Determination of Nonylphenol and Nonylphenol Ethoxylates in Powdered Milk Infant Formula by HPLC-FL

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Abstract

Linear nonylphenol (4-*n*-NP), nonylphenol isomers (NP), and NP short chain ethoxylated derivates ($NPEO_1$ and $NPEO_2$) show a well-reported endocrine disrupting activity due to their ability to mimic natural estrogens of living organisms. In this work, two methodologies were developed for the simultaneous extraction and determination of NP, 4-*n*-NP, long and short chain nonylphenol ethoxylates in commercial powdered milk infant formula using high performance liquid chromatography with fluorescence detection (HPLC-FL). Separation was performed on an Eclipse XDB-C_8 column (3 µm and 4.6 × 150 mm) in isocratic mode at 1 mL/min using a mixture of ACN–H_2O (65:35). In the first approach, milk powder was reconstituted according to the indications of the manufacturer. A saponification step was carried out to remove triacylglycerols and, subsequently, the obtained solution was solid-phase extracted. Following this procedure, quantitative recovery and high selectivity were achieved for 4-*n*-NP, whereas several interferences appeared at the retention times of $NPEO_x$, thus increasing limits of detection for these compounds.

The second approach consisted of a simple ultrasonic-assisted extraction of the milk powder (non-reconstituted sample) and subsequent determination by HPLC-FL. Different organic solvents were tested for the extraction, and among them acetonitrile (ACN) provided the best results. Quantitative recoveries were obtained for all the analytes in a single extraction step of 15 min using only 1 mL of ACN. Additionally, this approach was more selective than the reconstituted milk-based procedure. The limits of detection achieved ranged from 0.010 µg/g to 0.085 µg/g depending upon the analyte.

Introduction

Nonylphenol isomers (NP), linear nonylphenol (4-*n*-NP) and NP short chain ethoxylated derivates ($NPEO_1$ and $NPEO_2$) show a well-reported endocrine-disrupting activity (1,2,3,4) due to their ability to mimic natural estrogens of living organisms. Nonylphenol (NP) is used in many areas of the industry such as the production of lubricant additives, emulsifiers, hardeners, resins, certain plastics (i.e., polyvinyl chloride or polystyrenes), and non-ionic surfactants (5). Among them, the manufacture of industrial and household detergents, in which nonylphenol ethoxylates (NPEOx) are predominant, represents the 80% of the total demand of NP (6). The main problem associated to such huge industrial use is that both NP and its derivatives are continuously released into the environment and, consequently, these compounds have been detected in surface waters, soil, and sediments (7). In addition, the degradation of $NPEO_x$ detergents in the environment leads to the formation of NP and short chain ethoxylates. Also, the migration of residual NP from packages to food has been observed (8), and it is considered the prior source of contamination of food by NP. Food contamination with these endocrine disrupters is of major concern for human health, and particularly on a so sensitive endocrine system as that of children. There is not a specific legislation in the European Union about the presence of this kind of contaminant in infant food, and only limits for pesticides (0.01 mg/kg) have been established according to EU directive 1999/39/CE (9). In order to protect children's health, those limits are considered as a reference for other potentially harmful compounds, such as NP and its derivates.

Although literature on this topic is not very wide, the occurrence of NP in food-contact materials has been demonstrated (10), and there are several reports that have established the relation of migration rates with the type of packages and the lipid content of food (11,12,13). Several works have already studied the presence of endocrine disrupting compounds in foodstuff, and in 2002, Guenther et al. (14) established that NP was ubiquitous in different kinds of food products at the low µg/kg concentration level. Recent studies have also reported the presence of NP in composite food (15) at concentrations varying from 3.8 to 25 µg/kg depending upon the sample. Concerning milk, there are previous studies in which NP was analyzed among other endocrine disrupting chemicals by gas chromatography-mass spectrometry (MS) and liquid chromatography (LC)–MS–MS and it was found at a concentration of 27.1 µg/kg in milk infant formula (16) and 17.6 µg/kg in normal bottled milk (17). However, there is a lack of publications or scientific data regarding the incurrence of milk samples with the other previously-mentioned endocrine disrupting chemicals (4-*n*-NP,

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$NPEO_1$, and $NPEO_2$) and with their precursors ($NPEO_x$). Besides, the methods mentioned previously for the determination of NP required, after extraction by solid-phase extraction (SPE) (16) or by matrix solid-phase dispersion (17), a laborious clean-up step using large amount of organic solvents.

Thus, the aim of this work is the development of a simple analytical method, avoiding the use of complex techniques and equipments, for the simultaneous determination of NP, 4-*n*-NP, long and short chain nonylphenol ethoxylates in commercial powdered milk infant formula using high performance liquid chromatography coupled to a fluorescence detector (HPLC-FL), in order to establish a base for accurate human-exposure studies to these contaminants in the near future.

Experimental

Reagents

Nonylphenol Tech (CAS Nº 84852-15-3), $NPEO_{1+2}$ (Igepal CO-210, CAS Nº68412-54-4) and $NPEO_x$ (Tergitol x = 3 – 13, CAS Nº 127087-87-0) were purchased from Sigma-Aldrich (St Louis, MO) and 4-*n*-nonylphenol (CAS Nº 104-40-5) from Riedel-de-Haën (Seelze, Germany). All HPLC grade solvents were supplied by Scharlau S.L. (Barcelona, Spain). Sodium hydroxide was obtained form Panreac (Barcelona, Spain) and powdered octadecyl silica (Discovery DSC-18) from Supelco (Bellefonte, PA).

Instrumental

Saponification

Reaction was carried out in 15 mL screw cap tubes placed in an incubator S160D equipped with a roller mixer SRT1 purchased from Stuart (Barloworld Scientific, Staffordshire, UK).

Extraction equipment

Empty SPE glass columns (6 mL) with teflon frits were obtained from Supelco. Ultrasonic water bath (290W, 50/60 Hz, S 40 H Elmasonic) was supplied by Elma (Singem, Germany). Collection of extracts was performed using a vacuum manifold from Supelco.

A pH-meter Crison GLP 21 (Barcelona, Spain) was used for pH measurements.

HPLC-FL equipment

HPLC separations and determinations were carried out using a 1100 LC instrument equipped with a gradient pump, an autosampler and coupled to a programmable fluorescence detector from Agilent Technologies (Wilmington, DE). The chromatographic column used for analysis was an Eclipse XDB-C_8 column (3 µm and 4.6 × 150 mm) from Agilent Technologies.

LC–MS–MS

The LC–MS–MS chromatographic system consisted of a 1200 LC instrument and a 6410 triple quadrupole mass spectrometer from Agilent Technologies and was used for confirmatory purposes.

Procedures

Reconstituted milk-based method

Milk was reconstituted according to the indications of the manufacturer by addition of 30 mL of water (HPLC grade) at 40°C to each 4.3 grams of milk powder. Reconstituted milk samples were then spiked with a mixture of analytes. A saponification step was performed following a methodology previously described in the literature (18). Briefly, an aliquot of three grams of reconstituted milk was placed in a glass tube and 6 mL of a sodium hydroxide solution 0.4 M in a mixture ethanol–water (9:1) were added. Saponification was performed at 60°C during 30 min to remove triacylglycerols. Subsequently, the obtained solution was decanted and acidified with formic acid to pH = 4 for SPE onto 1 g of C_{18} (Discovery DSC-18). The adsorbent was previously conditioned with two column volumes of acetonitrile (ACN), one column volume of methanol (MeOH), 2 × 1 mL of methanol and 1 × 1 mL of ethanol–water (9:1). Analytes were eluted with 2 × 1 mL of methanol plus 2 × 1 mL of acetonitrile. Sample extracts were evaporated up to a volume of 500 µL under a nitrogen stream and then redissolved up to 2 mL of saponification solution indicated previously. Samples solutions were acidified with formic acid (pH = 4) before determination by HPLC-FL.

Powdered milk-based method

Milk samples were spiked by adding 0.3 mL of a solution of the analytes in acetonitrile per each 0.5 g of powdered milk and stored during 60 min at room temperature to allow solvent evaporation and sample equilibration. Subsequently, an aliquot of 0.5 g of powdered milk (non-reconstituted sample) was placed in an empty SPE glass column (6 mL) containing a teflon frit at the bottom and 1 mL of acetonitrile was added. Columns were immersed in an ultrasonic bath and extractions were performed at room temperature during 15 min. The obtained extracts were collected by filtration in the SPE vacuum manifold and diluted with 1 mL of H_2O. Subsequent determinations were then performed by HPLC-FL.

HPLC-FL analysis

Separation and quantitation was carried out by HPLC coupled to fluorescence detection (λ_{ex} = 226 nm, λ_{em} = 305 nm). Injection volumes of 100 µL were used and elution was performed in isocratic mode at 1 mL/min using a mixture of ACN–H_2O (65:35). Under these conditions the selected compounds could be determined in less than 15 min.

LC–MS–MS

LC–MS–MS was performed using an electrospray interface (ESI), nitrogen as drying gas (9 L/min) and nebulizer gas (40 psi), capillary voltage of 400 V, mobile phase flow-rate of 1 mL/min and injection volumes of 10 µL and 20 µL for NP and $NPEO_x$ respectively. The fragmentor voltage was programmed at 130 V for NP and 4-*n*-NP and 80 V for the ethoxylated derivates and the collision cell energy was varied according to the requirement of the different measurements. Other operating conditions were set as follows: for 4-*n*-NP and NP, LC separations were performed in isocratic mode with a mixture of purified water containing 0.04% acetic acid (35%) and acetonitrile (65%). The ESI interface operated in negative mode and the desolvation temper-

ature was 200°C. The identifying ion (*m/z* 219, $[M–H]^-$) and the confirmatory ions (*m/z* 133 and 147) using collision energy of 25 and 20 eV respectively were used for both NP and 4-*n*-NP detection. For long and short chain $NPEO_x$, LC separations were performed in isocratic mode with a mixture of ammonium formate 5 mM (35%)–acetonitrile (65%). The ESI interface operated in positive mode and the desolvation temperature was 300°C. The identifying ion (*m/z* 238 + 44x, $[M+NH_4]^+$) and the confirmatory ions (*m/z* 89 and 133) using collision energy of 20 eV, were used for $NPEO_x$ (x = 1–13) detection.

Results and Discussion

The chemical structure of the analytes under study is shown in Figure 1. Although they have the same one ring based structure, these compounds have a wide range of polarity, which can complicate their simultaneous extraction. The term nonylphenol is used to refer a complex mixture of ring and branch isomers, among which linear chain nonylphenol in position 4 (4-*n*-nonylphenol) can be included. These isomers are often named indistinctly despite that their hydrophobicity is quite different and that linear isomer is not usually contained in the nonylphenol standard technical mixture. Concerning the ethoxylated derivates, their polarity progressively increases with the number of ethoxy units. Thus, it is difficult to select *a priori* an appropriated technique to be used for the simultaneous extraction of this family of compounds. In this sense, two different strategies commonly used for the analysis of other contaminants in powdered milk samples (reconstituted or on non-reconstituted milk-based procedures) were tested in this work.

Reconstituted milk-based method.

In the first approach, milk powder was reconstituted according to the manufacturer and extraction of analytes was performed by SPE onto 1 g C_{18} cartridges. The maximum volume of sample to be used, without affecting analytes recoveries, was tested by loading different volumes of aqueous standards onto the cartridge. It was observed that the retention of analytes was quantitative up to volumes of 100 mL. Concerning the elution step, different combinations of methanol and acetonitrile as elution solvents were tested. First fractions of methanol were enough to elute the more polar analytes whereas small volumes of acetonitrile were required to achieve quantitative elution of 4-*n*-NP, the most hydrophobic analyte. However, several problems were encountered when the procedure was applied to the SPE of the analytes from the reconstituted milk samples. Firstly, it was observed the impossibility of percolating more than 2 mL of milk sample, because larger volumes provoked clogging of cartridges. For this reason, milk samples were 10-fold diluted with distilled water before loading into the cartridges. In this manner, up to 10 mL milk samples (100 mL diluted sample) could be loaded into the cartridge and all the analytes were quantitatively retained. Another observed problem was related with the elution of $NPEO_{1+2}$ and NP with the optimized mixture (2 × 1 mL of MeOH + 2 × 2 mL of ACN), since recoveries dropped to 41% and 15% respectively. This result was attributed to the high lipid content that is usually present in infant formulae to guarantee high nutritional value needed during children growing. It has been reported that the extraction of hydrophobic contaminants is highly dependent of the grade of skimming of milk (19). In this case, the obtained results suggest that both $NPEO_{1+2}$ and NP strongly interact with lipids that are irreversibly adsorbed in the C_{18} SPE cartridges, being thus less recovered in the elution fraction.

As an alternative, the possibility of performing a destabilization emulsion with methanol according to the procedure described by Casajuana et al. (16) was evaluated. Reconstituted milk aliquots of 10 mL were firstly mixed with methanol and immersed in an ultrasonic bath for 10 min. Then, the emulsion was diluted up to 100 mL with purified water, loaded onto de C_{18} cartridges and finally eluted following the optimized procedure. Results obtained showed an improvement of the recoveries obtained for $NPEO_{1+2}$ and NP in the elution fraction, suggesting that part of milk lipid content was broken during ultrasound agitation with methanol. However, at the same time, the presence of MeOH in the loading solution negatively affected retention of the more polar analytes ($NPEO_x$) and this procedure was finally discarded.

Disruption of fat content by saponification has proven to be a good alternative to improve the efficiency of the extraction of various contaminants such as polychlorinated biphenyls (19) or polycyclic aromatic hydrocarbons (18) from milk samples. Thus, this procedure was also evaluated in the present work. Saponification reaction was carried out according to the procedure described in the experimental section and the supernatant solution obtained was separated and acidified (pH = 4) before loading it onto the cartridge. In the first test, 2 g of reconstituted and spiked milk were saponificated with 4 mL sodium hydroxide 0.4 M in a mixture ethanol–water (9:1). The obtained results were very satisfactory since this procedure yielded quantitative recoveries for all the analytes in the elution fraction, suggesting that milk fat content was totally disrupted by saponification. Subsequently, and in order to increase the amount of sample analysed, a second study on the breakthrough volume of saponification mixture onto 1 g of C_{18} was performed. Volumes of saponification mixture equivalent to the double of sample

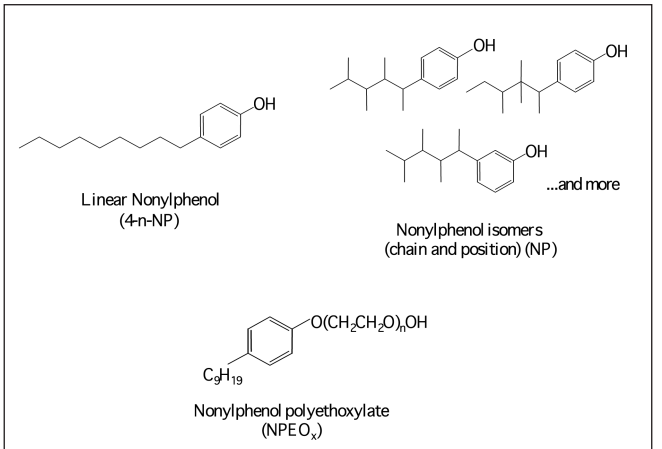

Figure 1. Chemical structures of the studied compounds.

aliquot were added to increasing amounts of reconstituted milk, and the obtained results are shown in Figure 2. Accordingly, the maximum sample amount was fixed in 3 g of reconstituted milk sample (6 mL of saponification solution added), since for higher amounts a decrease in the recoveries obtained occurred.

The analytical characteristics of this procedure are shown in Table I. As can be seen, both recoveries and relative standard deviations (RSDs) were very satisfactory allowing determination of all the selected compounds in one single run. However, several interferences appeared at the retention time of $NPEO_x$, which could not be eliminated by any of the washing solution tested (mixtures of MeOH–H_2O and buffer solutions of pH ranging from 6 to 10). Thus, the detection and quantitation limits obtained for these compounds were high in comparison to those achieved for $NPEO_{1+2}$, NP, and 4-*n*-NP.

Powdered milk based-method

In the second approach, solid–liquid extraction of the analytes directly form the milk powder (non-reconstituted) was performed. Experiments were carried out by ultrasonic-assisted extraction in small columns. This extraction format was previously developed in the laboratory and successfully employed for the analysis of nonylphenol and derivates in solid environmental samples (20).

All variables affecting the efficiency of the ultrasonic-assisted extraction (i.e., nature and volume of extracting solvent, the number of extractions) were studied and optimized. Firstly, different organic solvents (methanol, acetonitrile, dichloromethane hexane, and acetone) were tested, performing one single extraction of 15 min with 4 mL of solvent at room temperature. The obtained results are shown in Figure 3. As it can be observed, acetonitrile and methanol as extracting solvents provided very satisfactory results, allowing quantitative recoveries to be achieved for all the analytes under study. Thus, and in order to increase sensitivity, further optimizations were devoted to minimize the amount of organic solvent used without affecting the efficiency of the extraction step. Table II shows a summary of the different experiments performed using both extracting solvents and the results obtained in each case. It can be stated that, in general, the extractions performed using acetonitrile were more efficient that those performed with methanol. Extractions with acetonitrile enabled to achieve quantitative recoveries for all the analytes in one single extraction, using only 1 mL of extracting solution. Thus, and according to the obtained results, the definitive extraction procedure was set in one single extraction of 15 min at room temperature with acetonitrile (1 mL).

Figure 4 shows a comparison of the chromatograms obtained for spiked (NP and 4-*n*-NP: 1 µg/g; $NPEO_{1+2}$: 2 µg/g; $NPEO_x$: 10 µg/g) and non-spiked samples using the reconstituted milk-based method (Figure 4A) or the powdered milk-based method (Figure 4B). As it can be seen, the second procedure provided better results not only in terms of sensitivity but also of selectivity.

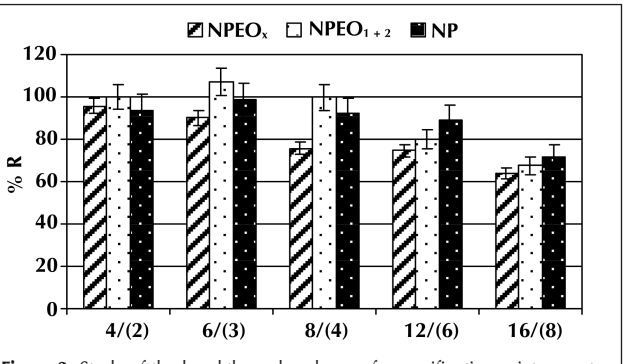

Figure 2. Study of the breakthrough volume of saponification mixture onto the C18 cartridges.

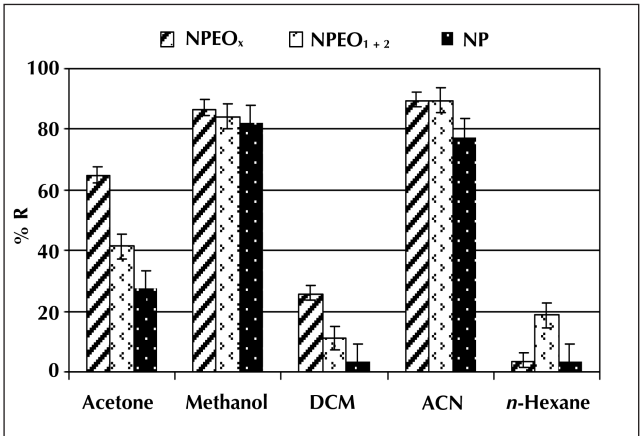

Figure 3. Recoveries obtained for NP, $NPEO_{1+2}$, and NPEOx after the ultrasonic-assisted extraction of powdered milk samples using acetone, methanol, dichloromethane (DCM), acetonitrile (ACN), and n-hexane.

Table I. Summary of the Recoveries (R), Relative Standard Deviations (RSDs), and Limits of Detection (LODs) Achieved Using the Reconstituted Milk-based Procedure (*n* = 3)

Parameter	$NPEO_x$	$NPEO_{1+2}$	NP	4-*n*-NP
R (%)*	96.8	94.0	92.7	89.2
RSD(%)	8.4	7.0	6.7	8.5
LOD (µg/g)†	0.89	0.48	0.51	0.047

* NPEOx: 1.0 µg/g NPEO1 + 2: 0.8 µg/g, NP and 4-*n*-NP: 0.4 µg/g.
† LODs were calculated as three times the average signal of the background noise in the analysis of three blank samples at the retention times of the target analytes.

Table II. Average Recoveries (%R) of the Analytes after Different Cycles of Extraction with MeOH and ACN

Volume (mL) (1st/2nd extraction)	MeOH					
	$NPEO_x$		$NPEO_{1+2}$		NP	
	1st ext.	2nd ext.	1st ext.	2nd ext.	1st ext.	2nd ext.
2 mL/2 mL	100	n.d.*	101	n.d.	68	21
2 mL/1 mL	97	n.d.	93	n.d.	72	17
1 mL/1 mL	85	n.d.	78	12.0	54	25
	ACN					
2 mL/2 mL	107	n.d.	106	n.d.	87	13
2 mL/1 mL	104	n.d.	102	n.d.	83	13
1 mL/1 mL	101	n.d.	110	n.d.	84	16

*not detected.

The analytical performance of the powdered milk-based method was established and is summarized in Table III.

Linearity

Method linearity was assayed by performing internal calibration curves using milk samples spiked with the analytes within a range (n = 6) from 0.015 to 1 µg/g for NP and 4-*n*-NP, 0.030 to 2 µg/g for $NPEO_{1+2}$, and 0.150 to 10 µg/g for $NPEO_x$. The detector response was linear in the range of concentration studied for all of the target compounds, and the correlation coefficient obtained by regression analysis varied from 0.991 to 0.997.

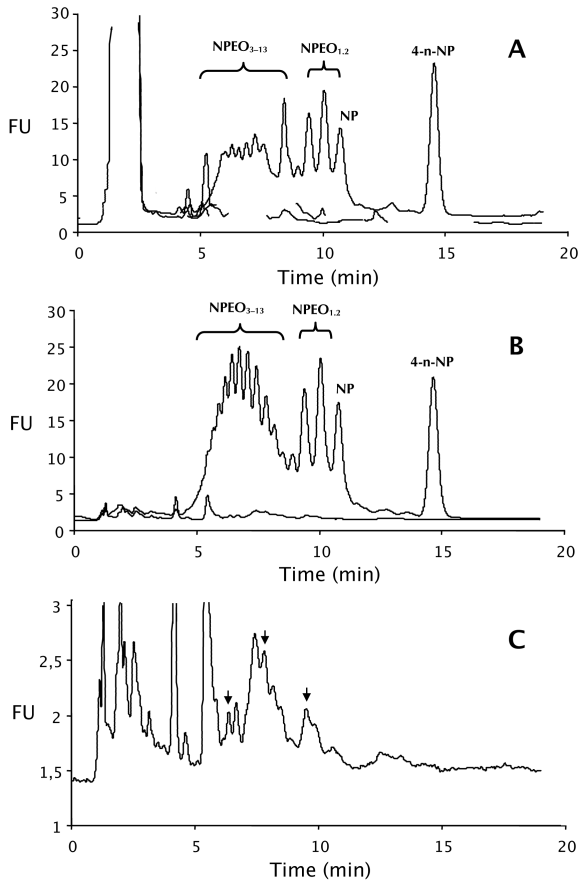

Figure 4. Chromatograms obtained for spiked (NP and 4-*n*-NP: 1 µg/g; $NPEO_{1+2}$: 2 µg/g; $NPEO_x$: 10 µg/g) and non-spiked samples using the reconstituted milk-based method (A), the powdered milk-based method (B), and the same chromatogram as that obtained for non-spiked samples using the powdered milk-based method with different y-axis scale (C).

Recovery and precision

Two different spiking levels close to the extremes of the previously determined operation range were selected for this study, and analysis were performed by triplicate. Precision was evaluated both in terms of retention times and peak area measurements after the extraction and chromatographic separation. The RSDs obtained for the retention times ranged from 0.1% to 0.7% depending on the compound, whereas for peak areas the RSDs were lower than 10.6% and 8.3% for both levels of concentration. Average recoveries of the analytes from spiked samples varied from 76.3 to 111.0 for the lower concentration, and 83.5 to 96.0 for the higher.

Limits of detection and quantitation

The limits of detection (LOD) were calculated as three times the average signal of the background noise obtained in the analysis of three blank powdered milk samples at the retention times of the corresponding analytes. The limits of quantitation were determined considering a value of 10 times the background noise. As it can be seen, the LOD achieved following this second approach were much better than those provided by the saponification based-method. It is remarkable the improvement obtained in the case of the ethoxylated derivates (see Table I) for whose values were reduced in more than one order of magnitude.

Selectivity

It is important to point out the fact that several peaks appeared in the chromatograms obtained for non-spiked samples (Figure 4C) at the retention times of the analytes under study. The fluorescence spectra of those unknown peaks were recorded and compared with those obtained for the corresponding standards using the software comparison tool. Three coincidences, (i.e., match factors higher than 950) were found (arrows in Figure 4C) corresponding to $NPEO_2$, $NPEO_5$, and $NPEO_{11}$, although at concentration levels below the LOD shown in Table III.

In order to confirm the presence of these compounds, samples were analysed LC–MS–MS. According to existing methodologies (21,22) the chromatographic separation conditions were slightly modified to allow effective ionization and fragmentation of the studied analytes in the mass spectrometer. For this purpose, both the mobile phase composition and ionization conditions were adjusted for each compound. The selection of *m/z* ions for the confirmation of the target compounds was based on bibliographic data (23) and they are summarized in Table IV. In the case of 4-*n*-NP and NP isomers, working in negative ESI mode with a 0.04% acetic acid solution instead of water in the mobile phase allowed to produce the identifying ion *m/z* 219 $[M–H]^-$ in the MS^1 and the confirmatory fragments 133 and 147 in MS^2. Concerning the ethoxylated derivates, it was necessary to use 5 mM ammonium formate in the mobile phase to produce identifying ions in MS^1 by adduct formation with ammonium $[M+NH_4]^+$, working in positive ESI mode. The selected confirmatory ions were *m/z* 89 and 133 in both cases. In these conditions,

Table III. Analytical Performance of the Powdered Milk-based Method.

Analyte	Linearity*		Spiking level 1†		Spiking level 2‡		LOD	LOQ
	Equation	r2	R(%)	RSD(%)	R(%)	RSD(%)	(µg/g)	(µg/g)
$NPEO_x$	Y = 235.5x + 98.82	0.996	111.0	10.6	96.0	8.3	0.085	0.250
$NPEO_{1+2}$	Y = 60.07x + 27.50	0.991	103.3	7.7	89.1	8.2	0.036	0.110
NP	Y = 23.42x + 16.35	0.995	80.2	5.9	83.8	4.5	0.016	0.050
4-n-NP	Y = 57.386x + 3.57	0.997	76.3	5.2	83.5	3.6	0.010	0.030

* Linear range: NP and 4-*n*-NP: 0.015–1.00 µg/g, $NPEO_{1+2}$: 0.030–2.00 µg/g, $NPEO_x$: 0.150–10.0 µg/g
† n = 3; NP and 4-*n*-NP: 0.020 µg/g, $NPEO_{1+2}$: 0.040 µg/g, $NPEO_x$: 0.200 µg/g.
‡ n = 3; NP and 4-*n*-NP: 1.00 µg/g, $NPEO_{1+2}$: 2.00 µg/g, $NPEO_x$: 10.00 µg/g.

milk samples were analyzed and unequivocal identification of $NPEO_2$, $NPEO_5$, and $NPEO_{11}$ was accomplished.

Conclusions

Two methodologies were developed for the simultaneous analysis by HPLC-FL of endocrine disrupters NP, 4-*n*-NP and $NPEO_{1+2}$, and long chain nonylphenol ethoxylates, in powdered milk infant formula. The first method was based on a saponification of lipid content in reconstituted milk samples followed by SPE onto C_{18} cartridges. This procedure allowed quantitative recoveries to be achieved but the detection limits were not satisfactory.

The non-reconstituted milk-based method provided greater selectivity and sensitivity than the previous approach. One-step ultrasound assisted extraction was enough to achieve quantitative recoveries for target analytes, allowing their determination in a precise and accurate manner with limits of detection low enough for the analysis of these contaminants at the concentration levels required. None of the analyzed samples were contaminated with NP or 4-*n*-NP, but the presence of $NPEO_2$, $NPEO_5$, and $NPEO_{11}$ was firstly evidenced by HPLC-FL and further confirmed by HPLC–MS–MS. From the results obtained it can be concluded that the developed procedure can be clearly used in routine laboratories for the quality control of powdered milk infant formula.

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