steps were optimized. Analyses in LC were carried out in reversed-phase separation system using seven columns with different stationary phases. In the CZE separations, application of non-coated and poly(acryl amide) coated capillaries was investigated. Addition of different cyclodextrins to the CZE background electrolyte was tested for the separation of possible position branch isomers of nonyl group of the surfactants, but the cyclodextrins did not significantly improve the separations. Finally, automated off-line 2D method with optimized conditions in both steps was applied to nonylphenol ethoxylates Serdox NNP samples, while the advantage of using two-dimensional system is in higher resolution of each NPE oligomers.

**Acknowledgements**

*Authors acknowledge the financial support by the Ministry of Education, Youth and Sports, project MSM 0021627502, and the Czech Science Foundation, project 203/09/P199.*

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