Fully automated on-line solid phase extraction coupled to liquid chromatography–tandem mass spectrometry for the simultaneous analysis of alkylphenol polyethoxylates and their carboxylic and phenolic metabolites in wastewater samples

Fully automated on-line solid phase extraction coupled to liquid chromatography–tandem mass spectrometry for the simultaneous analysis of alkylphenol polyethoxylates and their carboxylic and phenolic metabolites in wastewater samples

L. Ciofi[(a)], C. Ancillotti[(a)], U. Chiuminatto[(b)], D. Fibbi[(c)], B. Pasquini[(a)], M.C. Bruzzoniti[(d)], L. Rivoira[(d)], M. Del Bubba (corresponding author; phone number: +39-0554573326; fax number: +39-0554573385; e-mail address: delbubba@unifi.it)[(a)]

[(a)] *Department of Chemistry, University of Florence, Via della Lastruccia, 3 – 50019 Sesto Fiorentino, Florence (Italy)*

[(b)] *Sciex Italia, Viale Lombardia, 218, 20047 Brugherio, Monza Brianza (Italy)*

[(c)] *GIDA S.p.A., Via di Baciacavallo, 36, 59100 Prato, Italy*

[(d)] *Department of Chemistry, University of Turin, Via Pietro Giuria, 5 – 50019 Turin (Italy);*

Abstract

Three different sorbents (i.e. endcapped octadecylsilane, octasilane and styrene-N-vinylpiperidinone co-polymer) were investigated in order to develop an on-line solid phase extraction – liquid chromatographic tandem mass spectrometric method (on-line SPE-LC-MS/MS) for the simultaneous analysis of alkylphenols polyethoxylate (AP_nEOs, n=1-8), and corresponding monocarboxylate (AP_1ECs) and phenolic (APs) metabolites. The endcapped octadecylsilane was selected due to its full compatibility with a chromatographic approach, which allowed the elution of positively and negatively ionisable compounds in two distinct retention time windows, using a water-acetonitrile-tetrahydrofuran ternary gradient and a pellicular pentafluorophenyl column. On this SPE sorbent the composition of the loading/clean-up solution was then optimized in order to achieve the best recoveries of target analytes. Under the best experimental conditions, the total analysis time per sample was 25 minutes and method detection limits (MDLs) were in the sub-ng L^{-1} to ng L^{-1} range (0.0081-1.0 ng L^{-1}) for AP_nEOs with n=2-8, AP_1ECs and APs, whereas for AP_1EOs an MDL of about 50 ng L^{-1} was found. Using the mass-labelled compound spiking technique, the method performance were tested on inlet and outlet wastewater samples from three activated sludge treatment plants managing domestic and industrial sewages of the urban areas and the textile district of Prato and Bisenzio valley (Tuscany, Italy); in most cases, apparent recovery percentages approximately in the ranges of 50-110% and 80-120% were found for inlet and outlet samples, respectively. The on-line SPE-LC-MS/MS analysis of wastewater samples highlighted the presence of target analytes at concentrations ranging from few ng L^{-1} to thousands ng L^{-1}, depending on the compound and matrix analysed. AP_2ECs were also tentatively identified in outlet samples.

Keywords: On-line solid phase extraction-liquid chromatography-tandem mass spectrometry; alkylphenols polyethoxylates; alkylphenoxy carboxylates; alkylphenols; wastewater

1. Introduction

Alkylphenols polyethoxylates (AP_nEOs) are a well-known class of non-ionic surfactants that have been used as emulsifiers, dispersive agents, surfactants and/or wetting agents [1]; within the AP_nEO class, branched ethoxylate nonylphenols (NP_nEOs) with a number of ethylene oxide (EO) units up to 9-10, have been commonly used in cleaning processes [2].

AP_nEOs withstand degradation in wastewater treatment plants (WTPs), as well as in natural water bodies, mainly due to biological processes through a progressive shortening of the EO chain [3, 4].

Many evidences of the presence of AP_nEOs and their biodegradation derivatives have been reported in literature for different fresh water ecosystems, as a consequence of uncontrolled discharge in surface water or incomplete removal in WTPs [5-7]. Most persistent AP_nEO degradation metabolites are AP_nEO oligomers with 1-2 EO units and alkylphenols (APs), the latter being the final stage of the ethoxylate chain breakdown [8]; moreover, the formation of short-chain alkylphenoxy carboxylates (AP_nECs) have been highlighted during AP_nEO biodegradation [3]. These biotransformation products have been reported as more hydrophobic, more estrogenic and more persistent than the parent substances [7].

Based on the available ecotoxicological data, the European Community (EC) restricted the commercialization and the use of NP_nEOs and nonylphenols (NPs) [9]. Linear and branched NPs and octylphenols (OPs) have been included in the list of priority substances by the EC [10]. Moreover, 4-t-OP has been identified as a substance of very high concern because of its endocrine disrupting properties [11].

However, these compounds are currently used in many other recently developed countries for various industrial applications, including the treatment of semi-finished products (e.g. textiles), the manufacturing of which is then completed in European countries. Accordingly, the presence of these compounds in fresh water and wastewater still represents an important environmental issue and their monitoring in water samples should rely on analytical methods providing high sensitivity and selectivity, as well as high method throughput.

Liquid chromatography (LC), coupled to mass spectrometry (MS), has been extensively applied for the analysis of surfactants, including non-ionic ones, in different environmental matrices [12]. It is well-known that APs and AP_nECs are detected by MS under negative ionization (NI), whereas AP_nEOs must be analyzed in positive ionization (PI) mode, by monitoring the adduct ions produced in the MS source in presence of a suitable salt (typically, ammonium acetate or formate). Moreover, two LC runs are usually employed [13-16] since the elution of negatively (i.e. APs and AP_nECs) and positively (AP_nEOs) ionisable compounds in distinct retention time (Rt) windows is difficult to be achieved [17].

In order to improve the analytical throughput, a single run LC-MS/MS method has been proposed by Jahnke and co-workers [18], who overcame the lack in resolution among negatively and positively ionisable analytes, by employing the continuous MS polarity switching. This approach can be very useful when the analysis of a high number of positively and negatively ionisable compounds in a single chromatographic run is desired, even though it can involve signal aberrations, owing to duty-cycle problems, as well as classical matrix-dependent source phenomena which are enhanced by continuous and strong voltage variations [19].

An improvement of the analytical throughput in the analysis of these compounds has been also achieved by using the on-line solid-phase extraction (SPE) approach [20, 21]. However, two chromatographic runs were necessary for the determination of either positively or negatively ionisable analytes [21]; otherwise, the continuous polarity switching was adopted to overcome the lack of selectivity of the chromatographic method which showed a number of co-elutions among compounds detectable by PI and NI [20].

Very recently, a single run LC-MS/MS method for the simultaneous determination of AP_2EOs, AP_1EOs, AP_1ECs and APs, in which the positively and negatively ionisable analytes are eluted in two distinct Rt windows has been proposed by our team and successfully applied to the analysis of a wide range of water samples after off-line SPE [22].

Based on these considerations, the aim of this study is to develop an on-line SPE-LC-MS/MS method for the simultaneous determination of the above-mentioned analytes, as well as AP_nEOs with a higher number (n=3-8) of EO units, aiming at maximizing method sensitivity and throughput, without employing the continuous polarity switching approach. In this regard, key parameters of the method, such as the nature of the SPE sorbent phase and the eluent composition used during the extraction and clean-up phases, were investigated. Furthermore, the applicability of the method to the analysis of real samples was verified on wastewater collected in different WTPs, managing the sewage from a textile industrial district.

2. Materials and methods

2.1 Chemicals and materials

LC–MS grade methanol (MeOH), acetonitrile (ACN), water, formic acid (FOA), ammonia (NH_3 content > 25%), and inhibitor-free CHROMASOLV® tetrahydrofuran (THF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (resistivity > 18 MΩ) was obtained from a Milli-Q system (Millipore, Billerica, MA, USA), and MeOH gradient grade was purchased from VWR International (Fontenay-sous-Bois, France).

The unlabelled and labelled reference standards of target analytes used in this study are listed below; for each analyte the IUPAC name and the CAS registry number are following specified. 4-t-octylphenol (4-t-OP, purity 97%, CAS: 140-66-9), 4-n-octylphenol (4-n-OP, purity 99%, CAS: 1806-26-4), 4-(1-ethyl-1,4-dimethylpentyl)-phenol (4-NP, purity 99.9%, CAS 142731-63-3) and Alkylphenol Internal Standard Mix 7-solution for DIN EN ISO 18857-2 were purchased from Sigma-Aldrich. The internal standard solution contained the following mass labelled compounds: 4-tert-octylphenol ring $^{13}C6$ (4-t-OP$^{13}C6$, CAS: 1173020-24-0), 4-tert-octylphenol monoethoxylate ring $^{13}C6$ (4-t-$OP_1EO^{13}C6$, CAS: 1173019-48-1), 4-tert-octylphenol diethoxylate ring $^{13}C6$ (4-t-$OP_2EO^{13}C6$, CAS: 1173020-69-3), 4-(1-ethyl-1,4-dimethylpentyl)-phenol ring $^{13}C6$ (4-NP$^{13}C6$,

CAS: 1173020-38-6), 4-(1-ethyl-1,4-dimethylpentyl)-phenol monoethoxylate ring $^{13}C6$ (4-$NP_1EO^{13}C6$, CAS: 1173019-61-8) and 4-(1-ethyl-1,4-dimethylpentyl)-phenol diethoxylate ring $^{13}C6$ (4-$NP_2EO^{13}C6$, CAS: 1173019-36-7). Triton™ X-45 and IGEPAL® CO-520 technical mixtures were purchased from Sigma-Aldrich (St. Louis, MO, USA), containing mixture of oligomers of 4-t-octylphenols polyethoxylates (4-t-OP_nEOs, with n=1-11) and of 4-nonylphenols polyethoxylates (4-NP_nEOs, with n=1-11), respectively. 4-(1-ethyl-1,4-dimethylpentyl)-phenoxy-acetic acid (4-NP_1EC, purity 96.5%, CAS: 3115-49-9), 4-n-octylphenoxy-acetic acid (4-n-OP_1EC, purity 98.5%, CAS: 15234-85-2) and deuterated 4-nonylphenoxy-acetic acid (4-NP_1ECd2, purity 96.5%, CAS not available) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). 4-n-nonylphenol (4-n-NP, purity 99.9%, CAS: 104-40-5) was supplied by Riedel-de Haën (Seelze, Germany). Alkylphenol Internal Standard Mix 7 (1 µg mL^{-1} of each individual compound), 4-NP_1EC, 4-n-OP_1EC and deuterated 4-NP_1EC d2 (10 µg mL^{-1} each) were obtained as stock solutions in acetone and were stored in the dark at -20˚C. Stock solutions (1 mg mL^{-1}) of Triton™ X-45, IGEPAL® CO-520, 4-t-OP, 4-n-OP and 4-NP were prepared by dissolving 10 mg of standard into LC-MS MeOH in a 10 mL calibrated flask and were stored in the dark at -20˚C.

Phenex® RC membrane syringe filters (pore size 0.2 µm) were purchased from Phenomenex (Torrance, CA, USA).

The following SPE cartridges (20 x 2.0 mm), all purchased from Phenomenex, were used in this study: Strata® C18E 20 µm On-Line Extraction, Strata® C8 20 µm On-Line Extraction and Strata®-X 25 µm On-Line Extraction. The LC columns employed for the on-line SPE-LC-MS/MS analysis and the characterization of Triton™ X-45 and IGEPAL® CO-520 technical mixtures were the pellicular column pentafluorophenyl Kinetex® PFP (100 mm x 3 mm, 2.6 µm particle size) and the pellicular column Kinetex® Biphenyl (100 mm x 3 mm, 2.6 µm particle size), respectively. Both columns were purchased from Phenomenex.

Pre-dosed reagents for the determination of chemical oxygen demand (COD) were purchased from Hach Lange (Düsseldorf, Germany).

2.2 Tandem mass spectrometry

The on-line-SPE-LC system was coupled with a 5500 QTrap mass spectrometer (Sciex, Framingham, MA, USA), equipped with a Turbo V® interface by an electrospray (ESI) probe. MS/MS analysis was carried out using the Multiple Reaction Monitoring (MRM) mode by ESI both in NI and PI mode. The precursor and product ion pairs, as well as compound dependent parameters, were optimized by direct infusion of diluted standard solution and are reported in **Table 1**. The most intense and the second most intense (when detectable) MRM transition were used for analyte quantification and identification, respectively. Source dependent parameters were optimized in flow injection analysis at optimal LC flow and mobile phase composition, and were as follows: Curtain Gas 50, CAD Gas Medium, Temperature 500°C, Gas 1 50, Gas 2 60, Interface Heater ON and Ion Spray Voltage (IS) -4500 V in MRM(−) and 5500 V in MRM(+). The source parameters, with the only exception of IS, were kept constant during the whole chromatographic analysis. Instrument control during optimization of source dependent and compound dependent parameters was performed through the Analyst version 1.6.2 software (Sciex).

In order to confirm the identities of target analytes, criteria proposed by the Commission Decision 2002/657/CE [23] were adopted; this decision, applied to residues of veterinary medicinal products, provides added confirmation criteria in complex matrixes such as wastewater.

Quantification was performed using external calibration curves, after correction for apparent recoveries. For analytes showing chromatographic areas higher than the upper limits of the external calibration curves, the sample was properly diluted and submitted again to the on-line-SPE-LC-MS/MS analysis.

2.3 On-line SPE and chromatographic analysis

The on-line SPE-LC analysis was performed on a Shimadzu (Kyoto, Japan) chromatographic system, consisting of a low pressure gradient quaternary pump Nexera X2 LC-30AD (Pump 1) and three isocratic pumps LC-20AD XR, devoted to the on-line SPE procedure (Pump 2 and Pump 3)

and the delivery of the post-column PI promoter (Pump 4), respectively. A CTO/20AC thermostatted column compartment equipped with the above-mentioned PFP analytical column, a SIL-30AC auto-injector equipped with a 2 mL-sample loop, a DGU-20A 5R degassing unit and a CBM-20A module controller were also used; an HPLC six-port VALCO switching valve, housing the sorbent cartridge, was used for performing the on-line SPE loading and injection phases.

In the optimized conditions, the automatic procedure for sample analysis was performed as follows. The 2 mL loop was filled with the sample at 11 µL s^{-1} (filling time of the loop equal to 3 min). Meanwhile, the HPLC switching valve was set on the "load" position ("loading phase"), allowing the analytical column to be conditioned by Pump 1 with 97.5% of water modified with $1.0 \cdot 10^{-4}$ M FOA (eluent "A") and 2.5% of ACN/THF 10/90 (v/v) (eluent "B"), at a flow rate of 0.35 mL min^{-1}. When the loop filling was accomplished the elution program of Pump 1 started. Simultaneously, pumps 2 and 3 (dispensing water and methanol, respectively) delivered a water/methanol mixture 80/20 (v/v) through a high-pressure eluent mixer, at 1.5 mL min^{-1} for 3.5 min, thus loading the 2 mL sample onto the C18E SPE cartridge and washing out the matrix from the sorbent.

Subsequently, the HPLC valve was switched to the "injection" position ("injection phase"), thus permitting the SPE cartridge to be back-flushed by Pump 1 and target analytes transferred from the SPE cartridge to the analytical column for the chromatographic separation

The elution program performed by Pump 1 was the following: 2.5% B for 5.5 min, from 2.5% to 93.5% B in 9.5 min, 93.5% B for 3.5 min, return to the initial eluent composition and system re-equilibration in 3.5 min. Flow rate was 0.35 mL min^{-1}. The column compartment temperature was set to 25°C. These elution conditions were derived from the ones previously optimized for these analytes [22], with minor modifications.

After analyte transfer to the analytical column was completed (i.e. at the retention time of 12.5 min.), the valve was switched again to the "load" position and the cartridge washed for 5 min with methanol dispensed by Pump 3 at 1.5 mL min^{-1}. The cartridge was finally re-equilibrated before the successive analysis by eluting with the water/methanol mixture 80/20 (v/v).

During the whole LC run, the addition of a 300 mM ammonia solution in methanol (i.e. the positive-ionisation promoter) was post-column dispensed by Pump 4 at 40 µL min^{-1}, by means of a three-way connector.

Under the aforementioned experimental conditions, the whole duration of the chromatographic run was 22 min, which was divided in two periods: (i) from 0 to 15.7 min, MRM(+) for AP_nEO monitoring and (ii) from 15.7 to 22 min, MRM(−) for APs and AP_1ECs analysis. Total analysis time per sample, including loop filling was 25 min.

The whole functioning of pumps 1-4 and switching valve was automatically controlled by the Analyst version 1.6.2 software (Sciex).

2.4 Glassware cleaning and blank evaluation

Precautions were taken to avoid contamination in the laboratory. All glassware was cleaned before use by a double washing with a minimum quantity of hot mixture of chromic and concentrated sulphuric acid, followed by repeated rinsing with ultrapure water and gradient grade methanol, and finally dried in an oven at 130 °C for 1 hour.

The blank contribution of the whole analytical procedure was evaluated as following detailed. A sampling bottle previously cleaned as described above was filled with Milli-Q water; a Milli-Q water aliquot was then drawn by a polypropylene syringe, passed through an RC membrane and the eluate collected in an auto-injector vial and analysed by the aforementioned on-line SPE-LC-MS/MS method. Under these experimental conditions, the blank contribution for target analytes was null.

2.5 Site description and sample collection

Wastewater samples were collected (i) in two different WTPs (i.e. Baciacavallo and Calice facilities) devoted to the treatment of wastewater from the industrial textile district and the city of Prato (Tuscany, Italy) and (ii) in one WTPs (i.e. Cantagallo facility) treating the domestic and industrial wastewater from the civil and textile areas of Bisenzio valley (Tuscany, Italy). The

Baciacavallo and Calice WTPs consist mainly of biological oxidation and ozone tertiary treatment, after which the effluents are discharged in the Ombrone river. The Val di Bisenzio WTP is essentially based on biological oxidation and the effluents are discharged in the Bisenzio river.

In September 2015, three 24-h composite samples were collected at the inlet and the outlet of each WTP described above, using a Endress + Hauser ASP-PORT D2 autosampler (Reinach, Switzerland). All samples were collected using dark glass bottles previously .

Samples were immediately acidified to pH=2.4±0.1 with HCl 6 M. Sample aliquots of proper volume were then filtered on RC membrane syringe filters and stored in the dark at 4°C until analysis, which was performed in triplicate within 48 h after sampling.

2.6 Chemical oxygen demand

COD was spectrophotometrically determined according to the USEPA approved [24] method for wastewater analysis [25], using a DR/4000U UV-visible spectrophotometer (Hach), after sample filtration on RC membranes (porosity 0.2 µm), in order to be consistent with the procedure adopted for the on-line SPE-LC-MS/MS analysis.

2.7 Statistical analysis

All statistical analyses were performed using the SPSS software, version 17 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1 Optimisation of the on-line SPE step

3.1.1 Chromatographic separation and selection of type of sorbent

As previously stated, APs and AP_nECs must be analysed in NI mode, whereas AP_nEOs have to be detected in PI mode, typically in the presence of a source of ammonium to form positive adduct ions in the MS source. Accordingly, the chromatographic separation between the ethoxylated and

the carboxylic and phenolic species is necessary for their simultaneous, sensitive and precise determination [22].

In this study, the selection of the stationary phase to be employed for the on-line SPE process was based on the fulfilment of the following criteria: (i) high affinity for target compounds, in order to ensure high recoveries; (ii) compatibility with the previously obtained chromatographic conditions, which allow the elution of positively and negatively ionisable compounds in two successive Rt windows (see **Fig. 1**) [22].

Three different sorbents were investigated: (i) a surface-modified styrene-N-vinylpiperidinone co-polymeric phase (Strata®-X); (ii) an octyl (C8) functionalized silica-based sorbent and (iii) an end-capped octadecyl (C18) silica-based phase.

The use of a polymer modified with both hydrophilic and lipophilic groups has been previously investigated for the extraction of these compounds, with both off-line [18, 26] and on-line [20] SPE approaches. These polymers are characterized by good chemical stability (e.g. full compatibility with extreme pH values), but limited mechanical resistance (maximum tolerated backpressure equal to about 275 bar), compared to silica-based on-line SPE sorbents, for which the conservative backpressure limit suggested by the manufacturer (about 350 bar) is only due to the architectural characteristics of the cartridge.

In our previously optimized chromatographic conditions [22] the initial backpressure value was about 200 bar. This value increased with increasing the percentage of the organic eluent, according to the trend reported for the viscosity of water/THF mixtures as a function of their relative percentages [27]. More in detail, a maximum backpressure of about 350 bar is observed at Rt=13.5 min (i.e. 13.5 min after the start of the “injection phase”). In this regard, it should be noted that the complete desorption of target analytes from the Strata®-X cartridge, occurred at Rt≃13 min (see **Fig. 2A**), approximately corresponding to the maximum backpressure value observed in the presence of the analytical column, which is not compatible with the Strata®-X mechanical resistance. This finding forced us to exclude the surface-modified styrene-N-vinylpiperidinone co-

polymeric sorbent from the study. Conversely, the silica-based C8 and C18 sorbents are compatible with the backpressure values experimentally observed and were therefore investigated for the on-line pre-concentration of target analytes. In all the optimization steps, standard solutions in MilliQ water acidified to pH=2.4±0.1 with HCl 6 M were used.

When the C8 on-line SPE cartridge was employed, target analyte desorption occurred approximately between 9.5 and 13.0 min; in particular, a slightly higher retention was observed for AP_nEOs, compared to APs (**Fig. 2B**). Thus, an inverse selectivity was exhibited by the C8 SPE sorbent, in respect to the one obtained by the analytical column, in which compounds detected in PI are less retained than those revealed in NI.

Conversely, the use of the SPE C18 sorbent, produced a narrower desorption time window (i.e. from about 9.0 to 12.0 min) and did not exercise any selectivity during the desorption process (**Fig. 2C**), thus avoiding any negative influence on the chromatographic resolution of the analytical column.

The introduction of the on-line SPE step, using both C8 and C18 cartridges, did not change the elution order of target compounds, compared to that reported in **Fig. 1**. More in detail, phenolic and ethoxylate compounds with branched alkyl chain eluted before the isomers with linear alkyl chain (e.g. Rt 4-NP < Rt 4-n-NP) and octyl-derivatives before the corresponding nonyl-derivatives (e.g. Rt 4-n-OP < Rt 4-n-NP). Moreover, Rt values decreased with increasing ethoxylation degree (Rt AP_2EO < AP_1EO < AP) and AP_1ECs were the most retained compounds. However, when the C8 sorbent was employed, a partial co-elution of 4-NP_1EO (the last eluting analyte of the positively ionisable compound group) and 4-t-OP (the first eluting analyte of the negatively ionisable compound group) was observed, whereas the use of the C18 cartridge allowed to maintain the baseline resolution of the two analytes (**Fig. 3**). The different influence of SPE sorbents on chromatography could be ascribed to the aforementioned inverse selectivity of AP_nEOs and APs, as well as to the higher analyte retention, observed using the C8 phase, the latter implying the start of the analytical separation at significantly higher percentage of the organic solvent in the eluent.

Based on these considerations, the C18 cartridge was selected for the further optimisation of the on-line SPE process.

3.1.2 Recovery evaluation as a function of the loading solution composition

The goal of this optimisation step was to identify the best on-line SPE carrier phase composition that allowed for maximizing analyte retention during the sample loading. Based on our experiences [22, 28] water/methanol mixtures with relative percentages ranging from 90/10 to 60/40 (v/v) were used for the loading of 2 mL-aliquots of a standard solution in MilliQ water of target analytes (concentration of each standard: 500 ng L^{-1}; concentration of each technical mixture: 125 ng L^{-1}). Chromatographic analysis was performed with the PFP column and the elution gradient described in the paragraphs 2.1 and 2.3, respectively. For each composition of the on-line "loading phase", replicated analysis (n=5) were performed and the chromatographic areas were determined for each investigated compound.

Direct injections (n=5) of equivalent amounts of target analytes (i.e. 1 ng of each analytical standards and 250 pg of each technical mixtures) were also performed and mean values of chromatographic areas of each compound were determined. Accordingly, for a given compound, the percentage recovery of the "nth" replicated on-line SPE analysis was calculated as the percentage ratio between the peak area obtained in the on-line configuration and the mean area of the direct injection.

Fig. 4 A-C illustrates the mean percentage recovery values and corresponding standard deviations obtained for target analytes. The increase from 10% to 20% of the methanol percentage in the "loading phase" produced a general statistically significant raise of recovery values. A further increase of the amount of organic solvent (from 20 to 30%) translated into a decrease (in most cases statistically significant) of chromatographic areas for both 4-NP_nEOs (**Fig. 4A**) and 4-t-OP_nEOs (**Fig. 4B**), as well as for 4-NP and 4-NP_1EC, whereas 4-n-NP exhibit a constant recovery value (**Fig. 4C**). Conversely, for 4-t-OP, 4-n-OP and 4-n-OP1EC the increase of methanol percentage

from 20% to 30% gave rise to a significant improvement of the recovery values (**Fig. 4C**). Finally, when a water/methanol 60/40 (v/v) mixture was used, the recovery values of all target analytes underwent to a further decrease. The bell-shaped curve found herein for recovery values as a function of different methanol percentage in the "loading phase" was elsewhere observed for other organic compounds containing the phenolic moiety [28] and could be explained on the basis of the influence of the SPE eluent composition on: (i) sorbent activation and (ii) analyte partition between mobile phase and sorbent. More in detail, at the lowest organic solvent percentage, the stationary phase could be not adequately wetted, thus preventing an efficient interaction between the sample and the sorbent. Vice versa, when the highest percentage of organic solvent is present in the SPE eluent, the stationary phase is properly activated, but analytes are washed off due to the preferential partition of analytes in the mobile phase. The highest recovery values should be therefore observed when these two aspects are optimally balanced. For a given stationary phase, the best compromises of the two aforementioned effects is expected to be compound-dependent.

Based on the results described above, a water/methanol mixture 80/20 (v/v) was chosen as "loading phase". It should be noted that this value agreed with the one selected for off-line SPE performed manually on the same analytes and sorbent [22].

3.1.3 Loading volume

In order to evaluate the influence of the loading volume on the chromatographic response of target compounds, three different volumes (1.0, 1.5 and 2.0 mL) of 500 ng L^{-1} of each analytical standard and 125 ng L^{-1} of each technical mixture solutions in Milli-Q water were loaded onto the SPE cartridge and analysed using the pellicular PFP column, according to the elution gradient reported in the paragraph 2.3. The chromatographic areas were determined and plotted as a function of the loading volumes, evidencing a very good linear response in the range investigated (R^2=0.991–0.999, depending on the analyte considered). Thus, no saturation of the sorbent was evidenced and

breakthrough phenomena could be excluded. Based on these results, an injection volume of 2 mL has been selected and used for all successive analysis, in order to maximize sensitivity.

3.2 Characterization of Triton™ X-45 and IGEPAL® CO-520 technical mixtures

Since reference standards of AP_nEOs with n>2, certified for concentration and purity of each oligomer are not commercially available, for quantitative analytical purposes (i.e. evaluation of the linear dynamic range, detection and quantification limits, as well as the determination of target analytes in real samples), it is necessary to assess the percentage composition of each octyl and nonyl-derivative, constituting the Triton™ X-45 and IGEPAL® CO-520 technical mixtures, respectively. With this aim, an HPLC-DAD method has been developed, given that the molar extinction coefficient of each individual oligomer is expected to be not dependent on the length of the ethoxylate chain, whereas the ESI-MS response of alkylphenol polyethoxylates is a function of the ethoxylation degree. The former assumption has been confirmed comparing the relative responses of the mono and diethoxylate alkylphenols present in the Alkylphenol Internal Standard Mix 7 (data not shown).

The details regarding the HPLC-DAD method are available in the Electronic Supplementary Material section. With this method, the baseline separation of 4-t-OP_nEO oligomers was achieved (see Electronic Supplementary Material Fig. S1), whereas for NP_nEOs, only a partial resolution was observed, due to the presence of alkyl chain isomers in the IGEPAL® CO-520 technical mixture (see Electronic Supplementary Material Fig. S2). For the investigated technical mixtures, quantitative determination was allowed for oligomers with n=1-11 (see Electronic Supplementary Material Table S1). Both mixtures showed a predominance of AP_3EO and AP_4EO and a significantly lower abundance of the other oligomers, and especially of those with n=1 and n>6; moreover, for either octyl or nonyl technical standards, oligomers with n>8 were found to be less than 2.5% (see Electronic Supplementary Material Table S1). Accordingly, only AP_nEOs with n=1-

8 were considered for performance evaluation of the on-line-SPE-LC-MS/MS and its application to real samples, similarly to other literature studies [20].

3.3 Method performance evaluation

Method performance were evaluated by estimating method detection limits (MDLs), method quantification limits (MQLs), linearity, intra-day and inter-day precision via the replicated injection in the on-line SPE-LC-MS/MS system of standard solutions in Milli-Q water. **Table 2** summarizes the results obtained for these performance parameters.

4-NP was taken as reference compound for the quantification of branched isomers of nonylphenol, since, based on the results of a previous study [22], they showed the same MS response factor.

The MDLs were established by replicated injection (n=7) of decreasing concentrations of target compounds and were taken as the concentration that gave rise to a mean signal-to-noise ratio (s/n) equal to three. The MQLs were assessed by the same approach, but considering a s/n=10. Very good sensitivities, in the sub-ng L^{-1} range, were obtained for AP_nEOs with n=2-8, as well as for 4-NP and carboxylate derivatives (MDLs in the range of 0.0081-0.13 ng L^{-1}). MDLs of about 1 ng L^{-1} were obtained for linear alkylphenols and 4-t-OP, whereas, in agreement with findings previously observed by our team and other research groups, much higher MDLs values (50-55 ng L^{-1}) were observed for the monoethoxylate derivatives [22, 29, 21, 19, 20]. The linearity was evaluated by performing five replicated analysis of standard solutions in Milli-Q water at concentration levels ranging from MQLs to 1000 ng L^{-1} or 2500 ng L^{-1}, depending on the compound investigated, and obtaining in any case determination coefficients ≥0.9949.

Good intra-day and inter-day precisions of the on-line SPE-LC-MS/MS method, comparable with those reported in the other studies [21, 20], were also achieved. More in detail, intra-day and inter-day relative standard deviation percentages (RSD%), were respectively included in the ranges of 2.3-9.2% and 3.3-12%, as estimated by means of replicated injections (n=5) of (i) a 250 ng L^{-1}

solution in Milli-Q water for AP_nEOs with n=1-2, AP_1ECs and APs, and (ii) a 125 ng L^{-1} solution of Triton™ X-45 and Igepal® CO-520 technical mixtures in Milli-Q water for AP_nEOs with n>2.

3.4 Apparent recovery evaluation

One issue that must be addressed in method development is the influence of the matrix on the recovery from the SPE cartridge and the ionization process, the latter commonly identified as matrix effect (ME).

In fact, the partition of target compounds between the SPE stationary phase and the liquid sample, or the sample loading solution, can be affected by matrix components. Furthermore, the presence of matrix components co-eluting with target analytes may alter their ionization, thus affecting the sensitivity and accuracy of the method for the analysis of real samples.

Therefore, the evaluation of both these effects is of paramount importance for a reliable quantification of target compounds in real samples.

Accordingly, in this work the combination of the two effects was evaluated by determining the apparent recovery percentage (*AR%*) [30] of target analytes in wastewater samples collected at the inlet and outlet of each WTP. To this aim, each sample was spiked with the Alkylphenol Internal Standard Mix 7 and 4-NP_1ECd2 solution at a final concentration of 500 ng L^{-1}, and then subjected to the on-line SPE-LC-MS/MS method as described above; the peak areas observed were compared to those obtained in Milli-Q water at the same concentration level. *AR%* was defined according to the following equation [30]:

$$AR\% = \frac{A_{spiked}}{A_{standard}} \cdot 100$$

where A_{spiked} is the peak area of the mass-labelled compound in the spiked real sample (n=3), while $A_{standard}$ is the peak area of the mass-labelled compound in Milli-Q water (n=3).

Regarding those analytes for which the mass-labelled reference compounds are not available (AP_nEOs with n=3-8), AR% has been evaluated by spiking real samples with Triton™ X-45 and

Igepal® CO-520 technical mixtures at a final concentration of 250 ng L^{-1}. In this case AR% was calculated as follows [28, 31]:

$$AR\% = \frac{A_{spiked} - A_{unspiked}}{A_{standard}} \cdot 100$$

where A_{spiked} is the peak area of the target analyte in the spiked real sample (n=3), $A_{unspiked}$ is the peak area of the target analyte in the unspiked real sample (n=3) and $A_{standard}$ is the peak area of the target analyte in Milli-Q water (n=3).

Table 3 summarizes the results obtained. As expected, AR% was found to be matrix-dependent and compound-dependent. For each investigated compound, the AR% was found to be included approximately in the range 70-100% in all samples collected at the outlet of the WTPs investigated, with the only significant exception of 4-NP in the Cantagallo WTP (62%). Conversely, generally lower AR% values were found in the inlets of WTPs. These results are in agreement with the higher presence in the inlet samples of co-eluting matrix components, which can negatively affect recovery and, above all, ionization efficiency. In fact, it should be noted that a number of inorganic and organic compounds, such as salts, highly polar compounds and surfactants, have been identified as "matrix-endogenous suppressors" [32] and that wastewaters, including the one of Prato textile district, are rich in all these compounds [33, 34]. In this regard, an interesting correlation was evidenced when the mean AR% data of each analysed wastewater sample, were plotted as a function of the COD values determined in the same samples (R^2=0.54, P=0.095).

In order to enhance AR% observed in the WTP inlet samples, smaller injection volumes (i.e. 1.0 and 1.5 mL) were tested, highlighting the absence of any significant improvement in terms of recovery and/or reduction of matrix effect (data not shown). This finding is in agreement with the results of Stahnke and co-workers who reported that a logarithmic, rather than linear, relationship typically exists between matrix concentration and matrix effect [35]. Therefore, in order to significantly reduce the extent of matrix effect by diluting real samples, high dilution factors should be employed, with consequent negative effects on method sensitivity.

3.5 Comparison with previously published on-line SPE-HPLC methods

The main characteristics of the method herein proposed can be compared with those reported in the very few others papers adopting a similar instrumental approach [21, 20]. Vega-Morales et al. [20] proposed an on-line SPE-LC-MS/MS method for the fast determination (9 min) of 27 endocrine disrupting chemicals, including AP_nEOs with n=1-8 and APs, but not AP_1ECs. The MDLs achieved in this study for AP_nEOs and APs ranged from hundreds pg L^{-1} to low ng L^{-1}, with surprisingly low differences in sensitivity among AP_1EOs (MDLs=1.2-2.1 ng L^{-1}) and the other investigated compounds (MDLs=0.3-1.6 ng L^{-1}) (**Table 4**). Hence, MDLs found by Vega-Morales and co-workers resulted one-two magnitude orders higher than those achieved by us for APs and AP_nEOs with n=2-8, whereas for AP_1EOs an opposite trend was observed in the comparison of sensitivities. It should also be noted that in the study of Vega-Morales negatively and positively ionisable compounds did not elute in separated Rt windows, thus requiring the introduction of the continuous polarity switching. Moreover, the combined use of a polystyrene-divinylbenzene-N-vinylpyrrolidone terpolymer as SPE sorbent and methanol buffered with ammonia and ammonium acetate as desorption eluent, made necessary to perform a strong washing step based on hexane of the SPE cartridge, in order to eliminate all carryover effects; the implementation of a complex instrument configuration was therefore necessary in order to perform the aforementioned fast analyte determination [20].

Gorga et al. [21] investigated AP_nEOs with n=1-2, AP_1ECs and APs, reporting MDLs ranging from 0.01 to 59.4 ng L^{-1}, which were comparable with the ones found in our study (**Table 4**). It should be however noted that the recovery values obtained for Milli-Q water solutions of AP_nEOs with n=1-2 were quite low (range: 28-58%) and in the presence of a suppressive matrix effect, which is often present when real samples are analyzed [32], method sensitivity may be negatively affected. Moreover, two distinct chromatographic runs with change of the eluents were adopted for the determination of negatively and positively ionisable compounds, thus needing about 1 hour for the analysis of one sample.

3.6 Method application to real samples

The analysis of real samples was performed according to the identification and quantification criteria reported in the paragraph 2.2. As an example, **Fig. 5** shows the overlapped quantifier and qualifier MRM transitions, acquired for 4-NP_8EO (Fig. 5A-C), 4-NP_1EC (Fig. 5D-F) and 4-NP (Fig. 5G-I) in the inlet and outlet of the Baciacavallo WTP sample, in comparison with the corresponding standard solutions in Milli-Q water.

Table 5 summarizes the mean concentrations found for AP_nEOs with n=1-8, AP_1ECs and APs in the aforementioned wastewater samples collected in the inlet and outlet of the three investigated WTPs. Target compounds detected with s/n values in between 3 and 10 or not detected in real samples were reported as below MQL_{sample} or below MDL_{sample}, respectively. MQL_{sample} and MDL_{sample} were calculated by dividing MQLs and MDLs reported in **Table 2** for AR% values determined in each sample (**Table 3**). For analyte peaks with s/n>10, concentration values calculated by external calibration curves were corrected for apparent recoveries. The quantification of 4-t-OP_1EC was carried out assuming the same MS response of the linear isomer.

Linear alkylphenols were never detected in any samples investigated, in accordance with their absence in the industrial products commonly used in the textile Prato district. Conversely, branched AP_nEOs, AP_1ECs and APs were determined at concentrations higher than MQL_{sample} with few exceptions. More in detail, 4-t-OP_1EO concentrations resulted in most samples less than MQL_{sample} and in the outlet of Calice WTP even lower than MDL_{sample}; furthermore, 4-t-OP was below MQL_{sample} or MDL_{sample} in three out of the six investigated matrices (**Table 5**).

Interestingly, two peaks were detected at 17.06 and 17.11 min (**Fig. 6A-B**) by the diagnostic MRM transitions of 4-t-OP (205 → 133 and 205 → 134 Da) and 4-NP (219 → 133 and 219 → 147 Da). These peaks can reasonably be attributed to 4-t-OP_2EC and 4-NP_2EC, assuming for these analytes the same in-source fragmentation phenomenon elsewhere described for AP_1ECs [22], consisting in the loss of the ethoxy-acetic acid group, followed by the common dissociation pattern of the

corresponding branched APs. However, the tentative attribution has not been confirmed due to the lack of reference standards.

As repeatedly observed in previous researches [22, 34], also in this study NP_nEOs, NP_1ECs and NPs were found to be much more abundant than the corresponding octyl derivatives, in agreement with the higher utilization of the former in industrial worldwide produced AP_nEO mixtures [36]. The concentration profiles determined in the inlet samples of Cantagallo and Baciacavallo WTPs evidenced the prevalence of mono and diethoxylate derivatives, whereas in the Calice WTP influent the highest concentration was found for the monocarboxylates. The predominance of diethoxylate and monoethoxylate alkylphenols, as well as carboxylate derivatives, was also highlighted in other studies [21, 37], indicating that, for more degradable AP_nEOs, the shortening of the ethoxylic chain can occur even in the drainage system, especially if the wastewater has a domestic contribution and the sampling is performed during summer [8].

Total inlet concentrations of the investigated compounds ranged from few µg L^{-1} to few tens of µg L^{-1}, confirming their significant decrease in the domestic-industrial mixed wastewater of Prato, compared to data obtained before the adoption of the Directive 2003/53/EC for the restriction of use and commercialization of NP_nEOs [33, 34]. The results reported in **Table 5** clearly evidenced the removal obtained in the different WTPs for all target analytes with the only exception of carboxylates in the Cantagallo samples. The particular behaviour observed in this WTP is probably related to the absence of the ozonation as tertiary treatment stage, which is conversely present in both Baciacavallo and Calice facilities. It should also be stressed that the effluent NP concentrations were well below the water quality criteria indicated by EPA for freshwater (6.6 µg L^{-1}) [38]. Moreover, the effluent concentrations found for 4-NP (164-377 ng L^{-1}) and 4-t-OP (<1-539 ng L^{-1}) were compatible with environmental quality standards set by the European Community for inland surface water (annual average values of 300 ng L^{-1} and 100 ng L^{-1} for 4-NP and 4-t-OP, respectively) [10].

4. Conclusions

The procedure herein proposed provides a reliable analytical approach for the automated on-line extraction and LC-MS/MS simultaneous identification and determination of APnEOs (n=1-8) and some selected carboxylic and phenolic metabolites, with a total analysis time of 25 min per sample. The combination of on-line SPE and LC-MS/MS analysis allowed for obtaining detection limits in the sub-ng L^{-1} or low-ng L^{-1} levels, with the only exception of monoethoxylate alkylphenols, for which MDLs of 50-55 ng L^{-1} were found. These limits are generally lower than those recently achieved with other on-line SPE-LC-MS/MS methods [21, 20]. Moreover, elsewhere reported studies involved two distinct analysis for PI and NI compounds with total analysis time of about one hour [21], or used the continuous polarity switching approach [20], which may however show the drawbacks of a significant loss of sensitivity in real complex matrices, together with a lower precision in respect to the single polarity acquisition, since classical source phenomena, affecting the ionisation process (e.g. competitive interactions between the analyte and the matrix, redox and acid–base reactions) are emphasized by the strong voltage variations [19].

Based on the aforementioned considerations, this method is advantageous in terms of simplicity and/or sensitivity and/or analysis time, compared to previously published methods, also ensuring a high analytical throughput. It should also be underlined that the proposed method allows for analysing also carboxylate metabolites, which represent important degradation by-products in WTPs.

The method was successfully applied to the determination of target analytes in real wastewater samples of three activated sludge WTPs, treating domestic-industrial mixed sewages from the urban areas and the textile district of Prato and Bisenzio valley (Tuscany, Italy). Although this study has not been designed for environmental purposes, it is interesting to compare data herein obtained on real samples with those previously achieved in the same zone of Tuscany. Our results were in good agreement with those recently reported in the same area [22] and confirmed that target analytes are still present in the environment, even though at much lower concentrations than those highlighted in

the late nineties [33, 34], notwithstanding they were strongly restricted in European countries in the early 2000's.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. OSPAR Commision. Background Document on nonylphenol/nonylphenol ethoxylates. Background Document on nonylphenol/nonylphenol ethoxylates. 2009.
2. Talmage SS. Environment and Human Safety of Major Surfactants: Alcohol Ethoxylates and Alkylphenol Ethoxylates. vol Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.: CRC; 1994.
3. Ahel M, Giger W, Koch M. Behavior of alkylphenol polyethoxylate surfactants in the aquatic environment - I. Occurrence and transformation in sewage treatment. Water Res. 1994;28(5):1131-42.
4. Mann RM, Boddy MR. Biodegradation of a nonylphenol ethoxylate by the autochthonous microflora in lake water with observations on the influence of light. Chemosphere. 2000;41(9):1361-9.
5. Bennie DT. Review of the environmental occurrence of alkylphenols and alkylphenol ethoxylates. Water Qual Res J Can. 1999;34(1):79-122.
6. Soares A, Guieysse B, Jefferson B, Cartmell E, Lester JN. Nonylphenol in the environment: A critical review on occurrence, fate, toxicity and treatment in wastewaters. Environ Int. 2008;34(7):1033-49.
7. Ying G-G, Williams B, Kookana R. Environmental fate of alkylphenols and alkylphenol ethoxylates - a review. Environ Int. 2002;28(3):215-26.
8. Sacco C, Pizzo AM, Tiscione E, Burrini D, Messeri L, Lepri L et al. Alkylphenol polyethoxylate removal in a pilot-scale reed bed and phenotypic characterization of the aerobic heterotrophic community. Water Environ Res. 2006;78(7):754-63.
9. European Parliament and the Council. Directive 2003/53/EC of the European Parliament and of the Council of 18 June 2003 amending for the 26th time Council Directive 76/769/EEC relating to restrictions on the marketing and use of certain dangerous substances and preparations (nonylphenol, nonylphenol ethoxylate and cement). Official Journal of the European Union. 2003.
10. European Parliament and the Council. Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council. Official Journal of the European Union. 2008.
11. Agreement of the member state committee on the identification of 4-(1,1,3,3-tetramethylbutyl)phenol, (4-tert-octylphenol) as a substance of very high concern according to articles 57 and 59 of regulation (EC) 1907/2006 adopted on 9 December 2011. 2011.
12. Gonzalez S, Barcelo D, Petrovic M. Advanced liquid chromatography-mass spectrometry (LC-MS) methods applied to wastewater removal and the fate of surfactants in the environment. TrAC, Trends Anal Chem. 2007;26(2):116-24.

13. Gorga M, Insa S, Petrovic M, Barceló D. Analysis of endocrine disrupters and related compounds in sediments and sewage sludge using on-line turbulent flow chromatography–liquid chromatography–tandem mass spectrometry. J Chromatogr A. 2014;1352(0):29-37.
14. Vega-Morales T, Sosa-Ferrera Z, Santana-Rodriguez JJ. Determination of alkylphenol polyethoxylates, bisphenol-A, 17α-ethynylestradiol and 17β-estradiol and its metabolites in sewage samples by SPE and LC/MS/MS. J Hazard Mater. 2010;183(1-3):701-11.
15. Brix R, Postigo C, González S, Villagrasa M, Navarro A, Kuster M et al. Analysis and occurrence of alkylphenolic compounds and estrogens in a European river basin and an evaluation of their importance as priority pollutants. Anal Bioanal Chem. 2010;396(3):1301-9.
16. Andreu V, Ferrer E, Rubio JL, Font G, Picó Y. Quantitative determination of octylphenol, nonylphenol, alkylphenol ethoxylates and alcohol ethoxylates by pressurized liquid extraction and liquid chromatography–mass spectrometry in soils treated with sewage sludges. Sci Total Environ. 2007;378(1–2):124-9.
17. Vega Morales T, Torres Padrón ME, Sosa Ferrera Z, Santana Rodríguez JJ. Determination of alkylphenol ethoxylates and their degradation products in liquid and solid samples. TrAC, Trends Anal Chem. 2009;28(10):1186-200.
18. Jahnke A, Gandrass J, Ruck W. Simultaneous determination of alkylphenol ethoxylates and their biotransformation products by liquid chromatography/electrospray ionisation tandem mass spectrometry. J Chromatogr A. 2004;1035(1):115-22.
19. Houde F, DeBlois C, Berryman D. Liquid chromatographic-tandem mass spectrometric determination of nonylphenol polyethoxylates and nonylphenol carboxylic acids in surface water. J Chromatogr A. 2002;961(2):245-56.
20. Vega-Morales T, Sosa-Ferrera Z, Santana-Rodríguez JJ. Development and optimisation of an on-line solid phase extraction coupled to ultra-high-performance liquid chromatography–tandem mass spectrometry methodology for the simultaneous determination of endocrine disrupting compounds in wastewater samples. J Chromatogr A. 2012;1230(0):66-76.
21. Gorga M, Petrovic M, Barceló D. Multi-residue analytical method for the determination of endocrine disruptors and related compounds in river and waste water using dual column liquid chromatography switching system coupled to mass spectrometry. J Chromatogr A. 2013;1295(0):57-66.
22. Ciofi L, Ancillotti C, Chiuminatto U, Fibbi D, Checchini L, Orlandini S et al. Liquid chromatographic–tandem mass spectrometric method for the simultaneous determination of alkylphenols polyethoxylates, alkylphenoxy carboxylates and alkylphenols in wastewater and surface-water. J Chromatogr A. 2014;1362(0):75-88.
23. The Commission of the European Communities. implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. 2002;Official Journal of the European Communities.
24. USA Federal Register. 1980 April 21. p. 26811-2.
25. Jirka AM, Carter MJ. Micro semiautomated analysis of surface and waste waters for chemical oxygen demand. Anal Chem. 1975;47(8):1397-402.
26. Loos R, Hanke G, Umlauf G, Eisenreich SJ. LC-MS-MS analysis and occurrence of octyl- and nonylphenol, their ethoxylates and their carboxylates in Belgian and Italian textile industry, waste water treatment plant effluents and surface waters. Chemosphere. 2006;66(4):690-9.
27. Wohlfarth C. Viscosity of the mixture (1) water; (2) tetrahydrofuran. In: Lechner MD, editor. Supplement to IV/18. Landolt-Börnstein - Group IV Physical Chemistry: Springer Berlin Heidelberg; 2009. p. 797-9.
28. Ciofi L, Fibbi D, Chiuminatto U, Coppini E, Checchini L, Del Bubba M. Fully-automated on-line solid phase extraction coupled to high-performance liquid chromatography–tandem mass spectrometric analysis at sub-ng/L levels of selected estrogens in surface water and wastewater. J Chromatogr A. 2013;1283(0):53-61.

29. DeArmond PD, DiGoregorio AL. Rapid liquid chromatography-tandem mass spectrometry-based method for the analysis of alcohol ethoxylates and alkylphenol ethoxylates in environmental samples. J Chromatogr A. 2013;1305:154-63. doi:10.1016/j.chroma.2013.07.017.
30. Thompson M, Ellison SLR, Fajgelj A, Willetts P, Wood R. Harmonized guidelines for the use of recovery information in analytical measurement. Pure Appl Chem. 1999;71(2):337-48.
31. Salvador A, Moretton C, Piram A, Faure R. On-line solid-phase extraction with on-support derivatization for high-sensitivity liquid chromatography tandem mass spectrometry of estrogens in influent/effluent of wastewater treatment plants. Journal of Chromatography A. 2007;1145(1):102-9.
32. Trufelli H, Palma P, Famiglini G, Cappiello A. An overview of matrix effects in liquid chromatography–mass spectrometry. Mass Spectrom Rev. 2011;30:491-509.
33. Lepri L, Del Bubba M, Masi F, Udisti R, Cini R. Particle Size Distribution of Organic Compounds in Aqueous Aerosols Collected from Above Sewage Aeration Tanks. Aerosol Sci Technol. 2000;32(5):404-20.
34. Lepri L, Desideri P, Del Bubba M. Analysis of organic pollutants in a wastewater treatment plant. Ann Chim. 1997;87:317.
35. Stahnke H, Kittlaus S, Kempe G, Alder L. Reduction of Matrix Effects in Liquid Chromatography–Electrospray Ionization–Mass Spectrometry by Dilution of the Sample Extracts: How Much Dilution is Needed? Anal Chem. 2012;84(3):1474-82.
36. Petrovic M, Barceló D. Fate and Removal of Surfactants and Related Compounds in Wastewaters and Sludges. In: Barceló D, editor. Series Anthropogenic Compounds. The Handbook of Environmental Chemistry: Springer Berlin Heidelberg; 2004. p. 1-28.
37. Loyo-Rosales JE, Rice CP, Torrents A. Octyl and nonylphenol ethoxylates and carboxylates in wastewater and sediments by liquid chromatography/tandem mass spectrometry. Chemosphere. 2007;68(11):2118-27.
38. U.S. Environmental Protection Agency. Nonylphenol (NP) and Nonylphenol Ethoxylates (NPEs). Action Plan. [RIN 2070-ZA09]. 2010.

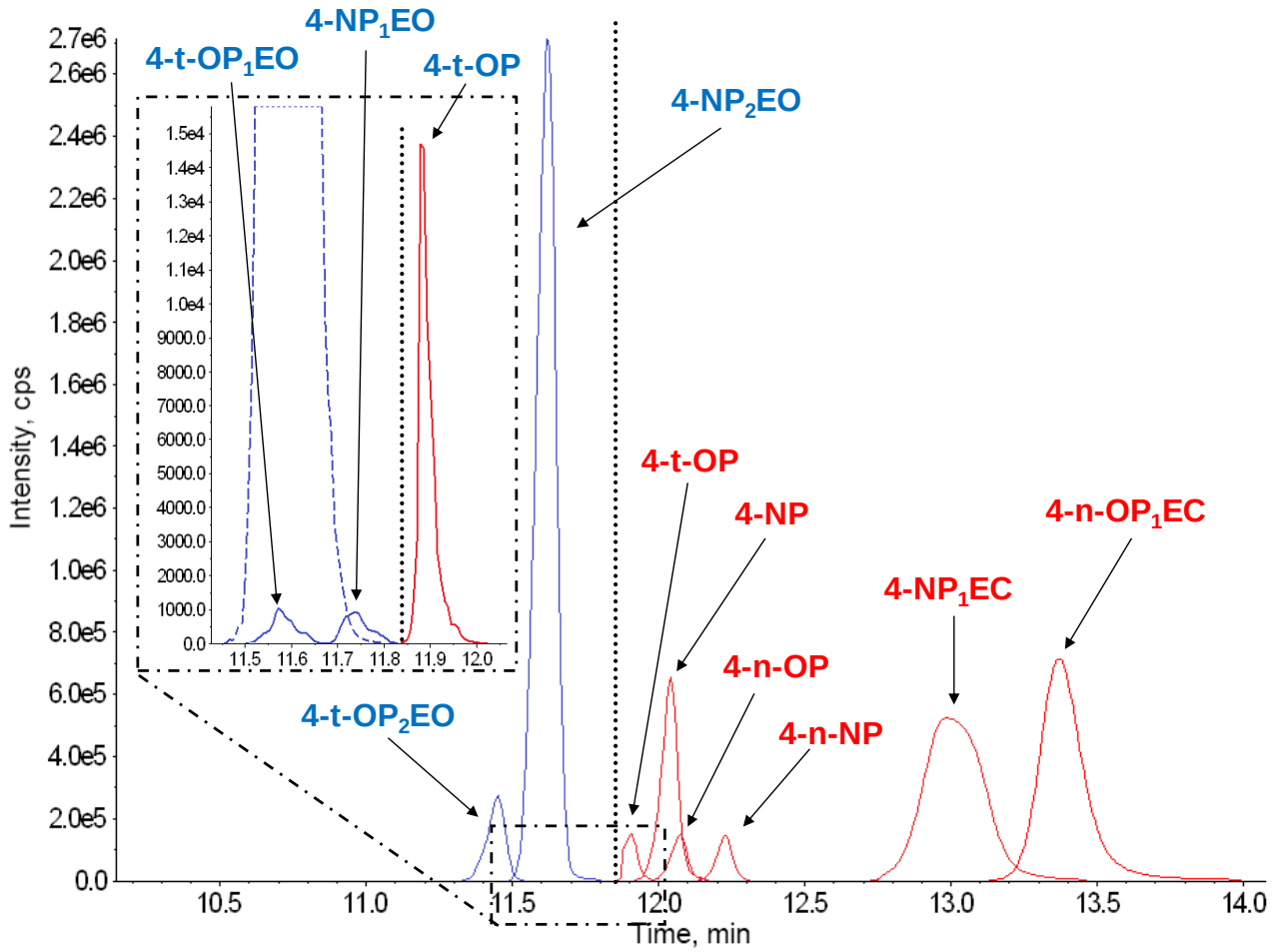

Figure 1 – Reconstructed MRM chromatogram of the quantifier transitions of the investigated alkylphenols polyethoxylates, alkylphenoxy carboxylates and alkylphenols obtained on a pentafluorophenyl pellicular column 100 x 3 mm, 2.6 µm particle size according to the elution gradient elsewhere reported [22]. The dotted line at 11.85 min indicates the shift from positive to negative polarity. Blue and red colours indicated compounds detected in positive and negative ionization, respectively. See paragraph 2.1 for acronym meaning.

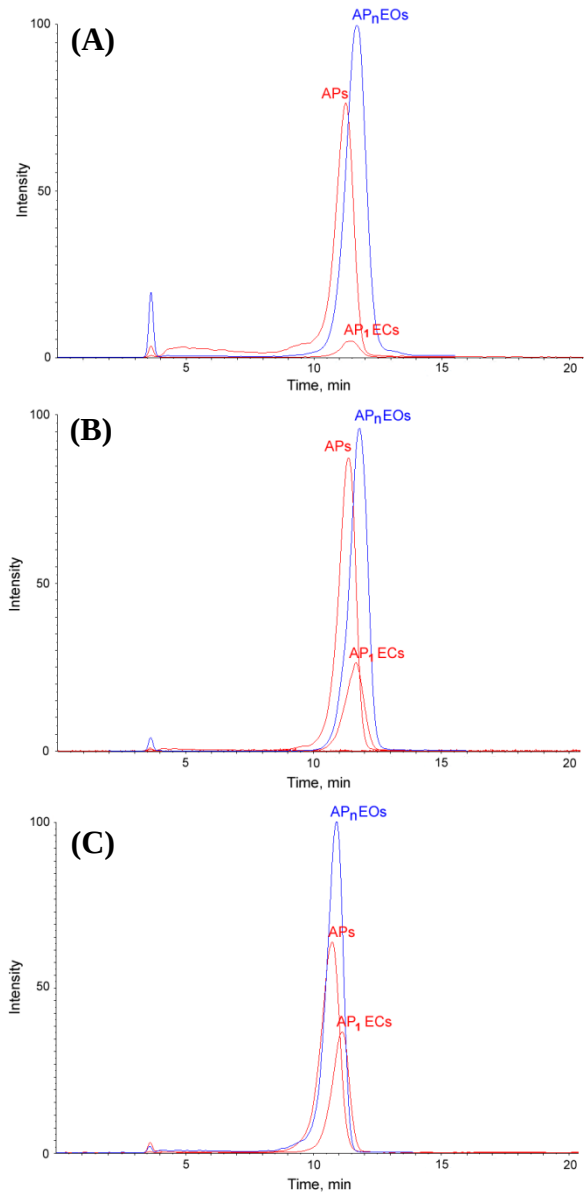

Figure 2 – Reconstructed chromatograms obtained from the individual elutions of alkylphenols polyethoxylates (AP_nEOs), alkylphenoxy carboxylates (AP_1ECs) and alkylphenols (APs) on: (A) Strata®-X; (B) Strata® C8, and (C) Strata® C18E SPE cartridges, in the absence of analytical column (see paragraph 2.3 for the details of the elution gradient). Blue and red colours indicated compounds detected in positive and negative ionization, respectively.

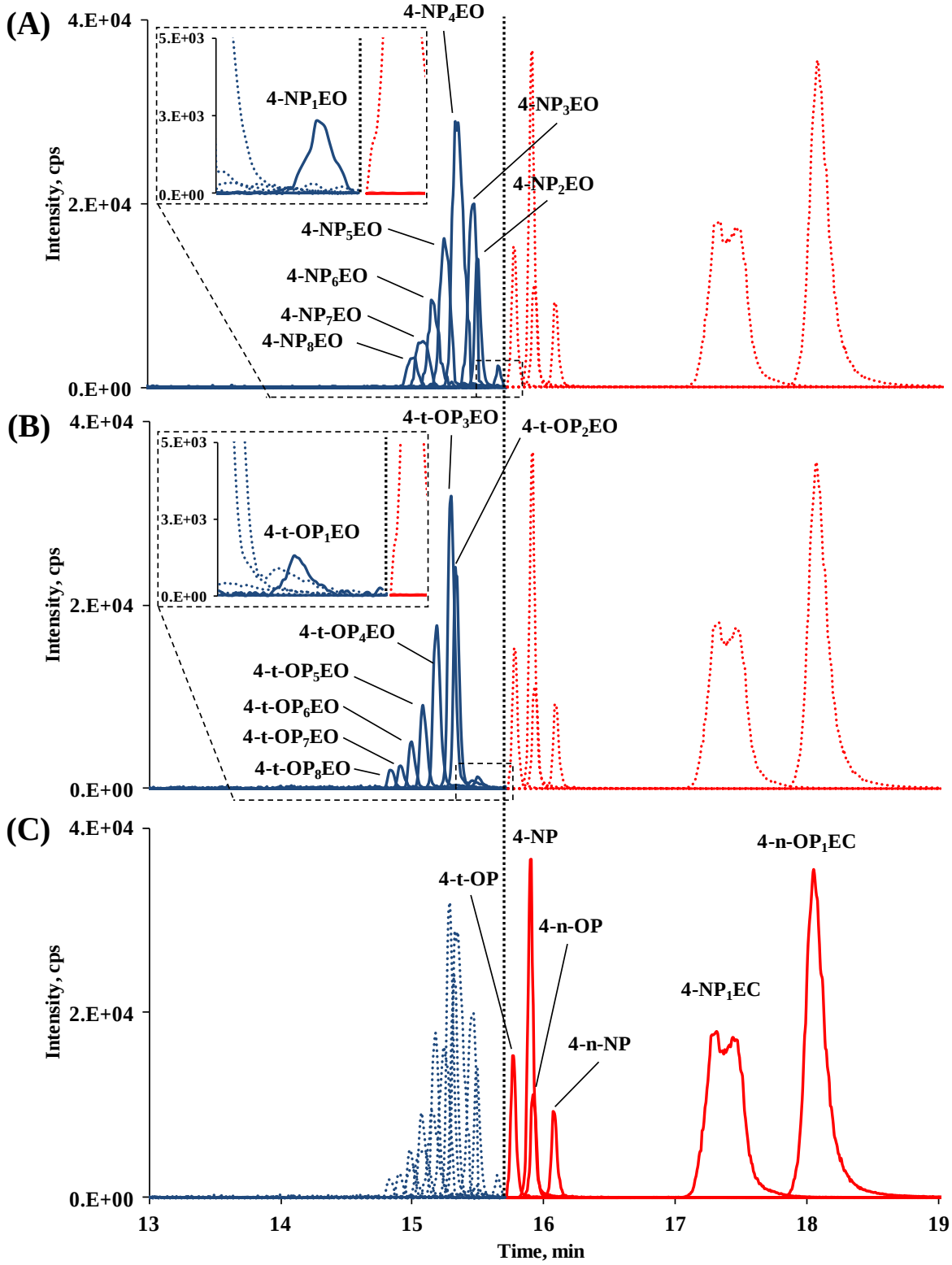

Figure 3 – Reconstructed MRM chromatogram based on the quantifier transitions illustrating the elution order on the resolution of target analytes: (A) branched 4-nonylphenol polyethoxylates (4-NP_nEOs, n=1-8); (B) 4-*tert*-octylphenol polyethoxylates (4-t-OP_nEOs, n=1-8); C) 4-*tert*-octylphenol (4-t-OP), 4-(1-ethyl-1,4-dimethylpentyl)-phenol (4-NP), 4-n-octylphenol (4-n-OP), 4-(1-ethyl-1,4-dimethylpentyl)-phenoxy-acetic acid (4-NP_1EC) and 4-n-octylphenoxy-acetic acid (4-

n-OP$_1$EC). The dotted line at 15.70 min refers to the shift from positive to negative polarity. Blue and red colours indicated compounds detected in positive and negative ionization, respectively.

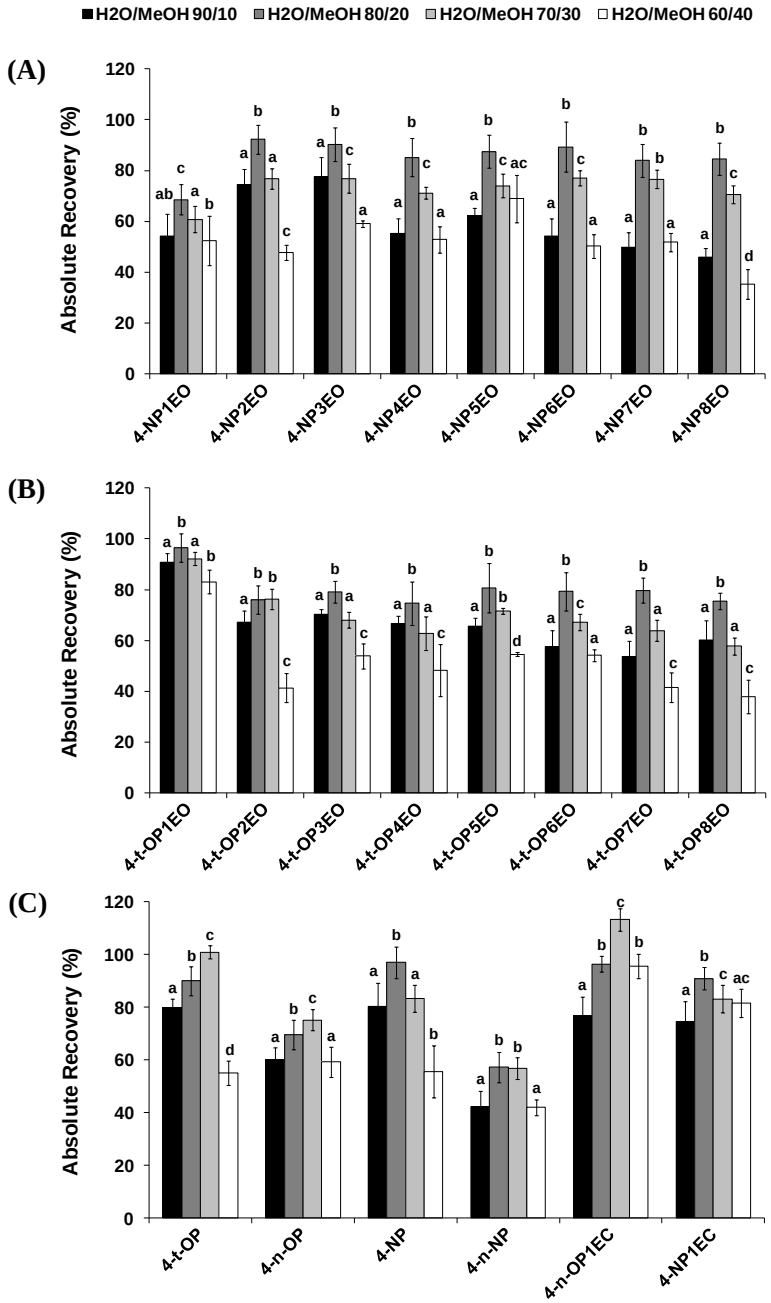

Figure 4 – Mean recovery values (n=5) of target analytes in MilliQ water loaded on the on-line Strata C18E cartridge as a function of the water/methanol relative percentage in the eluent mixture dispensed during the "loading phase". (A) branched 4-nonylphenol polyethoxylates (4-NPnEOs, n=1-8), (B) 4-*tert*-octylphenol polyethoxylates (4-t-OPnEOs, n=1-8); (C) 4-*tert*-octylphenol (4-t-OP), 4-(1-ethyl-1,4-dimethylpentyl)-phenol (4-NP), 4-n-octylphenol (4-n-OP), 4-(1-ethyl-1,4-dimethylpentyl)-phenoxy-acetic acid (4-NP$_1$EC) and 4-n-octylphenoxy-acetic acid (4-n-OP$_1$EC). Error bars represent standard deviations. Values with the same letter are not statistically different at 5% significance level according to the Dunnett T3 nonparametric test. Concentration of target analytes: 4-NP$_1$EO = 500 ng/L; 4-NP$_2$EO = 500 ng/L; 4-NP$_3$EO = 90 ng/L; 4-NP$_4$EO = 85 ng/L; 4-NP$_5$EO = 70 ng/L; 4-NP$_6$EO = 55 ng/L; 4-NP$_7$EO = 40 ng/L; 4-NP$_8$EO = 25 ng/L; 4-t-OP$_1$EO = 500 ng/L; 4-t-OP$_2$EO = 500 ng/L; 4-t-OP$_3$EO = 110 ng/L; 4-t-OP$_4$EO = 110 ng/L; 4-t-OP$_5$EO = 80 ng/L;

4-t-OP_6EO = 50 ng/L; 4-t-OP_7EO = 35 ng/L; 4-t-OP_8EO = 20 ng/L; 4-t-OP = 500 ng/L; 4-n-OP = 500 ng/L; 4-NP = 500 ng/L; 4-n-NP = 500 ng/L; 4-n-OP_1EC = 500 ng/L; 4-n-NP_1EC = 500 ng/L.

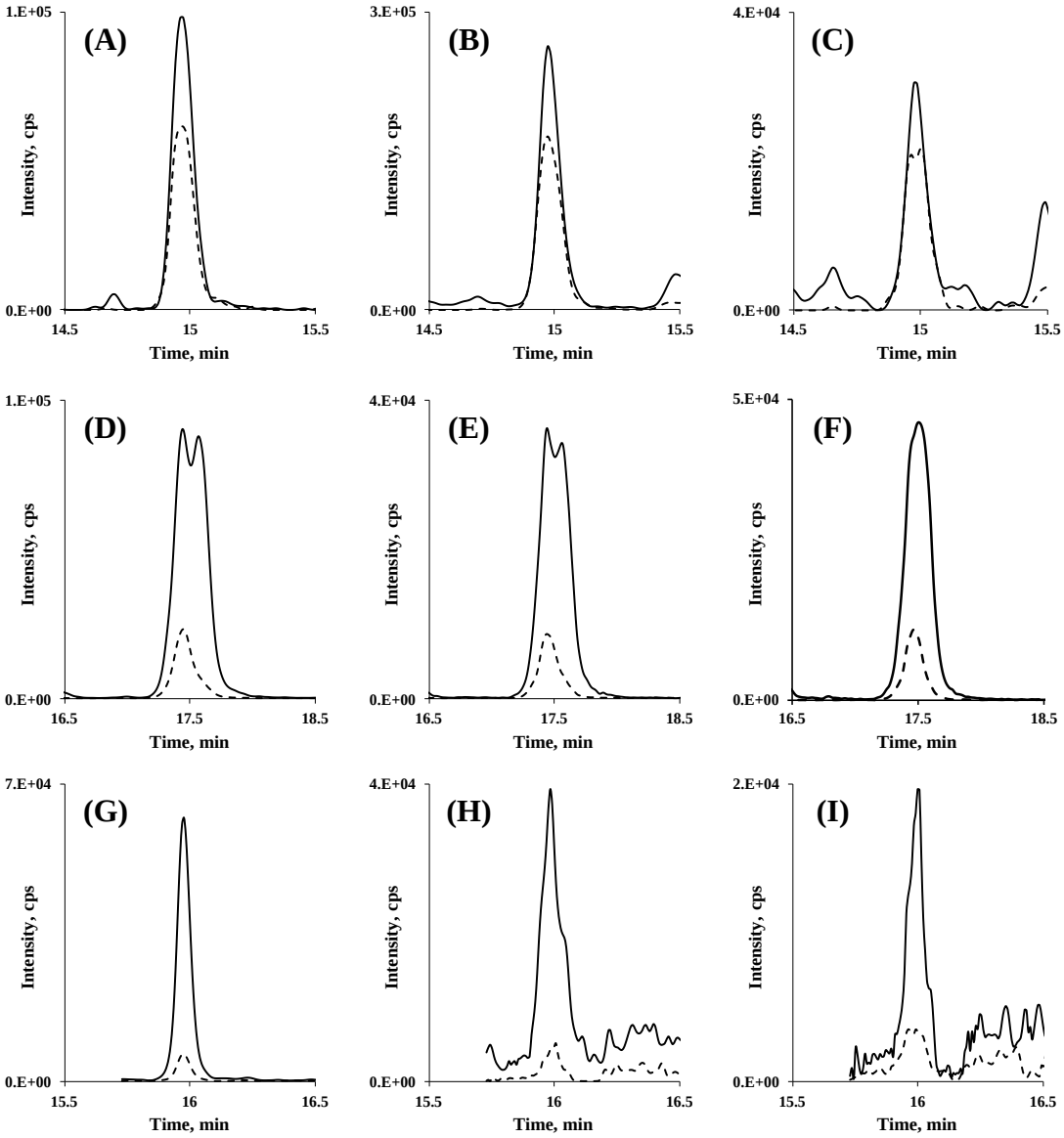

Figure 5 - Overlapped MRM quantifier (solid line) and qualifier (dotted line) transitions of selected compounds in Milli-Q water and in samples collected at the Baciacavallo WTP. Branched 4-nonylphenol octaethoxylate (4-NP_8EOs) in (A) Milli-Q water (130 ng L^{-1}), (B) Baciacavallo WTP inlet (672 ng L^{-1}) and (C) Baciacavallo WTP outlet (39 ng L^{-1}); branched 4-nonylphenol monocarboxylates (4-NP_1EC) in (D) Milli-Q water (500 ng L^{-1}), (E) Baciacavallo WTP inlet (363 ng L^{-1}) and (F) Baciacavallo WTP outlet (339 ng L^{-1}); branched 4-nonylphenols (4-NP) in (G) Milli-Q water (250 ng L^{-1}), (H) Baciacavallo WTP inlet (250 ng L^{-1}) and (I) Baciacavallo WTP outlet (171 ng L^{-1}).

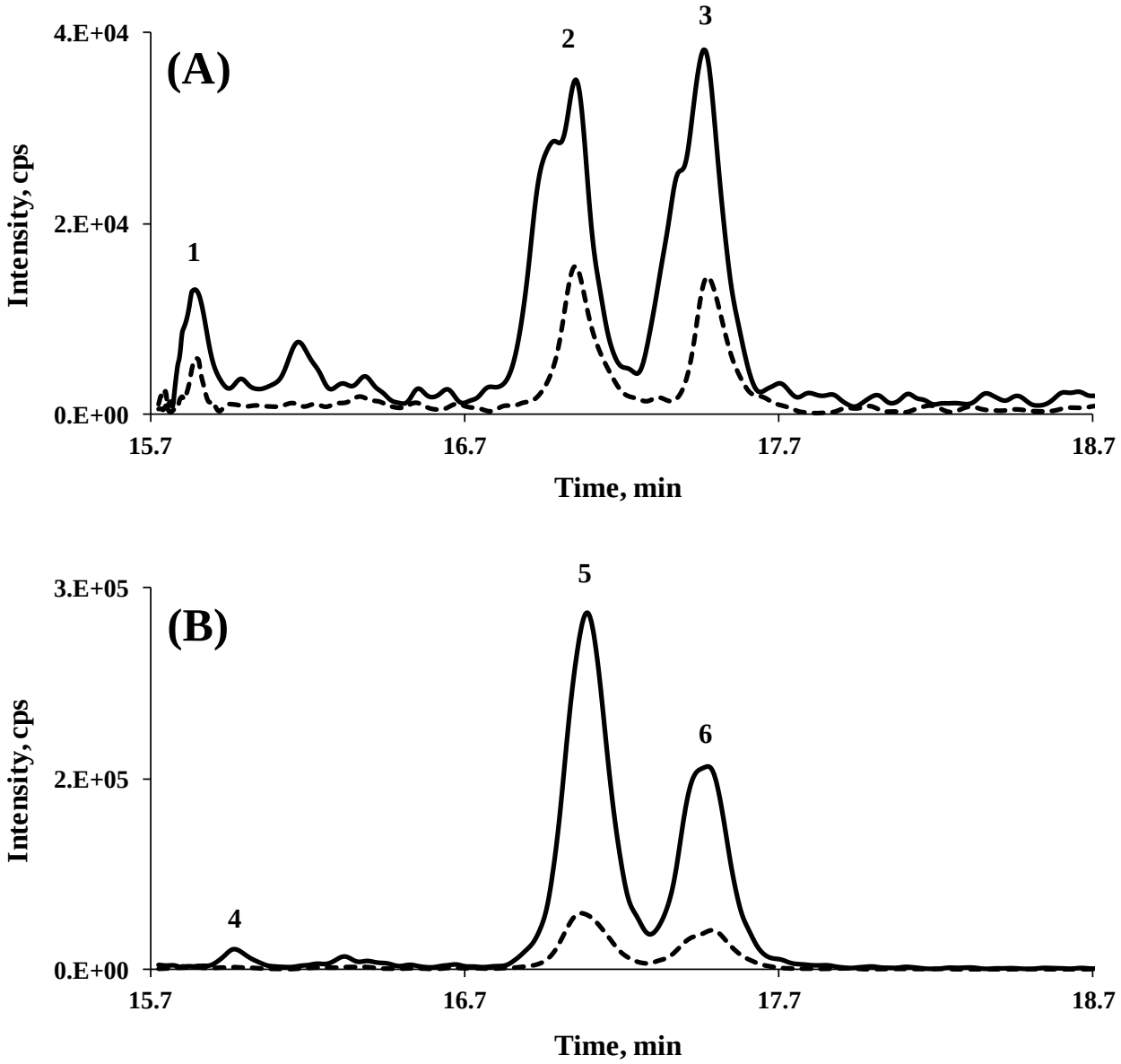

Figure 6 - Overlapped MRM quantifier (solid line) and qualifier (dotted line) transitions of: (A) 4-t-OP and (B) 4-NP in a sample collected in the Baciacavallo WTP outlet. Peak 1 (Rt=15.83 min): 4-tert-octylphenol (4-t-OP); peak 2 (Rt=17.05 min): tentatively identified as 4-tert-octylphenol dicarboxylate (4-t-OP_2EC); peak 3 (Rt=17.46 min): 4-tert-octylphenol monocarboxylate (4-t-OP_1EC); peak 4 (Rt=15.97 min): branched 4-nonylphenols (4-NP); peak 5 (Rt=17.09 min): tentatively identified as branched 4-nonylphenol dicarboxylates (4-NP_2EC); peak 6 (Rt=17.47 min): branched 4-nonylphenol monocarboxylates (4-NP_1EC).

Table 1
Optimized MS parameters for the investigated analytes. collision energy (CE, reported in bracket together with the related product ion); declustering potential (DP); entrance potential (EP); collision cell exit potential (CXP). See paragraph 2.1 for the meaning of target analyte acronyms.

Compound	Precursor Ion	Product Ions (CE)		DP	EP	CXP
		Quantifier Ion	Qualifier Ion			
4-t-OP_8EO[a]	576	133 (35)	121 (55)	50	5	1.5 – 1.5
4-t-OP_7EO[a]	532	133 (35)	121 (50)	25	5	1.5 – 1.5
4-t-OP_6EO[a]	488	133 (30)	121 (45)	15	5	1.5 – 1.5
4-t-OP_5EO[a]	444	121 (43)	315 (27)	35	5	1.5 – 2.5
4-t-OP_4EO[a]	400	271 (25)	121 (37)	33	5	3.0 – 1.5
4-t-OP_3EO[a]	356	227 (20)	121 (35)	35	5	2.5 – 1.5
4-t-OP_2EO[a]	312	183 (17)	121 (35)	35	3.5	2.0 – 1.0
4-t-$OP_2EO^{13}C6$[a]	318	189 (17)	127 (35)	35	3.5	2.0 – 1.0
4-t-OP_1EO[a]	268	113 (13)	251 (12)	30	3.5	1.5 – 2.5
4-t-$OP_1EO^{13}C6$[a]	274	113 (13)	257 (12)	30	3.5	1.5 – 2.5
4-n-OP_1EC[b]	263	205 (-26)	106 (-41)	-35	-10	-1.0 – 0.0
4-t-OP[b]	205	133 (-35)	134 (-28)	-45	-7	0.0 – 0.0
4-t-$OP^{13}C6$[b]	211	139 (-35)	140 (-28)	-45	-7	0.0 – 0.0
4-n-OP[b]	205	106 (-28)	-	-25	-2.5	0.0
4-NP_8EO[a]	590	133 (35)	291 (35)	55	6	3.0 – 3.5
4-NP_7EO[a]	546	133 (35)	529 (25)	60	6	3.0 – 4.0
4-NP_6EO[a]	502	359 (25)	121 (45)	48	6	1.5 – 4.5
4-NP_5EO[a]	458	315 (25)	121 (40)	45	6	1.5 – 3.5
4-NP_4EO[a]	414	271 (20)	121 (35)	40	6	3.5 – 1.5
4-NP_3EO[a]	370	227 (18)	121 (35)	35	6	2.5 – 1.5
4-NP_2EO[a]	326	183 (15)	121 (30)	33	5	2.5 – 2.5
4-$NP_2EO^{13}C6$[a]	332	189 (15)	127 (30)	33	5	2.5 – 2.5
4-NP_1EO[a]	282	127 (10)	265 (12)	33	2.5	2.0 – 3.0
4-$NP_1EO^{13}C6$[a]	288	127 (10)	271 (12)	33	2.5	2.0 – 3.0
4-NP_1EC[b]	277	219 (-27)	133 (-60)	-35	-9	-1.0 – 0.0
4-NP_1ECd2[b]	279	219 (-27)	133 (-60)	-35	-9	-1.0 – 0.0
4-NP[b]	219	133 (-43)	147 (-36)	-45	-12	0.0 – 0.0
4-$NP^{13}C6$[b]	225	139 (-43)	163 (-36)	-45	-12	0.0 – 0.0
4-n-NP[b]	219	106 (-30)	-	-45	-9	0.0

[a] monitored as $[M + NH_4]^+$ adduct ion
[b] monitored as $[M-H]^-$ ion

Table 2.
Method detection limits (MDLs), linearity range, coefficient of determination (R^2) and intra-day and inter-day repeatability (expressed as relative standard deviation percentages, RSD%) of the on-line SPE-LC-MS/MS method determined in standard solutions of target analytes in Milli-Q water See paragraph 2.1 for the meaning of target analyte acronyms.

Compound	MDL[a] (ng/L)	Linear Range (ng/L)	R^2	$RSD\%_{intra}$[b]	$RSD\%_{inter}$[b]
4-t-OP_8EO	0.015	0.05-1000	0.9985	7.8	9.6
4-t-OP_7EO	0.010	0.05-1000	0.9984	8.8	10
4-t-OP_6EO	0.015	0.05-1000	0.9987	3.6	4.8
4-t-OP_5EO	0.015	0.05-1000	0.9993	7.5	9.7
4-t-OP_4EO	0.011	0.05-1000	0.9996	5.6	7.4
4-t-OP_3EO	0.0081	0.03-1000	0.9998	3.7	5.1
4-t-OP_2EO	0.072	0.5-2500	0.9984	2.5	3.3
4-t-OP_1EO	55	200-2500	0.9994	6.9	8.5
4-n-OP_1EC	0.13	0.5-2500	0.9980	2.5	4.0
4-t-OP	0.85	5-2500	0.9985	3.5	4.9
4-n-OP	0.75	5-2500	0.9990	2.3	3.4
4-NP_8EO	0.031	0.2-1000	0.9972	7.1	9.5
4-NP_7EO	0.015	0.15-1000	0.9964	9.2	12
4-NP_6EO	0.025	0.15-1000	0.9954	8.1	11
4-NP_5EO	0.023	0.15-1000	0.9949	4.0	5.9
4-NP_4EO	0.032	0.2-1000	0.9982	7.7	9.4
4-NP_3EO	0.062	0.2-1000	0.9985	9.1	11
4-NP_2EO	0.049	0.25-2500	0.9987	3.7	5.0
4-NP_1EO	50	200-2500	0.9990	6.8	8.9
4-NP_1EC	0.051	0.25-2500	0.9976	2.6	3.9
4-NP	0.092	0.5-2500	0.9998	3.9	5.2
4-n-NP	1.0	5-2500	0.9972	4.8	6.1

[a] Signal-to-noise ratio = 3

[b] Evaluated for all compounds at a concentration equal to 250 ng/L, except for AP_nEOs with n>2 which have been evaluated at 125 ng/L of a Triton™ X-45 and Igepal® CO-520 technical mixtures (see Electronic Supplementary Material for more information).

Table 3.
Mean values (n=3) and standard deviation (in bracket) of apparent recovery percentages (AR%), evaluated in each matrix investigated. See paragraph 2.1 for acronym meanings.

Compound	Cantagallo		Baciacavallo		Calice	
	IN	OUT	IN	OUT	IN	OUT
4-t-OP_8EO	45 (6)	65 (5)	54 (9)	73 (7)	75 (6)	86 (6)
4-t-OP_7EO	36 (4)	67 (4)	44 (2)	75 (8)	69 (5)	84 (5)
4-t-OP_6EO	44 (5)	71 (8)	53 (6)	79 (4)	74 (4)	89 (8)
4-t-OP_5EO	45 (8)	77 (4)	54 (6)	87 (6)	78 (6)	91 (9)
4-t-OP_4EO	38 (7)	70 (6)	45 (3)	79 (7)	79 (5)	85 (7)
4-t-OP_3EO	37 (5)	72 (8)	44 (6)	81 (8)	75 (5)	81 (6)
4-t-OP_2EO $^{13}C6$	42 (6)	83 (2)	69 (7)	108 (5)	83 (7)	78 (5)
4-t-OP_1EO $^{13}C6$	54 (6)	89 (5)	75 (8)	94 (7)	91 (6)	83 (5)
4-OP_1EC	70 (3)	84 (7)	55 (7)	70 (4)	77 (4)	105 (8)
4-t-OP $^{13}C6$	59 (4)	65 (2)	75 (7)	92 (6)	64 (3)	80 (7)
4-NP_8EO	43 (4)	84 (10)	52 (3)	95 (4)	69 (5)	94 (8)
4-NP_7EO	52 (6)	74 (7)	62 (4)	83 (2)	81 (7)	89 (7)
4-NP_6EO	42 (5)	69 (5)	50 (4)	77 (5)	74 (6)	86 (8)
4-NP_5EO	46 (8)	80 (6)	56 (3)	90 (3)	78 (5)	94 (9)
4-NP_4EO	50 (4)	76 (4)	59 (2)	85 (8)	83 (8)	91 (7)
4-NP_3EO	49 (7)	75 (8)	58 (2)	84 (7)	85 (7)	95 (10)
4-NP_2EO $^{13}C6$	36 (2)	72 (5)	62 (7)	97 (7)	84 (9)	92 (5)
4-NP_1EO $^{13}C6$	45 (5)	87 (8)	68 (8)	91 (8)	89 (7)	95 (9)
4-NP_1EC d2	81 (2)	98 (4)	58 (4)	67 (5)	88 (8)	94 (8)
4-NP $^{13}C6$	54 (4)	62 (3)	71 (4)	88 (4)	58 (7)	82 (7)

Table 4
Main characteristics of the analytical method proposed herein, compared to the ones previously published and developed by using the on-line solid-phase extraction approach. See paragraph 2.1 for acronym meanings.

SPE sorbent	N. of runs	Analysis time (min)	MDL (ng/L)										[Reference]
			4-t-OP_1EO	4-t-OP_2EO	4-t-$OP_{3-8}EO$	4-NP_1EO	4-NP_2EO	4-$NP_{3-8}EO$	4-n-OP_1EC	4-NP_1EC	4-t-OP	4-NP	
C18	1[b]	25.0	55.0	0.07	0.008-0.020	50.0	0.05	0.05-0.30	0.13	0.05	0.85	0.09	This study
PS-DVB-NVP [a]	1[c]	9.0	2.1	1.2	0.7-1.6	1.2	0.5	0.3-1.1	n.i.[d]	n.i.[d]	1.8	1.3	[20]
C18	2	24.5[e]	16.0	0.01	n.i.[d]	59.4	0.012	n.i.[d]	0.062	0.03	0.13	0.012	[21]

[a] polystyrene-divinylbenzene-N-vinylpyrrolidone
[b] single polarity switch
[c] continuous polarity switch
[d] not investigated
[e] the analysis time is referred to one chromatographic run

Table 5.
Mean concentrations and standard deviation (in bracket) found for the target compounds in three 24-h composite samples collected in September 2015. All results are expressed in ng L^{-1}. See paragraph 2.1 for the meaning of target analyte acronyms.

Compound	Cantagallo		Baciacavallo		Calice	
	IN	OUT	IN	OUT	IN	OUT
4-t-OP_8EO	151 (24)	43 (3)	70 (1)	23 (1)	63 (5)	20 (2)
4-t-OP_7EO	167 (19)	57 (5)	82 (2)	29 (5)	63 (3)	41 (3)
4-t-OP_6EO	72 (4)	57 (4)	78 (1)	34 (2)	48 (4)	33 (2)
4-t-OP_5EO	60 (6)	64 (5)	73 (1)	44 (6)	42 (3)	41 (5)
4-t-OP_4EO	52 (3)	58 (3)	47 (2)	41 (6)	25 (2)	37 (3)
4-t-OP_3EO	106 (11)	61 (4)	88 (3)	37 (5)	33 (2)	41 (5)
4-t-OP_2EO	366 (29)	93 (9)	97 (12)	28 (7)	103 (8)	<0.1[a]
4-t-OP_1EO	285 (19)	<225[b]	<265[b]	<215[b]	<220[b]	<65[a]
4-t-OP_1EC	132 (12)	422 (37)	110 (12)	128 (12)	247 (18)	108 (11)
4-t-OP	<8[b]	<1[a]	221 (10)	539 (61)	<8[b]	14 (2)
4-NP_8EO	1584 (120)	102 (12)	660 (32)	37 (5)	534 (47)	89 (7)
4-NP_7EO	1321 (117)	187 (21)	550 (41)	68 (6)	447 (26)	120 (10)
4-NP_6EO	1578 (135)	320 (29)	650 (50)	109 (9)	398 (19)	221 (20)
4-NP_5EO	1305 (114)	440 (36)	641 (74)	144 (12)	337 (21)	269 (19)
4-NP_4EO	1327 (99)	708 (51)	697 (42)	205 (23)	297 (19)	461 (35)
4-NP_3EO	2161 (168)	815 (63)	1135 (126)	252 (18)	317 (25)	456 (37)
4-NP_2EO	6026 (464)	772 (52)	2746 (190)	257 (11)	565 (41)	355 (32)
4-NP_1EO	5460 (386)	754 (45)	2153 (215)	234 (17)	754 (43)	358 (27)
4-NP_1EC	1313 (110)	4084 (397)	352 (19)	330 (26)	1360 (124)	406 (45)
4-NP	830 (98)	377 (24)	247 (4)	164 (30)	<0.9[b]	228 (19)

[a] Method Detection Limit in real sample (MDL_{sample})
[b] Method Quantification Limit in real samples (MQL_{sample})

Manuscript: "Fully automated on-line solid phase extraction coupled to liquid chromatography–tandem mass spectrometry for the simultaneous analysis of alkylphenol polyethoxylates and their carboxylic and phenolic metabolites in wastewater samples" by L. Ciofi et al.

Supplementary Materials

S1. Characterization of Triton™ X-45 and IGEPAL® CO-520 technical mixtures

Since each OPnEO and NPnEO with n>2 are unavailable as analytical standards, Triton™ X-45 and IGEPAL® CO-520 technical mixtures have been employed to evaluate figures of merit of the method; however, it is known that the MS response of alkylphenol polyethoxylates is dependent on the ethoxylation degree.

In order to determine the percentage composition of each technical mixtures, HPLC-DAD analysis have been performed since each octyl and nonyl-derivative has a response factor independent from the length of the ethoxylate chain.

The HPLC-DAD analysis was performed on a Shimadzu (Kyoto, Japan) chromatographic system, consisting of a SIL-10AD autosampler, two isocratic pumps LC-10AD VP, a DGU-20A$_3$ degassing unit, a CTO-10AS thermostatted column compartment, a SPD-M10A diode array detector and a SCL-10A module controller. The analysis was carried out eluting with (A) MilliQ water and (B) MeOH, at a flow rate of 0.4 mL min^{-1}, according to the following elution gradient: 70% B for 2 min, from 70% to 97% in 54 min and a final isocratic for 10 min. The column compartment temperature was set to 40°C. The injection volume was set to 5 µL and detection was carried out at 225 nm. The separation was performed on a pellicular column Kinetex® Biphenyl (100 mm x 3 mm, 2.6 µm particle size).

Fig. S1 shows the chromatogram obtained for Triton™ X-45: the octylphenol ethoxylate oligomers were baseline resolved and were eluted in sharp peaks, suggesting a high isomeric purity of the precursor compound, i.e. 4-t-octylphenol. Conversely, the chromatograms obtained for the IGEPAL® CO-520 technical mixture (**Fig. S2**) showed that the nonylphenol ethoxylate homologues give rise to broader peaks together with shoulder and split peaks, highlighting the presence of chain and/or ring isomers. It should be noted that the HPLC-DAD method herein proposed is suitable for the analysis of AP$_n$EOs with n>11, since no loss in resolution was observed for the oligomers with longer ethylene oxide chain.

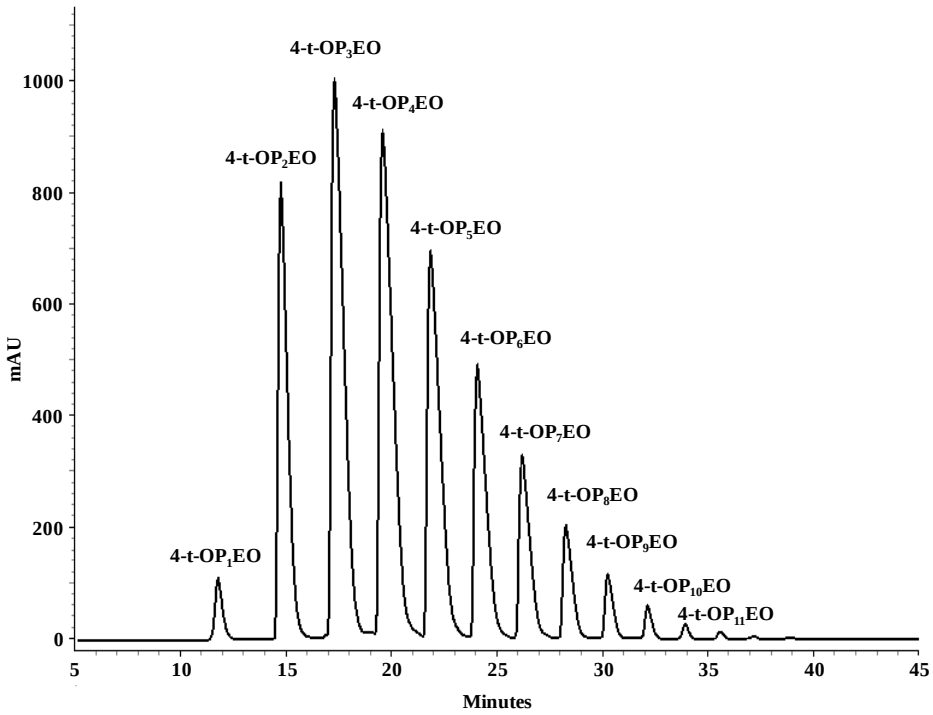

Figure S1 – Chromatogram of Triton™ X-45 (10 mg/ml) obtained under the experimental conditions described in paragraph S1. See paragraph 2.1 for acronym meaning.

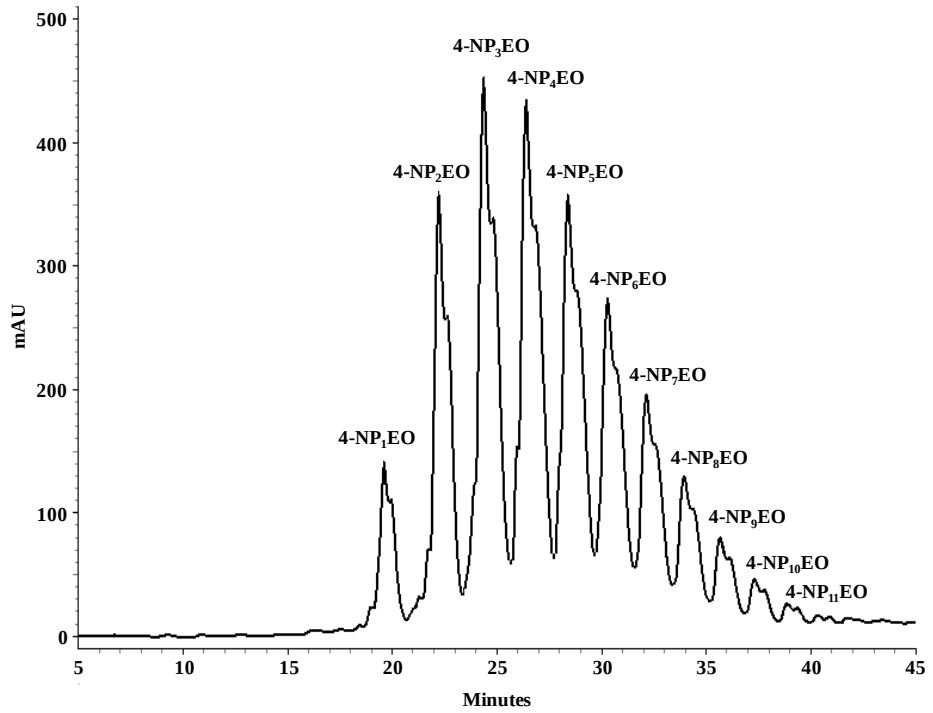

Figure S2 – Chromatogram of IGEPAL® CO-520 (10 mg/ml) obtained under the experimental conditions described in paragraph S1. See paragraph 2.1 for acronym meaning.

Table S1 summarizes the results obtained regarding the percentage composition of the two technical mixtures analyzed.

Table S1.
Percentage composition of technical mixtures.

	Triton™ X-45
Compound	Percentage composition
4-t-OP_1EO	1.57±0.08
4-t-OP_2EO	14.34±0.70
4-t-OP_3EO	22.38±0.85
4-t-OP_4EO	21.93±1.04
4-t-OP_5EO	16.04±0.43
4-t-OP_6EO	10.55±0.37
4-t-OP_7EO	6.52±0.29
4-t-OP_8EO	3.62±0.15
4-t-OP_9EO	1.82±0.10
4-t-$OP_{10}EO$	0.82±0.02
4-t-$OP_{11}EO$	0.42±0.01
	Igepal® CO-520
Compound	Percentage composition
4-NP_1EO	5.69±0.27
4-NP_2EO	14.56±0.54
4-NP_3EO	18.31±0.94
4-NP_4EO	17.56±0.77
4-NP_5EO	14.42±0.53
4-NP_6EO	11.02±0.33
4-NP_7EO	7.87±0.30
4-NP_8EO	5.26±0.22
4-NP_9EO	2.48±0.11
4-$NP_{10}EO$	1.98±0.04
4-$NP_{11}EO$	0.83±0.04