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Journal of Chromatography A, 1157 (2007) 227–236
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Column temperature as an active variable in the isocratic, normal-phase high-performance liquid chromatography separation of lipophilic metabolites of nonylphenol ethoxylates

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Received 1 January 2007; received in revised form 30 April 2007; accepted 2 May 2007
Available online 16 May 2007

This paper is dedicated to the memory of Prof. Dr. Daniel A. Batistoni.

Abstract

Normal-phase separation of technical grade nonylphenol (t-NP, about 90% 4-nonylphenol), 4-nonylphenol mono-ethoxylate (4-NP1EO) and 4-nonylphenol di-ethoxylate (4-NP2EO) was assessed, with the inclusion of column temperature as an active variable. The compound 2,4,6-trimethylphenol was evaluated for use as internal standard. Isocratic elution with 2-propanol/*hexanes* mixtures from an amino-silica column and spectrometric UV detection at 277 nm were employed. Technical nonylphenol presented a significant contribution from unknown substances that eluted with retention times similar to that of 4-NP1EO. GC–MS analysis of the unknowns allowed to identify them as isomers of 2-NP. The response of the system to joint variations in flow rate, eluent composition and column temperature was investigated by means of Doehlert statistical experimental design. A model for retention of the analytes as a function of the experimental variables was proposed, and separation selectivity was studied. Selection of the optimal working zone was made through desirability function (D) calculations. Potential co-elution of 2-NP isomers with 4-NP1EO was considered when optimizing the separation. The occurrence of a restricted region of the experimental space where baseline resolution of analytes, associated impurities and internal standard results feasible ($D \neq 0$) is apparent.
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Keywords: Nonylphenol ethoxylates; Lipophilic metabolites; Normal-phase HPLC; Column temperature; Separation selectivity

1. Introduction

Nonylphenol ethoxylates (NP*n*EO) are non ionic surfactants still widely employed in numerous countries as detergent products for industrial and domestic applications. Laboratory and field degradation studies indicate that bio-elimination after waste water processing in sewage treatment plants is incomplete, leading to lipophilic, strongly persistent decomposition products. They comprise mainly mixtures of branched isomers of 4-nonylphenol (4-NP), 4-nonylphenol mono- and di-ethoxylate (4-NP1EO, 4-NP2EO), and also their mono- and di-carboxylate analogues, the 4-nonylphenoxy acetic- and nonylphenoxyethoxy acetic acids (4-NP1EC, 4-NP2EC). All these compounds accumulate in aquatic environments, and they are reported to generate varied estrogenic responses in living organisms, even at very low concentration levels [1–6].

Different liquid chromatography methodologies have been exploited for the resolution of complex mixtures of the analytes [7]. In addition to reversed-phase methods [6,8–10], both gradient and isocratic elution, normal phase HPLC on amino-silica columns have been employed for separation and measurement of 4-NP, 4-NP1EO and 4-NP2EO [11–13]. In general, analyses are carried out either at ambient or slightly higher than ambient temperatures.

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0021-9673/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2007.05.038

It is generally assumed that variations in operating HPLC temperature will not produce the same magnitude of improvement in retention selectivity that can be achieved by changing traditional variables such as the properties of the eluent or by applying gradient elution [14]. However, the present availability of stable stationary phases able to operate at temperatures substantially higher than room temperature, and the straightforward implementation of the technique by employing column thermostats, have prompted the application to specific analytical situations. High performance liquid chromatography separations under isocratic conditions present the advantage of operation simplicity. In this context, column temperature could appear as an effective way of shifting separation selectivity, as well as improving efficiency and shortening analyses times [14,15].

Most of the reported applications of chromatographic columns operated at temperatures other than room temperature deal with reversed-phase separations [14–21]. Nonetheless, Rey and Pohl [22] demonstrated that column temperature is an effective variable to enhance the separation of monovalent and divalent species on a cation-exchange stationary phase. Similarly, Hatsis and Lucy [23] found relevant the differential effect of temperature on retention factors of various inorganic anions separated on two commercially available anion-exchange columns. More recently, a study on the influence of temperature on the separation of octylphenol ethoxylates in both reversed and normal phase systems with hydro-organic mobile phases has been reported [24].

In the present work, we have incorporated column temperature as an active variable in the normal-phase separation of technical grade NP (t-NP), 4-NP1EO and 4-NP2EO. The compound 2,4,6-trimethylphenol (TMP) was included for use as internal standard [8,12,13]. Changes in retention and selectivity arising from joint variations in flow rate, eluent composition and column temperature are investigated.

2. Experimental

2.1. Chemicals

All chemicals were used as received from the suppliers. *Hexanes* (95% *n*-hexane/5% branched isomers), cyclohexane and 2-propanol (2-PrOH) were HPLC grade solvents (Sintorgan, Buenos Aires, Argentina). 4-Nonylpehnol – technical grade (Fluka, Buchs, Switzerland), 4-NP1EO and 4-NP2EO (Promochem, Wesel, Germany), were used as analyte standards for the estrogenic metabolites. TMP was Riedel de-Haen.

2.2. Instrumentation

The liquid chromatography system consisted of a *SpectraSERIES P200* binary pump and a *SpectraSERIES UV100* UV/visible detector (Thermo Separation Products, USA); data were acquired and analyzed with the *Konikrom 5.2* software (Konik Instruments, Spain). Separations were performed in a 5 μm particle amino-silica column of 250 mm × 4.6 mm (APS-2 Hypersil with 10 mm × 4.0 mm guard-column, Thermo Electron Corporation, USA). Elution was carried out with 2-PrOH/*hexanes* mixtures. Eluent reservoir was kept at room temperature (20 °C) during all the experiments. Column temperature was controlled to within 0.1 °C using an Eppendorf CH-30 column heater and an Eppendorf TC-50 controller (Alltech, IL, USA), except for the experiments performed at room temperature, in which no thermostating was applied. Injection volume was 100 μl. Detection was carried out at 277 nm.

GC–MS analyses were performed with a *Shimadzu GC-17A* split-splitless gas chromatograph coupled to a *MS-QP5050A* mass spectrometer. Data were acquired and analyzed with the *Class-5000* software (Shimadzu Corporation, Kyoto, Japan) which includes the WILEY 229 spectral library. The capillary column used was a ZB1 of 60 m × 0.32 mm × 0.50 μm (100% methyl polisiloxane, Zebron, USA). Instrumental conditions were as follows: injector and detector temperature, 300 °C; oven temperature programming, 80 °C (1 min) – 6 °C min^{-1} – 280 °C (5 min); column pressure, 90.5 kPa; column flow rate, 1.8 ml min^{-1}; linear velocity, 34.9 cm seg^{-1}; split ratio, 10; total flow rate, 22.4 ml min^{-1}.

2.3. Procedures

Standard solutions were prepared by diluting and mixing in cyclohexane stock solutions c.a. 1000 mg l^{-1} of the independent compounds in this solvent, stored at 4 °C.

Solvent mixtures for HPLC elution were prepared every day. The column was allowed to equilibrate until stable baseline was achieved (approximately 30 min) at the beginning of daily operation and when changing eluent composition, flow rate or temperature.

Temperature studies were conducted from 25 to 60 °C. Temperatures lower than room temperature were not achievable with the column oven used. Temperatures higher than 60 °C were not examined so as to avoid possible damage to the column and the detector cell [24], and to preserve safety regarding the highly flammable solvents used for elution.

Collection of HPLC fractions for GC–MS analyses was made by means of a 3-way manual switching valve (Rheodyne 7030RV, USA). Collected eluates were injected in the gas chromatograph either directly or after partial evaporation of HPLC solvents under a stream of N_2 for sample concentration.

Experimental retention factors (k) of the eluting compounds were calculated as:

$$k = \frac{(t_r - t_0)}{t_0} \quad (1)$$

where t_r is retention time of the analyte (computed at the absolute maximum in the case of split peaks) and t_0 is the hold-up time (measured from the cyclohexane peak). From these parameters, selectivity factors (α) for a pair of compounds (a, b) were calculated as:

$$\alpha = \frac{k_a}{k_b} \quad (2)$$

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