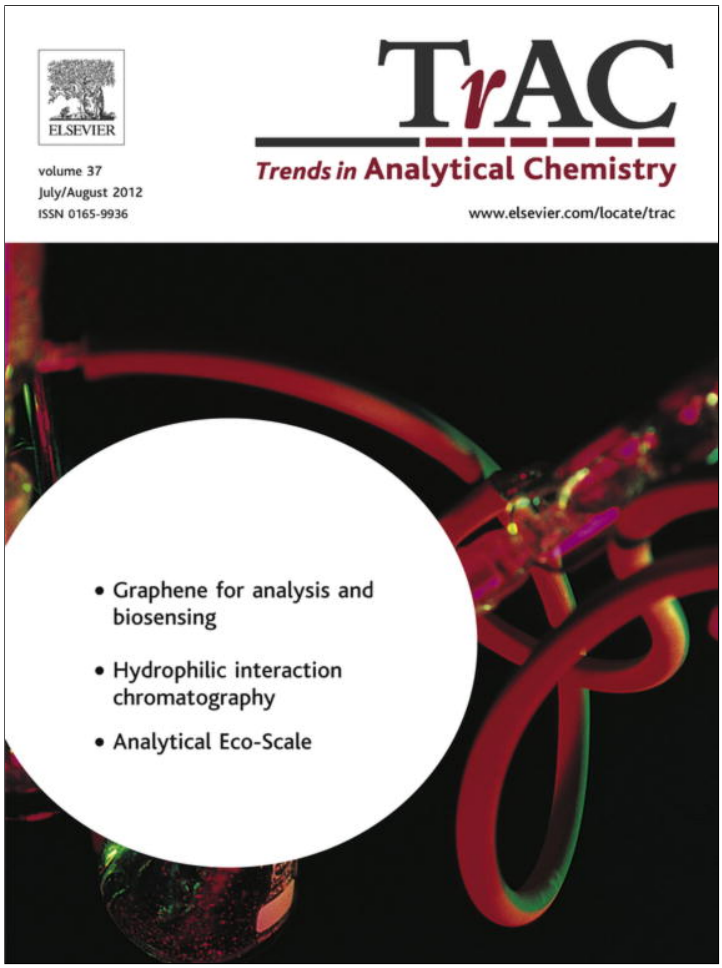

# Analytical chemistry and metrological issues related to nonylphenols in environmental health

S. Rabouan, A. Dupuis, A. Cariot, M. Albouy-Llaty, B. Legube, V. Migeot

**In recent decades, the development of environmental risks and their repercussions on health has led to environmental health being a field of scientific research in which interdisciplinarity is intrinsic.**

**This article on nonylphenols (NP) shows how exchanges and knowledge transfer involving chemists, biologists, pharmacists and physicians underscore the need to further the development of analytical methodologies. We spell out the difficulties encountered when selecting a reference material for the analysis of NP (i.e. multiplicity of isomers, and variability in the composition of batches for the same CAS Registry Number). As a result, we propose 353NP (CAS 186825-36-5) as the reference material (because of its high proportion in the industrial mixture and its pronounced estrogenic power).**


*Keywords:* 353NP; Environment; Estrogenic power; Exposure; Health; Nonylphenol; Reference material; Risk; Standard; Water

**S. Rabouan*, A. Dupuis, A. Cariot, M. Albouy-Llaty, V. Migeot,**
Université de Poitiers, CNRS-UMR 7285 IC2MP, Faculté de Médecine et Pharmacie (Departments of Analytical Chemistry, Pharmaceutics and Epidemiology), 6 rue de la Milétrie, 86034 Poitiers Cedex, France

**A. Cariot, A. Dupuis, M. Albouy-Llaty, V. Migeot,**
CHU Poitiers, Biology-Pharmacy-Public Health Pole, 2 rue de la Milétrie, BP 577, 86021 Poitiers Cedex, France

**B. Legube**
Université de Poitiers, CNRS-UMR 7285 IC2MP, Ecole Nationale Supérieure d' Ingénieurs, 1 rue Marcel Doré, 86022 Poitiers Cedex, France

*Corresponding author.
Tel.: +33 549 45 43 80;
E-mail: sylvie.rabouan@univ-poitiers.fr

## 1. Introduction

High-level official recognition of the influence of the environment on public health was accorded in 1994 at the Helsinki conference organized by the European office of the World Health Organization. A charter on environment and health was drawn up by the members. It describes rights and obligations of people, governments and other actors involved. It also formulates fundamental principles of general public interest and announces strategic elements and action priorities [1]. The European Environment and Health Strategy integrates information on the state of the environment, the ecosystem and human health, in order to render assessment of the overall environmental impact on human health more efficient. The main thrust of the strategy is to fill the knowledge gap on the link between environment and health, in a first phase focusing on a number of priority adverse health effects, including the endocrine-disruptive effects [2]. Based on human health data, 42 substances were listed as Category 1, including nonylphenols (NP) [3], which are continuously introduced into the aquatic environment by means of industrial, agricultural and municipal effluents. NP are found throughout the environment, particularly in water resources [4]. NP are of major concern to environmental public health due to their high potential for human exposure and their demonstrated toxicity. In animals, NP induce reproductive and developmental toxicity involving feminization, a decrease in male fertility and juvenile survival, malformation during embryo development, adverse effects on organ structure and weight, a decrease in embryonic survival and insufficient body growth [4,5].

From an environmental standpoint, the consequences of exposure to physical, chemical and biological aggressors are multiple and generally long-term, while the number of persons exposed is often exceedingly high. Moreover, the circumstances and the duration of exposure, be they in natural, domestic or professional environments, are hard to quantify. As a result, decision-makers are often compelled to take measures in situations characterized for the most part


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by the pervasive uncertainty of scientists. The consequences of their decisions may involve high economic stakes and raise questions of social acceptableness going above and beyond their strictly sanitary effects. The frame of reference existing at an international level is aimed at organizing scientific knowledge and bringing it to bear with regard to public decisions. In this respect, risk assessment (science-based) is clearly distinguished from risk management (policy-based) [6,7].

Classically, a risk-assessment approach comprises four distinct steps:

(1) hazard identification;
(2) definition of dose-response relationships;
(3) population-exposure assessment;
(4) characterization of the sanitary risks.

Step (3) is the subject matter of a methodology known as exposure science, and its objective in real-life situations is to identify and to characterize contact with toxic agents and their penetration into an organism. Unlike toxicology, it is based upon field observations. For that reason alone, exposure assessment is of great interest [8,9]. Human biomonitoring or biosurveillance is one component of exposure science, which revolves around analysis of environmental pollutants, or their metabolites, in the biological environment of the individuals involved [10–12].

With respect to NP, hazard identification has been established [13,14] and the definition of dose-response relationships is known in the animal through toxicological studies [13–16]. As for assessment of population exposure to NP, exposure science performs analyses of the pollutants in the environment of the individuals being studied and also carries out biomonitoring, which necessitates an appropriate choice of not only the biological sample (e.g., blood, urine, fat, or maternal milk), but also the molecule or metabolite involved. More generally speaking, when choosing, it is necessary to ensure that the risk-assessment approach maintains methodological continuity with regard to the contaminant in both natural and human biological environments, and thereby allows the data to contribute to the characterization of sanitary risks [Step (4)] through epidemiological studies.

The key aim of this article is to focus the scientist's attention on the analytical chemistry and metrological issues related to NP. First, we spell out the difficulties encountered when selecting a substance of reference for the analysis of NP, then propose 353NP (CAS 186825-36-5) as the substance of reference, and, finally, discuss this choice, in a case where the objective of assessing the presence of the NP in the environment so as to evaluate the quality of natural environments is transformed into the objective of evaluating human exposure to the self-same NP.

## 2. Generalities on nonylphenol analysis

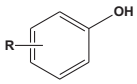
### *2.1. The nonylphenols present in the environment [4,14,17]*

NP ethoxylate (NPE) derivatives are one sub-set of a general group of compounds known under the name of alkylphenol ethoxylates. NPE are mass-produced chemical compounds. In Europe in 2002, as the result of the ethoxylation of NP with ethylene oxide, NP production neighbored 73,500 tons. The most widely utilized alkylphenols are 4-tert-octylphenol (OP, CAS 140-66-9) and 4-NPs (NPs, CAS 84852-15-3). For more than 40 years, NPE have been used as detergents, emulsifiers, wetting agents and dispersing agents, and they are found in a large, wide-ranging number of fields (e.g., textile transformation, pulp and paper treatment, paints, resins for protective coating, recovery of gas and petrol, manufacture of steel, anti-parasite products and energy production). They are also found in a wide array of consumer products, including cosmetics, cleaning products and paint. Lastly, NP can be used as adjuvants (antioxidants and softeners) in the manufacture of plastics (e.g., epoxy resins and PVC). There are also NPE transformation products, formed by means of microorganisms in the environment.

There exist no known natural sources of NP and NPE. The presence of these substances in the environment results solely from human activity. Release of NP and NPE into the environment may occur at different steps and stages of product life cycles, during primary NPE production, manufacture of products containing NPE and NP, utilization of the products and their elimination in wastewater-treatment facilities, septic tanks, town or factory landfill, or simply in the natural environment.

### *2.2. Naming nonylphenols*

In general, the name NP specifies neither the location nor the isomer of the nonyl radical (R), of which the general formula is as follows:




Industrial NP are manufactured via Friedel–Crafts alkylation of phenol with technical nonene ("propylene trimer": C9-olefins with varying degrees of branching). Due to the formation of carbocations during the acid-catalyzed process, the resulting NP mixture is highly complex, mostly containing para-substituted isomers with differently branched nonyl side chains [18,19]. The approximate composition of the technical mixture is: 3–6% *o*-NP, 90–93% *p*-NP and 2–5% decylphenol [20].

Besides, approximately 50–80 NP, with 550 possible isomers, are found in biological and other environmentally relevant matrices [21].

There exist numerous CAS numbers for the NP named, and the list in Table 1 is limited to the names pertaining to NP in para or predominantly para substitution – or to 4-NP – since they are relevant to environmental issues. Table 1 was drawn from the Sigma-Aldrich 2011 catalogue (sigma-aldrich.com), the French *Institut National de Recherche et de Sécurité* (INRS) [13], the Internet site of the Canadian Government [22], and the relevant ISO standards. In this article, we are not dealing with CAS 11066-49-2 (predominantly ortho substitution of the nonyl chain) or CAS 139-84-4 (predominantly meta substitution of the nonyl chain). Until 2009, references CAS 186825-39-8 and CAS 186825-36-5 were not commercially available in any catalog (regardless of the supplier). The study described below was carried out with a locally synthesized substance of reference 353NP (CAS 186825-36-5) (cf. sub-Section 3.1. Material and methods).

This recapitulative list underlines how much it matters that the user of NP pays particular attention to the exact product he wishes to employ. On this score, standard ISO18857-2:2009 explicitly mentions the fact {in Table 1, page 2 of the document [28]} that ''CAS numbers 104-40-5 (4-NP, straight chain) and 25154-52-3 (NP, straight chain) have also been incorrectly used to denote the isomer mixture CAS 84852-15-3''. The relevant standard indicates that ''the commercially produced NP are predominantly 4-NP with a varied and undefined degree of branching in the alkyl groups. This mixture of isomers falls under the CAS number 84852-15-3''. Issued the same year, standard ISO24293:2009 nonetheless describes the substance of reference to be used with regard to the concentration of 13 NP isomers corresponding to substitution isomers on the nonyl chain, which is itself in para substitution with regard to the phenol cycle and should be a product designated by the CAS number 25154-52-3 and described as a ''technical mixture of isomers'' {paragraph 4.8, page 3 of the document [24]}. Given this apparent contradiction, we have created chromatographic imprints by GC-MS laboratory analysis of each one of the two products:

(1) CAS 25154-52-3 (batch N°50720, supplier Dr Ehrenstorfer, ref C15629000); and,
(2) CAS 84852-15-3 (batch N° 1092230, supplier Fluka, ref 74430).

In our analysis, CAS product 84852-15-3 (in para substitution) corresponded to around 75% of CAS product 25154-52-3. It would consequently seem to be the case that the technical mixture with CAS number 25154-52-3 is indeed a mixture of substitution isomers on the cycle and on the chain, and not simply (Table 1) a mixture of molecules with linear radicals (as substitution isomers on the cycle). Conversely, CAS number 84852-15-3 presents a small percentage of substitution isomers on the cycle (other than in para substitution), as mentioned in the Sigma-Aldrich analysis reports: 90% minimum of ramified nonyl chain in position 4; in the impurities, substitution isomers on the cycle are once again reported, along with 0.2% of free phenol and to 3–4% of dinonylphenol. This observation corroborates the fact that industrial manufacture has not totally mastered the reproducibility of the batches, which are indeed complex and relatively variable according to the manufacturer and the batch number [25]. Moreover, in GC-MS laboratory analysis, the presence of 4*n*NP (linear radical, CAS 104-40-5) was sought but not found in the two products, a finding signifying concentrations below 5% for CAS 25154-52-3 and CAS 84852-15-3.

It bears mentioning that, in metrology in chemistry, the material (chemical substance) of reference must be of well-known and clearly-defined composition, and we have just observed that for NP:

(1) the degree of purity of the products with regard to their description is variable;
(2) it can be found out by examining the analysis report;
(3) all the isomers of the mixture are not necessarily subjected to dosage during the control.

### *2.3. References for nonylphenols*

The latest advances in monitoring chemical pollutants to assess water-quality status in accordance with the Water Framework Directive (WFD) and the attendant challenges were thoroughly reviewed in 2009 [26], and are listed in the Directive 2008/105/EC [27]. In the case of NP, the common position adopted by the European Parliament and the Council on 16 December 2008, on Environmental Quality Standards (EQSs) in the field of water policy and in amending Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and 2000/60/EC, was to propose CAS 104-40-5 (4*n*NP) as EQS (<0.3 μg/L when expressed as an annual average value, or <2 μg/L when expressed as a maximum allowable concentration) with the mention that ''Unless otherwise specified, it applies to the total concentration of all isomers'', even though the recommended standard method ISO 18857-2 [28] postulates CAS 84852-15-3 as the material of reference, whereas ISO 24293 [24] postulates CAS 25154-52-3. In the case of France and as a response to the questions put forward by the *Agences de l'Eau* (the French public establishments entrusted with implementing the WFD) concerning the CAS n° to be used, in December 2010 the *Institut National de l'Environnement Industriel et des Risques* recommended for use as a standard a substance internally codified by the *Service d'Administration National des Données et Référentiels sur l'Eau* (SANDRE 6598) and containing NP CAS 84852-15-3, CAS 25154-52-3 and CAS 104-40-5 [29].

Tables 2 and 3 list the different methods and substances used as references for the determination of NP in water and in complex matrices other than water, drawn from scientific literature published between 2002 and 2011. They show that the measurement benchmarks to be used may differ according to the studies in two significant respects:

(1) designation of the NP with no indication of the CAS n°; or,
(2) non-standardized expressions of results [i.e. variability with regard to the substance of reference (CAS n°) or to the means of calculation (with or without internal standard)].

As for dosage in an aqueous environmental sample, Table 2 illustrates the wide variety of calculation methods and substances selected as materials of reference, notwithstanding the harmonization texts established in 2006 with ISO standard 18857-1 (Water quality: Dosage of selected alkylphenols, part 1 published in 2006, updated in 2009) and then the ISO standard 24293 (Water quality: Determination of individual isomers of NP, published in 2009).

We consider the choice of substance of reference later in this article, particularly in the Discussion (Section 4).

## 3. Analysis of 353NP in the environment

### 3.1. Materials and methods

*3.1.1. Commercial chemicals and reagents.* Methanol, hexane, acetone, ethyl acetate, and dichloromethane of high analytical grade (Pestipur) quality were purchased from SDS (Peypin, France). Sodium thiosulfate, internal standard 4*n*-NP-2,3,5,6-D4, was purchased from Sigma-Aldrich, Inc. Water was pre-treated by a Purelab Prima and then purified by a Purelab classic (ELGA, Antony, France). Nitrogen alphagaz-1 was purchased from Air Liquide (Paris, France). 4-NP (diastereomer mixture of 4-(3,5-dimethylheptan-3-yl)phenol named 353NP) was custom synthesized by @rtMolecule (Poitiers, France). Purity was superior to 98%, and the ratio of diastereomer mixture synthesized in the present study was 353NP(E):353NP(G) = 45:55{according to the nomenclature proposed by Katase [25]}.

*3.1.2. Water samples.* Inflow and effluent water (IW and EW) samples were collected from the eight municipal drinking water-treatment plants (DWTPs) located in the French Poitou-Charentes region (n = 16). Sampling was performed on three different days and samples were pooled before analysis. IW samples were directly collected at the river surface above the DWTP and EW samples were collected at the outlet pipes of the DWTP. EW samples were stored with the addition of a reducer (5 mg of sodium thiosulfate/250 mL) in order to stop chlorination of target compounds, and all samples were kept frozen at −20°C until analysis.

*3.1.3. Preparation of standard solutions.* A 200-mg/L methanol stock solution of 353NP was stored at +4°C. Extemporaneously, the initial stock solutions were diluted in methanol/water 50/50 (v/v) in order to obtain working standard solutions at 2, 4, 8, 20 and 40 μg/L (MeOH-standard). Internal standard solution was prepared in methanol/water 50/50 (v/v) at 20 μg/L from initial stock solution (200 mg/L).

*3.1.4. Solid-phase extraction.* Prior to the extraction, 1.25 mL of methanol and 100 μL of the internal standard solution (50 μg/L) were added to an aliquot of 250 mL of water sample. Water samples were extracted by means of solid phase extraction (SPE). The SPE procedure for clean-up and concentration of water samples was performed using a glass C18 upticlean end-capped cartridge 200 mg (Interchim, Montluçon, France). Cartridges were conditioned with 4 mL of dichloromethane/hexane (50/50) and twice with 3 mL of methanol/acetone/ethyl acetate (2/2/1) (v/v/v) and then equilibrated with 5 mL of purified water. Water samples (250 mL) were passed through the wet cartridges, washed with 5 mL of purified water and dried for 15 min. For all of these different steps, flow rate was set at 10 mL/min. Elution was performed with a mixture of hexane/dichloromethane 50/50 (v/v) (2 × 2 mL) along with a mixture of methanol/acetone/ethyl acetate 2/2/1 (v/v/v) (2 × 2 mL) at a flow rate of 2 mL/min. Extracts were evaporated at 40°C to dryness under a gentle nitrogen stream. Residues were dissolved in 250 μL of a purified water/methanol (50/50) solution. Finally, 25 μL of extract were injected into a LC/MS/MS apparatus.

*3.1.5. LC/MS/MS analysis.* Concentration of 353NP was determined using an LC/MS/MS system consisting of an HPLC ultimate 3000 (DIONEX, Sunnyvale, USA) coupled to an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Forster City, CA, USA). The HPLC column was a supercosil ABZ 5 (3 μm particle size, 150 mm × 4.6 mm) (Supelco, St Louis, USA). Mobile-phase composition was methanol/water 85/15 (v/v), using an isocratic solvent program. The MS/MS was equipped with an atmospheric pressure chemical-ionization (APCI) interface, operating in negative-ionization mode. Quantitative analysis was carried out in the multiple reaction monitoring (MRM) mode, using two specific combinations of a precursor-product-ion transition for each compound. Retention times and precursor-product transitions along with their corresponding collision energies are shown in Table 4. MS/MS detector conditions were set as follows: ion-source gas 150 psig; probe temperature 550°C; curtain gas 20 psig; collision

**Table 1.** List of the names and CAS numbers of the different para-nonylphenols, drawn from the Sigma-Aldrich catalogs 2011, INRS, the Canadian Government Internet site, and ISO standards

| CAS Number | Usual name | Description |
|---|---|---|
| 104-40-5 | 4-nonylphenol<br>4-*n*-nonylphenol<br>*p*-nonylphenol | Linear radical |
| 84852-15-3 | Branched 4-nonylphenol | In para substitution, but as an isomer mixture with ramified radical (without linear radical)<br>*for Sigma-Aldrich 90% minimum in position 4 with mention of substitution isomers on the cycle.* |
| 25154-52-3 | Nonylphenol | Isomer mixture<br>according to *INRS FT249 and ISO 18857-2:2009 mixture of substitution isomers on the cycle of the linear nonyl radical.*<br>*according to ISO 24293:2009 isomer mixture in para substitution for the ramified nonyl radical.* |
| 90481-04-2 | Branched nonylphenol | Ramified radical (in isomer mixture), and mixture of substitution isomers on the cycle |
| 26543-97-5 | p-isononylphenol | Isononyl radical in para substitution |
| 87247-00-5 | *p*-tripropylenephenol | Tripropylene radical in para substitution |
| 186825-39-8 | 3E2-nonylphenol | 4-(2-ethyl-1-methylhexyl)phenol<br>*Available in catalog since 2009* |
| 186825-36-5 | 353-nonylphenol | 4-(1-ethyl-1,3-dimethylpentyl)phenol<br>*Available in catalog since 2009* |

gas 9 psig, entrance potential −5.0 V, nebulizer current −1.

*3.1.6. Method validation.* 353NP contamination may arise from laboratory accessories, reagent, SPE procedures and apparatus. In order to avoid such contamination, only pre-treated glassware (500°C, 5 h), Teflon seals and high-quality solvent were used throughout the study. Two kinds of blanks were performed. The first blank involved purified water with sodium-thiosulfate addition as the sample-loading step on which the SPE procedure was carried out; the second blank was obtained using an SPE procedure without the sample-loading step.

Linearity of the chromatographic response was assessed on three different days using standard curves including five calibration points in the range 2–40 ng/L. Recovery (R) was determined by analysis of n river water samples (exempt from target compounds) spiked at two different concentrations (20 ng/L and 40 ng/L) with 353NP (n = 10) and 4*n*NPD4 (n = 7). The recovery value included matrix effect and losses during SPE. The limit of detection (mLOD) and the limit of quantification (mLOQ) of the method were calculated, respectively, as three times and 10 times the signal-to-noise (S/N) ratio in blanks, corrected with the recovery. Intra-day and inter-day coefficients of variation were calculated for 20 ng/L. Sample concentrations were determined using the corresponding MeOH-standard curve calibration and corrected by the recoveries. The intra-day coefficient of variation was obtained with five replicates of quality-control (QC) sample at 20 ng/L. The inter-day coefficient of variation was obtained from experiments performed on three separate days with two replicates of QC sample at the same concentration. Accuracy was assessed by measuring the ratio between calculated ($C_{water}$) and theoretical values.

### *3.2. Results*

*3.2.1. Method validation.* 353NP was detected in blank extracts but at a level lower than the mLOQ. Regardless of whether purified water was loaded prior to SPE procedure, the results were similar. Moreover, no trace of 353NP was detected following direct injection of solvents. A small degree of contamination appeared as the extraction technique was being applied, and it was taken into account as we calculated the results. Recoveries for 353NP and 4*n*NPD4 were respectively 49% (RSD 19%) and 35% (RSD 58%). Calibration curves of 353NP provided adequate linearity, as shown by correlation coefficients greater than 0.99. The method provided mLOD and mLOQ values of 1.4 ng/L and 4.1 ng/L, respectively, for 353NP. Intra-day and inter-day variation obtained from QC of the 20 ng/L of 353NP were, respectively, 5.6% (n = 5) and 14.9% (n = 6), while accuracies were 98.3% and 89.2%, respectively, in intra-day or inter-day analyses.

*3.2.2. Water-sample analysis.* Appropriate internal QC were considered so as to evaluate the validity of the optimized analytical procedure and to check that no outliers occurred during routine analysis sequences. Signal specificity was systematically checked by comparison of each retention time and fragmentation ratio with the corresponding standard. Calibration curves were also regularly checked for linearity ($r^2 \geqslant 0.99$). Moreover, quantification of QC (20 ng/mL) was regularly performed during DWTP water-sample analysis (bias $\leqslant$11.0%, RSD$\leqslant$ 17.0%). 353NP was detected in concentrations in the range 13.5–124.9 ng/L in the IW and from <mLOD–59.4 ng/L in the EW (Fig. 1).

**Table 2.** The different reference nonylphenols used in the literature (examples) for assays in water (environment and drinking water)

| Ref. (year) | Technique | Nonylphenol of reference as mentioned in the publication | Supplier | Method with internal standard |
|---|---|---|---|---|
| [61] (2002) | GC/MS | CAS 25154-52-3 | Not mentioned | Absent |
| [62] (2005) | Not indicated | CAS 25152-52-3<br>CAS 104-40-5 | Not relevant | |
| [63] (2005) | GC/MS | 4- nonylphenol | Sigma-Aldrich | Bisphenol A-d16 |
| [64] (2005) | HPLC/(ESI)MS | Technical grade 4-nonylphenol | Aldrich | 4-heptylphenol (Aldrich) |
| [65] (2006) | | | | |
| [23] (**2006**) | GC/MS<br>**AFNOR – Europe**<br>**ISO 18857-1** | CAS 84852-15-3 | Not relevant | 4-*n*-nonylphenol (ring-$^{13}C_6$)<br>or<br>4-*n*-nonylphenol (*CAS 104-40-5)*<br>if absent from the sample to be dosed |
| [66] (2006) | GC/MS | Technical 4-nonylphenol (NP) | Riedel de Haen | 4-*n*-nonylphenol (*CAS104-40-5;* Riedel de Haen) |
| [67] (2007) | HPLC (Fluo) | 4-nonylphenol | Lancaster | 4-*n*-nonylphenol (*CAS104-40-5* ; Riedel de Haen) |
| [68] (2007) | HPLC/<br>(ESI)MS/MS | 4-nonylphenol CAS 84852-15-3 | Aldrich | 4-*n*-nonylphenol d8 (Dr Ehrenstorfer) |
| [42] (2008) | HPLC/<br>(ESI)MS/MS | 4-*n*-nonylphénol | Cil cluzeaux | Absent |
| [69] (2008) | **Laboratory (included 7) intercomparison study**<br>GC/MS without derivatization<br>or GC/MS with derivatization<br>or LC/MS/MS<br>or HPLC (Fluo) | 4-nonylphenol CAS 84852-15-3 | Not mentioned | 4-*n*-nonylphenol d8<br>Or 4-*n*-nonylphenol d6<br>Or 4-bromophenol<br>Or atrazine d5<br>Or without internal standard |
| [35] (2009) | **Interlaboratory trial (included 14) according to ISO 18857-2**<br>GC/MS with derivatization | NP | Sasol | 4-(3,6-dimethyl-3-heptyl)phenol (ring-13C6)<br>*Abbrev.*: 363 NP-13C6 |
| [28] (2009) | GC/MS<br>**ISO 18857-2** | CAS 84852-15-3 | Not relevant | 4-(3,6-dimethyl-3-heptyl)phenol (ring-13C6)Abbrev.: 363 NP-13C6 |
| [24] (2009) | GC/MS<br>**ISO 24293-1** | CAS 25154-52-3 | Not relevant | 4-*n*-nonylphenol (ring-$^{13}C_6$) |
| [53] (2010) | HPLC/<br>(ESI)MS/MS | Nonylphenol CAS 84852-15-3 | Not mentioned | 4-*n*-nonylphenol d8 (Dr Ehrenstorfer) |
| [70] (2010) | VALLME/HPLC (Fluo)<br>((VALLME = Vortex-Assisted Liquid–Liquid MicroExtraction) | Nonylphenol | Riedel de Haen Pestanal | Absent |
| [43] (2011) | LLLME/HPLC (Fluo)<br>(LLLME = Liquid–Liquid–Liquid MicroExtraction) | 4-*n*-nonylphenol | Alfa Aesar Johnson Matthey | Absent |
| [44] (2011) | LC/MS/MS | 4-*n*-nonylphenol | Supelco | Absent |
| [71] (2011) | GC/MS | NP | Fluka | Absent |

**Table 3.** The different nonylphenols of reference used in the literature (examples) for assays in complex matrices other than water

| Ref. (year) | Matrix | Technique | Nonylphenol of reference mentioned in the publication | Supplier | Method with internal standard/Quality control (QC) |
|---|---|---|---|---|---|
| [45] (2005) | Urine | GC/MS | 4-*n*-NP (linear) | absent | Isotope (name not indicated)/QC |
| [72] (2007) | Eggs and milk | LC/MS/MS | 4-*n*-NP technical purity NP | Tokyo Kasei Kogyo | absent |
| [73] (2008) | Breast milk | GC/MS | nonylphenol | ChemService | Dodecyl benzene |
| [74] (2009) | Soil | TD/GC/MS | 4-nonylphenol (technical mix) | Aldrich | absent |
| [75] (2009) | Breast milk and milk | GC/MS | 4-nonylphenols technical grade | Aldrich | cumylphenol |
| [76] (2010) | | | | | |
| [77] (2011) | Powdered milk | HPLC/MS/MS | NP | Aldrich | absent |

**Table 4.** MS/MS parameters

| Compound | Retention time (min) | MRM(*m/z*) | Declustering potential (V) | Collision energy (V) | Collision-cell exit potential (V) | Dwell time (ms) |
|---|---|---|---|---|---|---|
| 353NP (E+G) | 4.45 | 219–133 | −70 | −35 | −20 | 40 |
| | | 219–147 | −70 | −26 | −11 | 40 |
| 4*n*NPD4 | 6.05 | 223–110 | −80 | −40 | −30 | 40 |

## 4. Discussion

Analytical chemistry is a discipline whose key objective does not lie in the mere application of methods producing a numerical figure in exchange for an experimental sample [30]. Quite to the contrary, it plays an instrumental role in decision-making with regard to problems as wide-ranging and vital as medical diagnosis, QC for industrial products, protection of the environment, and environmental epidemiology. After all, risk characterization as defined by the National Research Council [7] is continually enhanced by studies emanating from research in three areas where measurement matters: the field, toxicology, and epidemiology. By basing presentations on analytical chemistry methodology in the form of a triad bringing together (a) the problem to be resolved, (b) how the problem is framed, and (c) the chemical information to be obtained [30], the issues in environmental health pertaining to NP may be understood as follows:

(a) in learning whether or not there are endocrine-disruptive effects to be observed in humans provoked by the NP present in the environment;
(b) to consider that 353NP is the NP isomer liable to be largely responsible for endocrine disruption, because it is largely present in the environment; and,

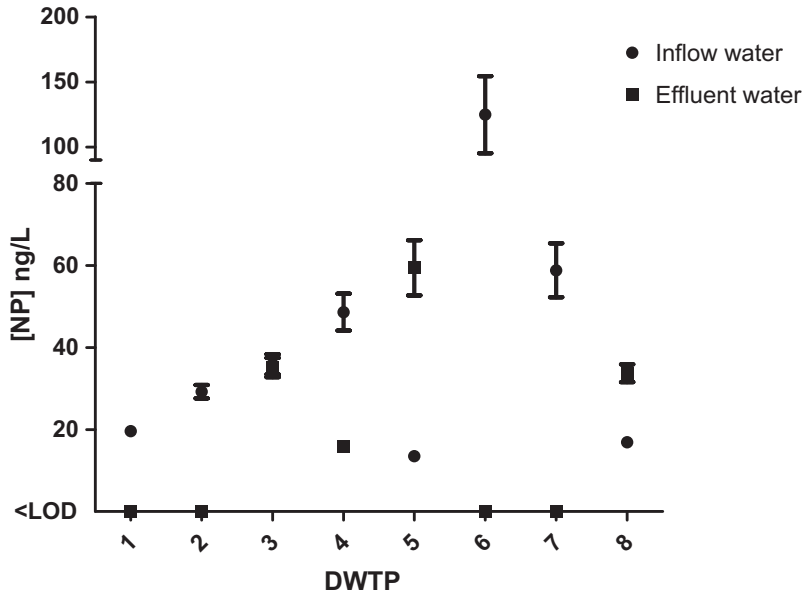



**Figure 1.** 353NP concentration in water (inflow and effluent of eight drinking-water-treatment plants).

(c) to perform 353NP analysis in environmental water and biomonitoring.

Since NP in the environment involve more than 100 substitution isomers [21], exhaustive analysis of these is hardly realistic. And when the final objective involves studying the endocrine-disruptive effects that may or may not be attributed to these molecules, the first priority is to decide on the molecule to be found in the environment and a biological sample. The choice of the right molecule is contingent on the model, which is tantamount to the way the problem is framed. The model is a tool designed to detect the NP isomer bearing the brunt of responsibility for endocrine-disruptive effects. Researchers have got to define this NP isomer, a hormonally active agent otherwise known as an endocrine disruptor, which predominates in technical NP mixtures and is found in the environment and biological samples. We set out the arguments in favor of the 353NP molecule (CAS 186825-36-5) in the following paragraphs, in which we respectively discuss the chromatographic separation and MS detection of NP, and the consequences of the modified objectives of NP analysis in the framework of environmental health on the NP-analysis benchmark reference. Lastly, we explain the final selection, along with the analytical procedure.

### *4.1. Chromatographic separation and MS detection*

Due to the similarities of the chemical and physical properties of the many NP isomers, complete separation and identification of individual isomers remains quite difficult, if not altogether impossible. Routine analysis using gas chromatography (GC) with an apolar capillary column yields separation of 13 p-NP isomers, which comprise more than 90% of the 4-NP isomers that are detectable in technical products and environmental samples in general [24,31]. One may note that no single 4*n*NP molecule appears in these technical mixtures (concentration below the mLOD). Much recent research has been focused on the application of highly sophisticated coupling systems to the isomer-specific determinations of NP. For example, the coupling of two-dimensional GC and mass spectrometry (GCxGC/MS) has led to the identification of 80–110 different isomers in technical NP from different manufacturers [21,32,33]. It should also be noted that there exist 211 possible constitutional isomers of p-NP out of a total of 550 possible compounds, taking into consideration the chiral C-atoms [18,21]. The composition of isomers in the proposed reference varies according to the batch numbers, and their fragmentation fails to yield the same fragments [19], so it becomes downright impossible to quantify with regard to the technical mixture, which consequently cannot function as a reference.

In the overall context of the WFD, the reliability [QC/quality assurance (QA)] and comparability of the analytical data gathered in a given environment are of paramount importance, as they lead to enhanced demonstration of the correspondence of the ensuing measurements to the established references [34], as occurred in 2009 for the ISO 18857-2 protocol, with the participation of 14 laboratories from four different countries in Europe and Canada [35].

The first approach (ISO protocol 18857-1:2006) adopted by the research community and the international measurement organizations is to proceed by GC/MS with regard to the sum of the isomer-peak areas: particular attention must be paid to substances that co-elute with NP and yield the same ion(s) because the co-elution can interfere in the determination and exert substantial influence on the final result. ISO protocol 18857-2:2009 (with CAS 84852-15-3 as the standard) is aimed at ensuring that interfering peaks are excluded from the sum of the areas, and limits inclusion to the peaks from the sample attributable to the multi-component analyte [28]. The second approach by GC/MS is proposed in ISO protocol 24293:2009 (with CAS 25154-52-3 as the standard), and it involves in quantifying specific isomers identified by their retention time along with two selected diagnostic ions; the specificity of the signal used must be verified.

The two choices are both satisfactory in terms of the objective of analysis proper to the ISO standards, which is to quantify NP in the environment [35]. But when the objective of a study is to assess sanitary risk by means of biomonitoring (assays in biological samples) and environmental analysis (water) in order to study their correlation, these two methods of analysis are no longer suitable, for reasons to be explained later.

### *4.2. The final objective of the study*

Using the preceding benchmarks, the sum of the signals recorded in the samples for the sought-after analytes is compared to the sum of the signals emanating from the mixture of reference and expressed in terms of concentration with regard to the substance of reference. If the proportions of the different isomers are the same in the samples of reference as in the unknown quantities, this mode of calculation engenders practically no evaluation error in determination of the NP content (in the form of a mixture). In reality, all relevant findings lead us to believe that the relative proportions of the different isomers to be found in unknown biological samples are bound to differ from those to be found in a reference mixture, which corresponds to the raw material utilized in industry ... and to the source of biological sample contamination! Three separate arguments lead to the same conclusion, as follows.

(1) The relative proportions of the different NP isomers vary from one manufactured batch to another, which means that the substance of reference is

not the same when the batch number has been modified (between two different manufacturers or even the same manufacturer).

(2) How each of these different isomers evolves in the environment cannot be known. All the literature tells us is that
  (2.1) NP would certainly appear to be the main product of degradation of NP ethoxylates;
  (2.2) does not undergo further transformation; and,
  (2.3) is strongly adsorbed onto the sludge solids [4].

As for the pattern of relative of NP-isomer concentrations, it has been found to vary from one environmental sample to the next [36,37]. The proportion of isomers in the environmental sample subjected to analysis will inevitably differ from the proportion in the substance of reference.

(2) The same goes for the proportions of the isomers in the biological samples subjected to analysis. No data in conjunction with bioconcentration for isomers such as ''partition coefficient'' ($K_{OW}$) are presently available. The rare published studies on the subject indicate nothing other than log $K_{OW}$ for the technical NP mixtures [38,39], and in a study on 353NP bioconcentration, it is pointed out that ''a correlation between the metabolism and the $K_{OW}$ seems to be unlikely, and that two mechanisms triggering the bioconcentration (partitioning between two phases and biotransformation)'' can be distinguished from each other and ''described both mechanisms with a simple weight-dependent bioconcentration model'' [40]. Another study mentions considerable algal bioaccumulation, but the material & methods section does not indicate the CAS n° used in NP [41].

To sum up, if the scientific community wishes to pose as an objective the comparison of studies seeking to draw a connection between environmental NP content and human-impregnation levels, it is necessary to use the same substance of reference for environmental analyses and biomonitoring. In absence of this condition, comparative studies are pointless, and information with regard to exposure science will remain limited.

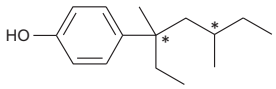
### 4.3. Selection

It is consequently necessary to seek a reference NP, present in the environment *per se* and in the biological environment, comprising a substance defined by its chemical formula and of known purity, in order to express the results in terms of a precisely defined molecule. In Table 1, CAS 84852-15-3, CAS 25154-58-3 and CAS 90481-04-2 are isomer mixtures and cannot be selected, nor can CAS 26543-97-5, CAS 87247-00-5 or CAS 104-40-5, which are indeed single substances, but have not been employed industrially in the production of ethoxylated NP, and are consequently of no relevance to studies of the environment or environmental health. Moreover, the 4*n*NP isomer (linear radical, CAS n° 104-40-5) is not detectable (<5%) in the technical NP mixtures used for industrial purposes, and research in surface water and drinking water ([42–44]) shows that the compound is absent or minimally present (<mLOQ), as is the case with concentrations in urine [45].

The substance of reference liable to be selected will be one of the branched isomers of the technical mixture used by industry (CAS n° 84852-15-3). Which one? Along with analytical development aimed at quantifying NP in the technical mixture, numerous researchers have studied the estrogenic power of the main isomers contained in industrial mixtures [25,46–50], specifically in 353NP [47,51,52]. Since isomer 3E2NP (CAS 186825-39-8, Table 1) is not one of the isomers studied by the authors consulted while this article was being written, it will not be taken into account. On the contrary, if the 353NP para-NP molecule (CAS 186825-36-5) has been selected as a reference in this work of ours, it is for the following reasons:

(1) it represents 12–20% of the isomer mixture CAS 84852-15-3, and, through the *MVLN* test, the 353NP molecule significantly contributes to the estrogenic effect with regard to CAS 84852-15-3;
(2) the 3E22NP para-NP molecule, which presents the most pronounced estrogenic power through the *YES* test, represents only 4–6% of the CAS 84852-15-3 mixture; and,
(3) 353NP has been shown to be present in living organisms using a radiolabeled NP isomer to investigate the bioconcentration in *Daphnia magna* [40].

The 353NP nomenclature corresponds to 4-(1-ethyl-1.3-dimethyl-pentyl)-phenol. It contains two asymmetrical carbons and is presented in the form of a diastereomeric mixture of 353NP(E) and 353NP(G) (according to the nomenclature proposed by Katase [25]):




Using the nomenclature proposed by Guenther, this is molecule 111, as described in the Electronic Supplementary Material [21].

### 4.4. Our analytical procedure

In the work presented in this article, we attempted to detect the presence of 353NP in environmental water and at the entrance to a DWTP, and we then analyzed the water coming out of the plant. Our goal was to propose a protocol for analysis and to evaluate the presence or the absence of this molecule in the

environment. High-purity solvents and glass – instead of plastics – were used and specific purification procedures were performed, but they did not avoid 353NP contamination, which was probably due to leaching effects during SPE. Other studies have also reported NP contamination in blanks [53]. In the present study, 353NP contaminations did not exceed the mLOQ, but, still, special attention needs to be paid when proceeding. During MS-parameter optimization, the negative-ionization mode produced higher signal intensity than the positive-ionization mode and a much better signal-to-noise ratio (S/N). APCI (atmospheric pressure chemical ionization) and ESI (electrospray ionization) interfaces have been compared using both a methanol solution of compound and spiked river water. The ESI interface produced higher sensitivity using methanol solution. Due to a significant matrix effect, the best results in spiked water were obtained using the APCI interface, which was consequently chosen for use in this study.

In this work, 4*n*NP-2,3,5,6-D4 was initially envisioned for use as the internal standard, based on the recommendation of 4*n*NP (ring-$^{13}C_6$) in the ISO24293:2009(E), and, because the presence of 4*n*NP has been reported in environmental samples [42,44], the unlabeled 4*n*NP cannot be employed as internal standard. However, as our method was drawn up, it became obvious that the physicochemical properties of 4*n*NP-2,3,5,6-D4 differ too pronouncedly from those of 353NP to be an acceptable choice. Not only is retention time appreciably longer, (6.05 min instead of 4.45 min for 353NP), but it presents a weak and excessively variable yield of extraction (35% with RSD of 58%). Others authors [54] have also shown that the structure and the shape of this molecule with a linear alkyl chain differ markedly from the isomers in the technical mixture, especially in the response factor by GC/MS, so they have proposed a 4-NP isomer with a secondary alkyl side chain (4-(2,6-dimethylhept-3-yl)phenol) to serve as internal standard. In the same way, ISO18857-2:2009(E) recommended the 4-(3,6-dimethyl-3-heptyl) phenol (ring-$^{13}C_6$) as internal standard. The results described herein consequently employ external calibration as a means of calculation, but use of an isotope-labeled 353NP (deuterium or carbon-13) is recommended in order to be able to calculate by means of internal calibration. In experimental conditions, the 353NP isomer was characterized by its retention time (4.45 min, diastereomers E+G co-eluted) and the *m*/*z* fragmentation 219–133 and 219–147.

In our water-sample analysis results, the presence of 353NP is evaluated in terms of inflow and effluence of DWTP producing from surface water. 353NP was found in all surface-water samples analyzed in this study at a level in the range 13.5–124.9 ng/L. Similar or different results are reported in the literature, but these comparisons should be put forward with caution, given the complications that we have mentioned about the NP standard to be used [53,55–58]. Concentration decreased in most of the effluent water samples (Fig. 1, Nr 1-2-4-6-7) from 2.2% to 100%, with a median of 100%. Authors from different countries reported similar results with overall elimination in the range 73–100% [56,58,59]. However, we found more 353NP in some EW samples (than in corresponding IW), probably because our EW and IW withdrawals were carried out at the same time in the DWTP, without taking into account (water) flow time in the plant and the (unknown) fluctuations in 353NP concentrations in the IW, but it is also possible that some 353NP was released during drinking-water production and transport [60].

As a general rule, NP quantification may be carried out by 353NP analysis. However, so far, the methods used in NP analysis have failed to yield adequately distinct results, and it would consequently be preferable to select a single, clearly identified isomer.

## 5. Conclusion

The analytical data gathered in an environmental perspective constitute the foundations of the European water-quality assessment system, as described in the WFD. In this context, reliability (QC/QA) and comparability of measurements are of paramount importance as they lead to enhanced demonstrations of the correspondence of ensuing measurements to the established references.

In this article, we have detailed the difficulties encountered in selecting a substance of reference in the case of NP determination (multiplicity of the isomers, variability in the composition of batches for the same CAS number). What is more, the references to NP used in today's environmental analyses (CAS 84852-15-3 and CAS 25154-52-3, respectively, in conjunction with standards ISO18857-2:2009 and ISO24293:2009) are not transposable in assays of this micro-pollutant in biological samples.

Classically, a risk-assessment approach involves four basic steps or stages, including the exposure assessment, which is meant to assess population exposure to NP, through analyses of the pollutant in the environment of the individuals studied and its biomonitoring. Here, the choice of the molecule to be analyzed at this stage has been shown to be the most pertinent with regard to 353NP (high proportion in the industrial mixture and pronounced estrogenic power). As a general rule, given that this choice should help to ensure that the risk-assessment approach will be part and parcel of a methodological continuum, it is necessary to carry out follow up in not only human biological samples, but also water; the data gathered will consequently contribute to the

characterization of sanitary risks [Step (4)] by means of epidemiological studies.

In this work, the concentration at the entrance and the outlet of the DWTP producing from surface water, showed that 353NP was found in all surface-water samples analyzed at a level of 13.5–124.9 ng/L, and also remained in some effluents (drinking water). Moreover, Preuss et al. have shown 353NP bioconcentration, using U-ring-$C^{14}$-labeled 353NP, in daphnies [40].

To conclude, while 353NP assays do not allow for quantification of all the NP, evidence of its presence in the environment and during biomonitoring can allow estimation of NP exposure. That is why the 353NP molecule (CAS 186825-36-5) can be recommended for the purpose of not only studies of the quality of environmental and drinking water, but also epidemiology studies, in which correlations are sought between presence of the contaminant in the environment and its presence in biological samples. As a result, the sizable number of environmental data gathered during analysis campaigns could be used in an epidemiological context and thereby enhance assessment of the public health risk with regard to NP from an environmental health standpoint.

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