A flow immunoassay for alkylphenol ethoxylate surfactants and their metabolites—questions associated with cross-reactivity, matrix effects, and validation by chromatographic techniques†

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This paper describes the application and evaluation of a competitive enzyme flow injection immunoassay (EFIIA) for screening of alkylphenol ethoxylate (APEO) surfactants in different water samples based on a generic immunoassay system previously developed (see E. Burestedt, C Nistor, U. Schagerlöf and J. Emnéus, *Anal. Chem.*, 2000, **72**, 4171–4177). The detection limits for octylphenol ethoxylates (OPEOs), nonylphenol ethoxylates (NPEOs), and nonylphenol (NP) were 0.5 $\mu\text{g l}^{-1}$, between 2 and 3 $\mu\text{g l}^{-1}$, and 50 $\mu\text{g l}^{-1}$, respectively, with a sample throughput of 6 h^{-1} (*i.e.*, for triplicate analysis of each sample). Different OPEOs and NPEOs were highly cross-reactive within the assay, with sensitivities in the same order of magnitude for all the ethoxylates tested, thus the result obtained by the EFIIA method could be used as an “alkylphenol ethoxylate index”. No or minor matrix effects with recoveries between 70–120% for the reference analyte NPEO₁₀ in tap, and surface water, and acceptable for rainwater, were observed. Influent and effluent surfactant containing wastewater samples were analysed by EFIIA, LC-MS, LC-Fluorescence (LC-FL), and a commercial microplate ELISA. High recoveries for different concentrations of APEO₁₀ spiked into a 200 times diluted raw influent and effluent wastewater were achieved with the EFIIA method, however, the found APEO content of the same diluted wastewater samples, before spiking, could not be correlated directly to the chromatographic result by any of the immunoassays, and the possible reasons for this are discussed. The same trend of decreasing APEO content from influent to effluent wastewater could, however, be followed for all methods employed.

Introduction

Alkylphenolethoxylate (APEO) surfactants are used in a variety of industrial applications, such as in the manufacturing of pulp and paper, textiles, paints and coatings, adhesives, leather products, rubber and plastics, as well as in metalworking (as lubricants) and agriculture (as emulsifiers, dispersants or adjuvants for some herbicides).¹ Their main use remains however as detergents, both for household and industrial applications, because of properties such as relative ionic insensitivity and high absorptive behavior.^{2–5}

Since APEOs are primarily used in aqueous solutions, they enter into the environment mainly through sewage and industrial wastewater treatment plants. Numerous biodegradation studies of APEOs have been performed, leading to results that showed variations from zero to complete biodegradation, depending on the experimental conditions used.^{6–9} The efficiency to remove alkylphenols (APs) and APEOs during wastewater treatment depends on the treatment conditions, *e.g.* 90–97% of APEOs from water can be (bio)degraded under certain conditions.¹⁰

The aquatic toxicity of the APEOs and their breakdown products has shown to be directly proportional with the hydrophobicity of the compound. Generally, APs are about one

order of magnitude more toxic than their long chained (> 10) ethoxylate counterparts. The toxicity of mono and di-ethoxylate APEOs is similar to the corresponding AP itself.¹ Even though both nonylphenol (NP) and nonylphenol monoethoxylate (NPEO₁) show relatively high toxicity for aquatic organisms (*i.e.*, their lethal dose varies between 5 to 500 $\mu\text{g l}^{-1}$ for different tested organisms),^{11,12} they are generally considered minimally toxic to mammals, although they can cause severe skin and eye irritation.¹ However, both APs and short-chain APEOs have proved to give reproductive and endocrine disruptive effects,^{13–21} therefore rapid detection and identification of APs and APEOs in the environment is nowadays a subject of great concern.

The chemical complexity of APEOs and APs (see the general structure in Fig. 1), which are mixtures of numerous isomers

Fig. 1 General structure of APEOs (a), APs (b), and the chemical structure of the APEO hapten derivative used for tracer synthesis in this work (c). R denotes the alkyl chain, *i.e.*, octyl- or nonyl- groups; n: 1–40 ethoxy groups.

† Electronic supplementary information (ESI) available: Cross reactivity data. See <http://www.rsc.org/suppdata/an/b3/b302110f/>

and oligomers, necessitates a rather complicated analytical methodology for their isolation, determination and identification in water environments. A simplification of the problem is represented by the development of methods for determination of surfactants as classes of compounds. Simple, relatively fast and inexpensive quantitative or semi-quantitative methods based on titrimetric or spectrophotometric techniques have commonly been used.^{22–25} Methods that depend on a spectrophotometric reaction do, however, not reliably reflect the concentration of either the individual surfactant types (non-ionic, anionic or cationic), or the concentration of individual compounds within a class. Major improvements have been made in the identification and quantification of surfactants by gas chromatography-mass spectrometry (GC-MS)^{26–28} and high-performance liquid chromatography (HPLC).^{29–35}

Although these analytical techniques are under constant improvement, extensive sample cleanup and pre-concentration is usually necessary before detection. As a direct result, the sample throughput is generally low and there is a long delay between the sample collection and the communication of results back to the sampling site. As a complement to already existing techniques, there is a continuous need for developing screening methods that should be technically simple and useful for routine analysis of a large number of samples. Immunoassay techniques are potential candidates for fulfilling these requirements due to the high selectivity of antibodies, the good sensitivity of such systems and the fact that antibodies can be raised against virtually any compound. The only APEO immunoassay existing today is to our knowledge a commercial enzyme linked immunosorbent assay (ELISA) kit available from Takeda Chemical Industries, Japan^{36–38} that also provides kits for other surfactants and environmental pollutants.³⁹ The results obtained by different chromatographic techniques and the Takeda immunoassay kit was compared in a recent inter-laboratory exercise for the determination of surfactants in water samples.⁴⁰

The aim of this work was to adapt a previously developed generic enzyme flow injection immunoassay (EFIIA)⁴¹ for determination of APs and APEOs, and to study its potential for selective determination of these analytes in different environmental waters as well as influent and effluent waters from wastewater treatment plants (WWTPs). The EFIIA results were compared with those obtained with a commercial ELISA, and two chromatographic methods.

Experimental

Reagents and chemicals

The anti-APEO antibody (monoclonal, from mouse) used in all experiments and the APEO hapten (*i.e.*, *p*-nonylphenoxy-pentaethoxylate succinic ester, see structure in Fig. 1c), were from Takeda Chemical Industries, Tokyo, Japan. The APEO hapten and β -GAL (Roche Biochemicals) were used for tracer synthesis.

Phosphate buffer saline (PBS, 100 mmol L⁻¹) stock solution was prepared according to the following: 80 g NaCl (Merck, Darmstadt, Germany), 2 g KCl (Merck), 14.3 g Na₂HPO₄ 2H₂O (Merck) and 3.43 g KH₂PO₄ (all from Merck, Darmstadt, Germany) were dissolved in 1 l water, and the pH was adjusted with either NaOH (Eka Nobel, Bohus, Sweden) or HCl (Merck). The substrate solution for determination of β -GAL activity of the tracers was made by dissolving 2-nitrophenyl- β -D-galactopyranoside (2-NPG, Sigma Chemical Co, St. Louis, MO, USA) in 25 mmol l⁻¹ phosphate buffer at pH 6.8, according to the protocol provided by the supplier of the enzyme. The β -GAL substrate carrier solution was prepared by dissolving 2-NPG in 10 mmol l⁻¹ PBS, pH 7.4. Polyoxyethylenesorbitan mono-

laureate (Tween 20, Sigma Chemical Co., St. Louis, MO, USA) was used as blocking reagent in all experiments to prevent unspecific protein adsorption in the system. A 0.1 M glycine (Sigma) buffer for regeneration of the protein G column was prepared by dissolution in water and pH adjustment to 2.0 with hydrochloric acid (Merck).

Nonylphenol decaethoxylate (NPEO₁₀) was kindly provided by Takeda Chemicals, as 1 mg l⁻¹ solution in water, containing 20% methanol. Nonylphenol (NP, Aldrich, Steinheim, Germany) was supplied as a technical mixture of branched isomers. All the other standards were from Kao Corporation (Barcelona, Spain).

For studying the influence of different metal ions the following analytical grade reagents were used: Hg(CH₃COO)₂ (Fluka, Chemie AG, Buchs, Switzerland), Cu₂Cl₂ (Fluka), MnCl₂ × 4H₂O (Merck), Al(NO₃)₃·9H₂O (Merck), and Zn(CH₃COO)₂·2H₂O (Merck).

Water produced in a Milli-Q system (Millipore, Bedford, MA, USA) was used for preparing all standard- and buffer solutions throughout the work.

Preparation of APEO tracers

Tracers with different hapten densities (*i.e.*, initial ratio 20, 50 and 100 moles APEO hapten derivative (Fig. 1c) per mole β -GAL) were synthesized according to the following procedure: *N*-hydroxysuccinimide activated APEO derivative was prepared by dissolving 2.16 mg (4 μ mole) of APEO hapten derivative, 2.3 mg (20 μ mole) of *N*-hydroxysuccinimide (NHS, Fluka) and 8.25 mg (40 μ mol) of *N,N'*-dicyclohexylcarbodiimide (DCC, Sigma) in 150 μ l of dry dimethylformamide (DMF, Fluka), the mixture was stirred and the reaction was allowed to proceed for 4 h at room temperature. The resulting precipitate was removed by centrifugation, while the supernatant containing the activated APEO derivative was used further for tracer synthesis. The tracer containing the initial ratio 100 moles APEO derivative per mole β -GAL was prepared by slowly adding 50 μ l of the NHS activated APEO derivative to 5.4 mg β -GAL dissolved in 450 μ l of 130 mM NaHCO₃ (Merck, Darmstadt, Germany). The tracers with initial molar ratios 50 and 20 APEO derivative per β -GAL were prepared by slowly adding 50 μ l of the NHS activated APEO derivative initially diluted 1 : 2 and 1 : 5, respectively, with dry DMF to 5.4 mg each of β -GAL dissolved in 450 μ l of 130 mM NaHCO₃. All three mixtures were gently stirred and allowed to react for 12 h at 4 °C.

The separation of β -GAL labelled APEO tracers from the non-conjugated NHS-activated APEO derivative was performed by dialysis (dialysis cassettes, MWCO 10,000 Da, Pierce Chemical Co., Rockford, IL, USA) against 2 l of 10 mM PBS pH 7.4 during 24 h at +4 °C.

Method and instrumentation

All affinity reactions were performed in off-line mode, by mixing the antibody, tracer and sample containing the analyte(s) until establishment of equilibrium. Then, the mixtures were introduced *via* a 50 μ l injection loop into the flow system (see Fig. 2) by a fully automated ASTED autosampler (233 XL, Gilson, Villiers le Bel) equipped with a Rheodyne six-port injection valve (model 7010, Berkeley, CA). The autosampler was controlled by a Gilson keypad including the software 720 Sampler Controller, version V2.01. A Peek column (0.3 × 0.5 cm) filled with protein G immobilized on Sepharose (High-Trap®, Pharmacia Biotech, Bromma, Sweden) was inserted into the flow system preceding the injection valve. A Gilson Minipuls 2 (Gilson) four channel peristaltic pump was used for pumping the carrier of 10 mmol l⁻¹ PBS, pH 7.4 also containing

Tween 20 (0.01%, v/v, if not otherwise stated), and the enzyme substrate carrier, containing 1.57 mg l^{-1} 2-NPG in PBS 10 mM, pH 7.4 also containing 2 mM MgCl_2 (Merck), as previously optimized in a similar system.⁴¹ The two flow carriers were mixed at the ratio 1 : 1 via a T connection, and the reaction catalysed by the eluted Ag- β -GAL fraction was allowed to take place in a knitted Teflon mixing coil of 1875 μl , placed into a in-house-built water bath, connected to the flow-cell of the UV-VIS detector (model 204, Spectraphysics, NY).

Antibody dilution curves. These were performed for each tracer first by incubation of different amounts of antibody [Ab] with an optimised, minimal and constant amount of tracer (working tracer concentration $[\text{Ag}^*_\text{w}]$, giving a signal to noise (S/N) ratio of about 150), after the total volume was adjusted to 0.5 ml. After 15 min incubation the solutions were injected into the flow system in Fig. 2. The antibody-bound tracer and analyte fractions (AbAg^* and AbAg) were trapped inside the protein G column and the free unbound tracer (Ag^*) eluted and monitored spectrophotometrically at 405 nm. The result was a decrease of the signal with increasing concentrations of antibody.

Analyte calibrations. These were obtained by mixing 450 μl of different concentrations of analyte [Ag] with the $[\text{Ag}^*_\text{w}]$ and the antibody concentration resulting in 50% binding of the tracer (working antibody concentration $[\text{Ab}_\text{w}]$), obtained from the antibody dilution curves) in a total volume of 0.5 ml. After incubation for 15 min, the reaction mixtures were injected into the flow system. The result was an increase of the signal with increasing concentrations of analyte.

Optimizations and interference studies

The optimisation of pH, ionic strength, methanol, and Tween 20 content in the assay mixture were performed by testing the influence of the studied parameter on: (1) the β -GAL activity, by incubating the $[\text{Ag}^*_\text{w}]$ for 15 min with different concentrations of H^+ , NaCl, methanol or Tween 20, then injection into the flow system (Fig. 2) and measurement of the residual enzyme activity; (2) the antibody binding in the absence of the analyte (zero dose), by mixing the $[\text{Ag}^*_\text{w}]$ and $[\text{Ab}_\text{w}]$ at different concentrations of the H^+ , NaCl, methanol, or Tween 20, incubating the mixture for 15 min, and then injection into the

flow system. The obtained signal was compared with the one obtained at optimal conditions.

Recovery tests and analysis of water samples

Tap, rain and surface (Höje Å, Lund, Sweden) water samples were analysed in the first week after sampling. The samples were stored at 4 °C and filtered through 0.45 μm filters (type HA, Millipore) before analysis.

Four samples were collected from the raw influent and the effluent of two WWTPs, namely Abrera and Martorell, located close to Barcelona, Spain. The samples were supplemented to a final concentration of 1% formaldehyde as a preservative, to avoid bacterial degradation of the organic matter during shipment. The samples were stored at 4 °C and filtered through 0.45 μm filters (type HA, Millipore) prior to analysis.

Analysis performed by EFIA and ELISA kit. The matrix effects of various water samples was tested by first buffering to a final concentration of 10 mM PBS (*i.e.*, by mixing the sample with 20 mM PBS in the ratio 1 : 1, v/v), then spiking each sample with NPEO₁₀ at the desired concentration. When the sample matrix considerably influenced the result (*e.g.*, the recovery of the spiked analyte was outside the range 70–120%)⁴² the sample was additionally diluted with 10 mM PBS prior spiking. The diluted water samples were further analysed in the same way as the reference solutions (*i.e.*, incubated with the optimum concentrations of antibody and tracer, *etc.*).

The calibration and sample analysis using the ELISA test kit was performed according to the instructions provided by the manufacturer (Takeda Chemical Industries). Briefly, the protocol consisted of the following steps: the reconstituted antigen–enzyme conjugate (*i.e.*, the APEO hapten in Fig. 1c labelled with horseradish peroxidase (HRP)) was mixed with either the standard solution or the water sample, and 100 μl of the resulting mixture was added into each antibody-coated well of an ELISA plate. After a 60 min incubation step, the solution was removed from each well, and the plate was washed with $3 \times 1 \text{ ml}$ of the reconstituted washing buffer. After drying the plate, and mixing the chromogen and substrate solutions, 100 μl of the resulting mixture was dispensed into each well of the plate. The reaction was stopped after 30 min of incubation at room temperature by adding 50 μl of the stopping solution. Quantification was performed measuring the absorbance of each sample

Fig. 2 Scheme and working principle of the EFIA system: The sample containing the mixture of analyte, antibody and tracer is incubated off-line until equilibrium (1), then injected into the main flow stream (2) that passes through the protein G column, where the antibody-bound fractions are trapped (3). The free analyte and tracer are eluted (4) from the column and mixed with the secondary flow containing the β -GAL substrate 2-NPG (5), forming the product 2-NP in the mixing coil (6), which is detected spectrophotometrically at 405 nm (7). Injection volume: 50 μl ; mixing coil volume: 1875 μl ; flow rate: 0.375 + 0.375 ml min^{-1} . Otherwise conditions are described in the Experimental section or specified in the different figure and table legends.

at 450 nm using an ELISA plate reader (EL800, Bio-Tek Instruments Inc.).

The recovery (R) values were calculated according to the eqn. (1):

$$R = \frac{C_{IA} - C_{IA,0}}{C_{spiked}} \quad (1)$$

where C_{IA} is the analyte concentration in the spiked sample evaluated by interpolation in the standard curve, $C_{IA,0}$ is the concentration of analyte in the sample without spiking (*i.e.*, this was different from zero only in the wastewater samples), and C_{spiked} is the calculated concentration of the analyte spiked into each sample.

Taking into account the possible matrix effects, the evaluation of the APEO-equivalent concentration in the wastewater samples by immunoassay was realized by a variant of the standard addition method: each sample was diluted with PBS at 5 different dilution levels (*i.e.*, the sample dilution factor was between 100 and 1000 times, v/v), and NPEO₁₀ was added to a final spiked concentration of 5, 10, 25, 50 and 100 $\mu\text{g l}^{-1}$ each. The APEO-equivalent concentration was evaluated by extrapolation of each new calibration to zero spiked analyte concentration, and the value obtained was multiplied with the sample dilution factor.

Liquid chromatography–mass spectrometry (LC-MS) and liquid chromatography–fluorescence (LC-FL).

Solid-phase extraction (SPE). Prior LC-MS experiments, the samples were pre-concentrated on LiChrolut C₁₈ (Merck, Darmstadt, Germany) SPE cartridges within 24 h after sampling in order to avoid any degradation of target compounds. All SPE experiments were performed using an automated sample preparation with extraction columns system (ASPEC XL) fitted to an external 306 LC pump for the dispensing of samples through the SPE cartridges and with a 817 switching valve for the selection of samples, all from Gilson (Villiers-le-Bel, France). Disposable 3 ml cartridge columns packed with 500 mg of LiChrolut C₁₈ sorbent from Merck were activated and conditioned first with 7 ml of methanol and then with 3 ml of HPLC water at a flow-rate of 1 ml min^{−1}. Samples (100 ml) were loaded at a flow rate of 5 ml min^{−1}. After pre-concentration, the sorbents were completely dried (30 min) to avoid hydrolysis using a Baker SPE 12g apparatus (J. T. Baker, Deventer, The Netherlands) connected to a vacuum system set at −15 psi. After drying SPE cartridges were wrapped in an aluminium foil and kept at −20 °C until analysis (maximum 1 month).

Cartridges were eluted with 2 × 4 ml of methanol. The eluted solutions were evaporated to dryness under a gentle stream of nitrogen and reconstituted with methanol to a final volume of 1 ml.

Before LC-FL analysis, 100 ml of each sample were enriched by SPE (LiChrolut EN, Merck) immediately after collection. Cartridges were eluted with 2 × 5 ml of methanol : dichloromethane (9 : 1, v/v) with 5 min between elution steps. The eluted solutions were reconstituted with acetonitrile : water containing 14 g l^{−1} sodium perchlorate (4 : 6, v/v) to a final volume of 0.5 ml.

LC-MS and LC-FL experiments. For the LC-MS experiments, the HPLC system consisted of an HP 1100 autosampler having a 100 μl loop and an HP 1090 A LC binary pump both from Hewlett Packard (Palo Alto, CA, USA). The HPLC separation was achieved on a 5 μm , 250 × 4 mm id C₁₈ reversed phase column (LiChrospher 100 RP-18) preceded by a guard column (4 × 4 mm, 5 μm) of the same packing material from Merck. The injection volume was set at 25 μl and the flow rate was 1 ml min^{−1}.

Nonionic surfactants (OPEOs and NPEOs), detected in positive ionisation (PI) mode, were separated by gradient elution using the following mobile phase: solvent A methanol/ acetonitrile (50 : 50, v/v) and solvent B water, both acidified with 0.5% of acetic acid. The following solvent programming was used: initial conditions were held linear at 30% A for 3 min, then linearly increased to 90% A in 10 min, then to 100% A in 10 min, finally kept isocratic for 10 min.

Compounds detected in negative ionisation (NI) mode (polyethoxylene carboxylate alkylphenols (APECs) and APs) were separated by ion pair chromatography. The solvent A consisted of acetonitrile/water (80 : 20, v/v) and solvent B was water, both containing 5 mM acetic acid and 5 mM triethylamine. The gradient was held linear at 50% A for 5 min and then increased to 100% A in 15 min, which was held constant for additional 10 min.

Detection was carried out using a HP 1040 M diode array UV-VIS detector coupled in series with an LC-MS HP 1100 mass-selective detector, equipped with an atmospheric-pressure ionization source with an electrospray (ESI) interface, under the following operating conditions: (PI/NI): drying gas flow 12/11 l min^{−1}; drying gas temperature 375/325 °C; nebulizer pressure 55/50 psi, capillary voltage 4500/3500 V and fragmenter voltage 60/80 V.

Diagnostic ions used for the identification of APEOs were those corresponding to $[M + Na]^+$: m/z 273–933 ($\Delta m/z$ 44) (OPEO₁–OPEO₁₅), m/z 287–947 ($\Delta m/z$ 44) (NPEO₁–NPEO₁₅). The extracts were fortified with 25 μM sodium acetate (5 μl of 5 mM aqueous solution) prior analysis in order to avoid the possible reduction in APEO ionisation due to insufficient metal ion availability.

NPEC₁, octylphenol (OP), nonylphenol (NP) and linear benzene sulfonate (LAS) surfactants with a chain length of 10–13 carbon atoms were detected under NI conditions as $[M - H]^-$, m/z 263 (OP₁EC), m/z 277 (NP₁EC), m/z 205 (OP) m/z 219 (NP), m/z 297 (C₁₀LAS), m/z 311 (C₁₁LAS), m/z 325 (C₁₂LAS), and m/z 339 (C₁₃LAS).

Quantitative analysis was performed in a selected ion-monitoring mode (SIM) using external calibration. The identification of target compounds was done in a full scan mode (m/z 100–1000) by matching the retention time and mass spectrum with authentic standards.

APs, APEOs and LAS were also analysed by LC-FL. The HPLC system was a Waters Alliance 2690 type (Waters). The separation was achieved on a 5 μm , 250 × 4 mm id C₁₈ reversed phase column (LiChrospher 100 RP-18) preceded by a guard column (4 × 4 mm, 5 μm) of the same packing material from Merck. The injection volume was set at 40 μl , the flow rate was 1 ml min^{−1} and the following gradient was applied: Acetonitrile (solvent A) and water containing 14 g l^{−1} NaClO₄ (solvent B): 0–3 min 60% B, 23 min 30% B, 26 min 10% B, 30 min 60% B, 32 min 60% B. Detection was carried out using a Waters Fluorescence Detector 474 at an excitation wavelength of 225 nm, and an emission wavelength of 295 nm. Quantification was achieved by interpolation of chromatographic areas from calibration curve built for each target compound ($r^2 > 0.999$).

For comparative purposes, the results obtained by LC-MS and LC-FL were also expressed as an approximate “APEO index”, taking into consideration the relative mean cross-reactivity of different NPEOs, OPEOs, NPECs and NP tested in the EFIIA. The calculation was performed according to the eqn. (2):

$$\text{NPEO}_{10} \text{ equiv.} = 1.1 \times ([\text{NPEOs}] + [\text{NPEC}_2]) + 2.3 \times [\text{OPEOs}] + 0.02 \times ([\text{NP}] + [\text{NPEC}_1]) \quad (2)$$

NPEO₁₀ equiv. is the calculated “APEO index”, expressed in number of equivalent nonylphenol decaethoxylate concentration ($\mu\text{g l}^{-1}$), while [NPEOs], [NPEC₁], [OPEOs], and [NP] are the corresponding concentrations (in $\mu\text{g l}^{-1}$) of the total

nonylphenol polyethoxylates, polyethoxylene carboxylate alkylphenols with $x = 1$ or 2 ethoxy groups, total octylphenol polyethoxylates, and nonylphenol, respectively.

Results and discussion

EFIIA optimization

The flow rate, substrate concentration/reaction time, pH, temperature, concentration of salt and blocking agent were optimized and found to give similar optimum conditions as previously found, proving the generic nature of this type of system.¹ All experiments were thus performed at room temperature with a carrier of 10 mM phosphate buffer at pH 7.4, containing 75 mM NaCl, and 0.01% (v/v) Tween 20 (PBST), a substrate carrier, containing 1.57 mg l⁻¹ 2-NPG and 2 mM MgCl₂ in PBST, both delivered at a flow rate of 0.375 ml min⁻¹ with a β -GAL-substrate reaction time of 2.5 min and product detection at 405 nm.

Hapten density. β -GAL is a large protein (MW 540 kDa) with many possible conjugation sites, so that the number of APEO groups per enzyme molecule can vary depending on the initial molar ratio used between reactants. The antibody recognition of the tracers, synthesized starting from different amounts of APEO hapten derivative and β -GAL (*i.e.*, 20, 50 and 100 moles APEO derivative per mole β -GAL), was studied. The best recognition was observed for the tracer having the highest hapten density, and the affinity for the tracer decreased with the decrease in the APEO derivative/ β -GAL molar ratio (results not shown). The enzyme activity was, however, negatively affected by the increase in hapten density (*i.e.*, 73%, 40% and 22% from the initial β -GAL activity was recovered for the tracers with the initial density of 20, 50 and 100 moles hapten per mole β -GAL, respectively), probably because of the changes in the folding of the protein structure and alteration of the β -GAL catalytic site when many APEO residues are covalently coupled to the protein molecule. Since the tracer obtained from an initial APEO derivative density of 100 moles APEO derivative per mole β -GAL led to the lowest antibody consumption for the assay development (results not shown), this was the tracer considered for further experiments as a compromise between the β -GAL activity and antibody recognition.

Content of methanol in the sample. APEOs exhibit a number of interesting properties related to their solution phase behaviour, and their "cloud point" is probably one of the most important for their use as surfactants. The "cloud point" is defined as the temperature above which an aqueous solution of water-soluble non-ionic surfactant becomes turbid,¹ and its value is strongly influenced by pH, salt content and concentration of surfactant. In normal aqueous environment, the "cloud point" decreases with the decrease in ethoxylate chain length (*e.g.*, from 63 °C for nonylphenol decaethoxylate, NPEO₁₀, to 20 °C for nonylphenol heptaethoxylate, NPEO₇, and from 65 °C for Triton X-100, OPEO₁₀ to 22 °C for Triton X-114, OPEO₈, while the APEOs with a number of ethoxylate chains less than 6 are virtually insoluble at room temperature).⁴¹ It is obviously very important for immunoassay development to increase the solubility of the APEOs in order to enhance their interaction with the corresponding antibody and to improve the assay sensitivity. One way to achieve this is by addition of small amounts of a polar organic solvent, in which APEOs are highly soluble.¹

Fig. 3 shows the effect of methanol content in the sample on both the signal for the β -GAL activity of the tracer alone (index 1) and the signal for the antibody-tracer recognition, with the contribution from the tracer signal alone subtracted (index 2).

For the assay under study the increase in the concentration of the methanol leads to a corresponding enhancement of the β -GAL activity up to around 20%, a point where the signal levels off. The antibody-tracer recognition follows the same trend until about 20% methanol, after which the antibody binding of the tracer is inhibited. In a different experiment, an NPEO₁₀ calibration curve was performed at different concentrations of methanol. The increase in the concentration of methanol causes an improvement in IC₅₀, however the precision of the measurement is also strongly affected (see Table 1), which in turn affects the final sensitivity of the assay.⁴³ This can be due to that polar organic solvents disrupt both the antibody-tracer recognition and the protein G-antibody interactions (which is why they are often employed as components of the buffers used for "regeneration" of protein G-based affinity columns), so that the amount of bound tracer is lower and the separation of the free tracer fraction from the antibody-tracer complex is less efficient. A concentration of 5% methanol in the sample was considered to be sufficient in order to obtain satisfactory immunoassay sensitivity and precision, as well as good separation of the labelled fractions.

EFIIA characteristics

The signal given by all tracers was stable at room temperature for at least 4 h of continuous operation at room temperature, after which a slow decrease of the signal with time was observed. However, when a new tracer solution was prepared from the stock solution (kept at 4 °C), the initial signal was regained. Due to dispersion in the EFIIA, decrease in the signal

Fig. 3 Influence of the methanol content in the sample on the β -GAL activity of the tracer (1) and antibody binding (2) [Ab]_w = 0.8 mg l⁻¹, and [Ag*]_w = 320 μ g l⁻¹. Carrier: 10 mM phosphate buffer pH 7.4, containing 150 mM NaCl, and 0.01% (v/v) Tween 20 (PBST). Substrate carrier: 1.57 mg l⁻¹ 2-NPG in PBST, also containing 2 mM MgCl₂. Otherwise, conditions as in Fig. 2.

Table 1 Effect of the methanol content in the sample on the immunoassay sensitivity (*i.e.*, IC₅₀) and precision. Reference analyte: NPEO₁₀; antibody concentration 0.8 mg l⁻¹. CV is the mean coefficient of variation. Conditions: as in Fig. 3

Methanol (% v/v)	IC ₅₀	CV (%)
0	115	1.2
5	36.5	3.1
10	28.4	7.4
15	16.4	11.2
20	3.5	35.5

of about 10% was observed when the β -GAL tracers were passed through the protein G column in comparison with the signal obtained when the column was by-passed.

Evaluation of the optimised immunoassay and cross-reactivity studies. The EFIIA was evaluated in terms of sensitivity, precision and selectivity with the main characteristics for the reference analytes NPEO₁₀ and NP shown in Table 2. The assay shows a good precision within the quantification range, and the limit of detection (LOD) for both NPEO₁₀ and NP is satisfactory for application as screening method for environmental water samples.

The sensitivity is generally better (*i.e.*, the IC₅₀ value in nM decreases), when the ethoxylate chain length is increased, and is superior for an OPEO than a NPEO with the same ethoxylate chain length (see the electronic supplementary information (ESI) to the paper for details[†]). However, from the analytical point of view, an interesting aspect is that the analytes can be separated in two different classes as a function of their sensitivity and CR in the assay: (1) APEOs and APECs with more than one ethoxylate group (CR $\approx$ 100%, calculated from

IC₅₀ values in $\mu\text{g l}^{-1}$)(thus an “APEO index”), and (2) APs and APEC with one ethoxylate group (CR $\approx$ 2%).

Effect of the sample matrix components on the immunoassay

Table 3 shows the influence of different water sample matrices on the sensitivity and precision of the EFIIA for NPEO₁₀. As seen, the sensitivity was similar in PBST, tap and surface water and with good recoveries, while the one performed in rainwater was about 30% more sensitive. The recovery in rainwater was still within the acceptable limits (between 70–120%) as recommended by the guidelines published by US EPA for analysis of environmental samples.⁴² Thus, the developed immunoassay could be used without any pre-treatment step for the estimation of APEO content in these environmental water samples.

When even more complex wastewaters were analysed, a dilution of the samples of 100–200 times with the carrier buffer was necessary prior analysis in order to minimise the matrix effects and to obtain recovery values of spiked NPEO₁₀ between 70–120%. The obtained recoveries for different spiked concentrations were between 70–113% (Table 4) for the EFIIA and when the spiked real concentrations were plotted *vs.* the found values, the slopes varied between 0.95–1.1, as shown in Fig. 4. The wastewater matrix influenced less the results obtained using the commercial ELISA kit, with recoveries within 70–120% obtained for sample dilution factors of only 10–50 (*v/v*) (results not shown). The reason for less pronounced matrix effects in the ELISA is due to that here the bound tracer is measured, *i.e.*, there is a time separation between sample application and detection, whereas in the EFIIA the matrix elutes simultaneously with the detected tracer, and thus the EFIIA is more prone to interferences.

The content of APEOs, APECs and APs in the unspiked influent and effluent of two WWTPs was evaluated both by the developed EFIIA, the commercial microplate ELISA, and by chromatographic techniques. The concentration of the target analytes determined by LC-MS and LC-FL are presented in Table 5. The NPEO content in the Abrera and Martorell influents is generally high, and the concentrations are considerably reduced in the effluents due to the chemical and biological treatment in the two WWTPs.

Table 2 The main characteristics of the EFIIA for NPEO₁₀ and NP. Conditions: as in Fig. 3

	NPEO ₁₀			NP
	Assay 1	Assay 2	Assay 3	
Calibration points (<i>N</i>)	13	11	11	14
Replicates	3	3	3	3
LOD ^a / $\mu\text{g l}^{-1}$	2.40 $\pm$ 0.2	2.55 $\pm$ 0.2	2.38 $\pm$ 0.2	51 $\pm$ 2
DR ^b / $\mu\text{g l}^{-1}$	5–250	5–250	5–250	100–5000
IC ₅₀ ^c / $\mu\text{g l}^{-1}$	26.5 $\pm$ 0.5	25.1 $\pm$ 0.4	24.9 $\pm$ 0.4	963 $\pm$ 39
% CV ^d intra-assay	1.43	1.27	1.27	1.44
% CV ^d inter-assay	2.68			nd

^a The limit of detection (LOD) was estimated as the concentration of the analyte that gives a change in signal equal to 3 times the standard deviation of the signal at zero dose (*i.e.*, 1.03% for 21 replicates). ^b The dynamic range (DR) was approximated between the analyte concentrations that led to a relative change in signal of 20 and 80%, respectively, when compared with the zero dose. ^c Inhibition concentration at 50% binding (IC₅₀) is the analyte concentration which leads to 50% change in signal *vs.* the response in the absence of the analyte. ^d The mean CV (%) was calculated in each case only within the dynamic range of the response. nd—not determined.

Table 3 Effect of the sample matrix on the immunoassay sensitivity and precision of spiked NPEO₁₀. Abbreviations: as in Table 2. Conditions: as in Fig. 3

Sample	IC ₅₀ / $\mu\text{g l}^{-1}$	CV (%)	Spiked Concentration/ $\mu\text{g l}^{-1}$				
			10	25	50	70	100
			Recovery (%)				
Standard buffer	24.5	1.44	100.0	100.0	100.0	100.0	100.0
Tap water	24.8	1.96	103.1	101.8	98.6	91.8	118.2
Surface water	23.5	3.45	97.4	91.0	112.0	106.7	113.3
Rain water	16.7	4.50	114.8	86.9	108.4	86.1	100.1

Table 4 Recovery of NPEO₁₀ from spiked wastewater samples analysed by EFIIA

Sample	Dilution <i>v/v</i>	Slope (see Fig. 4)	Spiked concentration/ $\mu\text{g l}^{-1}$				
			5	10	25	50	100
			Recovery (%)				
Martorell, influent	200	1.06	70.0	85.0	108.0	105.0	105.2
Martorell, effluent	200	0.96	110.0	80.0	95.2	104.0	95.0
Abrera, influent	200	1.10	84.0	83.0	107.2	113.0	108.6
Abrera, effluent	200	0.95	88.0	109.0	102.4	91.2	106.2

The APEO index (expressed in $\mu\text{g l}^{-1}$ as NPEO₁₀, equivalents) evaluated by the different methods (the APEO index for chromatographic data was calculated according to eqn. (2)) was significantly higher for the immunoassays than for the chromatographic methods (see Fig. 5), and the effect of overestimating the APEO content was dependent on the immunoassay format employed. As already reported, these differences cannot be due to the high content of LAS (Table 6), since these compounds do not cross-react with the APEO binding in ELISA.⁴⁰ The difference between the EFIIA and ELISA, in addition to the difference in assay format, is due to two different enzyme labels being used, *i.e.*, β -GAL in EFIIA and HRP in microplate ELISA. A pre-cleaning step by solid phase extraction (SPE) is recommended by the ELISA kit manufacturer to reduce the matrix effects. However, some previous studies have demonstrated a reduction of the analyte recovery from samples analysed by immunoassay after an SPE step *vs.* without sample pre-treatment.^{44,45}

Since the recoveries for NPEO₁₀ in the immunoassays were quite good, the overestimation of the APEO content by the two immunoassays *vs.* the chromatographic methods (Fig. 5), might be due to the methods actually measuring different things, *e.g.*, the CR of the immunoassays have only been tested on structurally related compounds and with those standards that were available. In principle the CR could be substantially greater, however the testing of all compounds by any method is technically impossible. Moreover, the accuracy of determining APEOs by chromatographic methods is highly dependent on the

availability of the necessary standard materials, the similarity between the oligomer composition in the reference and the samples analysed (*i.e.*, the relative response factor of individual oligomers generally depends on the molecular weight of the compound analysed and the possible co-elution of the analytes), and the complexity of the sample matrix. The results seen in Fig. 5, obtained by the different methods (*i.e.*, EFIIA, ELISA, LC-MS and LC-FL), simply reflect these differences. Nevertheless, a similar trend from influent to effluent wastewater of the two WWTPs can be seen for all methods, showing a decrease in APEO index during the treatment process.

Conclusions

This paper presents the application and evaluation of an EFIIA for determination of AP and APEOs in different water matrices. Despite good recoveries for the reference analyte NPEO₁₀ in matrices such as tap, surface, and rainwater, as well as in diluted wastewaters, this paper demonstrates the problems associated with immunoassay techniques in general when trying to correlate sample analysis results with those obtained with chromatographic reference methods, also previously observed.^{46,47}

The EFIIA and a commercial ELISA kit substantially overestimated the content of APEOs and APs in wastewaters when compared to results obtained by LC-MS and LC-FL. Although the quantitative correlation between the methods was relatively poor, it was shown that the immunoassays could be

Fig. 4 Recovery of NPEO₁₀ from spiked wastewater samples by EFIIA. Spiking levels: 5; 10; 25; 50, and 100 $\mu\text{g l}^{-1}$ NPEO₁₀. Conditions: as in Fig. 3.

Fig. 5 Quantification of the NPEO₁₀ equivalents in wastewater samples with EFIIA, ELISA, LC-MS, and LC-FL. Conditions: for EFIIA as in Fig. 3, otherwise, see text for details.

Table 5 Analysis of wastewater samples by LC-MS and LC-FL. Conditions, as specified in the Experimental section. Other symbols: nd: not detectable; LAS—linear alkylbenzene sulfonate. All results are in $\mu\text{g l}^{-1}$.

Compound	Martorell				Abrera			
	Influent		Effluent		Influent		Effluent	
	LC-MS	LC-FL	LC-MS	LC-FL	LC-MS	LC-FL	LC-MS	LC-FL
NPEOs	335	208	23	25	2460	844	26.6	24
OPEOs	4.2	nd	0.63	nd	20	nd	0.54	nd
NPEC ₁	108	nd	6.3	nd	635	nd	7.6	nd
OPEC ₁	nd	nd	1.9	nd	15	nd	nd	nd
OP	nd	nd	nd	nd	1.20	nd	nd	nd
NP	1.8	58	1.2	1.8	24	748	nd	2.75
Total LAS	1922	2062	24.6	46	1310	1223	45.1	43

applied for a qualitative evaluation of cleaning efficiency in WWTPs.

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References

- 1 R. M. Wainheimer and P. T. Varineau, in *Nonionic Surfactants—Organic Chemistry*, ed. N. M. van Os, Marcel Dekker, Inc., New York, 1998, **vol. 72**, pp. 39–85.
- 2 M. Wentz, *Detergency Theory and Technology*, Marcel Dekker, New York, 1987.
- 3 R. Osberghaus and F. Kresse, US Patent 4,648,882, Henkel KGaA, 1987.
- 4 L. F. Schwartz, US Patent 4,588,516, 1986.
- 5 C. L. Merrill and D. L. Wood, US Patent 5,034,158, Shell Oil Co., 1991.
- 6 L. Vaicum, M. Cicei and L. Stefanescu, *Stud. Epurarea Apelor*, 1976, **17**, 27.
- 7 H. H. Tabuk and R. L. Bunch, *Proceedings of the Purdue Industrial Waste Conference*, 1981, **vol. 36**, pp. 888.
- 8 V. W. Saeger, R. G. Kuehnelt, C. Linck and W. E. Gledhill, *Technical Report ES-80-SS-46*, Monsanto Industrial Chemicals Company, St. Louis, MO, 1980.
- 9 A. Marcomini, P. D. Chapel, T. Lichtensteiger, P. H. Brunner and W. Giger, *J. Environ. Qual.*, 1989, **18**, 523.
- 10 C. G. Naylor, *Text. Chem. Color.*, 1995, **27**, 29.
- 11 S. S. Talmage, *Environmental and Human Safety of Major Surfactants, Alcohol Ethoxylates and Alkylphenol Ethoxylates*, Lewis, Boca Raton, FL, 1994.
- 12 L. T. Brooke, D. Thompson, M. Kahl, D. Cox, R. L. Spehar and R. Erickson, presented at the *15th Annual Meeting of the Society of Environmental Toxicity and Chemistry*, Denver, CO, 1994.
- 13 A. M. Soto, H. Justica, J. W. Wray and C. Sonnenschein, *Environ. Health Perspect.*, 1991, **92**, 167–173.
- 14 R. White, S. Jobling, S. A. Hoare, J. P. Sumpter and M. G. Parker, *Endocrinology*, 1994, **135**, 175–182.
- 15 E. J. Abraham and L. S. Frawley, *Life Sci.*, 1997, **60**, 1457–1465.
- 16 B. Jimenez, *TrAC, Trends Anal. Chem.*, 1997, **16**, 596–606.
- 17 A. Tabata, S. Kashiwada, Y. Ohnishi, H. Ishikawa, N. Miyamoto, M. Itoh and Y. Magara, *Water Sci. Technol.*, 2001, **43**, 109–116.
- 18 P. A. Behnisch, K. Fujii, K. Shiozaki, I. Kawakami and S. Sakai, *Chemosphere*, 2001, **41**, 977–984.
- 19 L. Magliulo, M. P. Schreiberman, J. Cepriano and J. Ling, *Neurotoxicol. Teratol.*, 2002, **24**, 71–79.
- 20 J. P. Sumpter and S. Jobling, *Lancet*, 1993, **342**, 124–125.
- 21 T. K. Jensen, J. Toppari, N. Keiding and N. E. Skakkebaek, *Clin. Chem.*, 1995, **41**, 1896–1901.
- 22 H. Tanaka, K. Tsuji and K. Konishi, *Anal. Lett.*, 1974, **7**, 425–436.
- 23 S. Motomizu and Y. H. Gao, *Microchem. J.*, 1994, **49**, 326–439.
- 24 E. Y. Khmel'nitskaya, I. V. Gorbunova and B. N. Kolokolov, *J. Anal. Chem.*, 1995, **50**, 735.
- 25 P. Madhu, K. Seshiah, K. R. Mohan, V. K. Naidu and K. Janardhanam, *Res. J. Chem. Environ.*, 1998, **2**, 35–37.
- 26 P. de Voogt, K. de Beer and F. van der Wielen, *TrAC, Trends Anal. Chem.*, 1997, **16**, 584–595.
- 27 D. T. Bennie, C. A. Sullivan, H.-B. Lee, T. E. Peart and R. J. Maguire, *Sci. Total Environ.*, 1997, **193**, 263–275.
- 28 O. P. Heemken, H. Reincke, B. Stachel and N. Theobald, *Chemosphere*, 2001, **45**, 245–259.
- 29 M. Ahel, *Bull. Environ. Contam. Toxicol.*, 1991, **47**, 586–593.
- 30 A. Sweetman, *Water Res.*, 1994, **28**, 343–353.
- 31 T. C. G. Kibbey, T. P. Yavaraski and K. F. Hayes, *J. Chromatogr., A*, 1996, **752**, 155–165.
- 32 A. T. Kiewiet and P. de Voogt, *J. Chromatogr., A*, 1996, **733**, 185–192.
- 33 M. Zhao, F. van der Wielen and P. de Voogt, *J. Chromatogr., A*, 1999, **837**, 129–138.
- 34 M. Nasu, M. Goto, H. Kato, Y. Oshima and H. Tanaka, *Water Sci. Technol.*, 2001, **43**, 101–108.
- 35 M. Naasner, M. Mergler, K. Wolf and I. Schuphan, *J. Chromatogr., A*, 2002, **945**, 133–138.
- 36 Y. Toyoda, F. Masanori, G. Yasuhiro, M. Kenichiro and F. Shigeru, European Patent EP0834557A1, Takeda Chemical Industries, Ltd., Japan, 1998.
- 37 Y. Goda, S. Fujimoto, Y. Toyoda, K. Miyagawa, M. Ike and M. Fujita, *Yosui to Haisui*, 1998, **40**, 769–774.
- 38 S. Fujimoto and Y. Goda, *Nippon Rinsho*, 2000, **58**, 2491–2494.
- 39 Takeda Chemical Industries, Life-Environment Company, 2001, url: <http://www.takeda.co.jp/l-e/elisa/index-e.html>.
- 40 M. Castillo, J. Riu, F. Ventura, R. Boleda, R. Scheduling, H. F. Schröder, C. Nistor, J. Emnéus, P. Eichhorn, T. P. Knepper, C. C. A. Jonkers, P. de Voogt, E. González-Mazo, V. M. León and D. Barceló, *J. Chromatogr.*, 2000, **889**, 195–209.
- 41 E. Burestedt, C. Nistor, U. Schagerlöf and J. Emnéus, *Anal. Chem.*, 2000, **72**, 4171–4177.
- 42 IUPAC, Applied Chemistry Division on Agrochemicals, *Pure Appl. Chem.*, 1995, **67**, 2065–2088.
- 43 R. Ekins and P. Edwards, *Clin. Chem.*, 1997, **43**, 1824–1831.
- 44 A. Abad, M. J. Moreno, R. Pelegri, M. I. Martinez, A. Sáez, M. Gamón and A. Montoya, *J. Chromatogr., A*, 1999, **833**, 3–12.
- 45 M.-J. Moreno, A. Abad, R. Pelegri, M.-I. Martinez, A. Sáez, M. Gamón and A. Montoya, *J. Agric. Food Chem.*, 2001, **49**, 1713–1719.
- 46 D. B. Baker, R. J. Bushway, S. A. Adams and C. Macomber, *Environ. Sci. Technol.*, 1993, **27**, 562–564.
- 47 D. Zaruk, M. Comba, J. Struger and S. Young, *Anal. Chim. Acta*, 2001, **444**, 163–168.