Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1046 (2004) 83–88
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of 4-*tert.*-octylphenol and 4-nonylphenol in laboratory animal feed sample by stir bar sorptive extraction followed by liquid desorption and column-switching liquid chromatography–mass spectrometry with solid-phase extraction

Migaku Kawaguchi[a], Shima Takahashi[a], Fumie Seshimo[a], Norihiro Sakui[a,b], Noriya Okanouchi[a], Rie Ito[a], Koichi Inoue[a], Yoshihiro Yoshimura[a], Shun-ichiro Izumi[c], Tsunehisa Makino[c], Hiroyuki Nakazawa[a,*]

[a] *Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan*
[b] *Yokokawa Analytical Systems, Inc., Hachioji Site, 9-1 Takakura-Cho, Hachioji City, Tokyo 192-0033, Japan*
[c] *Department of Obstetrics & Gynecology, School of Medicine, Tokai University, Bohseidai, Isehara City, Kanagawa 259-1193, Japan*

Received 29 April 2004; received in revised form 17 June 2004; accepted 22 June 2004

Abstract

A novel analytical method has been developed for the determination of 4-*tert.*-octylphenol (OP) and 4-nonylphenol (NP) in laboratory animal feed samples, which involves stir bar sorptive extraction (SBSE) followed by liquid desorption (LD) and column-switching liquid chromatography–mass spectrometry (CS-LC–MS) with solid-phase extraction (SPE). The method required correction by stable isotopically labeled surrogate standards, deuterium 4-*tert.*-octylphenol (OP-d) and [$^2H_5$] 4-(1-methyl)octylphenol (m-OP-d_5). A feed sample was homogenized with methanol by ultrasonication. After centrifugation, the supernatant was subjected to extraction for 120 min at room temperature (25 °C) using a stir bar coated with polydimethylsiloxane. After the extraction, the analyte was desorbed from the stir bar by LD using acetonitrile. Then, the liquid sample was analyzed by CS-LC–MS with SPE. The average recoveries from laboratory feed samples spiked with OP and NP at 20 ng g^{-1} were 99.5 and 103.8%, respectively, with correction using the added surrogate standards. The limits of quantification were 1 ng g^{-1} for OP and 5 ng g^{-1} for NP in feed sample. The measurement of OP and NP in commercial laboratory animal feed samples resulted in the detection of sub ng g^{-1} NP

Keywords: Stir bar sorptive extraction; Extraction methods; Liquid desorption; Octylphenol; Nonylphenol; Alkylphenols; Phenols; Endocrine disruptors

1. Introduction

Endocrine disruptors (EDs) have elicited great concern worldwide because of their adverse effects on wildlife and human health. In vivo assays have shown that very small amounts of EDs may influence wild animals and humans.

Alkylphenolpolyethoxylates (APEOs) comprise the major class of non-ionic surfactants and have a variety of industrial and commercial applications. Alkylphenol (AP), 4-*tert.*-octylphenol (OP) and 4-nonylphenol (NP) are the degradation products of APEOs and have been shown to exist in seawater, river water and sewage wastewater [1–10]. In vitro assays have shown their ability to induce estrogenic activities [11–13]. Moreover, the effects of OP and NP on animals have been shown by in vivo assays [14–20]. OP and NP are

* Corresponding author. Tel.: +81 3 5498 5763; fax: +81 3 5498 5062.
E-mail address: nakazawa@hoshi.ac.jp (H. Nakazawa).

0021-9673/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.chroma.2004.06.066

postulated to exist in animal breeding environments; therefore, we are concerned about the contamination of these compounds in animal feed. To this end, we analyzed OP and NP in animal feed samples.

In this study, we show that OP and NP can be simultaneously measured in laboratory animal feed samples. Recently, a new sorptive extraction technique using a stir bar coated with polydimethylsiloxane (PDMS) was developed and called stir bar sorptive extraction (SBSE) [21]. Depending on their octanol/water partition coefficients, the compounds are extracted and enriched. Examples of successful applications are the determination of volatiles, semivolatiles, stale-flavored carbonyl compounds, pesticides, mycotoxin, polycyclic aromatic hydrocarbons and dicarboximide fungicides in various samples [22–36]. In addition, SBSE has been used successfully in the determination of OP and NP in water samples [30] and human biological samples [31]. In most of those studies, thermal desorption (TD) is generally used for the desorption of the analyte from the PDMS stir bar [22–31] and analysis is carried out by gas chromatography–mass spectrometry (GC–MS). In addition, liquid desorption (LD) by an organic solvent has been attempted [32–36], and the organic solvent is analyzed by high-performance liquid chromatography (HPLC).

On the other hand, column-switching (CS) HPLC coupled with solid-phase extraction (SPE) by direct injection analysis has been developed and successfully employed for the determination of organic species in biological samples [37-39]. This method is able to be performed on-line sample preparation. Moreover, a large sample volume can be injected, thereby increasing sensitivity. In our previous study, we determined OP and NP by column-switching liquid chromatography–mass spectrometry (CS-LC–MS) with SPE in human urine samples [40].

The aim of this study was to develop a novel analytical method that involves SBSE followed by LD and CS-LC–MS with SPE. By performing two different sample preparations, the refining efficiency was increased. Then, the determination of OP and NP in laboratory animal feed samples by SBSE followed by LD and CS-LC–MS with SPE was performed.

2. Experimental

2.1. Materials and reagents

4-*tert.*-Octylphenol and 4-nonylphenol (mixture) of environmental analytical grade were purchased from Kanto, Tokyo, Japan. Deuterium 4-*tert.*-octylphenol (a mixture in which the hydrogen of OP was replaced with 1-12 deuterium) (OP-d) and [$^2H_5$]4-(1-methyl)octylphenol (m-OP-d_5) were purchased from Hayashi, Osaka, Japan. The chemical structures are shown in Fig. 1. Methanol of

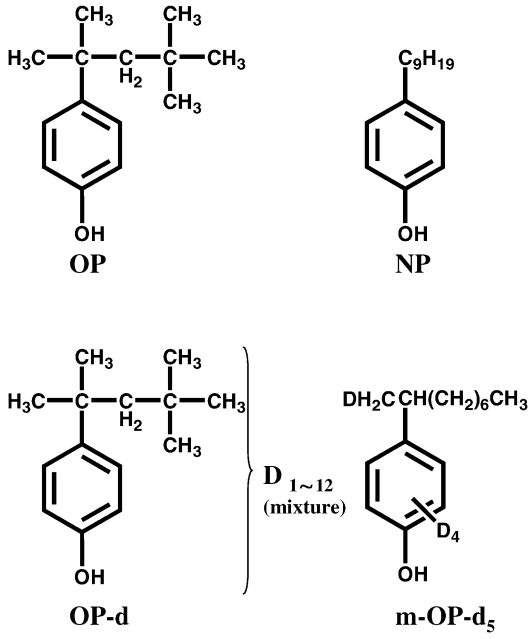

Fig. 1. Chemical structures of OP, NP, OP-d and m-OP-d_5.

pesticide grade was purchased from Wako, Osaka, Japan. The water purification system used was a Milli-Q gradient A 10 with an EDS polisher (Millipore, Bedford, MA, USA). The EDS polisher was a new filter purchased from Millipore, Japan.

2.2. Standard solutions

Stock solutions (1.0 mg ml^{-1} in methanol) of OP and NP were prepared. Concentration solutions (1–100 ng ml^{-1} for OP and 5–100 ng ml^{-1} for NP) were prepared as required by the addition of purified water with specific amounts of surrogate standard. Six-point calibrations were performed daily for all samples with surrogate standards.

2.3. Laboratory animal feed samples

Laboratory animal feed samples were purchased from three companies. All samples were stored at 4 °C prior to use. The feed samples for mouse breeding were used. The sample form was powder type.

2.4. Instrumentation

Stir bars coated with 500-μm thick (24 μl) PDMS (Twister: the magnetic stirring rod is incorporated in a glass jacket and coated with PDMS) were obtained from Gerstel (Mullheim an der Ruhr, Germany). The stir bars could be used more than 50 times with appropriate re-conditioning. The liquid chromatography–mass spectrometry (LC–MS) system was an Agilent LC–MSD Superior Line (Agilent Technologies, Palo Alto, USA) equipped with an electrospray

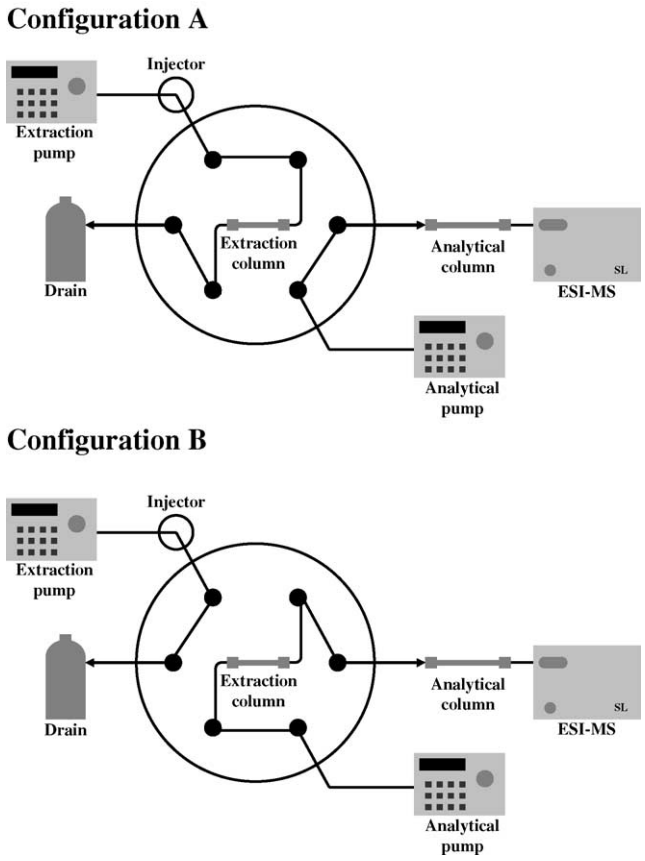

Fig. 2. Schematic of the CS-LC–MS with SPE system.

ionization (ESI) source. An Agilent pump was used to induce flow to elute the sample from the extraction column and to perform the separation on the analytical column. A Shimadzu LC-10AS Pump (Shimadzu, Kyoto, Japan) was used to induce flow through the extraction column to load and wash the sample and to equilibrate the extraction column. A Mightysil RP-18 GP (100 mm × 2.0 mm, 5 μm) analytical column with a guard column (Mightysil RP-18 GP, 5 mm × 2.0 mm, 5 μm) from Kanto Chemical Industries, Ltd. was used for separation. A TSK precolumn BSA-ODS/S (conjugated bovine serum albumin) extraction column (10 mm × 4.6 mm, 5 μm) from Tosoh (Tokyo, Japan) was used for cleanup and concentration.

2.5. CS-LC–MS conditions

The column-switching system, as depicted in Fig. 2 (Configuration A), was used for the injection of liquid sample. After 100 μl of the sample was injected by an auto sampler, it was loaded onto the extraction column by flowing methanol–water (1:9, v/v) at a rate of 0.5 ml min^{-1} using pump A for 3 min. While the extraction column was directed to waste during the 3-min run, the sample was extracted and purified on the on-line extraction column. The impurities were removed and OP and NP were retained on the extraction column. After on-line extraction for 3 min, the position of the switching valve was changed (see Fig. 2, Configuration B). This configuration connected the back-flashing extraction column to the analytical column and the MS detector in the flow path of pump B. The column oven was maintained at 40 °C for LC separation. The separation was carried out using a gradient mobile phase of 0.02% ammonium acetate in water–acetonitrile (v/v) at a flow rate of 0.2 ml min^{-1}. The gradient program is shown in Table 1. The effluent from the analytical column was directed to the ESI–MS. After elution for 12 min, the switching valve was returned to its original position (see Fig. 2, Configuration A). The total run time for the assay of the sample mixture was 15 min. The working conditions for ESI–MS were as follows: drying nitrogen gas was maintained at 350 °C and introduced into the capillary at a flow rate of 12 l min^{-1}; the capillary was held at a potential of 3500 V relative to the counter electrode in the positive and negative ion modes. The fragmentor voltage was 130 V for OP and NP during the chromatographic run. When working in the selected-ion monitoring (SIM) mode, *m/z* 205, 209, 219 and 224 ions were assigned to $[M-H]^-$ of OP, OP-d, NP and m-OP-d_5 in the negative ion mode, respectively.

Table 1
Time program for CS-LC–MS with SPE

Time		Mobile phase:	
min	Event	Column position[a]	Ammonium acetate in water acetonitrile (%, v/v)
0.0	Sample injection	Configuration A	25 : 75
3.0	Column switching	Configuration B	25 : 75 (Gradient)
5.0			5 : 95
12.0	Stop analysis Column switching	Configuration A	25 : 75 (Stepwise)
15.0	Next analysis[b]		

[a]Configurations A and B are shown in Fig 1.
[b]The mobile phase was switched for 3 min to re-equilibrate the column.

2.6. Sample preparation

A 5 g aliquot of the sample was weighed and placed inside a 50-ml glass tube. After the surrogate standards were added, the mixture was homogenized with 10 ml of methanol by sonication for 15 min. Then, the sample was centrifuged for 10 min at 1000 × *g*. Five milliliter of the supernatant was poured into a headspace vial and diluted with pure water to make a total volume of 20 ml. The stir bar was added and the vial was crimped with septum. SBSE of the sample was performed at room temperature for 120 min while stirring at 1000 rpm. Then, the stir bar was placed inside a 2-ml vial with a 250-μl insert vial that was filled with 200 μl of acetonitrile. Desorption of the analyte was performed with an ultrasonic device for 10 min. After desorption, the stir bar was removed by a magnetic rod. Then, the liquid sample was analyzed by CS-LC–MS with SPE.

Table 2
Concentrations of OP and NP in laboratory animal feed samples

Laboratory animal feed sample	OP (ng g^{-1})[a]	NP (ng g^{-1})[b]
A	N.D.	19.5
B	N.D.	14.7
C	N.D.	N.D.

[a] N.D. indicates OP concentrations lower than 1 ng g^{-1}.
[b] N.D. indicates NP concentrations lower than 5 ng g^{-1}.

3. Results and discussion

3.1. Optimization of analytical conditions

3.1.1. Optimal time for SBSE

The most important parameter affecting SBSE is the extraction time. Therefore, the optimum extraction times by SBSE followed by LD and CS-LC–MS with SPE for OP and NP spiked in animal feed samples at 50 ng g^{-1} were investigated. The extraction time was investigated from 10 to 150 min. The extraction time profiles (equilibration curves) of OP and NP using a PDMS stir bar were determined by LD and CS-LC–MS with SPE, and are shown in Fig. 3. From the extraction efficiencies, OP and NP were found to reach equilibrium after approximately 120 min. Therefore, this condition was used for the determination of OP and NP.

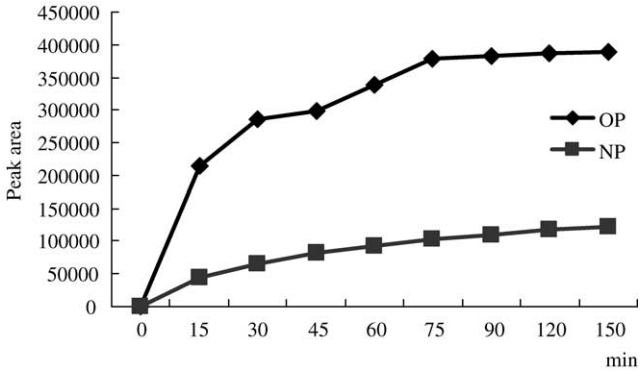

Fig. 3. Extraction time profiles of OP and NP in animal feed samples using PDMS stir bar. A 5 g aliquot to which 50 ng g^{-1} standard solutions were added was placed inside a glass tube, and this was homogenized with 10 ml of methanol by sonication for 15 min. Then, the sample was centrifuged for 10 min at 1000 × *g*. Five milliliter of the supernatant was poured into a vial and diluted with pure water to make a total volume of 20 ml. The stir bar was added and the vial was crimped with septum. SBSE of the sample was performed at room temperature for 10–150 min while stirring at 1000 rpm. Then, the stir bar was placed inside a 2-ml vial with a 250-μl insert vial that was filled with 200 μl of acetonitrile. Desorption of the analyte was performed with an ultrasonic device for 10 min. After desorption, the stir bar was removed by a magnetic rod. The liquid sample was analyzed by CS-LC–MS with SPE.

3.1.2. Optimal conditions for LC–MS

In the mass analysis using ESI–MS with flow-through injection analysis of standard solutions in the negative ion mode ([M−H]$^-$), OP, NP, OP-d and m-OP-d$_5$ exhibited peaks at *m*/*z* 205, 219, 209 and 224, respectively (Fig. 4). Many ion peaks were observed in the vicinity of *m*/*z* 209 for OP-d surrogate standards. However, because *m*/*z* 205 which is the

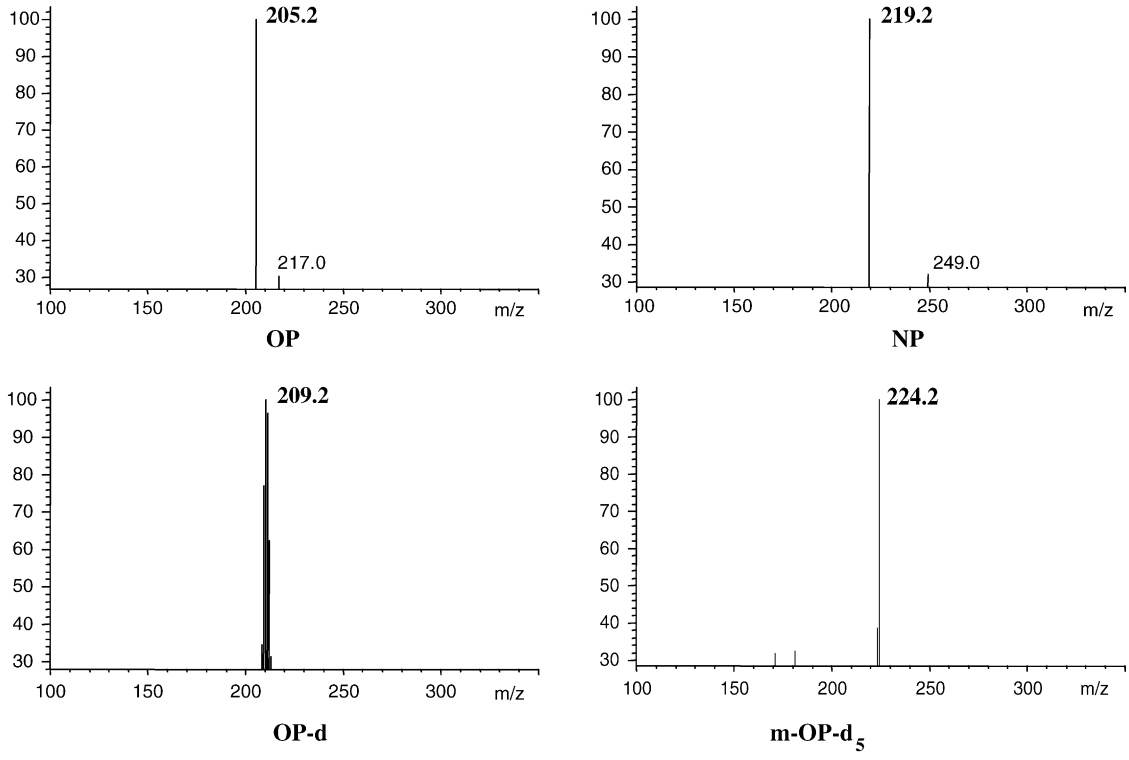

Fig. 4. Mass spectrum of OP, NP, OP-d and m-OP-d$_5$.

monitoring ion of OP was not observed, measurement could be performed satisfactorily by using OP-d as surrogate standards. The most important parameters affecting LC–MS, namely, fragmentor voltage and mobile phase, were investigated. In order to establish the optimum fragmentor voltage for the detection of OP and NP, the signals of *m*/*z* 205 and 219 ions versus fragmentor voltage were investigated. The optimal fragmentor voltages were 130 V in the negative ion mode for OP and NP standard solutions. The ionization of samples at the LC–MS interface is affected by the mobile phase; hence, a mobile phase containing a volatile acid or salt is used frequently. In this case, the responses were measured using 0–0.1% ammonium acetate in water–acetonitrile (v/v) as the mobile phase. The responses of OP and NP were increased by the addition of 0.02% ammonium acetate to the mobile phase. Optimization of LC–MS conditions gave a maximum response in 0.02% ammonium acetate at 130 V for OP and NP in the negative ion mode.

3.2. Validation of SBSE followed by LD and CS-LC–MS with SPE

The calculated limits of detection (LODs) of OP and NP in the animal feed samples were 0.2 and 1 ng g^{-1}, respectively, for SBSE followed by LD and CS-LC–MS with SPE detection with the ratio of the compound's signal to the background signal (S/N) of 3. In addition, the limits of quantification (LOQs) calculated when S/N > 10 were 1 and 5 ng g^{-1} for OP and NP, respectively. Chromatograms of blank animal feed sample were shown in Fig. 5. Moreover, chromatograms of animal feed samples spiked at the LOQ levels were shown in Fig. 6. The peak area ratio with respect to each surrogate standard was plotted, and the response was found to be linear over the calibration range with a correlation coefficient (r^2) of 0.999.

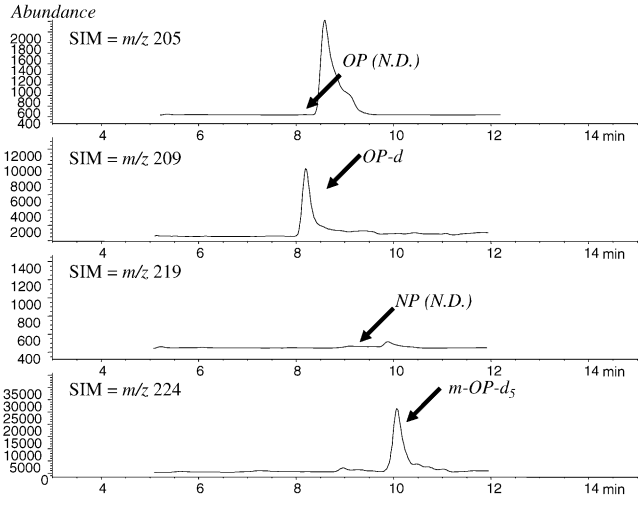

Fig. 5. Chromatograms of OP, NP and surrogate standards in blank laboratory animal feed sample subjected to SBSE followed by LD and CS-LC–MS with SPE.

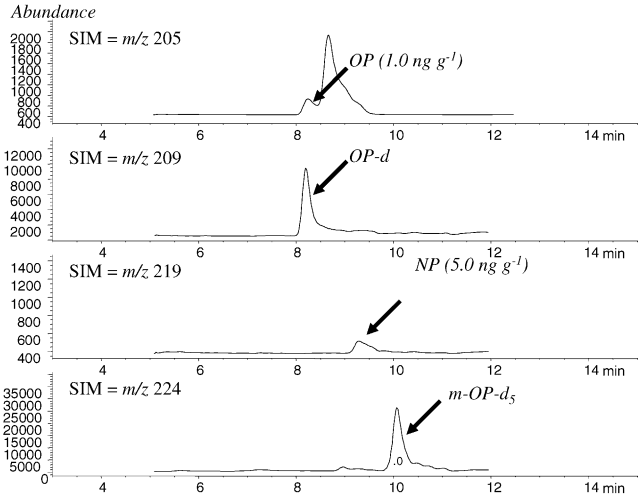

Fig. 6. Chromatograms of OP, NP and surrogate standards in laboratory animal feed sample spiked at the LOQ levels subjected to SBSE followed by LD and CS-LC–MS with SPE.

We investigated whether the recoveries of OP and NP (20 ng g^{-1}) from the laboratory animal feed samples could be determined by SBSE followed by LD and CS-LC–MS with SPE. The average recovery of OP was 99.5 with 4.3% (RSD). On the other hand, the average recovery of NP was 103.8 with 5.8% (RSD). The results show that the method enables the precise determination of OP and NP, and can be applied to the detection of those compounds in animal feed samples.

3.3. Application

We measured the concentrations of OP and NP in three animal feed samples. The results of NP and OP determination in the samples were shown in Table 2. Using this method, OP

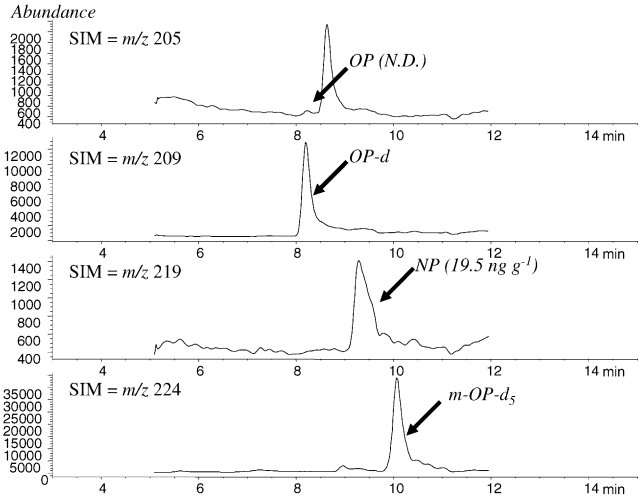

Fig. 7. Chromatograms of OP, NP and surrogate standards in laboratory animal feed sample A subjected to SBSE followed by LD and CS-LC–MS with SPE.

could not be detected in any of the feed samples, whereas NP could be detected in two of the animal feed samples. The detection range of NP was from N.D. (<5 ng g^{-1}) to 19.5 ng g^{-1}. The typical chromatograms of laboratory animal feed sample A are shown in Fig. 7. The results indicate that some of the animal feed samples were contaminated by NP. NP is widely used as a material for the synthesis of non-ionic surfactants that are used in the production of additives in plastics. If those plastics were used in collecting or preparing commercial animal feed samples, contamination might arise from them, and laboratory animals might ingest feed samples contaminated with NP. Therefore, in order to evaluate the influence of low doses of NP, it is necessary to monitor the possible sources of contamination of this compound in the animal breeding environment.

4. Conclusions

The determination of trace amounts of OP and NP in laboratory animal feed samples using SBSE followed by LD and CS-LC–MS with on-line SPE was investigated. Refining efficiency was improved by performing two different sample preparations. The analyte was efficiently extracted by SBSE. After LD, CS-LC–MS with SPE enabled refining and concentration, thereby leading to the high sensitivity of the method. The quantification limits were 1 ng g^{-1} for OP and 5 ng g^{-1} for NP. The average recoveries were 99.5–103.8% in the animal feed samples spiked with OP and NP at 20 ng g^{-1} with correction using the added isotopically labeled surrogate standards. This simple, accurate and highly sensitive method is expected to have potential applications in various feed samples.

Acknowledgments

This study was supported by Health Sciences Research grants from the Ministry of Health, Labour and Welfare of Japan, and Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

[1] M.H.I. Comber, T.D. Williams, K.M. Stewart, Water Res. 27 (1993) 273.
[2] P. Braun, M. Moeder, St. Schrader, P. Popp, P. Kuschk, W. Engewald, J. Chromatogr. A 988 (2003) 41.
[3] R. Espejo, K. Valter, M. Simona, Y. Janin, P. Arrizabalaga, J. Chromatogr. A 976 (2002) 335.
[4] R. Jeannot, H. Sabik, E. Sauvard, T. Dagnac, K. Dohrendorf, J. Chromatogr. A 974 (2002) 143.
[5] M. Petrovic, S. Tavazzi, D. Barcelo, J. Chromatogr. A 971 (2002) 37.
[6] M. Petrovic, S. Lacorte, P. Viana, D. Barcelo, J. Chromatogr. A 959 (2002) 15.
[7] M. Petrovic, A. Diaz, F. Ventura, D. Barcelo, Anal. Chem. 73 (2001) 5886.
[8] P.L. Ferguson, C.R. Iden, B.J. Brownawell, Anal. Chem. 72 (2000) 4322.
[9] A. Diaz, F. Ventrura, M.T. Galceran, Anal. Chem. 74 (2002) 3869.
[10] R.J.W. Meesters, H.Fr. Schroeder, Anal. Chem. 74 (2002) 3566.
[11] A.M. Soto, H. Justicia, J.W. Wray, C. Sonnenschein, Environ. Health Perspect. 92 (1991) 167.
[12] D.H. Han, M.S. Denison, H. Tachibana, K. Yamada, Biosci. Biotechnol. Biochem. 66 (2002) 1479.
[13] J. Schwaiger, U. Mallow, H. Ferling, S. Knoerr, Th. Braunbeck, W. Kalbfus, R.D. Negele, Aquat. Toxicol. 59 (2002) 177.
[14] S.C. Laws, S.A. Carey, J.M. Ferrell, G.J. Bodman, R.L. Cooper, Toxicol. Sci. 54 (2000) 156.
[15] J. Odum, I.T. Pyrah, A.R. Soames, J.R. Foster, J.P. Van Miller, R.L. Joiner, J. Ashby, J. Appl. Toxicol. 19 (1999) 367.
[16] H.C. Cunny, B.A. Mayers, K.A. Rosica, J.A. Trutter, J.P. Van Miller, Regul. Toxicol. Pharmacol. 26 (1997) 172.
[17] C. De Jager, M.S. Borman, G. Van der Horst, Andrologia 31 (1999) 99.
[18] C. De Jager, M.S. Borman, J.M. Oosthuizen, Andrologia 31 (1999) 107.
[19] S.A. Ferguson, K.M. Flynn, K.B. Delclos, R.R. Newbold, Neurotoxicol. Teratol. 22 (2000) 583.
[20] T. Nagao, K. Wada, H. Marumo, S. Yoshimura, H. Ono, Reprod. Toxicol. 15 (2001) 293.
[21] E. Baltussen, P. Sandra, F. David, C. Cramers, J. Microcol. Sep. 11 (1999) 737.
[22] P. Sandra, B. Tienpont, F. David, J. Chromatogr. A 1000 (2003) 299.
[23] V.M. León, B. Álvarez, M.A. Cobollo, S. Muñoz, I. Valor, J. Chromatogr. A 999 (2003) 91.
[24] J.C.R. Demyttenaere, J.I.S. Martinez, R. Verhé, P. Sandra, N.D. Kimpe, J. Chromatogr. A 985 (2003) 221.
[25] J.C.R. Demyttenaere, R.M. Moriña, P. Sandra, J. Chromatogr. A 985 (2003) 127.
[26] N. Ochiai, K. Sasamoto, S. Daishima, A.C. Heiden, A. Hoffmann, J. Chromatogr. A 986 (2003) 101.
[27] C. Blasco, G. Font, Y. Pico, J. Chromatogr. A 970 (2002) 201.
[28] B. Kolahgar, A. Hoffmann, A.C. Heiden, J. Chromatogr. A 963 (2002) 225.
[29] P. Sandra, B. Tienpont, J. Vercammen, A. Tredoux, T. Sandra, F. David, J. Chromatogr. A 928 (2001) 117.
[30] M. Kawaguchi, K. Inoue, M. Yoshimura, R. Ito, N. Sakui, H. Nakazawa, Anal. Chim. Acta 505 (2004) 217.
[31] M. Kawaguchi, K. Inoue, N. Sakui, R. Ito, S. Izumi, T. Makino, N. Okanouchi, H. Nakazawa, J. Chromatogr. B 799 (2004) 119.
[32] P. Popp, C. Bauer, L. Wennrich, Anal. Chim. Acta 436 (2001) 1.
[33] C. Blasco, G. Font, Y. Picó, J. Chromatogr. A 970 (2002) 201.
[34] B. Niehus, P. Popp, C. Bauer, G. Peklo, H.W. Zwanziger, Int. J. Environ. Anal. Chem. 82 (2002) 669.
[35] P. Popp, C. Bauer, A. Paschke, L. Montero, Anal. Chim. Acta 504 (2004) 307.
[36] C. Blasco, M. Fernández, Y. Picó, G. Font, J. Chromatogr. A 1030 (2004) 77.
[37] R. Tritter, M. Ehrlich, T.J. Galla, R.E. Horch, K. Kummerer, J. Chromatogr. B 775 (2002) 127.
[38] P. Molander, A. Holm, E. Lundanes, D.R. Hegna, E. Ommundsen, T. Greibrokk, Analyst 127 (2002) 892.
[39] X. Guo, T. Fukushima, F. Li, K. Imai, Analyst 127 (2002) 480.
[40] K. Inoue, M. Kawaguchi, F. Okada, N. Takai, Y. Yoshimura, M. Horie, S. Izumi, T. Makino, H. Nakazawa, Anal. Chim. Acta 486 (2003) 41.