Endocrine-disrupting nonylphenols

Determination in food samples and isomer-specific analysis

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Bonn, 2026

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der

Rheinischen Friedrich-Wilhelms-Universität Bonn

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Tag der Promotion: 08.06.2026

Erscheinungsjahr: 2026

Summary

Nonylphenols (NPs) are one of the most widely recognized environmental pollutants. They are also listed as one of the 13 priority hazardous substances by the European Union. NPs are mainly used as raw materials for producing nonylphenol ethoxylates (NPEOs), highly cost-effective surfactants with exceptional performance. They are also widely used in various industrial, institutional, commercial, and household appliances, such as detergents, emulsifiers, wetting and dispersing, antistatic, demulsifiers, and solubilizers. Owing to their extensive use, NPEOs are found in considerable amounts in sewage treatment works where they are incompletely degraded into NPs.

Consequently, sewage treatment works are the primary source of NPs in the environment. Since NPs were first synthesized in 1940, their use and production have increased almost exponentially. NP production in the US reached 154,200 tons in 2016, 73,500 tons in Europe, 16,500 tons in Japan, and 16,000 tons in China.

The European Parliament and Council of the European Union adopted Directive 2003/53/EC in 2003, amending Directive 76/769/EEC of the European Parliament and Council, to restrict the marketing and usage of certain dangerous substances and preparations, including NPs, NPEOs, and cement. According to the directive, using NPs and NPEOs at concentrations ≥0.1% by mass is prohibited in industries such as pesticide formulations, industrial cleaning, textile production, and metalworking. This EU directive was implemented in response to the work by Guenther et al. (2002), who reported NPs in relevant concentrations in all examined foods commonly available in the shops and supermarkets of Germany. The widespread consumption of precursor compounds of NPs worldwide has resulted in these estrogen-active compounds in a widespread variety of commercially available food products.

Interestingly, no efforts were made to evaluate the effectiveness of Directive 2003/53/EC regarding the level of NPs in food from German shops and supermarkets, their daily intake by German adults, or the presence of NP carboxylates (NPECs). The present thesis discusses the evaluation of NPs in the foods examined by Guenther et al. (2002). We measured the daily intake of NPs among German adults following the implementation of the 2003/53/EC Directive.

We initially examined the concentrations of NPs and NPECs. We then analyzed them using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS) in the same German food basket analyzed before the restriction.

We detected NPs in 22 of 24 food samples, whereas NPECs were below the detection limit in all 24 samples. The concentration of NPs on a fresh weight ranged from 0.1 to 6 μg kg^{-1}. Based on the consumption rates of food in Germany and the concentrations of NPs found in the food samples, the daily NP intake for a German adult was determined to be 2.7 μg day^{-1}, approximately one-third of the 2002 intake.

We also assessed NPs in selecting foods from different countries using HPLC–MS. We detected NPs in 8 of the 12 food samples. Regarding fresh weight, the concentration of NPs varied between 0.5 and 18.9 μg kg^{-1}. The NP content was the highest in coffee samples. An isomer-specific analysis could be conducted using two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC–TOF–MS), offering a superior resolution to GC–MS. It is characterized by the fact that the components of the investigated sample, which are present in a mobile phase, leave the first chromatographic column and are then collected by a modulator, which is the central component of the GC×GC system, and fractionally dosed into the second column. There is a difference in the polarity of the stationary phase between both columns, which increases selectivity.

GC×GC–TOF–MS can provide information about the qualitative and quantitative composition of the samples characterized by complex matrix compositions. Thirteen pure isomers of NPs were used as standards for cross-referencing NP isomers. In the coffee samples, two main NP isomers were identified: 4-[3-ethyl-1,1-dimethylpentyl]-phenol and 4-[1-ethyl-1,3-dimethylpentyl]-phenol, indicated as NP_{128} and $NP_{111a,b}$, respectively (Juelich Nomenclature).

The results showed that NPs remain ubiquitous in foods at relevant concentrations, indicating that further investigation is necessary, particularly for isomer-specific aspects. Our study showed that the GC×GC–TOF–MS system with a liquid nitrogen cryogenic modulator is appropriate for analyzing NPs under isomer-specific conditions. If further optimized, it can also be used for future routine food control analysis.

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Abbreviations

NPs	Nonylphenols
IUPAC	International Union of Pure and Applied Chemistry
NPECs	Nonylphenol carboxylates
NPEOs	Nonylphenol ethoxylates
HPLC-MS	High-performance liquid chromatography coupled with tandem mass spectrometry
GCxGC-TOFMS	Two-dimensional gas chromatography coupled with time-of-flight mass spectrometry
EPA	U.S. Environmental Protection Agency
HPLC	High-performance liquid chromatography
GC	Gas chromatography
MSD	Mass spectrometer detector
t-NP	Technical Nonylphenols
ERs	Estrogen receptors
YES	Yeast Estrogen Screen
RP	Relative Potency
^{2}D GC	2-dimensional gas chromatography
EC20	20% maximal effective concentration
EC50	50 % maximal effective concentration
β	Beta
γ	Gamma
α	Alpha
NP1EC	Nonylphenoxyacetic acid
NP2EC	Nonylphenoxy ethoxy acetic acid
NP1EO	Nonylphenol mono ethoxylate
NP2EO	Nonylphenol die-ethoxylate
EDCs	Endocrine-disrupting chemicals

S/N	Signal-to-noise ratio
^{1}D	First dimensional column
^{2}D	Second dimensional column
MR	Modulation Ratio
PM	Modulation period
FID	Flame ionization detector
VOCs	Volatile Organic Compounds
CFT	Capillary Flow Technology
MS	Mass Spectrometers
LN_2	Liquid Nitrogen
CO_2	Carbon Dioxide
SPME	Solid-Phase Microextraction
LOD	Limit of Detection
LOQ	Limits of Quantitation
APEOs	Alkylphenol Ethoxylates
EU	European Union
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
IS	Internal Standard
ACN	Acetonitrile
Na_2SO_4	Sodium Sulfate
$MgSO_4$	Magnesium Sulfate
HCl	Hydrochloric Acid
DSPE	Dispersive Solid Phase
PSA	Primary Secondary Amine
GCB	Graphitized Carbon Black
PET	Polyethylene Terephthalate Syringe Filter
ESI-	Negative Electrospray Ionization

SD	Standard Deviation
RSDs	Relative Standard Deviations
MRM	Multiple Reaction Monitoring
WWTPs	Wastewater Treatment Plants
MPs	Microplastics
TNPP	Tris-(Nonylphenol)-Phosphite
ER	Estrogen Receptor
RPE	Relative Proliferative Effect
17β-E2	Estrogen 17β-Estradiol
TDI	Tolerable Daily Intake
^{2}D GC	2-dimensional gas chromatography

1. Introduction

Nonylphenols (NPs) have a molecular formula of $C_{15}H_{24}O$ and a molecular mass of 220 g mol^{-1}). They have a branched molecular structure comprising phenol and a hydrocarbon tail, typically in the para position (Figure 1.1). At room temperature (20–25 °C), NPs exist as a pale-yellow viscous liquid with a density of 0.6–0.95 g/mL, a low melting point of –8 °C, a high boiling point of 304 °C, and vapor pressure of 1.33 Pa. It is soluble in common polar organic solvents such as acetonitrile and methanol but only slightly soluble in water (4.90 mg L^{-1} at 25 °C). Aqueous solutions of NPs behave as weak acids with an acid dissociation constant (p*K*a) of 10.7.[1-3]

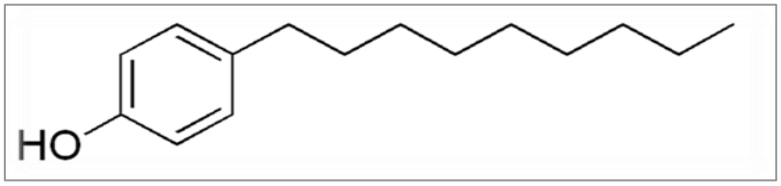


Figure 1.1: Non-branched isomer of 4-*n*-nonylphenol (NP_1).

1.1 Industrial Production and Applications of NP Isomers

NPs have numerous isomers, which can be synthesized at industrial scales through three common routes, including Friedel-Crafts alkylation of anisole and nonyl bromide, directly synthesized from nonanol and phenol by Friedel-Crafts alkylation, and the synthesis of NP isomers using 4-benzyloxyacetophenone as a precursor.[4]

(1) Friedel-Crafts alkylation of anisole and nonyl bromide (Figure 1.2a) Lalah et al. (2001) reported reacting anisole with three-bromo-3,6-dimethylheptane in Lewis acid (BBr_3) to obtain NP_{112} in a 47.3% yield. It has been found that ^{14}C-radiolabeled anisole can be used to prepare the standard isomer ^{14}C-NP_{121}. Although 3-bromo-3,6-dimethylheptane is not commercially available, it can be prepared by hydrolyzing butanone and 1-bromo-3-methylbutane using the Grignard reaction. Using the alkyl bromide and anisole reaction, Thiele et al. (2004) synthesized NP_{194} and NP_{36} in an 86:14 ratio and NP_{35} and NP_{143} in a 75:25 ratio. [5,6]

(2) Directly synthesized from nonanol and phenol by Friedel-Crafts alkylation (Figure 1.2b). In this pathway, hexyl bromide is first prepared, and then magnesium or lithium, 2-butanone, or acetone are added to produce the corresponding nonanol. Since the synthesized product is a conventional mixture, purifying it using n-hexane/ethyl acetate ester column purification to

remove ortho and dialkylated byproducts is necessary. There were numerous isomers of NP synthesized in this pathway, including radiolabeled isomers, such as NP_9, NP_{35}, NP_{36}, NP_{37}, NP_{38}, NP_{65}, NP_{96}, NP_{110}, NP_{111}, NP_{112}, NP_{119}, NP_{128}, NP_{152}, NP_{193}, NP_{194}, ^{13}C-NP_{38}, ^{13}C-NP_{112},^{14}C-NP_{38}, ^{14}C-4-NP_{112}.[7-11]

(3) Synthesis of NP isomers using 4-benzyloxyacetophenone as a precursor (Figure 1.2c). It was found that Uchiyama et al. (2008) were able to obtain the corresponding tert-nonanol by using the Grignard reaction with heptyl bromide and magnesium (or the Barbie reaction with heptyl bromide and lithium) combined with 4-benzyloxyacetophenone. After removing the benzyl protecting group using $(CH_3)_3Al/TiCl_4$, NP_{128}, NP_{37}, NP_{119}, and NP_{35} are obtained. The exact pathway was used by Katase et al. (2008) to synthesize NP_{128} and NP_{37}.[12,13]

According to Boehme et al. (2010), NPs are divided into tertiary carbon isomers, quaternary carbon isomers, and quaternary carbon isomers to yield 28 isomers. NPs with quaternary α-carbon have been obtained by Friedel-Crafts alkylation of anisole with tertiary nonyl bromides and demethylation with BI_3. Types include NP_9, NP_{35}, NP_{36}, NP_{37}, NP_{94}, NP_{95}, NP_{119}, NP_{128}, NP_{65}, NP_{111}, NP_{112}, NP_{170}, NP_{152}, NP_{194}, NP_{143}, as a result of coupling precursor p-methoxy phenyl magnesium bromide with ketones, NP_2, NP_3, NP_{10}, NP_{14}, NP_{30}, NP_{41}, NPs bearing a quaternary carbon were synthesized starting from 4′ methoxy iso-butyrophenone, obtained NP_{15}, NP_{51}, NP_{39} and NP_{98}.[14]The synthetic pathways for the three types of isomers are similar to the pathway (1), which begins with anisole or its derivatives as a precursor. In order to synthesize different NP isomers, different nonyl-branched chain structures must be constructed. Both common isomers and radiolabeled isomers can be prepared using all three synthesis methods, but rearrangement products may occur during the process. It is, therefore, necessary to purify the product after synthesis. Although NP mixtures can be separated at the analytical scale, it may need to be improved. Kim et al. (2004) used high-performance liquid chromatography to fractionate and purify technical NP, yielding 14 NP isomers.[15] The individual fractions may, however, still contain other NP isomers as impurities, affecting the quality of subsequent biological tests.[4]

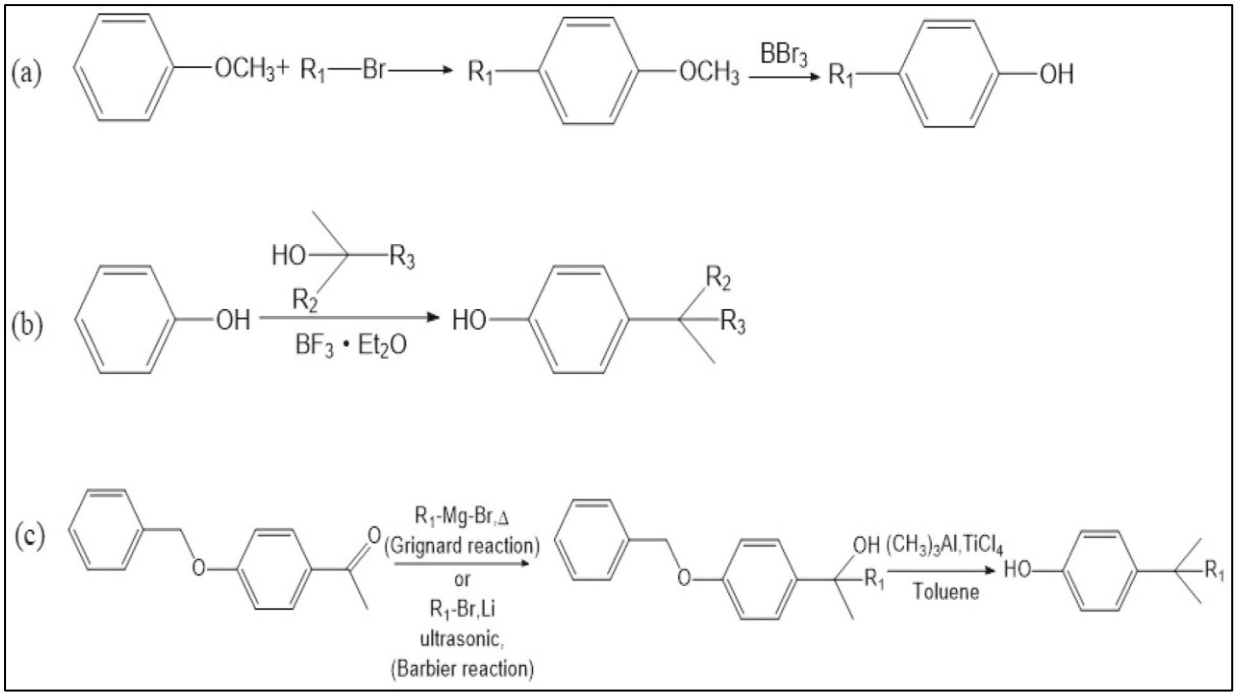
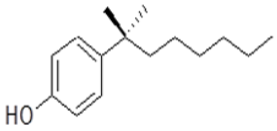
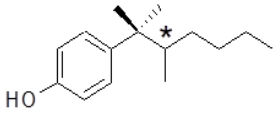
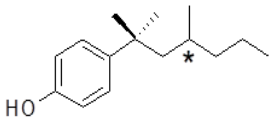
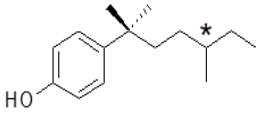
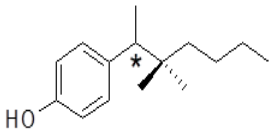
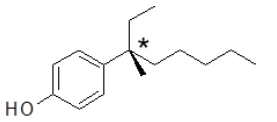
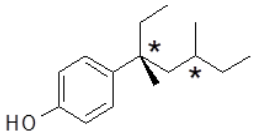
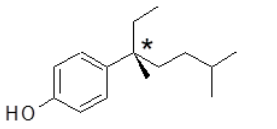
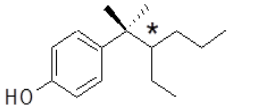
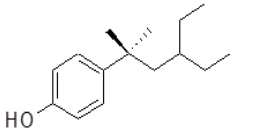
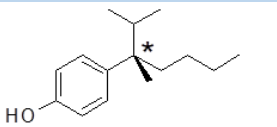


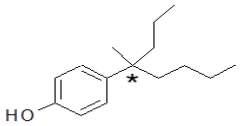
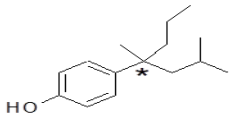
Figure 1.2: Synthetic pathways of NP isomers.[4]

The NP isomers differ either by the degree of branching of the alkyl chain or by the position of the alkyl tail on the phenyl ring. The predominant NP isomers comprise 4- or para-substituted mono-alkylphenols. Although theoretically, there can be at least 211 constitutional isomers of NPs, only <50% of these have been detected in environmentally relevant matrices. In addition, only 20 of these constitutional isomers of NPs are frequently detected in most of the environmental matrices.[16-18]

Due to numerous possible isomers of NPs, a hierarchical numbering system called Juelich Nomenclature was explicitly developed to identify NP isomers. Following the International Union of Pure and Applied Chemistry (IUPAC) rules, the Juelich Nomenclature covers 550 possible NP isomers by exploring their chirality (Table 1.1). NPs are used as (1) excipients for formulating various pesticides, (2) lubricant oil additives, (3) catalytic agents for curing epoxy resins, (4) detergents in industrial laundries, and (5) intermediates to produce NPEOs. NPEOs are essential starting materials for industrial and consumer products, including detergents, surfactants, wetting agents, dispersing agents, defoaming agents, de-inking agents, and antistatic agents. For example, tris(4-NP) phosphite, both branched and linear, is an antioxidant agent for stabilizing polymers, including polyethylene resins, vinyl polymers, polyolefins, and styrenics. According to the US Environmental Protection Agency (EPA) (2010), the demand for NPs in the US was approximately 380 million pounds in 2010 and is continuously growing.[18-21]

Table 1.1: Names, structures, and side chain lengths of some isomers of representative 4-nonylphenols (4-NPs).[22]

Juelich Number	Structure	IUPAC Name	Side Chain Length
NP$_9$		4- [1,1-dimethylheptyl] phenol	7
NP$_{35}$		4-[1,1,2-trimethylhexyl] phenol	6
NP$_{36}$		4-[1,1,3-trimethylhexyl] phenol	6
NP$_{37}$		4-[1,1,4-trimethylhexyl] phenol	6
NP$_{39}$		4-[1,2,2-trimethylhexyl] phenol	6
NP$_{65}$		4-[1-ethyl-1-methylhexyl] phenol	6
NP$_{111a,b}$		4-[1-ethyl-1,3-dimethylpentyl] phenol	5
NP$_{112}$		4-[1-ethyl-1,4-dimethylpentyl] phenol	5
NP$_{119}$		4-[2-ethyl-1,1-dimethylpentyl] phenol	5
NP$_{128}$		4-[3-ethyl-1,1-dimethylpentyl] phenol	5
NP$_{143}$		4-[1-isopropyl-1-methylpentyl] phenol	5

NP_{152}		4-[1-methyl-1-n-propylpentyl] phenol	5
NP_{194}		4-[1,3-dimethyl-1-n-propylbutyl] phenol	4

1.1.1 Estrogenic Activity of NP Isomers

Both in vitro and in vivo experiments have showed that NP isomers exhibit a greater estrogenic activity owing to their strong binding affinities to estrogen receptors (ERs). The estrogenic activity of NPs was incidentally discovered by Soto et al. (1991) while using polystyrene plasticware in routine clinical laboratory procedures. They reported that polystyrene tubes release a chemical substance with estrogenic activity, which they identified as an NP. It has also been reported that NPs encourage cell proliferation and progesterone receptor gene expression in human adenocarcinoma cells (MCF-7 cells) and cell proliferation in ovariectomized rat endometrium.[23]

Subsequent studies have demonstrated the isomer-specific estrogenic activity of NPs using fractions of tertiary branched alkyl-chain isomers of 4-NP (t-NP) and other synthetic linear NP isomers. Kim et al. (2004) fractionated t-NP into seven parts with individual estrogenic ligands using gas chromatography (GC). They evaluated the individual fractions' estrogenic activity through a yeast estrogen screen (YES) bioassay. The in vitro study demonstrated that one of the seven estrogenic ligands (NP_{37}) exhibited the highest estrogenic activity compared to that shown by t-NP or other estrogenic ligands of NP isomers. In a follow-up study, Kim et al. (2004) further separated t-NP into 14 fractions containing estrogenic ligands and tested their estrogenic activity. [9,10,12,13,15,24-29]

Surprisingly, NP_{119} exhibited the highest estrogenic activity with a relative potency (RP) several times higher than the other fractions, including that of NP_{37}, which was initially shown to exhibit superior estrogenic activity.[15] In another study, Kim et al. (2005) calculated seawater samples' relative total estrogenic activity (presumably contaminated with NPs), which was shockingly 2.7–3.0 times higher than the experimental values.[24]

However, researchers have also acknowledged that the unexpectedly high estrogenic activity exhibited by NP isomers in seawater is likely due to the inaccurate quantification of these isomers following GC's partial peak separation of their fractions. This high estrogenic activity

could also be because of the possible co-elution owing to the use of high-performance liquid chromatography (HPLC) or preparative GC. Hence, a lack of well-characterized synthetic references for NP isomers warrants more research to help improve similar analytical studies. Lu and Gan (2014) have listed the relative estrogenic potencies of synthetic 4-α-quaternary NP isomers using YES, yeast two-hybrid, MVLN cell, and E-screen bioassay techniques. The RP values determined from the minimal effective concentration (MEC) and EC50 or EC20 (50% or 20% maximal effective concentration, respectively) showed marked differences even for identical NP isomers. For instance, the range of calculated RP values for NP_{112} (0.16–1.87) and NP_9 (0.023–0.95) showed huge disparities.[30]

Most synthetic 4-α-quaternary NP isomers exhibit generally lower estrogenic activity than those exhibited by t-NP, except for NP_{128} and NP_{119}. These findings indicate that some unknown t-NP isomers might show considerably higher estrogenic activities than those currently identified t-NP isomers. Furthermore, α-tertiary isomers of t-NP, which can be considered a minor component, may contribute even more to the total estrogenic activity of t-NP than that initially indicated by their relative proportions in t-NP.[22]

Shioji et al. (2006) screened 11 isomers of 4-α-t-NP and identified two with higher RP values: NP_{176} (RP = 153) and NP_{210} (RP = 4.8).[19] Gabriel et al. (2008) identified three isomers of 4-α-t-NP, where NP_{70} exhibited the highest RP (approximately 1.7). It is necessary to confirm the occurrence of such highly estrogenic 4-α-t-NP isomers in environmental samples. However, it is practically impossible to synthesize all NP isomers and test their estrogenic activities because of their large numbers. The most feasible way to evaluate the estrogenicity of NP isomers is by understanding the relationship between their structures and estrogenic activities.[25]

Shioji et al. (2006) screened various isomers and congeners of NPs and made the following observations: (1) the alkyl chain must be of suitable length for optimal estrogenicity; (2) as branched alkyl chains tend to be crowded, bulkiness at β- and γ-carbons is responsible for exceptionally high estrogenicity at these positions; (3) presence of branched alkyl chains at α-carbon is associated with weak estrogenicity, and (4) NP isomers with α substituents exhibit reduced estrogenicity.[28] Preuss et al. (2006) suggested that α-substituents determine the degree of estrogenicity of NPs. For example, the α-ethyl-α-methyl isomer exhibited markedly higher estrogenic activity than the α-dimethyl isomers.[9]

These observations by Shioji et al. (2006) and Preuss et al. (2006) were later validated by Gabriel et al. (2008), who reported that NP isomers containing a side chain length of 4–6 carbon atoms exhibited the highest estrogenic activities. Rabinowitz and Little (2011) calculated the relative binding affinities of NP isomers to the estrogen receptor through a computational approach. They demonstrated that of the 15 strongest binding NP isomers, 13 were α-secondary isomers with no substitution next to the phenyl ring. Furthermore, substitution at the α-position tended to decrease the overall estrogenicity of an NP isomer. Hence, it can be suggested that minor components such as 4-α-tertiary NPs could greatly contribute to the overall estrogenic activity of t-NP isomers.[9,25,28]

Interestingly, Ying et al. (2012) reported that the following NP isomers can inhibit the release of testosterone in Leydig cells, though with varying efficacies: NP_{112} > NP_{65} > NP_{38} > NP_{111} > *t*-NP > 4-*n*-NP (NP_1). This anti-testosterone effect differed from the observed estrogenicity order, suggesting that different mechanisms mediate the estrogenicity and steroidogenesis of NP isomers.[29] According to Preuss et al. (2010), differences in the affinity of estrogen receptors, activation degree of estrogen receptors, or activation/deactivation state of non-receptor-mediated translations could explain the observed isomer-specific estrogenicity of NP isomers. Consequently, Preuss et al. (2010) categorized the following five NP isomers, $NP_{111a,b}$, NP_{112}, NP_{65}, NP_{37}, and t-NP, as partially estrogenic (<100% estrogenicity), based on the MVLN cell bioassay results. In contrast, they categorized NP_{38}, NP_9, and NP_1 as non-estrogenic because they exhibited similar binding affinities but lacked estrogenic activity.[31] Cormio et al. (2011) conducted a yeast androgenic screen (YAS) bioassay and demonstrated that 4-NP isomers, including NP_{112}, NP_{111}, NP_{65}, and $NP_{1,}$ did not exhibit any estrogenicity. However, the identical NP isomers showed steroidogenesis in the androgenic competition assay (anti-YAS) and maintained the same order as that obtained through the YES bioassay: NP_{111} > NP_1 > NP_{65} > NP_{112}.[32]

Given that some NP isomers possess at least one chiral carbon (center), it is essential to consider possible activity differences between enantiomers and diastereomers of each NP isomer.[10,27] Interestingly, Makino et al. (2008) noted significant differences between three diastereomers ($NP_{110\ a,b}$, $NP_{111a,b}$, and $NP_{193a,b}$) and two enantiomers (S-NP_{194} and R-NP_{194}); however, they reported little differences between S-NP_{119} and R-NP_{119}. All NP isomers possessing a β-methyl group substituent in the benzene ring generally exhibit high estrogenic activity, 2–4 times their corresponding diastereomers.[10]

Saito et al. (2007), using methods and materials similar to those of Makino et al. (2008), confirmed the enantiomeric specificity of NP isomers. *S*- and *R*-NP_{119} enantiomers exhibited equal estrogenic activity with an RP value of 4.5, whereas *S*- and *R*-NP_{194} exhibited different estrogenic activities (with RPs of 0.79 and 0.45, respectively).[27] These differences in the isomeric estrogenic activity indicate that it is generally not very useful to evaluate the risk of t-NP. Thus, constitutional isomers and even stereoisomers should be carefully considered to determine NP isomers' estrogenic activity accurately.

1.2 NPs in Food

Landfilling with sludge from NPEO-contaminated sewage or wastewater-treatment plants (WWTPs) often results in leachates contaminating groundwater and aquatic ecosystems. The purification of raw water sourced from NPEO-contaminated groundwater and other aquatic systems may result in drinking water that is highly contaminated with NPs. The unique physicochemical properties of NPEOs make them highly persistent in the environment, leading to their bioaccumulation in the biological environment. For example, NPEOs and their major metabolites (NPs and NPECs) have extremely low partition coefficients. As a result, they exhibit a high affinity to organic matter and tend to bioaccumulate in various plant and animal tissues. This bioaccumulating ability allows NPEOs, NPs, and NPECs to enter the food chain, exposing humans to their harmful effects.[33-35]

Although consuming contaminated foodstuffs is a major route through which humans are exposed to NPEOs, NPs, or NPECs, these contaminants in food samples have not been widely analyzed by global food safety agencies. Some of the available studies on the topic have reported NP concentrations in foodstuffs to be between 0.1 and 100 μg kg^{-1} f.w. (food weight). In drinking water, the levels of NPs tend to range from non-detectable concentrations (0.3 μg/L) to <7.7 ng/L in different countries.

Guenther et al. (2002) were the first to report substantial concentrations of NPs (0.1–19.4 μg kg^{-1} fresh weight) in various commonly consumed foodstuffs sampled from supermarkets in Germany. A similar study in Taiwan revealed that frozen seafood contained the highest levels of NPs, particularly oysters (235.8 μg kg^{-1}) and salmon (123.8 μg kg^{-1}). Hence, it is evident that NPs can bioaccumulate in seafood. High levels of NPs have also been reported in seafood

products from various geographical regions, including Asia, Europe, and North America. Studies from Sweden, Spain, and Florida have reported detectable levels of NPs (ranging from 5 to 50 μg kg^{-1} f.w.) in fruits and vegetables. These studies also reported the highest levels of NPs in carrots, pumpkins, apples, and citrus fruits. Another study evaluated the levels of NP bioaccumulation in the roots, stems, and leaves of lettuce and collards and reported significant variations in different parts of the plant (from 0.22 to 927 μg kg^{-1}).[36-45]

Surprisingly, NPs (ranging from 15 to 300 ng L^{-1}) have also been detected in purified mineral water packed in bottles of varying polymer materials. Another similar study in Italy reported NP levels of 7.7–84 ng L^{-1} in bottled mineral water and the water from public drinking fountains of the country. In commercial soft drinks, including juices, energy drinks, and carbonated drinks, the concentrations of NPs have been reported to be similar to those in bottled mineral water. It raises the question of whether the daily intake of NPs in humans through consuming contaminated foodstuffs and drinking water is within safe limits. [46-48]

1.2.1 Daily Intake of NPs

Various studies conducted in different countries have made reasonable attempts to calculate the average daily intake of NPs from different food sources in adult populations. For example, in Germany, Guenther et al. (2002) determined the daily intake of NPs from food sources to be 7.5 μg day^{-1}. In the USA, consuming contaminated vegetables and fruits is associated with an NP intake of 17.3 μg day^{-1}. In Italy, the consumption of contaminated seafood is responsible for a daily NP intake of 12 μg day^{-1}, mainly owing to the consumption of fish from the Adriatic Sea. Assuming a seafood intake of 31.8 μg day^{-1}, Italian adults have a hypothetical NP intake of 4.7 μg day^{-1} . In Swedish adults, a higher daily NP intake of 27 μg day^{-1} was calculated based on the total NP levels detected in a typical Swedish food basket. In Taiwan, 31.4 μg day^{-1} of NP has been reported among Taiwanese adults owing to the high concentration of NPs in commonly consumed foodstuffs. Similar studies in Germany have reported NP intakes of 0.23–0.65 μg day^{-1} kg^{-1} body weight among infants and babies because of the high concentration of NPs in commonly used infant/baby formula and foods. Even though the EU has strict restrictions in place for NPEOs and NPs, the maximum NP daily intake for babies is still 3.94 μg kg^{-1} day^{-1} even when these babies were exclusively breastfed.[36,37,42, 44,49-51]

1.3 NPs in Water and Environmental Samples

The occurrence and concentrations of NPs in the environment are associated with increased anthropogenic activities, particularly mechanized agriculture, increased sewerage treatment capacity producing vast quantities of sludge, and landfilling with solid wastes or sludge containing NPs. Because of their unique physicochemical characteristics, especially poor solubility and high hydrophobicity, NPs exhibit low bioavailability in sediments and water. Consequently, they can resist microbial biodegradation and persist in various environmental matrices for decades (Figure 1.2). NPEOs with a chain length of <5 ethoxylates are water-insoluble and lipophilic, whereas those with a chain length of >5 ethoxylates are water-soluble and hydrophilic.[34,52-55]

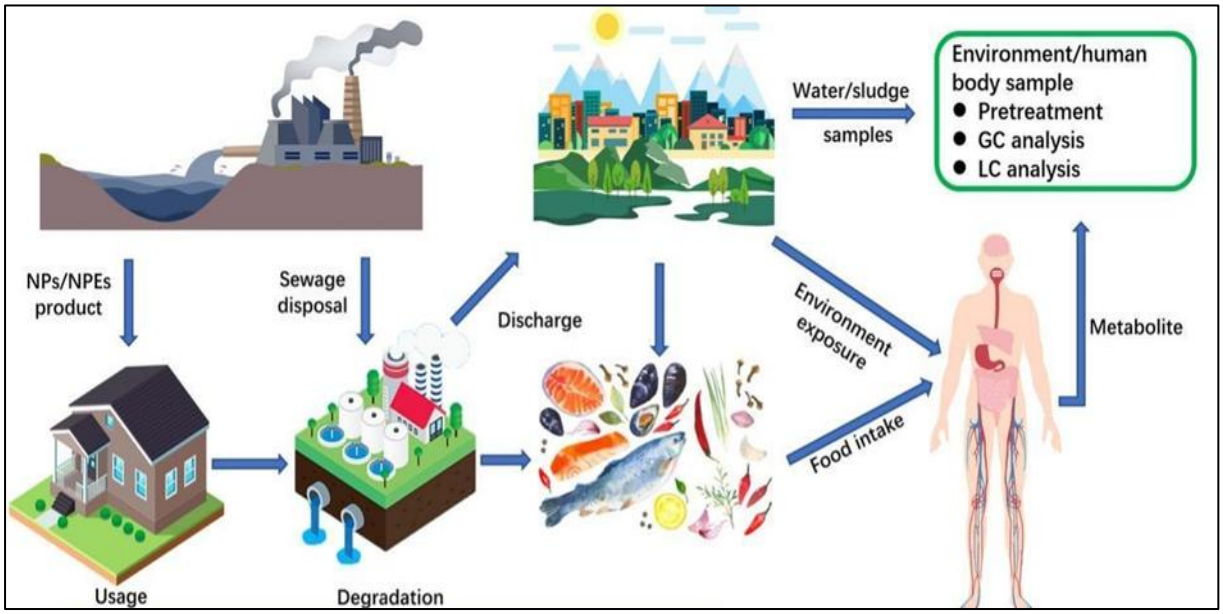


Figure 1.3: NP isomers in environmental samples.[4]

1.3.1 Environmental Biodegradation

Nonylphenol ethoxylates (NPEOs) are nonionic surfactants ubiquitously found in various environmental matrices owing to their widespread agricultural and industrial applications, including in household consumer goods.[56]

NPEO-based industrial and domestic products are first reconstituted into aqueous preparations before they can be used. They are then discharged into industrial and municipal wastewater and sewage treatment plants. Pesticides formulated with NPEOs often seep into the soil, from where they are either absorbed by plants and/or washed downstream through surface runoff to sewers, rivers, and other water bodies. In sewerage treatment plants, the biodegradation of NPEOs into the corresponding chemical constituents is a complex process comprising both aerobic and anaerobic steps involving several microorganisms at different stages.[57] In the initial phase of microbial degradation of NPEOs, the ethoxy chains of NPEOs cleave into several chemical fragments, including NPs, nonylphenoxy acetic acid (NP1EC), NP mono-ethoxylates, and NP diethoxylates.[58]

Aerobic biodegradation of NPEOs involves several aerobic bacteria that can use NPEO as the sole carbon and energy source. Aerobic bacteria use NPEOs as a carbon and energy source and decompose them into carboxylated metabolites (nonylphenoxy carboxylates), particularly nonylphenoxyacetic acid (NP1EC), NP mono-ethoxylates, and NP diethoxylates. However, *Bacillus* sp. LY has been demonstrated to possess both heterotrophic nitrification and aerobic denitrification capabilities for one-step degradation of NPEOs without forming nonylphenoxy carboxylates, particularly NP1EC nonylphenoxy ethoxy acetic acid (NP2EC) as intermediates. The primary aerobic degradation products of NPEOS include NP1EC, NP2EC, nonylphenol mono ethoxylate (NP1EO), and nonylphenol diethoxylate (NP2EO), which are highly persistent breakdown products with weak estrogenic activities. However, these estrogenic breakdown products undergo anaerobic breakdown via the mechanisms described below. NPEOs found in sewers, sludge, and aquatic environments could undergo further anaerobic biodegradation to form NPs and short-chain NPEOs.[59,60]

Several anaerobic bacteria, including *Pseudomonas, Klebsiella, Achromobacter, Staphylococcus, Sphingomonas, Ochrobactrum, Castellania, Variovorax, Cupriavidus, Ralstonia, Bacillus, and Psychrobacter* mediate anaerobic degradation of NPEOs. The most efficient anaerobic degraders of NPEOs include *Pseudomonas fluorescens* (strain Yas2) and

Klebsiella pneumoniae (strain Yas1), which use NPEOs as the sole carbon source. The anaerobic mechanism first involves cleaving ether bonds, yielding alkyl–phenol derivatives with short-chain NPEOs such as mono- and diethylated compounds (NP1EO and NP2EO, respectively). They are further cleaved to yield ethoxyacetic and acetic nonylphenol acid (NP1EC and NP2EC, respectively) intermediates. In the second step, the ethoxylated chains undergo ω-carboxylation to produce different polyethoxylated derivatives, with the predominant derivatives being diethoxylated species ($NPEO_2C$). The third step involves simultaneous cleavage or shortening of ethoxylated and alkylic chains to form propyl and heptyl diethoxylated compounds, which are finally oxidized without shortening to yield the corresponding carboxylic acids.[61]

The anaerobic biodegradation mechanism and the efficiency of NPEOs in sewage, sludge, or aquatic environments are influenced by multiple factors, including the chemical nature of the terminal electron acceptor, the initial concentrations of NPEO substrates, organic matter content, process temperature, and enzymatic inhibition by toxic intermediates. First, adding various electron acceptors, including sulfates and nitrates, effectively enhances the anaerobic biodegradation of NPs and NPEOS. Second, a high initial concentration of NPs and NPEOs inhibits the subsequent anaerobic biodegradation because of the high toxicity potential on the anaerobic. Third, anaerobic biodegradation in aquatic environments requires relatively high temperatures and low organic matter content. High organic matter shields NPs and NPEOS from enzymes or anaerobic bacteria responsible for biodegradation, while low temperature increases the activation energy required for anaerobic biodegradation.[59]

1.3.2 NPs in Water, Sediments, and Soil

NPs are chemical contaminants in almost all environmentally relevant matrices, including industrial effluent water, sewers, influents, and sludge from water treatment plants, rivers, lakes, oceans, groundwater, soil, and soil sediments. They were first detected in the early 90s in the groundwater sampled from different field sites near Zagreb, Croatia, with a mean concentration of 0.1 μg L^{-1}. Germany has reported significantly increased levels of NPs in groundwater, with the latest studies reporting the levels to be between 0.001 and 3.85 μg L^{-1}.[62-65]

The increase in groundwater contamination by NPs is attributed to increased landfilling activities (which increases the concentration of landfill leachates), increased surface runoffs from agricultural lands, and seepage from domestic septic tanks or sewer systems. The concentrations of NPs in river water typically range from 0.7 to 15 μg L^{-1}.[66,67]

Anthropogenic activities are associated with increased concentrations of NPs in groundwater (from 0.001 to 3.85 μg L^{-1}). However, these observed concentrations of NPs are time-specific, as significant seasonal variations have been reported. For example, increased microbial biodegradation and photocatalytic decomposition (induced by sunlight) of NPEOs into NPs have been observed in the summers.[58,68-71] Other seasonal factors, including the flow rate and sedimentation rate of rivers and effluent-discharge channels (influenced by the particle size), also contribute to seasonal variations in the degradation of NPEOs. In North America, Europe, and Asia, NPs in aquatic environments are associated more with sedimentation than dissolution.[53]

1.3.3 In Human Body

NPs have been detected in several human biological tissues and fluid samples, including blood/serum, amniotic fluid, urine, adipose tissue, liver, breast milk, placental/umbilical cord or serum, and semen.[67] They have also been quantified in blood samples, with results ranging from non-detectable levels to 15.17 μg L^{-1}. In an Indian population, the screening of phenolic endocrine-disrupting chemicals (EDCs) from the maternal blood plasma revealed that NPs were detectable with a mean concentration of 8.91 μg L^{-1}. Other human tissues with detectable NPs and metabolites include adipose tissues, breastmilk, urine, and hair. The maximum concentration of NPs detected in the adipose tissue was 85 μg kg^{-1} of lipids.[58,74-77]

NPs of up to 0.3 μg L^{-1} have been detected in human breast milk. The highest concentrations of NPs have been reported in the breastmilk samples of Italian women (32 μg L^{-1}), owing to their high consumption of seafood, mainly fish, which are highly contaminated with NPEOs. Fish feeding on contaminated plant materials and sediments may effectively bioaccumulate high levels of NPs, resulting in their high concentration in humans. Pomerania, located on the southern shore of the Baltic Sea in Central Europe, has one of the highest concentrations of NP-polluted foodstuffs, including seafood. As a result, NPs have been detected in high concentrations, ranging from 5.4 μg kg^{-1} to as high as 23,829.5 μg kg^{-1} dry weight, in the hairs of the residents of Pomerania.[36,51,78-80]

NPs have also been reported from the urine samples of both healthy and patient groups. An analysis of the urine samples of women with uterine leiomyoma revealed the mean NP concentrations of 2.77±2.22 μg L^{-1}. Urine samples obtained from textile and housekeeping workers exposed to alkylphenols revealed detectable NPs, with a mean concentration of 6.25±4.83 μg L^{-1}. The mean urinary concentrations of NPs in the Korean adult population are reported to be 3.7 μg L^{-1}. Similar NP levels have been reported for the Chinese adult population (1.69–27.8 μg L^{-1}).[81,82]

1.4 Overview of Comprehensive Two-Dimensional Gas Chromatography Basic Principles

Chromatography allows for rapidly resolving as many analytes as possible. A practical and comprehensive separation can be achieved using GC×GC, which exhibits complementary separations on both columns. The overlapped analyte peaks on the 1D column can be resolved on the 2D column. (Figure 1.3).

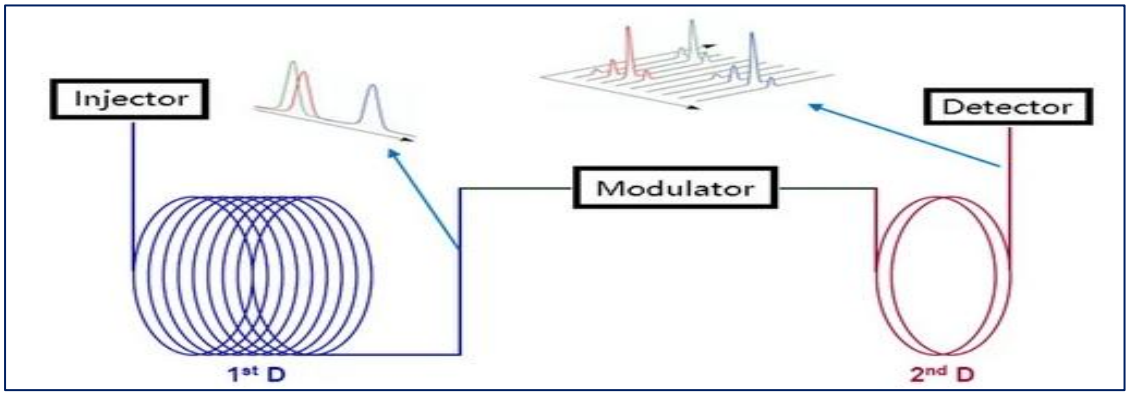


Figure 1.4: Basic operating scheme of GC×GC.[83]

A meaningful analysis of GC×GC data (single sample runs and datasets) greatly depends on the instrument design and performance (separation and detection). Various chemometric data analysis approaches can be implemented based on the results obtained from optimized GC×GC separations. With GC×GC, the aim is to utilize the capacity of 2D peaks fully. Using a sizeable ^{2}D peak capacity allows the extraction of more chemical information. In addition to improving the peak capacity, narrow peak widths also improve other peak characteristics, such as the signal-to-noise ratio (S/N).[84,85] Other properties must also be considered when optimizing the ^{1}D and ^{2}D peak widths related to the appropriate sampling density of ^{1}D separation using a modulator.[84] The sampling density (also known as the modulation ratio, MR) is the ratio between the ^{1}D peak width and the duration of the ^{2}D separation period. To achieve adequate

sampling density while maximizing the ^{2}D peak capacity, phase modulation (PM) should be conducted based on the ^{1}D peak width. The PM chosen must be able to provide a sampling density of ~2–4. A broader 1D peak width will result if the sampling density is reduced. In contrast, when the ^{1}D separation is under-sampled, ^{1}D resolution is reduced, and a potential loss in quantitative precision is noted. A practical method for simultaneously optimizing both ^{2}D peak capacity and sampling density is to first optimize the peak capacity of ^{1}D separation, generating narrow peak widths of 2–6 S, followed by a relatively short PM time of 1–2 s to obtain the optimal sampling density of 2–4. However, some applications may benefit from increasing the ^{2}D peak capacity by employing a relatively long PM (58 s).[86-92]

Typically, with such a longer PM, the ^{1}D peaks are either under-sampled or relatively broad (10−20 s), resulting in a trade-off between a reduction in the ^{1}D peak capacity and the overall ^{2}D peak capacity. To maximize the ^{2}D peak capacity of GC×GC, it is critical to select the right column, choose the right carrier gas flow rate, and program the correct temperature. In addition to the composition of stationary phases, column dimensions, and phase thickness, numerous column types have been developed recently, including ionic liquid columns and application-specific columns.[87] A sufficiently long orthogonal column set is utilized in all applications, regardless of the number of stationary phase combinations available, to achieve complementary separations on both columns.

A nonpolar 1D column is usually combined with a polar ^{2}D column. However, combining a polar 1D column and a nonpolar 2D column may be more beneficial for some applications. In GC×GC separations, the inner diameter of the column and the phase thickness significantly affect the separation and the loading-capacity efficiency. In most cases, it is preferred to use the best flow rate possible, which is determined by the length and inner diameter of the column. The temperature programming and flow rates should also be complementary to achieve the optimal separation.[88,94-99]

Significant advancements have been made in GC×GC detector technology. The detector should provide 10–20 scans per peak to obtain sufficient data for advanced chemometrics analysis.[94] However, this is a challenge for detectors generally used with GC×GC systems, where the ^{2}D separation peak widths are narrow (typically 100–250 ms).[100]

Detectors are classified based on whether they produce a single data point (univariate) or a vector of data points (multivariate) at each detected time point. The flame ionization detector

(FID) is an example of a univariate detector used with GC×GC. FID is used to detect ions produced during the combustion of organic compounds in a hydrogen flame. Identification of analytes depends solely on matching the ^{2}D retention times between a given analyte peak in a sample relative to a known peak in a standard, despite the system being simple, robust, and capable of operating at a collection frequency that is more than sufficient.[84]

With multivariate detectors, such as mass spectrometers, it is possible to obtain both ^{2}D retention times and spectral data for "fingerprinting" and identifying analytes based on known spectral databases. Because TOF–MS can have high scan rates (up to 500 spectra per second), it is the most commonly used mass spectrometry (MS) technology for GC×GC. In general, four key performance parameters are used to determine whether MS is appropriate for an application: mass accuracy, mass resolving power, sensitivity, and acquisition speed. However, for applications requiring GC×GC, the acquisition speed must also be sufficient to obtain the required data density for successful data analysis. Thus, many MS instrument types are incompatible with such GC×GC instruments.[84]

1.4.1 Modulators

The modulator is considered the "heart" of GC×GC systems. It transfers the eluate between the ^{1}D and ^{2}D separators at a specified time interval, called the modulation period. A modulator must repeatedly and precisely trap or collect the eluate before reinjecting it onto a ^{2}D column while preserving the integrity of ^{1}D separation. Three modulation technologies are available: thermal, valve-based, and flow-based.[101]

1.4.1.1 Thermal Modulation

Thermal modulation, the most commonly used technique, involves rapidly heating a ^{2}D column and trapping the analytes as they elute from the ^{1}D column. Thermal modulators can be of three general types: resistively heated trap, heated sweeper, and cryogenic focus. The latter comprises a longitude movable trap and a jet trap. In most cases, the jet trap is used, which utilizes a combination of hot and cool jets or strategically placed cryogenic gas jets. Commercially available thermal modulators have been demonstrated to be highly reliable. In addition, recently, we have produced more straightforward and cost-effective thermal modulators. Cryogen-free thermal modulators are gaining increasing popularity in both

commercial and research sectors. Cryogenic modulation can be simplified by eliminating the need for liquid nitrogen (LN_2) or carbon dioxide (CO_2).

Thermal modulation can be used in a broader range of applications. Both ZOEX and LECO Corporations have manufactured closed-cycle refrigerator-type modulators. ZOEX Corporation has introduced the ZX2 thermal modulator, which uses a closed-cycle refrigerator/heat exchanger to create a two-stage loop modulator capable of modulating C_7 and above.[102-105] J&X Technologies introduced the first thermal modulator based on commercial thermoelectric cooling, which allows tailoring the modulation to a specific range of volatility, ranging from C_8 to C_{40}. Despite being relatively new, this modulator can have various industrial applications (Figure 1.4). A consumable-free single-stage modulator using coated stainless-steel capillary traps has also been developed. To complete the desorption step, a capacitive discharge power supply is operated to resistively heat the trap, while cooled ceramic pads are employed to complete the cooling process.[106-108].

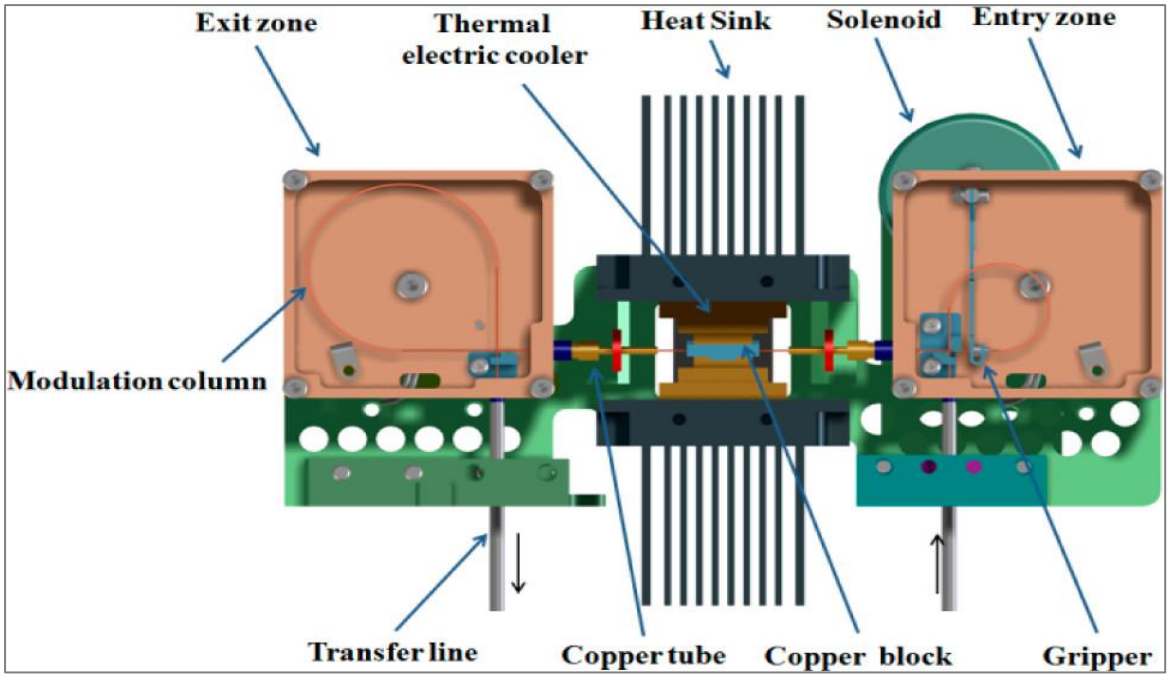


Figure 1.5: Key components of the thermal modulator designed by J&X Technologies based on thermoelectric cooling.[109]

A cost-effective approach to thermal modulation, in the form of a "Do-It-Yourself" interface, was introduced using two low-cost commercially available components.[110] The segmented loop-based thermal modulator is simple, does not require cryogens, and has many applications.

Although cryogen-free modulators might be more suitable for academic research and private-sector applications, the use of cryogens in thermal modulators remains prevalent.

The single-stage jet trap modulator is the simplest form of thermal modulator because it does not require moving parts. However, it is often plagued by various breakthroughs (e.g., not all ^{1}D eluate is trapped). Gorecki et al. demonstrated that adding silica wool to restrict the jet point can increase the trapping efficiency by slowing down the flow during the desorption stage to prevent the breakthrough (Figure 1.5).[111] The analytical performance of the restricted modulator is comparable to that of a quad-jet modulator.

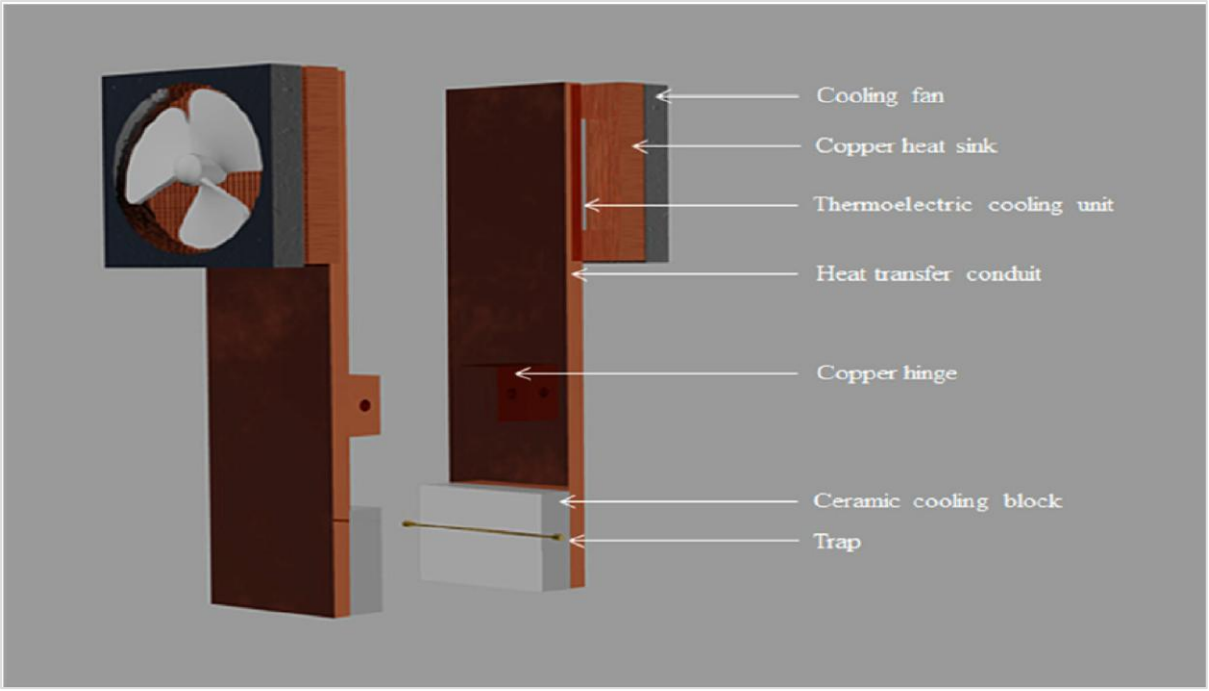


Figure 1.6: Thermal modulator with a single-stage consumable-free design.[107]

Loop-based modulation was introduced to override the difficulties associated with single-stage modulation. In loop-based systems, one set of jets traps and then desorbs the eluate, which travels around the loop before being trapped and desorbed onto the ^{2}D column. In GC×GC, the carrier gas velocity at the point of reinjection (desorption) is an important parameter. For loop-based thermal modulation, the ^{1}D and ^{2}D separations are serially coupled (Figure 1.6). Thus, the ^{2}D flow rate is directly influenced by the length, diameter, and flow rate of the ^{1}D flow. A bleed line can be added at the end of ^{1}D between the outlet of the modulator loop and the ^{2}D column to optimize the reinjection conditions. Other studies have used a similar technique to improve the separation on a ^{2}D column.[112-115]

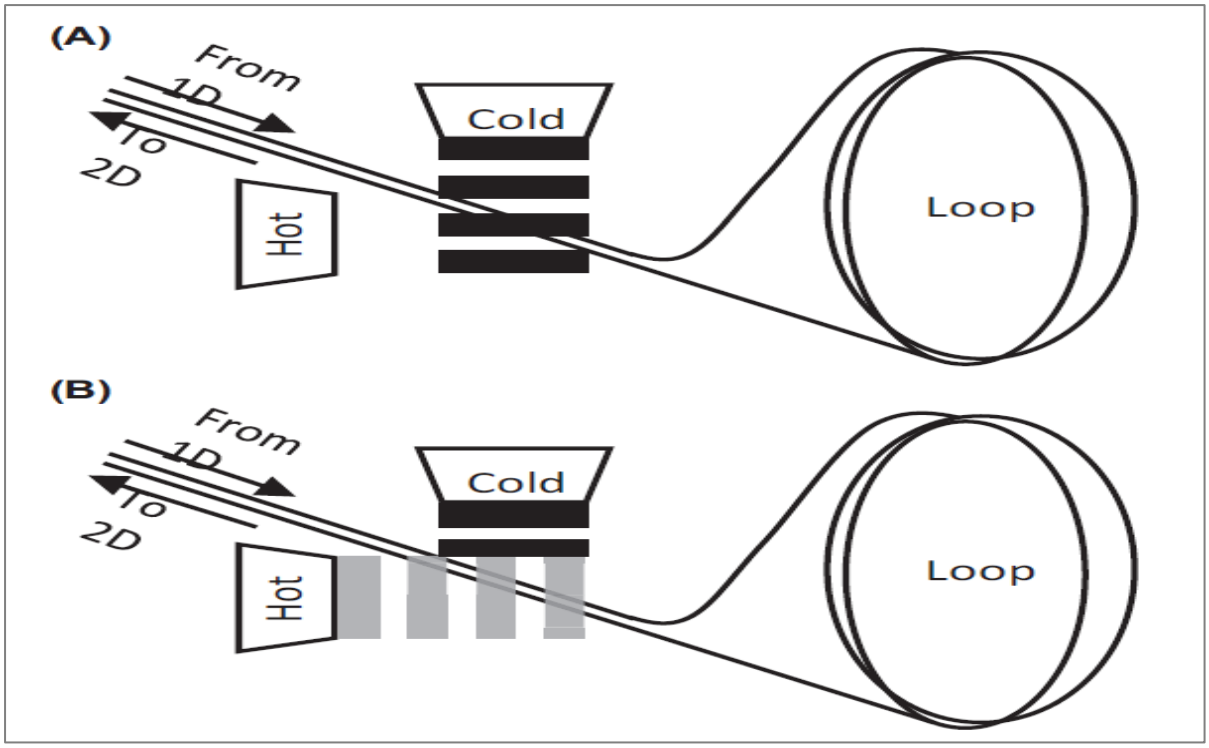


Figure 1.7: Modulation process of the dual jet loop modulator. A looped capillary column passes through both jets to provide dual-stage modulation using only two jets. Cold jets create two parallel cold spots in the loop upstream and downstream. The trapped analytes are periodically remobilized using the hot jet in each section of the column. Owing to the upstream cold spot, the analytes released along with the potential breakthrough are refocused in the downstream cold spot, while those released from the downstream cold spot are injected into the ^{2}D column.[116]

1.4.1.2 Valve-Based Modulation

Valve-based modulators collect the ^{1}D eluate in a short collection loop instead of using thermal zones to trap it. The ^{1}D eluate is then flushed and directed onto the ^{2}D column. Valve-based modulators can modulate compounds across a range of boiling points (e.g., C_1 to C_{40}+), are relatively simple in design, and require minimal consumables. Although GC×GC instruments with valve-based modulators are not as sensitive as those with thermal modulators, they require high flow rates on ^{2}D columns for the collection loops to be flushed quickly. Valve-based modulation was first performed for GC×GC using a diaphragm valve.[117] The ^{2}D peak widths obtained were relatively narrow, with excellent $^{2}t_R$ reproducibility. However, only 10% of the eluate reached the detector, resulting in a loss of sensitivity. The valve had a temperature limit

of 175 °C. The modulation performance can be improved by implementing a sample loop and using higher flow rates for ^{2}D separation.[118] The diaphragm valve technology for modulation in GC×GC is being continuously improved. A commercially available valve that can replace the temperature-sensitive O-ring with a perfluoro elastomer-based O-ring has recently been proposed to overcome the temperature limit of 175 °C (Figure 1.7), affording reliable performance to up to 325 °C.[119]

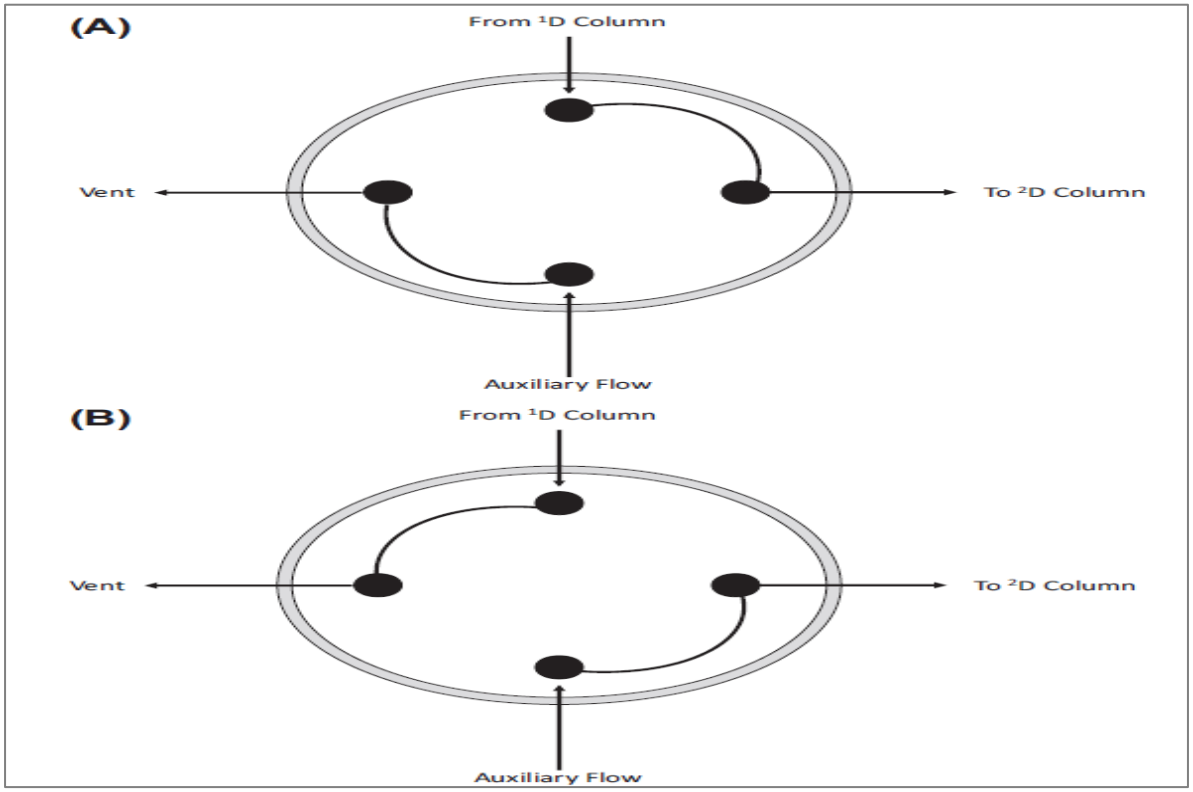


Figure 1.8: Diaphragm valve modulator showing (A) the effluent entering the secondary column and (B) the primary effluent venting to the atmosphere during the second separation.[116]

Although only 30% of the injected material makes it to the FID detector, the detection sensitivity is still eight-fold higher than that obtained using ^{1}D-GC under the same conditions. GC×GC with high-temperature diaphragm valve modulation is compatible with TOF–MS detection at a 3 mL/min flow rate for ^{2}D separation.[119] A high-temperature diaphragm valve is used for comprehensive three-dimensional gas chromatography coupled TOF–MS detection (GC×GC–TOF–MS). The modulators of GC3 comprised quad jets of LN_2 with a high-temperature diaphragm valve.[120]

1.4.1.3 Flow Modulation

Flow modulation has been gaining increasing popularity as a modulation method for GC×GC. Flow modulation is similar to valve-based modulation, except for the coupled two-column flows. Seeley (2006) pioneered a flow modulation design in which 100% of the ^{1}D eluate was transferred to the ^{2}D column for detection. Many subsequent fluidic modulator designs were based on this original design. The critical event that precipitated the broader adoption of flow modulation was the introduction of capillary flow technology (CFT) by Agilent Technologies. Many companies subsequently introduced their commercial versions of the flow modulator as well.[115] SepSolve recently introduced the INSIGHT valve-based reverse fill/flush modulator (Figure 1.8).[84,121,122]

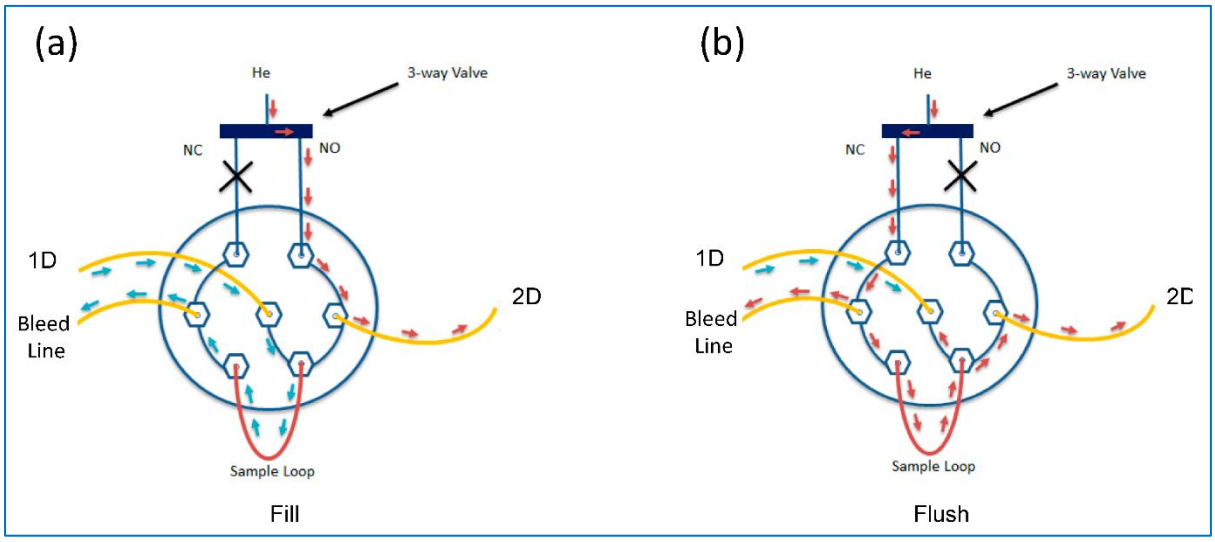


Figure 1.9: (a) Flow path of Insight RFF flow modulator fill stage; (b) flush stage.[123]

1.4.1.4 Modulator Comparisons

The first thermal and flow modulation comparisons were reported in 2011 when a quad jet thermal modulator capable of modulating LN_2 was compared with the CFT modulator designed by Agilent Technologies.[124] Modulation of compounds with $<C_{10}$ is better accomplished with a differential flow modulator, whereas compounds with $>C_{10}$ are more effectively focused and modulated using a liquid cryogen modulator. Both techniques could adequately separate light crude oil. However, each modulator must use different instrumental parameters (e.g., flow rates) for effective separation.

In recent years, SepSolve INSIGHT flow modulators have been successfully used for the GC×GC analysis of volatile organic compounds (VOCs) using cryogenically modulated GC×GC–TOF–MS techniques adapted for reverse fill/flush flow modulators. It is possible to modulate the flow of VOCs down to C_4 to achieve separations similar to those obtained by cryogenic modulation.[122] Because the flow splitting was used, only 20% of the injected sample reached the TOF–MS; nevertheless, a limit of detection (LOD) of 1−10 ppb was achieved.[84]

1.4.2 GC × GC-TOF-MS Used in this Work

A gas chromatograph 7890 B (Agilent Technologies, Palo Alto, CA, USA) with liquid nitrogen cryogenic modulation (consumable-free ZX2 thermal modulator) has been used in this study as part of the GC×GC–TOF–MS analysis. For cryo focus, liquid nitrogen was used at a 7.5 mL/min flow rate. Following elution from the first dimension (^{1}D) column, heated air was released into the second dimension (^{2}D) column to release and reject these compounds. The modulation period was 4 seconds, and the duration of the hot jet was 0.40 seconds. A 30 m × 0.25 mm i.d. ×0.25 μm df DB-WAX Ultra Inert (Agilent Technologies, Bellefonte, PA, U.S.A.) was coupled to a 1.7 m × 0.10 mm i.d. × 0.10 μm df MEGA-17 MS FAST (MEGA s.n.c., Legnano, MI, IT) as the second dimension. Both columns eluted compounds according to their polarity, with lower polarities eluting earlier than those with higher polarities.[125]

Mass spectrometry is commonly used to identify analyte peaks. This study used time-of-flight mass spectrometry (TOF-MS) (Markes International Ltd, Llantrisant, RCT, UK) because of its fast acquisition speed and full scanning range. Standard electron ionization (EI) involves an energy of 70 eV. Due to the unique design of the ion source, electrons do not cluster around the filament. The result is a reduction in spectral noise, a reduction in fragmentation, an increase in selectivity, and an increase in molecular ion intensity. To identify specific isomers of NPs coffee samples, hard-ionization-energy mass spectra (70 eV) were utilized in conjunction with GC x GC separation and resolution.[123]

1.5 Objective and outline of the study

The main objectives of the present study were to analyze the endocrine-disrupting NPs and NPECs in food samples, determine the daily intake of NPs for German adults after the implementation of Directive 2003/53/EC[126], and identify NPs isomers in the tested food samples.

Besides the introduction (chapter 1), the thesis consists of three chapters, each concerned with one aspect of the initial hypothesis. Guenther et al. (2002) study showed that NPs could be detected in relevant concentrations in 0.1-19.4 µg kg^{-1} in all examined food from shops and supermarkets in Germany. Moreover, the daily intake for an adult was calculated to be 7.5 µg.[36] Following that, the Directive 2003/53/EC was formulated in 2003 and implemented in all EU countries by 2005.[126] Furthermore, (NPECs) are present in many environmentally relevant matrices, such as influent and effluent water from sewage treatment plants, river water, seawater, surface water, groundwater, drinking water, and sediments and soil.[58] However, research on the determination of NPECs in food samples has not been widely conducted. This project's research tested the presence of NPECs in the same food samples.

NPs exhibit estrogenic activity depending on their structural features and degree of branching and bulkiness. Some NP isomers showed isomer-specific biodegradation behavior in an activated sludge bioreactor, enriching recalcitrant isomers and potentially having different estrogenic activities. The endocrine-disrupting effects of NPs include harmful impacts on the human body, such as the reproductive, immune, and nervous systems. Young animals and humans are susceptible to EDCs during their development phases. [127-130]

Hence, it is necessary to consider the nonylphenol problem from an isomer-specific viewpoint, including food and health studies. Until now, only total nonylphenol concentrations were analyzed in foodstuffs. The final part of the research addressed which NPs isomers could be identified in coffee samples. Therefore, Thirteen NP isomers have been injected into the GC x GC-TOF-MS system and used as a cross-reference library to identify NP isomers based on the molecular ion (m/z 220), characteristic fragment ions, and retention time on the coffee samples. The application of GC x GC Tof-Ms provides sufficient separation of a complex mixture of food samples, allowing us to identify the NPs isomers in coffee samples. Until this work, identifying a single isomer of NPs in food samples has not been achieved.

The final chapter of the thesis presented the conclusion (chapter 4). It explains the research questions' experimental results and suggests future studies regarding the isomer-specific analysis of NPs in food samples.

2. Determination of endocrine-disrupting nonylphenols and nonylphenol carboxylates by high-performance liquid chromatography-tandem mass spectrometry: Levels in German food after restriction

This chapter is adapted from N. Aashed and K. Günther, Determination of endocrine-disrupting nonylphenols and nonylphenol carboxylates by high-performance liquid chromatography-tandem mass spectrometry: Levels in German food after restriction, Analytical Letters, DOI: 10.1080/00032719.2021.1956515.

2.1 Introduction

Alkylphenol ethoxylates (APEOs) are nonionic surfactants with widespread applications in the agricultural, industrial, and domestic sectors.[56] For ease of handling, they are generally employed in their aqueous forms; however, using such aqueous preparations increases the potential of their discharge into municipal and industrial wastewaters and, eventually, into sewage treatment plants. The biodegradation of APEOs during sewage treatment is a complex process involving several microorganisms and different stages. In particular, the microorganisms metabolize the ethoxy chains of APEOs to form several products, including alkylphenols, alkylphenoxy acetic acid, and alkylphenol mono- and diethoxylates. These products are endocrine-disrupting chemicals that are persistent and accumulate in the environment, thereby posing severe threats to human health. Furthermore, nonylphenols (NPs) and nonylphenol carboxylates (NPECs) are present in many environmentally relevant matrices, such as influent and effluent water from sewage treatment plants, river water, seawater, surface water, groundwater, drinking water, and sediments and soil.[33,36,52,58,131-135]

Among the various APEO metabolites, NPs are the most dangerous and present a high risk to human health.[136-138] The European Union (EU) Water Framework Directive has listed NPs as a priority hazardous substance. NPs exhibit estrogenic activity depending on their structural features and degree of branching and bulkiness.[6,9,14,25,57,127,139,140]

To identify the individual NP isomers among the 211 possible constitutional isomers of NP, a numbering system for the individual NP isomers (Juelich Nomenclature) was developed. A hierarchical system was used to number the isomers in adherence with the International Union of Pure and Applied Chemistry (IUPAC) guidelines. This system covers 550 possible compounds and considers the chirality of the C-atoms.[18]

In 2003, Directive 2003/53/EC was passed, which amended Council Directive 76/769/EEC of the European Parliament and the Council of the European Union and relates to the restrictions on the marketing and usage of certain dangerous substances and preparations, such as NPs, NPEOs, and cement.[126] The directive established that using NPs and NPEOs at concentrations equal to or higher than 0.1% by mass is prohibited in industrial applications such as pesticide formulations, industrial cleaning, industrial textile production, and metal-work industries.[58,126] The reason for implementing this EU directive was the research by Guenther et al. (2002), which showed that nonylphenol could be detected in relevant concentrations in all examined food from shops and supermarkets in Germany.[36] The long and intensive worldwide consumption of precursor compounds of nonylphenols has led to the ubiquitous presence of estrogen-active compounds in commercially available foods.

Although diet is a significant route of exposure for humans, research on the analysis of NPECs in food samples has not been widely conducted. The presence of NPs in many food items, including packaged food purchased from supermarkets in Germany and commercial whole milk in Spain, among others, have been analyzed for the presence of NPs. Seafood and various edible marine species in Asia, Europe, and North America, as well as vegetables and fruits from Sweden, Spain, and the USA, have also been analyzed for the presence of NPs. NPs have also been detected in commercially bottled water, drinking water from public fountains in 35 cities across Italy, bottled mineral waters, and commercial soft drinks.[36,38,39,41-44,46-48,108,129,141-144]

The average daily intake of NPs according to the concentrations measured in foods and the expected consumption rates were determined to be between 0.067 and 0.370 $\mu g\ kg^{-1}\ day^{-1}$ for adults (assuming a body weight of 60 kg) and 0.36–0.60 $\mu g\ day^{-1}$ from bottled drinking water.[36,39,40,42,44,46,132]

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been employed for the analyses of drinking water, foods, and other environmental samples to reveal the presence of NPs and NPECs.[54,130,144,145]

The primary objective of this study was to reinvestigate the presence of NPs in the foods examined by Guenther et al. (2002) and the daily intake of NPs, which were obtained from German markets following the implementation of the 2003/53/EC Directive in 2005.[36,126] The secondary objective was to examine whether NPECs are present in the same food samples.

2.2 Materials and Methods

2.2.1 Standards Reagents, Chemicals, Food Samples, and Materials

Ultrapure water (18.2 MΩ·cm) obtained from a Milli-Q apparatus (Merck Millipore, Darmstadt, Germany) was used throughout the work. Technical-grade NPs (a mixture of NP isomers) were obtained from Fluka (85%, Buchs, Switzerland). The $^{13}C_6$–NP_{112} (^{13}C in benzene ring) internal standard (IS) was acquired from Sigma-Aldrich (ring-$^{13}C_6$ 99%, Darmstadt, Germany), and the standard for analysis of NPECs, $^{13}C_1$–$NP_{112}EC$ (^{13}C in the carboxylic group), was obtained from company ChemCollect (Wuppertal, Germany). The numbering of the nonylphenols (NP_{112}) was done according to the internationally used Juelich Nomenclature (Guenther et al. 2005). Acetonitrile (ACN, 99.9%), methanol (99.9%), and acetic acid (≥99.8%) were of the LC-MS grade and were purchased from VWR Chemicals (Langenfeld, Germany). Cyclohexane and isooctane (99.5%) were of the LC-MS grade and were obtained from Promochem (Wesel, Germany). Assay-grade sodium chloride (NaCl) was purchased from Fisher BioReagents (USA), and anhydrous sodium sulfate (Na_2SO_4, ≥99%) and magnesium sulfate ($MgSO_4$, ≥99%) was obtained from Roth (Karlsruhe, Germany). Hydrochloric acid [HCl; 37% (w/w)] was acquired from AnalaR Normapur (Paris, France). The QuChERS dispersive solid phase extraction (DSPE) clean up Kit contained a mixture of $MgSO_4$ (150 mg), primary secondary amine (PSA,50 mg), end-capped C18 sorbent (C18E,50 mg), and graphitized carbon black (GCB,50 mg)) and was obtained from Phenomenex (Aschaffenburg, Germany), polyethylene terephthalate (PET) syringe filter (0.20 µm) was purchased from Macherey-Nagel (Dueren, Germany).

Table 2.1: Packaging materials of the investigated foodstuffs.

Glass	Plastic bag/Plastic foil	Paper	Plastic beaker (polypropylene)	Aluminum foil/Aluminum tube	Beverage paper carton	Can
Peanut's cream	Liver sausage	Sugar	Lard	Mayonnaise	Dairy products	Tuna
Gooseberry marmalade	Spinach	Butter	Fresh cheese		Orange Juices	Pineapple
Beer	Apples	Tea			Pasta	
	Tomatoes					
	Potatoes					
	Bread					
	Coffee					
	Chicken					
	Chocolate					

2.2.2 Food Sample Preparation

All foodstuffs were purchased from German retail stores and packed using typical procedures (Table 2.1) before storage at either 4 °C or 25 °C until required for analysis. Non-solid foodstuffs, including pastes and liquid samples, were extracted without pre-treatment. Solids were homogenized before extraction using an Ultra-Turrax 18/10 (IKA-Werk, Staufen, Germany) or a Moulinex Moulinette AD56 (Spain).

2.2.3 Nonylphenols Extraction

The steam distillation apparatus designed by Veith et al. (1977) was fitted to a 2 L distillation flask (bottom) and a reflux condenser (top) and was used to extract the NPs from the food samples by applying the continuous steam distillation/solvent extraction technique.[146] A solution of NaCl (40 g) in water (600 mL) acidified with 2 mL HCl was used as the extraction solvent. The purification process consisted of boiling the condensate in the Veith apparatus with a cyclohexane/isooctane mixture (20 mL, 1:1 v/v) for 5 h using heating mantels obtained from Witeg (Wertheim, Germany). Fresh solvents were used to replace the aqueous and organic phases. The food sample of interest (10 g) was suspended in the distillation flask with the extraction solvent, and the IS (10 µL, $^{13}C_6$–NP_{112} in methanol (0.5 µg mL^{-1})) was added. The extraction process required 5 h using the cyclohexane/isooctane mixture (20 mL). The phases were then collected in a volumetric flask (100 mL). The organic layer was separated, dried using anhydrous Na_2SO_4, filtered, and concentrated under a stream of nitrogen until complete dryness was achieved. The dried residue was re-dissolved in methanol (1 mL), and the samples were stored in the dark at 4 °C before duplicate analysis. Prior to the extraction of the food samples, the required procedural blanks (extraction solvent with 10 µL IS) were run.

2.2.4 Clean up and Extraction of the Carboxylates

NPECs were extracted following a modified version of a previously reported extraction process [147]. A solution of $^{13}C_1$–NP_{112}EC in methanol (0.5 µg/mL) weighed into a 50 mL polypropylene tube was used as the IS. The IS (10 µL) was spiked into the homogenized sample (10 g), and ACN (10 mL) containing 1% v/v acetic acid was added along with Milli-Q water (5 mL). The resulting mixture was shaken in a rotary shaker end-over-end for 1 h. A mixture of salts (6 g $MgSO_4$ and 1.5 g NaCl) was added and shaken for 1 min. Centrifugation was carried out at 7000 rpm for 7 min, and an aliquot (1.5 mL) of the ACN layer was transferred into a 2 mL polypropylene microtube for matrix removal. This tube contained a mixture of $MgSO_4$ (150 mg), PSA (50 mg), C18 (50 mg), and GCB (50 mg). The extract was then filtered through a PET syringe filter into an autosampler vial for LC-MS/MS analysis.

2.2.5 LC-MS/MS Analysis

A Shimadzu HPLC (LC-20AD, Kyoto, Japan) equipped with an AB Sciex QTrap 4500 (Darmstadt, Germany) was used to identify and quantify the NPs and NPEC.

2.2.5.1 Nonylphenols

A Phenomenex Luna C18 column (3 µm; 150 × 2.0 mm i.d, Phenomenex, Aschaffenburg, Germany) was used at 60 °C for HPLC analysis. A binary pump system was utilized for the chromatographic separation process. The mobile phase comprised solvent A (methanol) and B (water). A linear gradient program with 50% A at 0 min, 95% A from 0 to 16 min, holding at 95% A for 10 min, and return to 50% A over 0.2 min was used, and the HPLC column was reconditioned for an additional 5 min with 50% A prior to subsequent sample introduction. The extract (5 µL) was introduced into the column at a flow rate of 0.2 mL min^{-1}. MS detection was performed using a Turbo Ion Spray® ionization source, and tuning was performed for negative electrospray ionization (ESI-) analysis. The block source temperature was set at 650 °C, and the values for the gas flow were as follows: curtain gas, 25 psi; nebulizer gas, 40 psi; turbo gas, 60 psi; and collision gas, 40 psi. The remainder of the parameters were as follows: delustering potential (−85 V), entrance potential (−10 V), collision exit potential (−9 V), and ion-spray voltage (−4000 V). Tandem MS in the multiple reaction monitoring (MRM) mode alternating between the two transition reactions for NPs (219 → 133 and 219 → 147 *m/z*) and its IS (225 → 139 and 225 → 153 *m/z*) with a dwell time of 150 ms was conducted for the quantitative analysis. Analyst® software version 1.4.2 was used to perform data processing.

2.2.5.2 Nonylphenol Carboxylates

HPLC analysis was conducted at 25 °C using a Phenomenex Synergi Polar-RP 80A, 4 µm; 250 × 3.0 mm i.d. column (Phenomenex, Aschaffenburg, Germany). The mobile phase comprised solvent A (ACN) and solvent B (water containing 0.1% acetic acid). The linear gradient program was 35% A at 0 min, 75% A from 0 to 25 min, held at 95% A for 2.50 min, and returned to 35% A over 2.50 min. The HPLC column was reconditioned for the extraction with 35% A for 2.50 min. After that, 5 µL of extracts were injected into the column at a flow rate of 0.4 mL min^{-1}. MS detection and tuning were performed as described above. The block source

temperature and gas set values were the same as those used in the abovementioned NPs analyses, except for the collision gas and the decluttering potential, which were set at 60 psi and −80 V, respectively. Tandem MS in the MRM mode alternating between the two transition reactions for NPECs (277 → 218 and 277 → 133 *m/z*) and its IS (278 → 219 and 278 → 134 *m/z*).

2.2.6 Calibration and Response Factors and Quantification

The isotopic dilution approach was used to quantify NPs and NPECs (analyte/IS area ratio (= *y*)) versus the analyte/IS concentration ratio (= *x*). Six calibration levels in the 0–50 ng mL^{-1} range were used. Each level contained IS at a concentration of 5 ng mL^{-1}. NPs and NPECs exhibited excellent linearities, with correlation coefficients of R > 0.999. The reagent assay was systematically measured with each food sample because NPs are ubiquitous, and nanogram-per-liter-level background contamination can occur from solvents and the laboratory environment. The final equation used for expressing the concentration of NPs (or NPECs) in μg kg^{-1} is as follows:

$$\text{NPs or NPECs} = \left(\frac{\frac{Aa}{Ais} - I}{S}\right) x\ c(IS), \quad (1)$$

where A_a is the peak area of NPs or NPECs in the blank or the sample; A_{is} is the peak area of $^{13}C_6$–NP_{112} or $^{13}C_1$–$NP_{112}EC$; *I* and *S* are the intercept and slope of the regression lines, respectively; and *C(IS)* is the concentration of the internal standard. The final concentrations of NPs in the food sample were calculated as follows:

The concentration of NPs in the food sample − concentration of NP in the blank = the final concentration of NPs in the food sample. (2)

2.3 Results and Discussion

2.3.1 Recoveries and Limits of Detection

An IS, $^{13}C_6$–NP_{112} or $^{13}C_1$-$NPEC_{112}EC$ (10 μL), was added to each food sample (duplicate extraction), and the appropriate procedural blank was used to validate the analytical procedure. The procedural blanks yielded efficiencies ranging from 90 to 100% with relative standard deviations (RSDs) of ≤4.2%, whereas the different food samples presented high variations, with recoveries of 16–127% and RSDs of 4–17%. Foodstuffs with high-fat contents, such as butter and eggs, exhibited low recoveries (<25%). Consequently, the NPs concentration

calculations were rectified based on the IS recoveries. Procedural blanks were spiked with $^{13}C_1$–$NPEC_{112}EC$ for NPECs analysis and $^{13}C_6$–NP_{112} for NPs analysis to obtain mean blank values of 5 ± 0.11 and 5 ± 0.05 ng for the NPs and NPECs, respectively, after which the limits of detection (LOD) were calculated. The LODs were 0.14 ng for NPs and 0.08 ng for NPECs (mean blank value + (3 × SD)). The limits of quantitation (LOQ) were 0.12 ng for NPECs and 0.17 ng for NPs (mean blank value + (6 × SD)). Figure 2.1 shows the LC-MS/MS chromatograms for NPs in pineapple, $^{13}C_6$–NP_{112} in pineapple, and $^{13}C_1$-$NPEC_{112}$ in apples, and no NPECs were detected in any food samples that were tested.

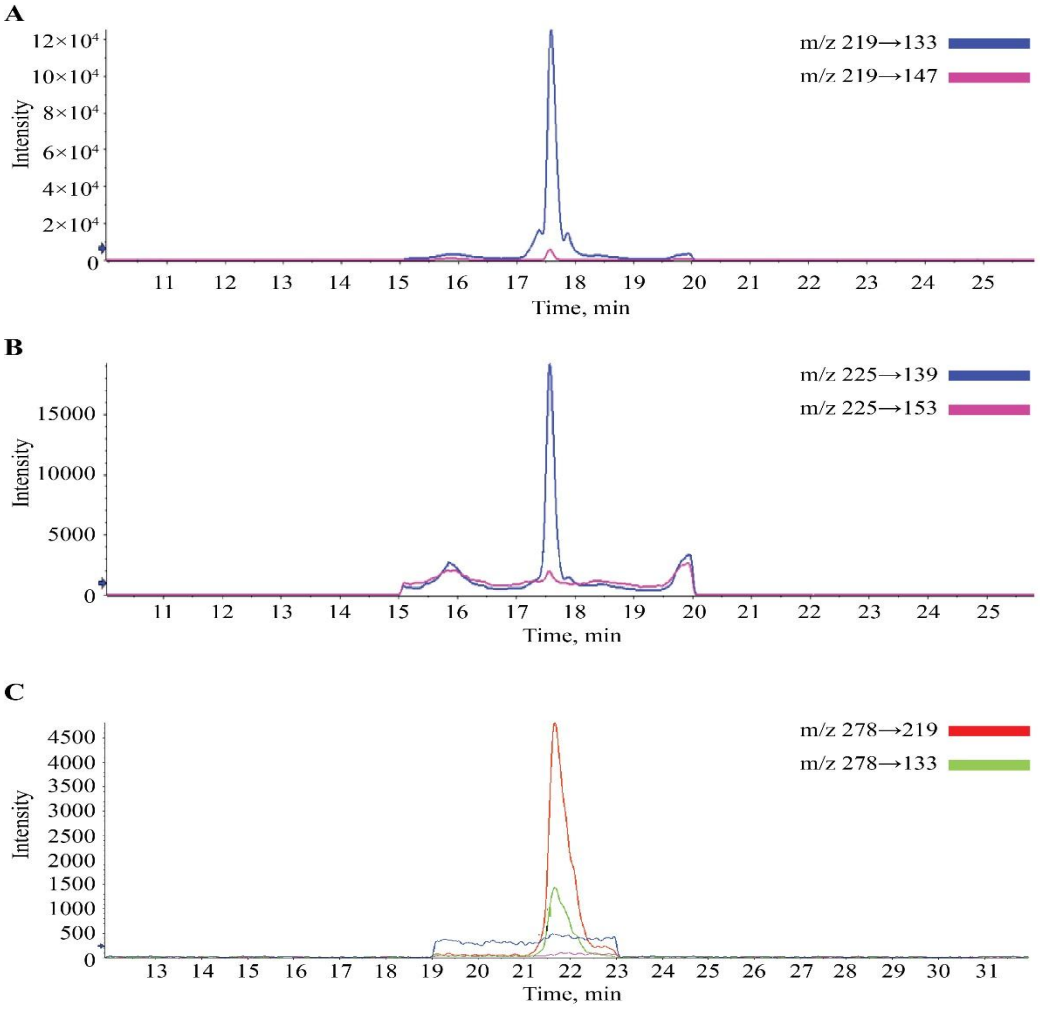


Figure 2.1: (A) LC-MS/MS chromatograms for NPs in pineapples (MRM mode: 219 → 133 and 219 → 147). LC-MS/MS chromatograms for the internal standard (IS) (B) $^{13}C_6$–NP_{112} in pineapples (MRM mode: 225 → 139 and 225 → 153), and (C) $^{13}C_1$–$NP_{112}EC$ in apples (MRM mode: 278 → 219 and 278 → 133). Here, 10 g of food sample was spiked with 10 µL of IS concentration (0.5 µg/mL^{-1}).

2.3.2 Presence of Nonylphenols in the Food Samples

Among the 24 food categories analyzed, NPs were detected in 22 samples ranging from 0.1 to 6.3 µg kg^{-1} on a fresh weight basis (Figure 2.2). These concentrations are lower than those reported by Guenther et al. (2002) (i.e., 0.1–19.4 µg kg^{-1}); however, our findings show that NPs remain in most food samples, even after 14 years of the implementation of the 2003/53/EC Directive in 2005.[36,126]

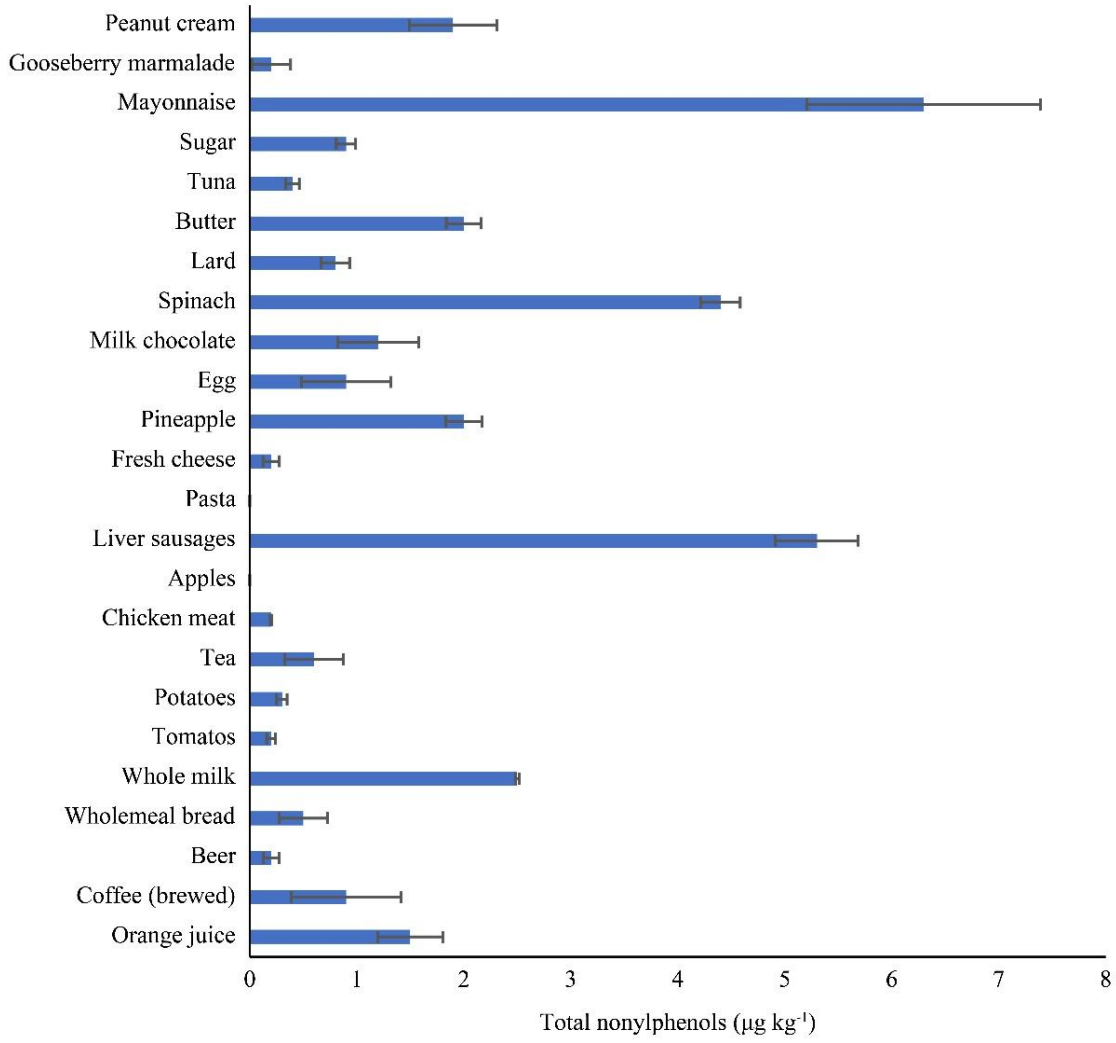


Figure 2.2: NPs concentrations in food samples from Germany were selected based on the 24 different food types. Concentrations (sum of all NPs isomers, mean values with standard deviations, n = 2) are expressed on a fresh weight basis.

Among the various samples examined, mayonnaise, liver sausages, and spinach presented the highest concentrations of NPs at 6.3, 5.4, and 4.4 µg $kg^{-1,}$ respectively, whereas the levels in pasta and apples were below the LODs. Compared to our previous findings [36], the present results show that the NPs levels in apples decreased from 19.4 µg kg^{-1} to below the LOD. The levels of NPs in other foodstuffs also decreased, including those of butter (from 14.4 to 2 µg kg^{-1}), lard (from 10.2 to 0.8 µg kg^{-1}), milk chocolate (from 14.1 to 1.2 µg kg^{-1}), and tomatoes (from 18.5 to 0.2 µg kg^{-1}). However, the concentration of NPs in spinach was nearly three times higher than that reported previously [36]. The difference between the packaging materials used in the work by Guenther et al. (2002) (i.e., paper packaging) and the materials used in this work (i.e., plastic bags) could account for the differences in the NPs levels.

Indeed, food packaging materials are a significant source of NPs, which are generated by the decomposition of tris(nonylphenol) phosphite (TNPP). TNPP is a phosphite stabilizer used to enhance the performance of certain polymer resins, particularly those constructed from polyethylene (PE), utilized in food contact applications. The migration limit for TNPP is specified at 30 mg kg^{-1} in foods, as per the provisions of EU Commission Regulation 10/2011 within the geographical limits of the EU. However, studies have established that TNPP migration occurs at milligram-per-kilogram levels. [20,148,149]

Despite this, EU regulations have established that NPs and NPEOs are permitted in industrial applications at concentration levels ≤0.1% by mass. Currently, NPEOs emissions alone have been calculated to be well above 11 kilotons per year within the EU [58], and thus, this could also be a possible source of NPs in foods. Moreover, many imported food products can be purchased in German supermarkets, with some originating from countries where EU regulations may not be applicable. Therefore, there is an urgent requirement for stricter legislation regarding the regulation of APEOs. Ideally, APEOs should be banned and/or replaced with less hazardous substances.

2.3.3 Daily Nonylphenols Intake

Figure 3 illustrates the average food composition consumed by an adult in Germany. Thus, a German adult's average daily intake of NPs from food sources was calculated as 2.7 µg day^{-1} from the data presented in Figures 2.2 and 2.3. Notably, the daily intake of NPs has reduced by more than half compared to that reported by Guenther et al. (2002), where a daily intake of 7.5 µg was determined. In the USA, the daily intake of NPs through vegetables and fruits alone was determined as 17.3 µg day^{-1}, while a level of 12 µg day^{-1} was determined from fish obtained from the Adriatic Sea in Italy. Considering the seafood intake level of 31.8 g day^{-1} for an adult in Italy, the daily intake of NPs was determined to be 4.7 µg day^{-1}, whereas a higher value of 27 µg day^{-1} was determined for a Swedish food market basket. [36,44,142]

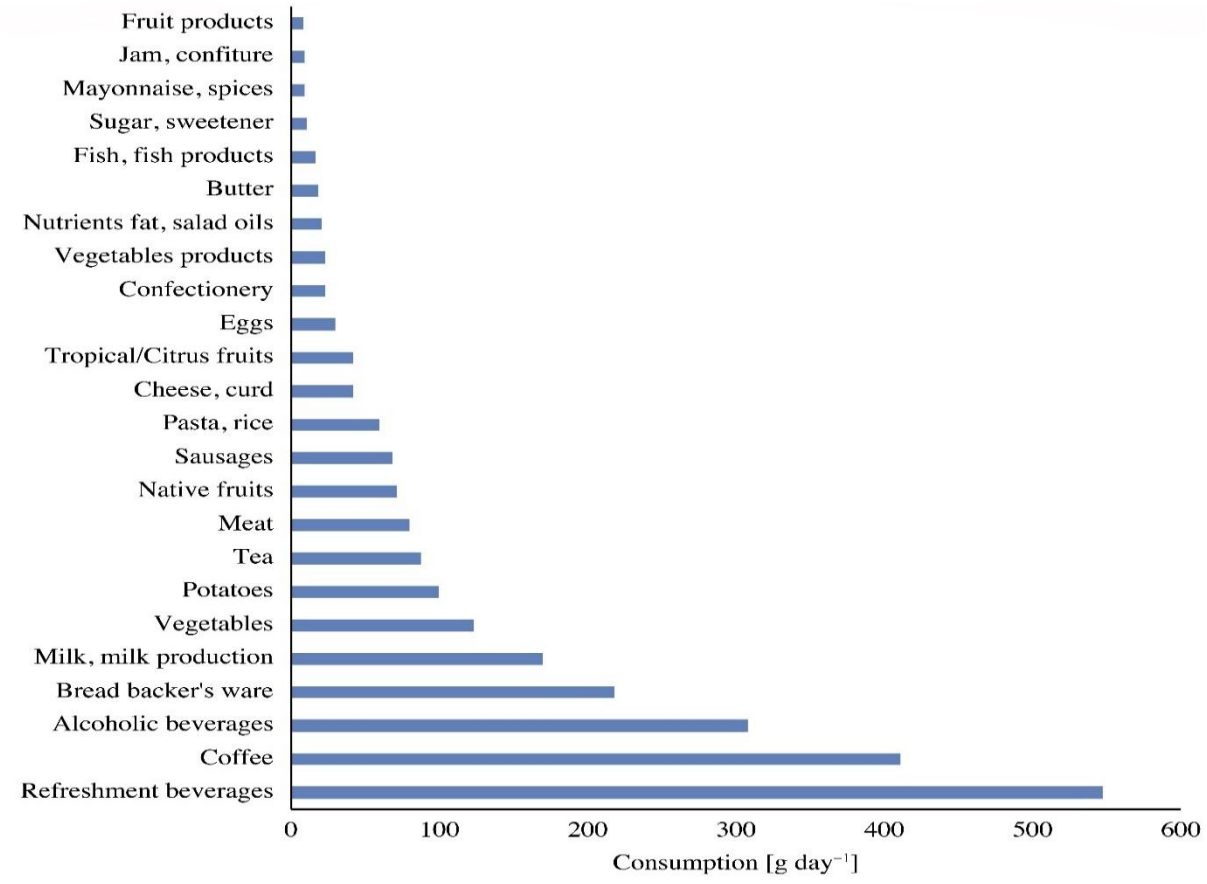


Figure 2.3: The average composition of food consumed daily by German adults is based on a total of 2493 g day^{-1}.[36]

Another German study reported that the daily NPs intake was 0.23–0.65 μg day^{-1} kg^{-1} body weight for babies.[50] A Taiwanese study showed that the daily intake of NPs in commonly consumed foodstuffs was 31.4 μg day^{-1}.[37] Moreover, in Italy, after introducing restrictions for NPEOs and NPs in the EU, an investigation was performed on human breast milk, and the maximum NP daily intake for babies was determined to be 3.94 μg kg^{-1} day^{-1}.[51]

2.3.4 Presence of Nonylphenol Carboxylates in Food Samples

NPs and NPECs are recalcitrant compounds ubiquitous in the environment. NPECs are formed when short-chain NPEOs undergo biodegradation because of the carboxylation of the terminal alcohol group in the presence of oxygen; this usually occurs during the post-secondary stage in wastewater treatment plants (WWTPs).[131,150]

However, the NPs concentrations in the final effluents obtained from WWTPs are more accurate indicators of the plant's efficiency because NPs are the most abundant APEOs derivative in the final effluent, and concentrations up to 343 μg L^{-1} have been reported.[60]

To date, several studies have established methods for determining the concentrations of NPECs in environmental samples. For example, Petrovic et al. (2003) analyzed the water at the outlet of a treatment plant in Spain and observed that it contained NPs and NPECs at levels of 85 and 12 ng L^{-1}, respectively.[151] A few other studies in Belgium and Italy entailed the analyses of the concentrations of NPs and NPECs in surface waters and effluents from the textile industry. They concluded similar ranges of 0.01 to 3.6 μg L^{-1}.[131]

Moreover, a study conducted in the Back River in Baltimore, USA, found the concentration of NPECs to be 1.2 μg L^{-1}.[134] In the foods examined in the present study, all concentrations of NPECs were below the LODs of the method developed here. It is thus clear that these compounds do not play a role in the food basket studied.

2.4 Conclusion

Directive 2003/53/EC was formulated in 2003 and implemented in all EU countries by 2005. The present study aims to determine the amount of NPs and NPECs in foodstuffs in Germany 15 years after the directive took effect to compare the determined levels with those measured in similar foods by Guenther et al., whose work prompted the directive. It can be confirmed that NPECs do not have serious concerns about foods, as all investigated samples were below the LOD. However, 22 out of the 24 foodstuffs assessed contained NPs, with levels ranging from 0.1 to 6.3 µg kg^{-1}, which were significantly lower than those reported by Guenther et al. (2002) for the same food samples (i.e., 0.1 to 19.4 µg kg^{-1}).

The intake of nonylphenol can now be calculated from the NPs contents in food determined in this study and the daily consumption rates. It amounts to 2.7 µg/day. About one-third of the intake of 7.5 µg/day was determined in the study before the restriction.[36]

However, these compounds remain in detectable concentrations in almost all commercial foods examined. Thus, future studies must examine other potential sources of NPs contamination to support additional regulation and remediation. Furthermore, isomer-specific analysis and evaluation of the NPs contents in foods will be crucial in the future since the estrogenicity of each isomer varies significantly.

It should also be noted that NPs have been found in relevant concentrations in human blood in many studies.[58] Moreover, there are often few restrictions on using NPs and NPs precursors outside Europe. Hence, the global food trade contributes to the continued problem with this endocrine disruptor in the food chain and the human organism. Therefore, further research in this area is necessary.

2.5 Authors Contribution Statement

This chapter is adapted from the research article N. Al Rashed and K. Günther, Determination of Endocrine-Disrupting Nonylphenols and Nonylphenol Carboxylates by High-Performance Liquid Chromatography-Tandem Mass Spectrometry: Levels in German Food after Restriction, Analytical Letters, DOI:10.1080/00032719.2021.1956515. For this research article, the following paragraphs specify the individual contributions of each author.

Author 1; N. Al Rashed: Conceptualization, conceived and designed the experiments, performed the experiments, analysis, and interpretation of data, performed the calculations, writing (original draft preparation), writing (review and editing), designing the figures, and literature review.

Author 2: Prof. Dr. K. Günther: Writing (review and editing), supervision.

3. Determination of nonylphenol in selected foods and identification of single isomers in a coffee sample by comprehensive two-dimensional gas chromatography-time of flight mass spectrometry

This chapter is adapted from N. Al Rashed, Christoph Gerlach, and Klaus Guenther, Determination of Nonylphenol in Selected Foods, and Identification of Single Isomers in a Coffee Sample by Comprehensive Two-Dimensional Gas Chromatography-Time of Flight Mass Spectrometry, Analytical Letters, Volume 56, Issue 16 (2023), doi.org/10.1080/00032719.2023.2180018.

3.1. Introduction

Nonylphenol ethoxylates (NPEOs) are among the most widely used non-ionic surfactants in the industrial production of cleaning products, textiles, petroleum, pulp and paper, and pesticide formulations. NPs are produced by the anaerobic biodegradation of NPEOs. NPs are present in many environmentally relevant matrices, such as influent and effluent water from sewage treatment plants, river water, seawater, surface water, groundwater, drinking water, sediments, and soil.[33,36,53,57,58,131-134]

Furthermore, NPs are present in many foodstuffs, including packaged food purchased from supermarkets in Germany and commercial whole milk in Spain. Seafood and various edible marine species in Asia, Europe, and North America, as well as vegetables and fruits from Sweden, Spain, and the USA, have also been analyzed for the presence of NPs. NPs have also been detected in commercially bottled water, drinking water from public fountains in 35 cities across Italy, bottled mineral water, and commercial soft drinks. Notably, consuming food contaminated with NPs is a direct source of exposure for humans.[36,38,39,42-44,47,48,129,142,143,154,155]

Another unintended source of NPs is the metabolization of plastics and microplastics (MPs) in the human body into plastic-derived endocrine disrupting compounds (EDCs), which either remain at stable concentrations in tissues or are excreted in urine within 24 h through phased enzymatic chemical reactions in the liver. For example, tris-(nonylphenol)-phosphite (TNPP) - a stabilizer in many bulk polymers in the percentage range - forms endocrine-disrupting nonylphenols upon decomposition. Ingesting food and drinks contaminated with plastic components is the main route for plastic components to enter the human body. Annually, an average of between 39,000 and 52,000 plastic particles are consumed.[157-159]

MPs have been detected in drinking water sources at low concentrations. Analysis of beer samples in Germany reported approximately 2–79 fibers/L and 12–109 fragments/L. An analysis of 21 samples of table salts from different brands in Spain was performed to determine MP-like particles, and it was found that fragments of polypropylene (PP) and polyethylene (PE) were found in all tested samples.[160]

NPs in human tissues, breast milk, and urine have been reported to influence semen quality. Maternal blood and amniotic fluid samples were collected from 53 Indian pregnant women and the plasma and urine of housekeeping workers. Other human tissues include adipose tissue and breast milk. A study in northern Taiwan demonstrated an association between decreased semen quality and high urinary NPs in men. Men's average urinary NPs level was 1.53 µg/g of creatinine.[36,51,75-77,161]

Theoretically, 211 constitutional NP isomers exist. Furthermore, many isomers have up to three chiral C atoms per molecule, and enantiomers have also been considered. To identify individual NP isomers among the 211 constitutional isomers, a numbering system for individual NP isomers (Juelich Nomenclature) was developed. According to the International Union of Pure and Applied Chemistry (IUPAC) guidelines, a hierarchical system was used to number isomers. This system covers 550 possible compounds and considers the chirality of C atoms. Moreover, Thiele et al. 2004 classified NP isomers in the technical mixture into six groups due to the identical mass spectra (Figure 3.1).[6,18]

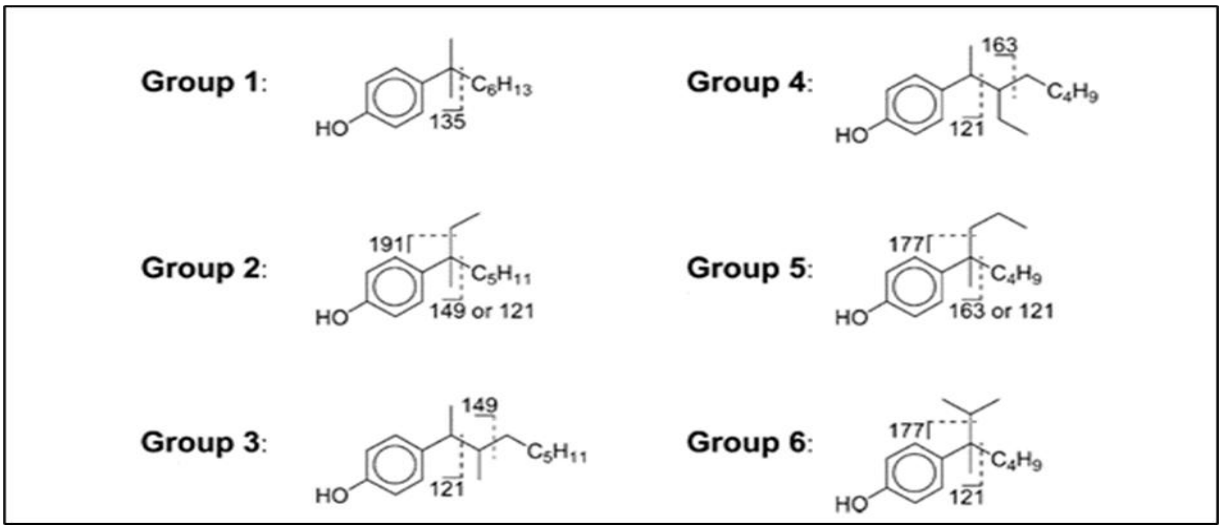


Figure 3.1: Classification of NP isomers of the technical mixture into six groups due to identical mass spectra.[6]

The European Union (EU) Water Framework Directive has listed NPs as a priority hazardous substance. The endocrine-disrupting effects of NPs include harmful impacts on the human body, such as the reproductive, immune, and nervous systems. Young animals and humans are susceptible to EDCs during their development phases. Several studies have shown that NPs bind to both estrogen receptor isoforms (ERα and ERβ) and compete with natural estrogen 17β-estradiol (17β-E2). Moreover, NPs exhibit estrogenic activity depending on their structural features and degree of branching and bulkiness.[9,14,25,127,129,130,135,139,140]

Some NP isomers showed isomer-specific biodegradation behavior in an activated sludge bioreactor, enriching recalcitrant isomers [128], potentially having different estrogenic activities. It is well known that enantiomers may selectively interact with biological systems because they are enantioselective, too. Previous studies reported the enantioselective degradation of two chiral NP isomers in water.[139] A difference in the estrogenic potency of a chiral NP isomer's S and R enantiomers was also described. The S-enantiomer exhibited a slightly higher relative proliferative effect (RPE) at a concentration that caused maximal proliferation than the R-enantiomer. These results indicate that enantioselective effects may play a role in the binding process between NPs and the estrogen receptor (ER).[135]

Moreover, NPs are risk factors that can trigger or even exacerbate allergic diseases.[158] As NPs possess low solubility, high hydrophobicity, and estrogenic activity, they can accumulate in the human body and trigger allergic diseases.[52] NPs raise considerable concerns regarding maternal exposure during pregnancy, even at low doses. Complications in pregnancy, such as implantation failure and fetal loss, result from an unbalanced cytokine network at the maternal-fetal interface.[130,163]

A recent biomonitoring study investigated schoolchildren's exposure to nonylphenol in Saudi Arabia, Indonesia, and Thailand. For this purpose, the specific metabolites hydroxy nonylphenol and oxo-nonylphenol were examined in urine samples. The specific metabolites were found 100% in Saudi (n=108) and Indonesian (n=89) children. The daily intake of nonylphenol was calculated from the urine concentrations of the metabolites. The mean daily intake for Saudi Arabian children is 0.36 5 μg kg^{-1} bw*d, and from Indonesia, 0.47 μg kg^{-1} bw *d. According to the Danish Environmental Protection Agency, the maximum values were close to the preliminary tolerable daily intake (TDI) of 5 μg kg-1 bw*d.[164] As a further development to this Danish TDI value, it is urgent to develop values for tolerable daily intakes

that consider the critical isomer-specific aspects of the nonylphenol problem. This will be an essential task for the different national food control authorities worldwide in the future.

The results of this study show that the presence of nonylphenol is not only ubiquitous in food but also in the human organism.[36] Therefore, global monitoring programs for food and the medical field are urgently needed in the future. As the individual nonylphenol isomers differ significantly in their estrogenicity, the investigations must be carried out on an isomer-specific basis.

Comprehensive two-dimensional (^{2}D) gas chromatography (GC × GC) is well suited for monitoring complex mixtures of hydrophobic organic contaminants in environmental matrices.[165] Two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC–TOF–MS) has been proposed as a promising technology for the isomer-specific determination of NPs.[18] Ieda et al. (2005) reported 102 peaks in technical nonylphenol (t-NP) and measured two NP isomers in river water samples by GC × GC-TOF-MS.[166] Moeder et al. (2006) used GC × GC –TOF–MS to improve the separation of NP isomers and their biodegradation products.[167] Eganhouse et al. (2009) demonstrated a significant advantage of GC × GC–TOF–MS over GC/MS for the quantitation of specific NP isomers and described the potential of this method to reveal previously undetectable NPs.[125]

The study's objective was to determine the presence of NPs in selected foodstuffs from different countries and the isomer-specific analysis of NP in a selected sample by a special GC x GC-TOF-MS equipped with a liquid nitrogen consumable-free cryogenic/thermal modulator. A reference library for key isomers of nonylphenols synthesized in previous studies should be created on this system.[14] The background for these investigations was the need to establish an isomeric-specific analysis of nonylphenols in environmental, food, and human matrices in the future with a relatively simple and cost-effective system of instrumental analysis.

3.2 Materials and Methods

3.2.1 Standards Reagents, Chemicals, Food Samples, and Materials:

Ultrapure water (18.2 MΩ·cm), obtained from a Milli-Q apparatus (Merck Millipore, Darmstadt, Germany), was used throughout the study. Technical-grade NPs (a mixture of NP isomers) were obtained from Fluka (85 %; Buchs, Switzerland). The $^{13}C_6$–NP_{112} (^{13}C in benzene ring) internal standard (IS) was acquired from Sigma-Aldrich (ring-$^{13}C_6$, 99 %, Darmstadt, Germany). NPs were numbered according to the internationally applicable Juelich Nomenclature.[6] Acetonitrile (ACN, 99.9 %) and methanol (99.9 %) of LC-MS grade were purchased from VWR Chemicals (Langenfeld, Germany). Cyclohexane and isooctane (99.5 %) of LC-MS grade were obtained from Promochem (Wesel, Germany). Assay-grade sodium chloride (NaCl) was purchased from Fisher BioReagents (USA), and anhydrous sodium sulfate (Na_2SO_4, ≥99 %) was obtained from Roth (Karlsruhe, Germany). Hydrochloric acid [HCl; 37 % w/w)] was acquired from AnalaR Normapur (Paris, France). Polyethylene terephthalate (PET) syringe filters (0.20 µm) were purchased from Macherey-Nagel (Dueren, Germany).

3.2.2 Food Samples Preparation:

All foodstuffs were purchased from German and Saudi retail stores and packed using typical procedures (Table 3.1) before storage at either 4 or 25 °C until analyses were performed. Nonsolid foodstuffs, including pastes and liquid samples, were extracted without pretreatment. The solids were homogenized before extraction using an Ultra-Turrax 18/10 (IKA-Werk, Staufen, Germany) or Moulinex Moulinette AD56 (Spain).

Table 3.1: Packaging materials of the investigated foodstuffs and the country of origin.

Food sample	Packaging materials	Country of origin
Milk	Beverage paper cartons	Saudi Arabia
Fruit juice	Beverage paper cartons	Saudi Arabia
Arabic coffee	Plastic bag/Plastic foil	Ethiopia
Apples	Plastic bag/Plastic foil	Germany, Italy, and Chile
Chocolate	Plastic bag/Plastic foil	Italy
Tea	Paper	Seri Lanka
Wheat flour	Paper	Saudi Arabia
Dates	Plastic	Saudi Arabia
Fava beans	Can	United States of America
Cheese	Foil	France

3.2.3 Nonylphenols Extraction:

The steam distillation apparatus designed by Veith and Kiwus (1977) was fitted to a 2 L distillation flask (bottom) and a reflux condenser (top) and was used to extract the NPs from the food samples by applying the continuous steam distillation/solvent extraction technique.[146] A solution of NaCl (40 g) in water (600 mL), acidified with 2 mL HCl, was used as the extraction solvent. The purification process involved boiling the condensate in a Veith apparatus using heating mantels obtained from Witeg (Wertheim, Germany). Prior to the extraction of food samples, a blank test run (extraction solvent with 10 µL IS) was performed. The food sample of interest (10 g) was suspended in the distillation flask with the extraction solvent, and the IS (10 µL, $^{13}C_6$–NP_{112} in methanol (0.5 µg mL−1)) was added to the suspension. The extraction process was performed for five hours using a cyclohexane/isooctane mixture (20 mL, 1:1 v/v). The phases were collected in a volumetric flask (100 mL), and fresh solvents were used to replace the aqueous and organic phases. The separated organic layer was dried using anhydrous Na_2SO_4, filtered, and concentrated under nitrogen flow until completely

dry. The dried residue was re-dissolved in methanol (1 mL), and the samples were stored in the dark at four °C before duplicate analysis.

3.2.4 LC-MS/MS Analysis for Nonylphenols:

A Shimadzu HPLC system (LC-20AD, Kyoto, Japan) with an AB Sciex QTrap 4500 (Darmstadt, Germany) was used to identify and quantify NPs. A Phenomenex Luna C18 column (3 µm; 150 × 2.0 mm i.d, Phenomenex, Aschaffenburg, Germany) was used at 60 °C for HPLC. A binary pump system was used for chromatographic separation: the mobile phase comprised solvent A (methanol) and solvent B (water). A linear gradient program was used with 50 % A at 0 min, 95 % A from 0 to 16 min, holding at 95 % A for 10 min, and returning to 50 % A over 0.2 min. The HPLC column was reconditioned for an additional 5 min with 50 % A prior to subsequent sample analysis. The extract (5 µL) was introduced into the HPLC column at a flow rate of 0.2 ml min^{-1}. MS detection was performed using a Turbo Ion Spray® ionization source, and tuning was performed for negative electrospray ionization (ESI) analysis. The block source temperature was set at 650 °C, and the values for the gas flow were as follows: curtain gas, 25 psi; nebulizer gas, 40 psi; turbo gas, 60 psi; and collision gas, 40 psi. The remaining parameters were as follows: delustering potential (−85 V), entrance potential (−10 V), collision exit potential (−9 V), and ion spray voltage (−4000 V). Tandem MS in the multiple reaction monitoring (MRM) modes was performed by alternating between the two transition reactions for NPs (219 → 133 and 219 → 147 *m/z*) and the corresponding IS (225 → 139 and 225 → 153 *m/z*) with a dwell time of 150 ms, for quantitative analysis. Analyst® software version 1.4.2 was used to perform data processing.

3.2.4.1 Calibration and Response Factors and Quantification:

The isotopic dilution approach was used to quantify NPs [analyte/IS area ratio (= *y*)] versus the analyte/IS concentration ratio (*x*). Six calibration levels were used in the 0–50 ng mL−1 range. Each level contained IS at a concentration of 5 ng mL^{-1}. The NPs exhibited excellent linearity with good correlation coefficients (R > 0.999). Since NPs are ubiquitous, nanogram-per-liter-level background contamination can occur from solvents and the laboratory environment. The reagent assay was systematically measured along with each food sample. The final equation used to express the concentration of NPs (µg kg^{-1}) was as follows:

$$\text{NPs} = \left(\frac{\frac{A_a}{A_{is}} - I}{S}\right) \times C(IS), \quad (1)$$

A_a is the peak area of the NPs in the blank sample or test sample, A_{is} is the peak area of $^{13}C_6$–NP_{112}, I and S are the intercept and slope of the regression lines, respectively, and $C(IS)$ is the concentration of the internal standard.

The final NP concentration in the food samples was calculated as follows:

$$[Final\ NP]_{FS} = [NP]_{FS} - [NP]_{BS}, \quad (2)$$

Where $[\text{Final NP}]_{FS}$ represents the final NP concentration in the food sample, $[NP]_{FS}$ represents the measured NP concentration in the food sample, and $[NP]_{BS}$ represents the measured NP concentration in the blank sample.

3.2.5 Identifying NPs Isomers in Coffee Samples

3.2.5.1 NPs Isomers:

The acronyms for the various NP isomers used in this study are based on the well-known, internationally accepted numbering system for this class of substances.[18] The 13 NP isomers, including NP_9 (4-[1,1-dimethylheptyl]-phenol), NP_{36} (4-[1,1,3-trimethylhexyl]-phenol), NP_{37} (4-(1,1,4-trimethylhexyl)-phenol), NP_{39} (4-[1,2,2-trimethylhexyl]-phenol), NP_{65} (4-[1-ethyl-1-methylhexyl]-phenol), NP_{94} (4-[1,1,3,3-tetramethyl-pentyl]-phenol), NP_{119} (4-[2-ethyl-1,1-dimethylpentyl]-phenol), NP_{128} (4-(3-ethyl-1,1-dimethyl-pentyl) phenol), NP_{143} (4-[1-isopropyl-1- methyl pentyl] phenol), NP_{170} (4-[1-ethyl-1,3,3-tri-methylbutyl]-phenol), NP_{194} (4-[1,3-dimethyl-1-propylbutyl]-phenol), $NP_{111a,b}$ (4-[1-ethyl-1,3-dimethylpentyl]-phenol) present as a 44.9/55.1 mixture, and a mixture composed of 20 % NP_{143} (4-[1-isopropyl-1-methyl pentyl] phenol) and 80 % NP_{35} (4-[1,1,2-trimethylhexyl]-phenol).[14] The concentration of NP isomers standards was adjusted at 2 ng ml^{-1}.

3.2.5.2 Solid Phase Microextraction (SPME) Fiber:

Coated divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 µm) SPME-coated fiber (Supelco, Bellefonte, PA, USA) was used.

3.2.5.3 Sample Preparation:

The conditions of the SPME extraction method were slightly modified, as Basheer et al. (2005) described .[168] Before SPME extraction, samples were held at an incubation temperature of 70 °C for 5 min without stirring. The extraction was performed at 70 °C for 35 min. After sampling the headspace with a mixed fiber, the compounds were desorbed using an SPME Liner (Restek, 0.75 mm x 6.35 mm x 78.5 mm) at 270 °C for 5 min. The fibers were reconditioned in a bakeout station for 20 min at 270 °C under nitrogen flow before each analysis to avoid carryover.

3.2.5.4 GC×GC–TOF–MS Analysis:

All chromatographic separations were achieved using an Agilent 7890 B gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a liquid nitrogen cryogenic modulator (consumable-free ZX2 thermal modulator) coupled to a time-of-flight mass spectrometer (Markes International Ltd, Llantrisant, RCT, UK). Liquid nitrogen was utilized for cryo focusing (Cryo focusing was accomplished using liquid nitrogen at a flow rate of 7.5 mL/min). After elution from the first dimension (^{1}D) column, heated air was released to release and reject these compounds onto the second dimension (^{2}D) column. A modulation period of 4 s and a hot jet duration of 0.40 s were used. A 30 m × 0.25 mm i.d. × 0.25 µm df DB-WAX Ultra Inert (Agilent Technologies, Bellefonte, PA, U.S.A.) was coupled to a 1.7 m × 0.10 mm i.d. × 0.10 µm df MEGA-17 MS FAST (MEGA s.n.c., Legnano, MI, IT) as the second dimension.

The separation was accomplished using the following temperature program: ^{1}D oven ramp: 50 °C (3 min), 10 °C min^{-1} to 130 °C (3 min), 5 °C min^{-1} to 200 °C (30 min), 10 °C min^{-1} to 230 °C (25 min), and 10 °C min^{-1} to 250 °C (15 min); 2D oven ramp: 135 °C (3 min), 10 °C min^{-1} to 175 °C (40 min), and 10 °C min^{-1} to 205 °C (5 min). The injector was operated at 270 °C in splitless mode. Helium was utilized as the carrier gas at a constant flow of 1.0 mL min^{-1}. The transfer line was maintained at 250 °C, and the analytes were ionized by operation in the electron impact (EI) mode at −70 eV. The ion source temperature was 250 °C. Chromatograms were recorded in full scan mode (EI, *m/z* 50-350). All samples were analyzed in duplicate.

3.3 Results and Discussion

3.3.1 Recoveries and Limits of Detection:

An IS, $^{13}C_6$–NP_{112} (10 µL), was added to each food sample (extraction was performed in duplicate), and the appropriate blank test was used to validate the analytical procedure. The blank tests yielded extraction efficiency of the IS ranging from 90 to 100 % with relative standard deviations (RSDs) ≤4.2 %, whereas the different food samples presented high variations, with recoveries of 16–127% and RSDs of 4–17%. Consequently, the NP concentration calculations were corrected based on IS recoveries. Blank test samples were spiked with $^{13}C_6$–NP_{112} for NP analysis to obtain mean blank values for the NPs, after which the limits of detection (LOD) were calculated. The LODs were 0.14 µg kg^{-1} for NPs (5 mean blank value + (3 * SD)). The limits of quantitation (LOQ) were 0.17 µg kg^{-1} for NPs (5 mean blank value + (6 * SD)). Figure 3.2 shows the LC-MS/MS chromatograms for NPs in coffee and $^{13}C_6$–NP_{112} in coffee.

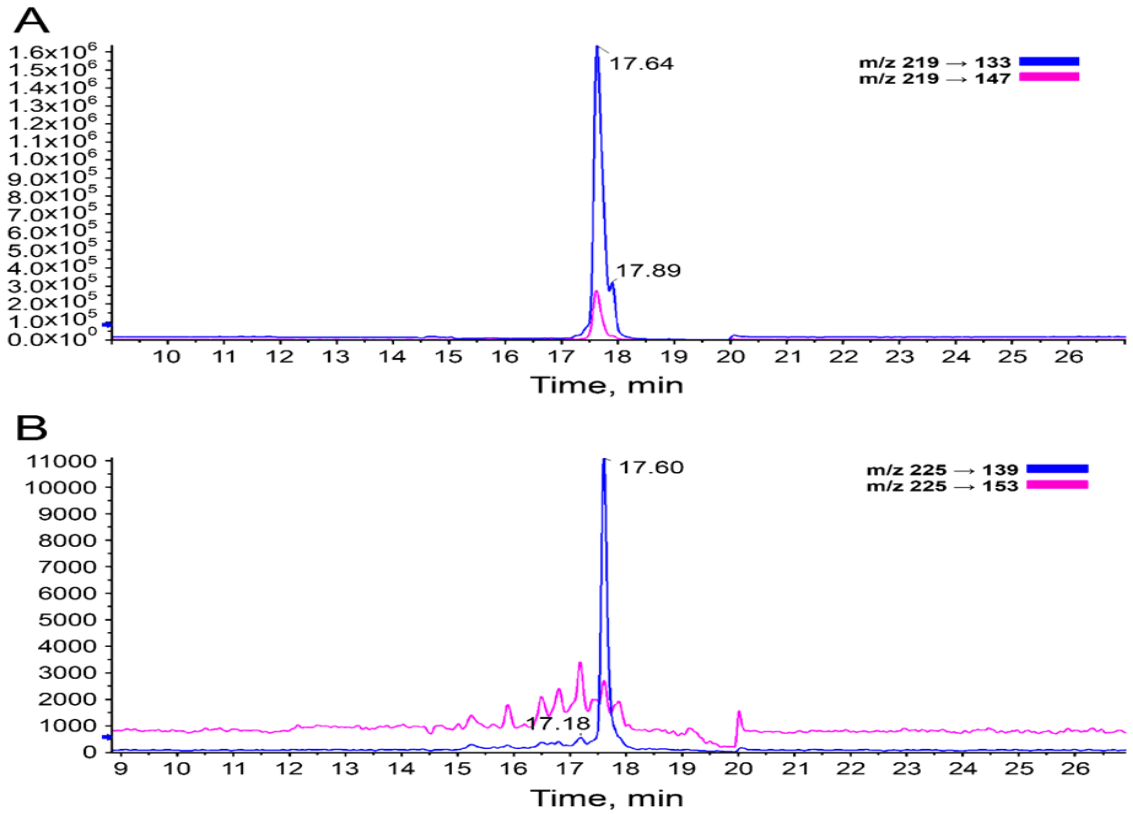


Figure 3.2: (A) LC-MS/MS chromatograms for NPs in Arabic coffee (MRM mode: 219 → 133 and 219 → 147). (B) LC-MS/MS chromatograms for the internal standard (IS) $^{13}C_6$–NP_{112} in Arabic coffee (MRM mode: 225 → 139 and 225 → 153).

3.3.2 Presence of Nonylphenols in the Food Samples:

Between the 12 analyzed food samples from different countries, NPs were detected in 8 samples ranging from 0.5 to 18.9 µg kg^{-1} on a fresh weight basis (Figure 3.3). Among the various samples analyzed, Arabic coffee, Chilean apples, Saudi dates, and Italian apples presented the highest concentrations of NPs at 18.9, 8.3, 1.6, and 1.0 µg kg^{-1}, respectively. Medium concentrations of NPs at 0.72, 0.68, 0.67, and 0.55 µg kg^{-1} were detected in fava beans, chocolate, tea, and wheat flour, respectively, whereas the concentrations in cheese, milk, fruit juice, and German apple were below the LODs.

Compared to previous findings, our results demonstrate how foods grown in different regions can reflect the NP levels of those regions and be an indicator of that region's legislative laws regarding the use of NPEOs in commercial/consumer products.[42,44,154]

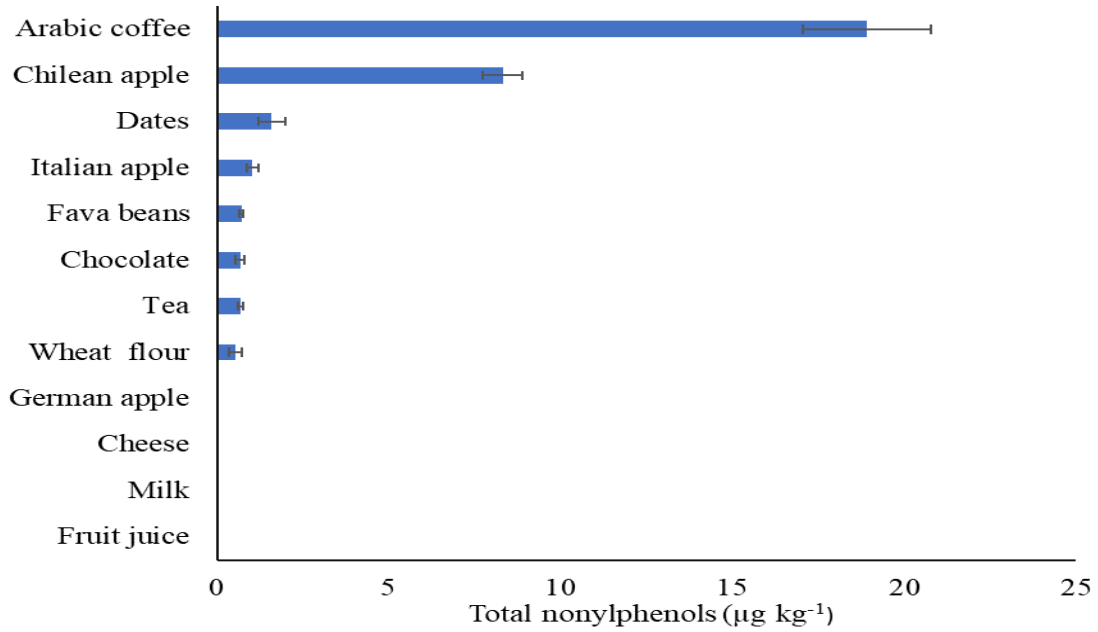


Figure 3.3: NP concentrations in food samples, concentrations (sum of all NPs isomers, mean values with standard deviations, n = 2) are expressed on a fresh weight basis. The limit of detection (LOD) was 0.14 µg kg^{-1}.

For instance, Arabic coffee is typically grown in the Middle East or other African regions (in Ethiopia). As far as we know, there are no regulations for NPEOs or NPs in most African countries except South Africa [169]. Therefore, a high possibility of detecting a high concentration of NPs was probable. The difference in the NP levels of apples from different regions (Germany, Chile, and Italy) indicates how well the legislative laws that forbid or restrict the use of NPEO products in regions around the globe are enforced. German and Italian apples show low NP concentrations of (<=1 μg kg^{-1}), while apples from Chile show a considerably high concentration (>8 μg kg^{-1}). This can be attributed to Germany and Italy being member states of the EU, which instated a directive in June 2003 to restrict the use and marketing of toxic substances, particularly NPEOs and their toxic derivatives [58]. This directive was implemented by all EU member states, with Germany taking it a step further and placing an industrial ban on the use of NPEOs in 2005.[58,126] The effects of this ban are evidenced by the NP levels of apples from Germany, which fell below the LOD values of the test. Although Italian apples have lower NP levels than Chilean apples, their NP levels are much higher than those of German apples, as Italy's textile industry still actively uses NPEO products.

3.3.3 Analyzing the Single Isomers on GC×GC–TOF–MS:

This study used GC×GC–TOF–MS with a highly polar/moderately polar dual-column system. The elution of compounds on both columns was based on the polarity of compounds, with lower polarity compounds eluting earlier than those with higher polarities.[125] For cross-referencing and identifying, 13 pure NP isomers, namely NP_9, NP_{35}, NP_{36}, NP_{37}, NP_{39}, NP_{65}, NP_{94}, $NP_{111\ a,b}$, NP_{119}, NP_{128}, NP_{143}, NP_{170}, and NP_{194}, were used (Figures 3.4-3.16).
Based on a comparison of mass spectra and relative retention times to previous data, it can be confirmed that 7 NPs isomers (NP_9, NP_{35}, NP_{36}, NP_{37}, NP_{94}, NP_{119}, and NP_{128}) out of 13 belonged to *group 1* isomer with its α,α-dimethyl structure, which exhibited base peaks at *m*/*z* 135 without significant fragment ions between *m*/*z* 137 and 220.[6,17,112]

Furthermore, 3 NPs isomers (NP_{65}, $NP_{111\ a,b}$, and NP_{170}) were classified in the *group 2* isomers with an α-ethyl-α-methyl structure. The base peak is *m/z* 149, and 2 specific fragment ions of *m/z* 191 and *m/z* 121 (Figures 3.9,3.11, and 3.15). The cleavage of the ethyl group substituted by the methyl group at the α-C atom caused *m*/*z* 191. In contrast, the loss of the pentyl group with methyl substituents on the β-carbon resulted in *m*/*z* 149. The ion with *the m/z* 121 peaks

is a fragment formed through the loss of a β-carbon with a final cleavage of the ethyl and pentyl groups from the α-C atom.[6,17,22]

The NP_{39} isomer (Figure 3.8) represents *group 4* isomers with a base peak of 121 m/z. According to Thiele et al. 2004, only the base peak at m/z 121 ([M - C_7H_{15}] +) stabilized the tropylium ion, indicating that alkyl groups at the β-C atom had no stabilizing effects. Consequently, alternative structures for group 4 isomers must comprise α-substituted C atoms. This would explain the formation of m/z 163 for group 4 isomers but would not have explained the formation of m/z 121.

The spectra of NP_{194} indicate that the isomer belongs to *group 5* with an α-methyl-α-*n*-propyl structure. This isomer had a base peak at *m*/*z* 121 and two major peaks at *m*/*z* 163 and 177, as illustrated in (Figure 3.16). Finally, NP_{143} (Figure 3.15) represents the *group 6* isomers and had spectra with an α-methyl-α-isopropyl configuration. The base peak was at *m*/*z* 121 and had a significant peak at *m*/*z* 177 because of the cleavage of the isopropyl group.[6]

Table 3.2 lists the resulting retention times for the different NP isomers on the first and second columns, the groups, and the fragment ions on the GC x GC-TOF-MS setup applied in this study.

Table 3.2: GC × GC/TOF-MS details of NPs peaks of the NP isomers.

NP isomers	1st Retention time (min)	2nd Retention time (sec)	Group No	Fragment ion *m/z*
NP_9	64.97	1.35	1	135
NP_{35}	64.72	1.32	1	135
NP_{36}	61.37	1.23	1	135
NP_{37}	62.37	1.23	1	135
NP_{39}	64.77	1.28	4	121
NP_{65}	64.72	1.31	2	121, 149 and 191
NP_{94}	64.32	1.32	1	135
NP_{111a}	62.52	1.27	2	121, 149 and 191
NP_{111b}	62.92	1.27	2	121, 149 and 191
NP_{119}	63.87	1.32	1	135
NP_{128}	62.27	1.26	1	135
NP_{143}	64.07	1.30	6	121 and 177
NP_{170}	61.82	1.28	2	121, 149, and 191
NP_{194}	60.82	1.27	5	121, 163 and 177

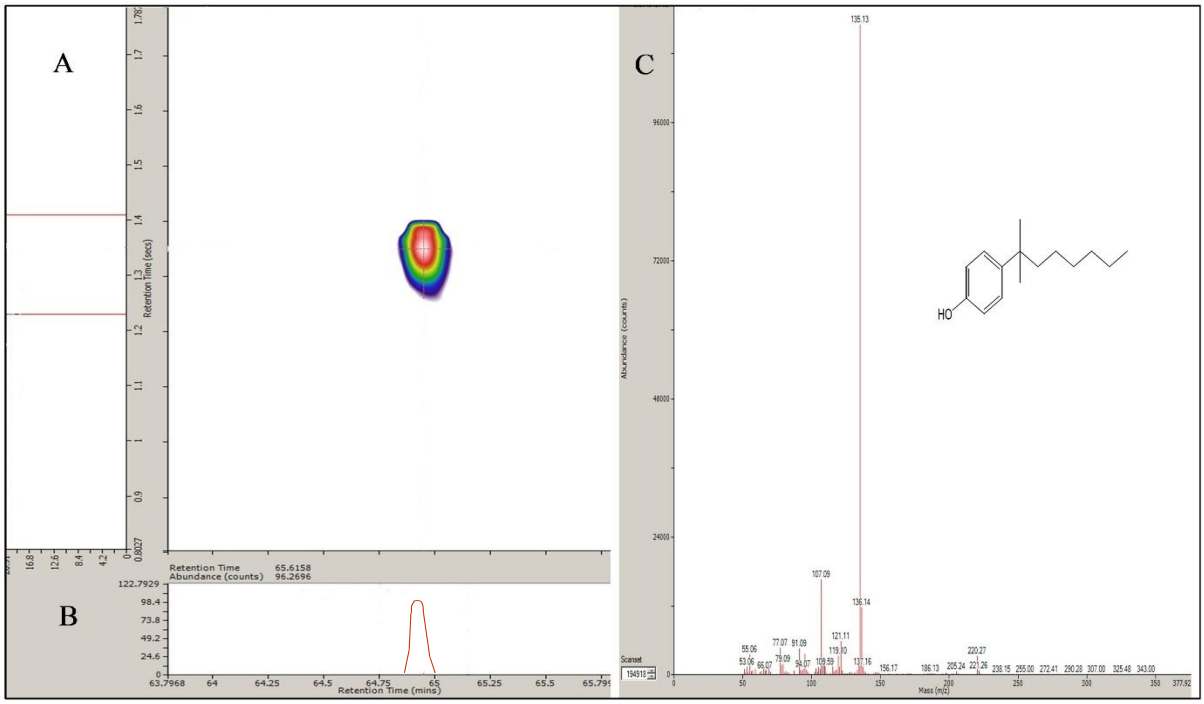


Figure 3.4: GC x GC-Tof-MS chromatogram of NP_9(concentration = 2 ng/mL^{-1}) separated using DB-WAX as the first dimension column (D1) and MEGA-17 MS FAST as the second dimension column (D2); (A) the retention time in the D2 column ; (B) the retention time in the D1 column; (C) The mass spectrum and chemical structure of NP_9.

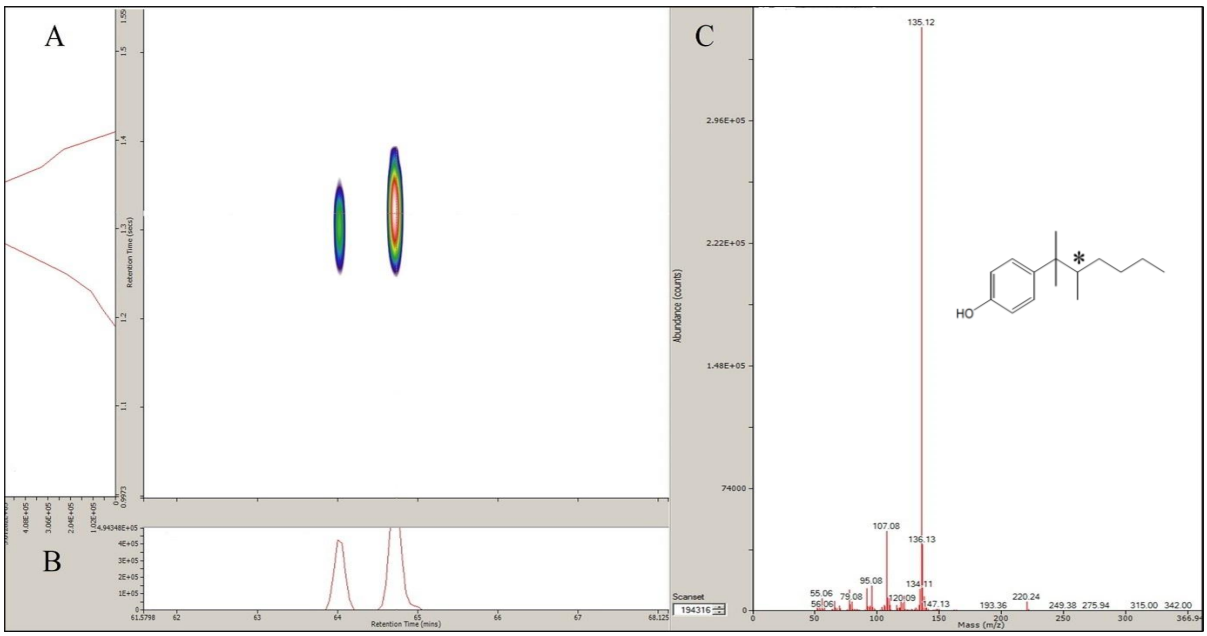


Figure 3.5: GC x GC-Tof-MS chromatogram of the NP_{35} (the red bulb, concentration = 1.60 ng/mL^{-1}) separated using DB-WAX as the first dimension column (D1) and MEGA-17 MS FAST as the second dimension column (D2); (A) the retention time in the D2 column ; (B) the retention time in the D1 column; (C) The mass spectrum and chemical structure of NP_{35}.

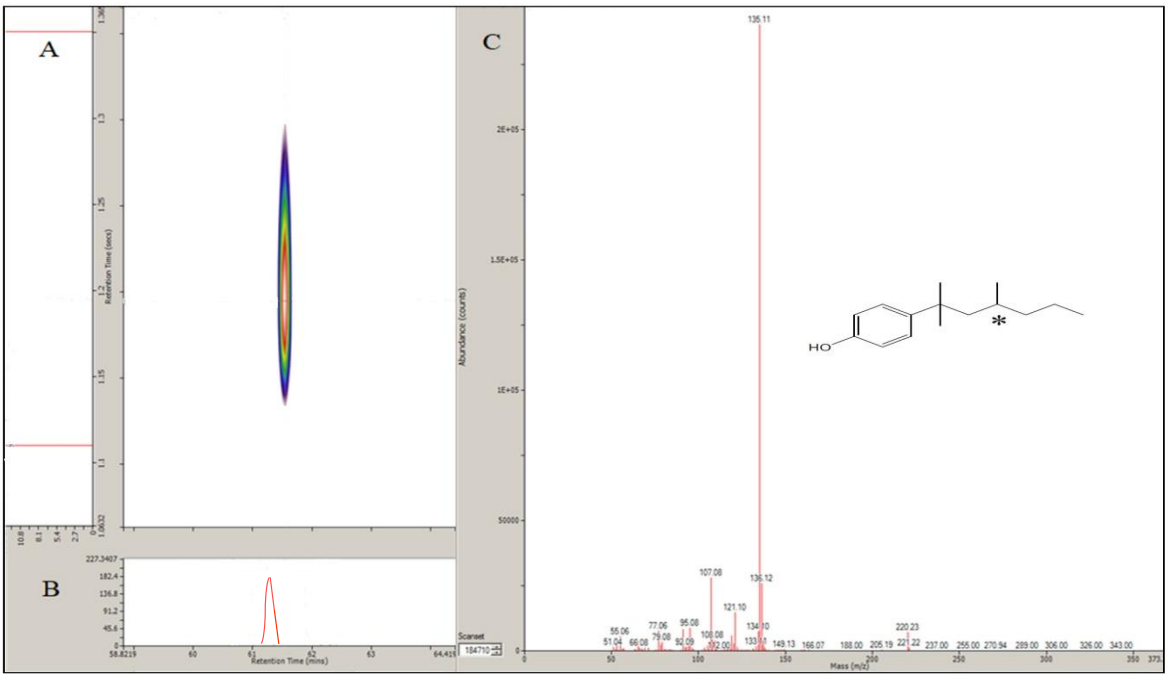


Figure 3.6: GC x GC-Tof-MS chromatogram of NP_{36} (concentration = 2 ng/mL^{-1}) separated using DB-WAX as the first-dimension column (D1) and MEGA-17 MS FAST as the second-dimension column (D2); (A) the retention time in the D2 column; (B) the retention time in the D1 column; (C) The mass spectrum and chemical structure of NP_{36}.

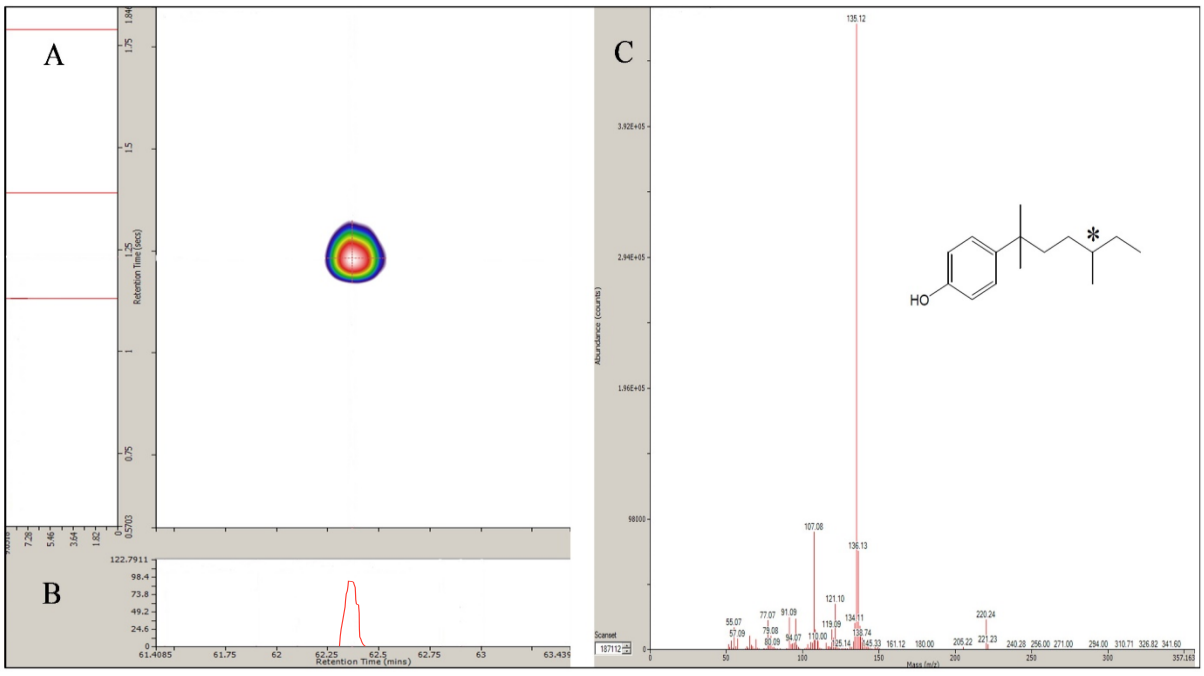


Figure 3.7: GC x GC-Tof-MS chromatogram of NP_{37} (concentration = 2 ng/mL^{-1}) separated using DB-WAX as the first-dimension column (D1) and MEGA-17 MS FAST as the second-dimension column (D2); (A) the retention time in the D2 column; (B) the retention time in the D1 column; (C) The mass spectrum and chemical structure of NP_{37}.

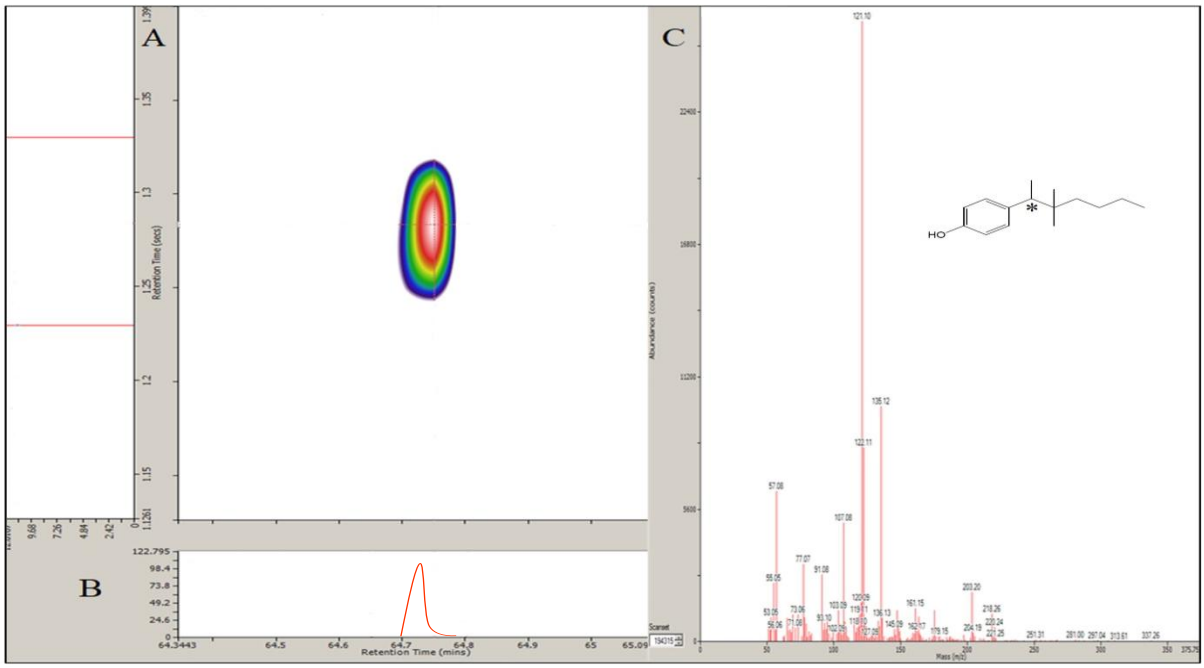


Figure 3.8: GC x GC-Tof-MS chromatogram of NP_{39} (concentration = 2 ng/mL^{-1}) separated using DB-WAX as the first dimension column (D1) and MEGA-17 MS FAST as the second dimension column (D2); (A) the retention time in the D2 column ; (B) the retention time in the D1 column; (C) The mass spectrum and chemical structure of NP_{39}.

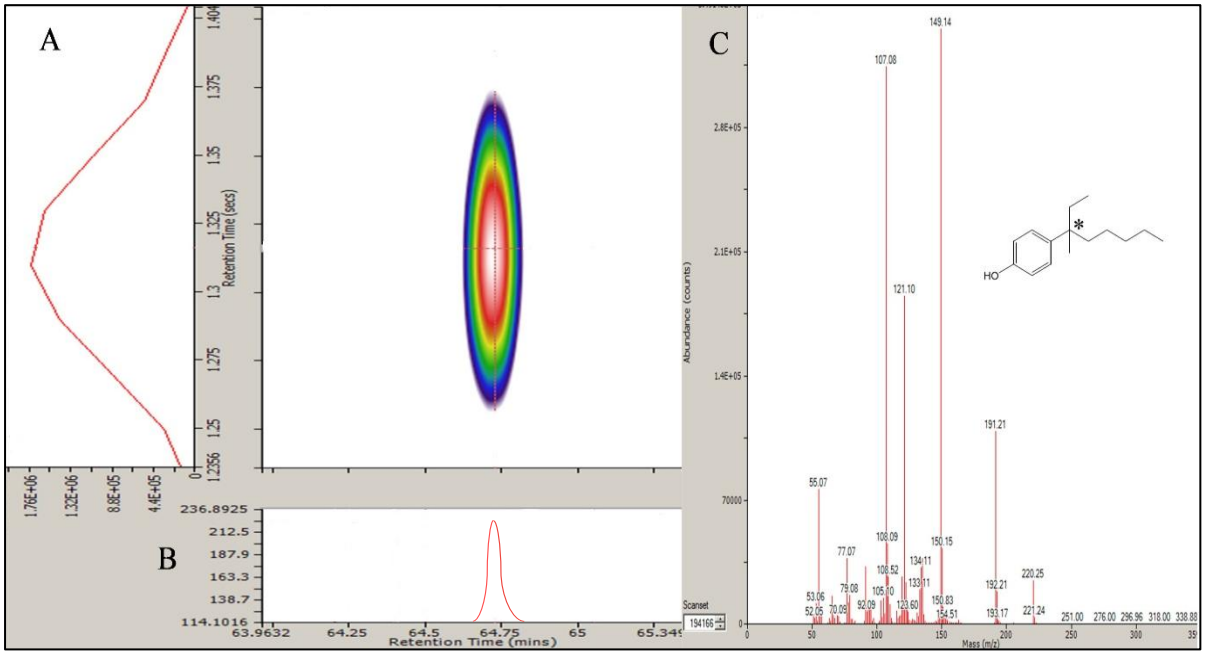


Figure 3.9: GC x GC-Tof-MS chromatogram of NP_{65} (concentration = 2 ng/mL^{-1}) separated using DB-WAX as the first dimension column (D1) and MEGA-17 MS FAST as the second dimension column (D2); (A) the retention time in the D2 column ; (B) the retention time in the D1 column; (C) The mass spectrum and chemical structure of NP_{65}.

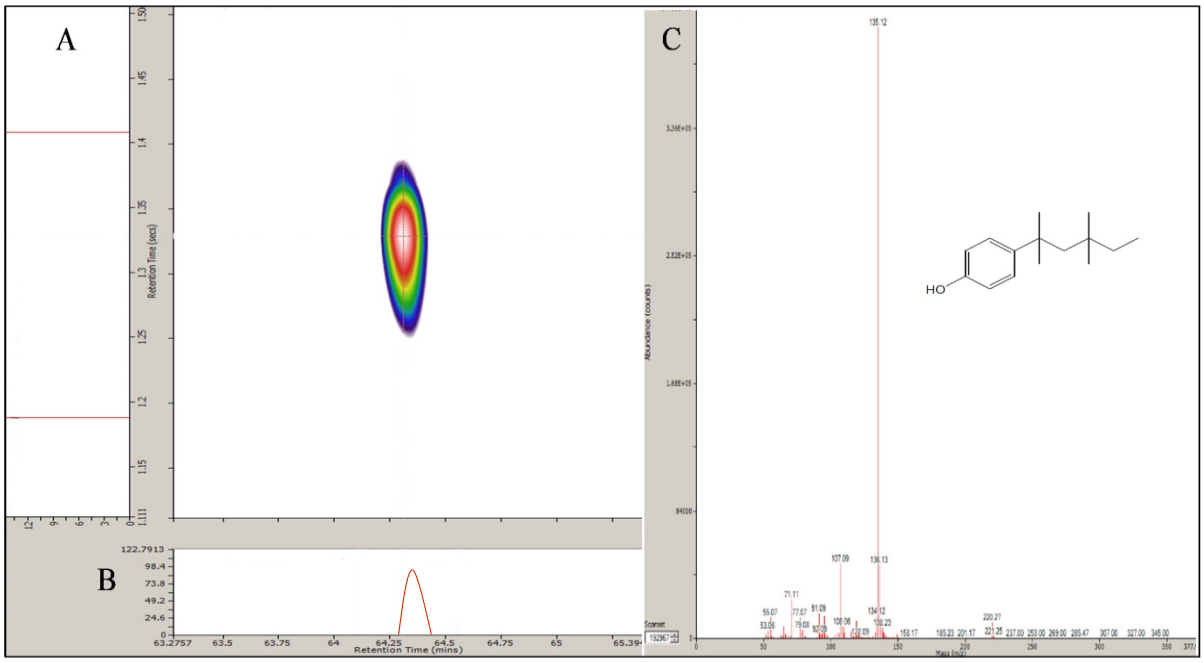


Figure 3.10: GC x GC-Tof-MS chromatogram of NP_{94} (concentration = 2 ng/mL^{-1}) separated using DB-WAX as the first dimension column (D1) and MEGA-17 MS FAST as the second dimension column (D2); (A) the retention time in the D2 column ; (B) the retention time in the D1 column; (C) The mass spectrum and chemical structure of NP_{94}.

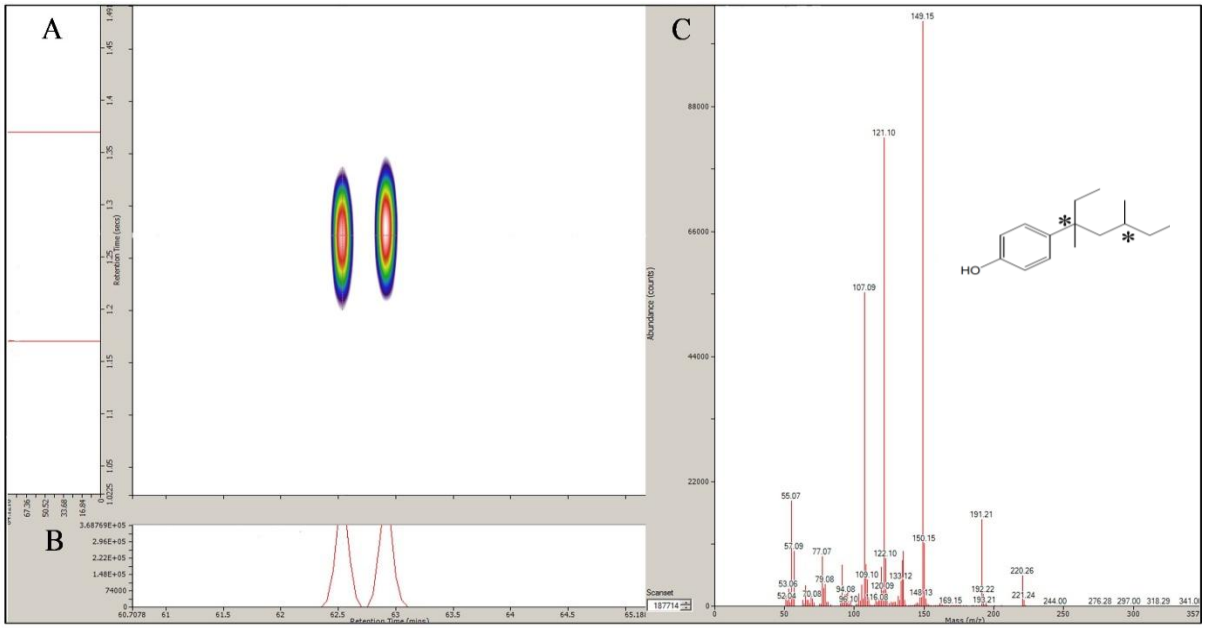


Figure 3.11: GC x GC-Tof-MS chromatogram of $NP_{111a,b}$ (concentration = 2 ng/mL^{-1}) separated using DB-WAX as the first dimension column (D1) and MEGA-17 MS FAST as the second dimension column (D2); (A) the retention time in the D2 column ; (B) the retention time in the D1 column; (C) The mass spectrum and the chemical structure of $NP_{111a,b}$.

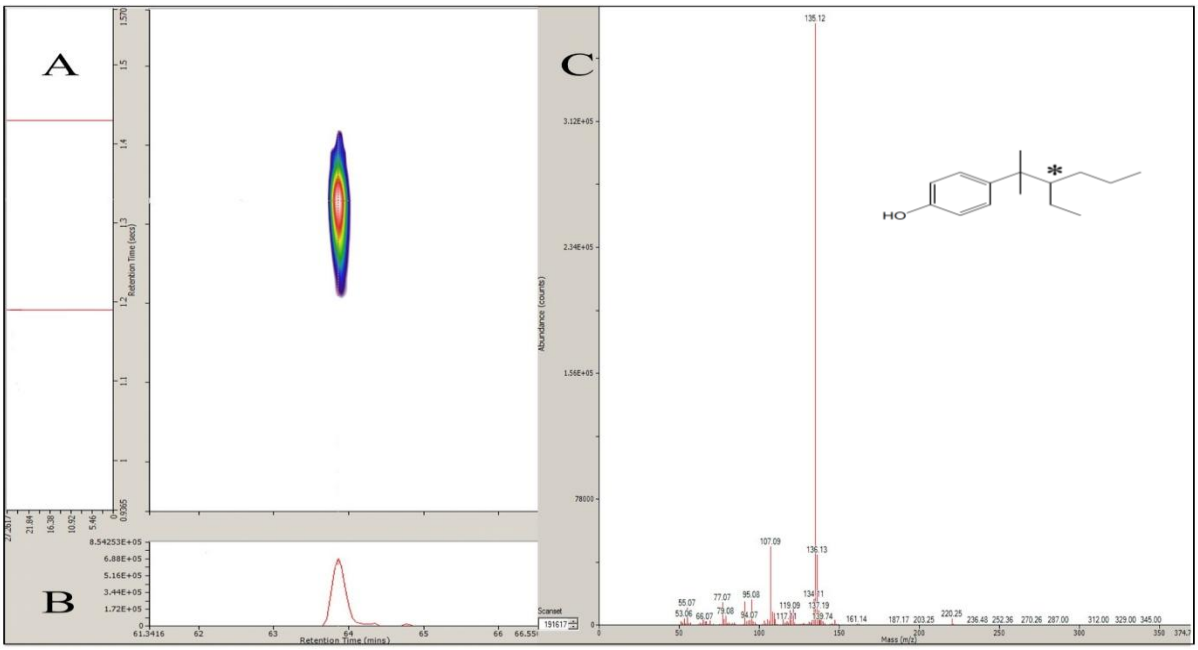


Figure 3.12: GC x GC-Tof-MS chromatogram of NP_{119} (concentration = 2 ng/mL^{-1}) separated using DB-WAX as the first dimension column (D1) and MEGA-17 MS FAST as the second dimension column (D2); (A) the retention time in the D2 column ; (B) the retention time in the D1 column; (C) The mass spectrum and chemical structure of NP_{119}.

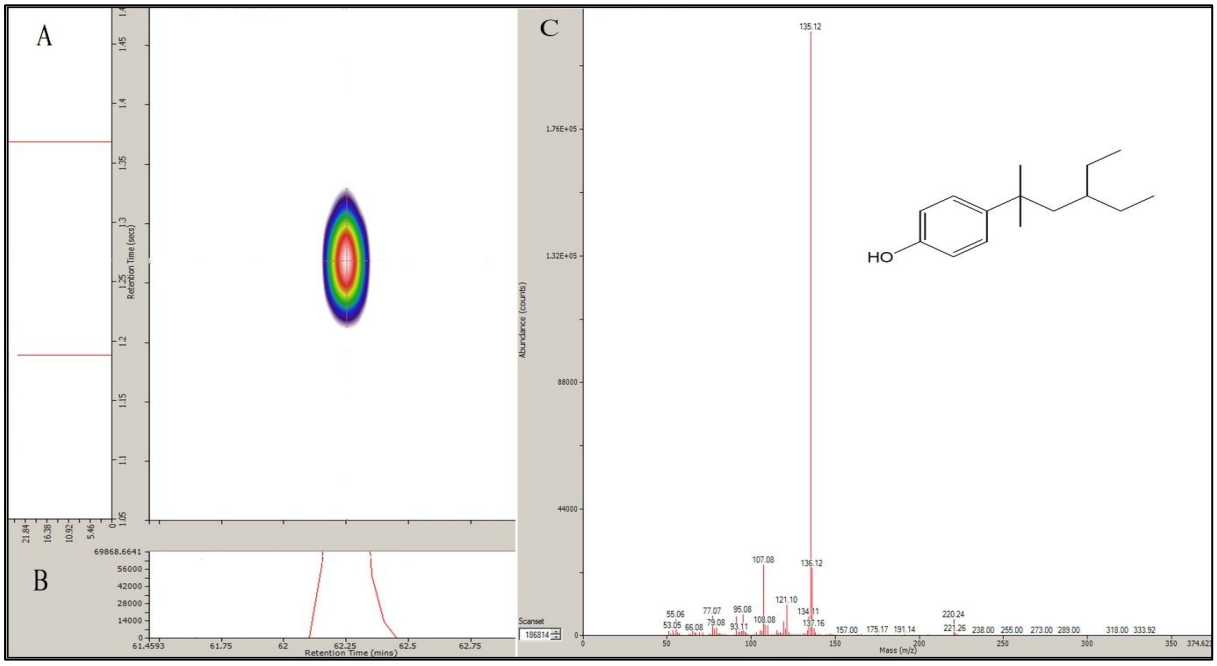


Figure 3.13: GC x GC-Tof-MS chromatogram of NP_{128} (concentration = 2 ng/mL^{-1}) separated using DB-WAX as the first dimension column (D1) and MEGA-17 MS FAST as the second dimension column (D2); (A) the retention time in the D2 column ; (B) the retention time in the D1 column; (C) The mass spectrum and chemical structure of NP_{128}.

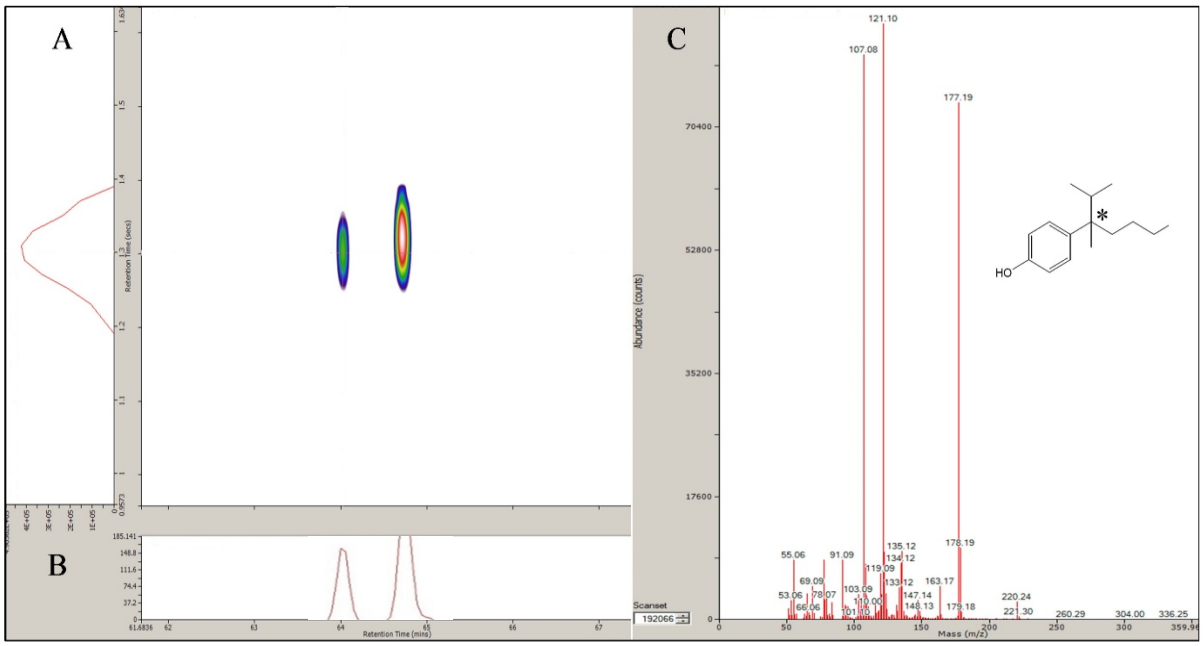


Figure 3.14: GC x GC-Tof-MS chromatogram of the NP_{143} (the green bulb, concentration = 0.40 ng/mL^{-1}) separated using DB-WAX as the first dimension column (D1) and MEGA-17 MS FAST as the second dimension column (D2); (A) the retention time in the D2 column ; (B) the retention time in the D1 column; (C) The mass spectrum and chemical structure of NP_{143}.

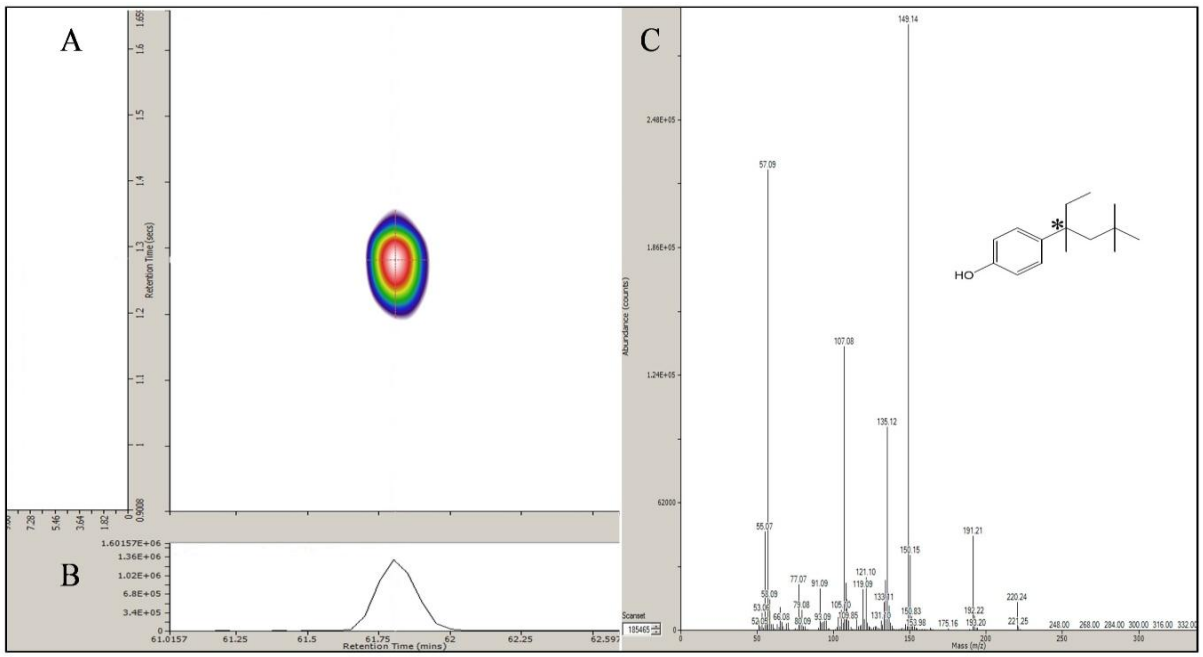


Figure 3.15: GC x GC-Tof-MS chromatogram of the NP_{170} (concentration = 2 ng/mL^{-1}) separated using DB-WAX as the first dimension column (D1) and MEGA-17 MS FAST as the second dimension column (D2); (A) the retention time in the D2 column ; (B) the retention time in the D1 column; (C) The mass spectrum and chemical structure of NP_{170}.

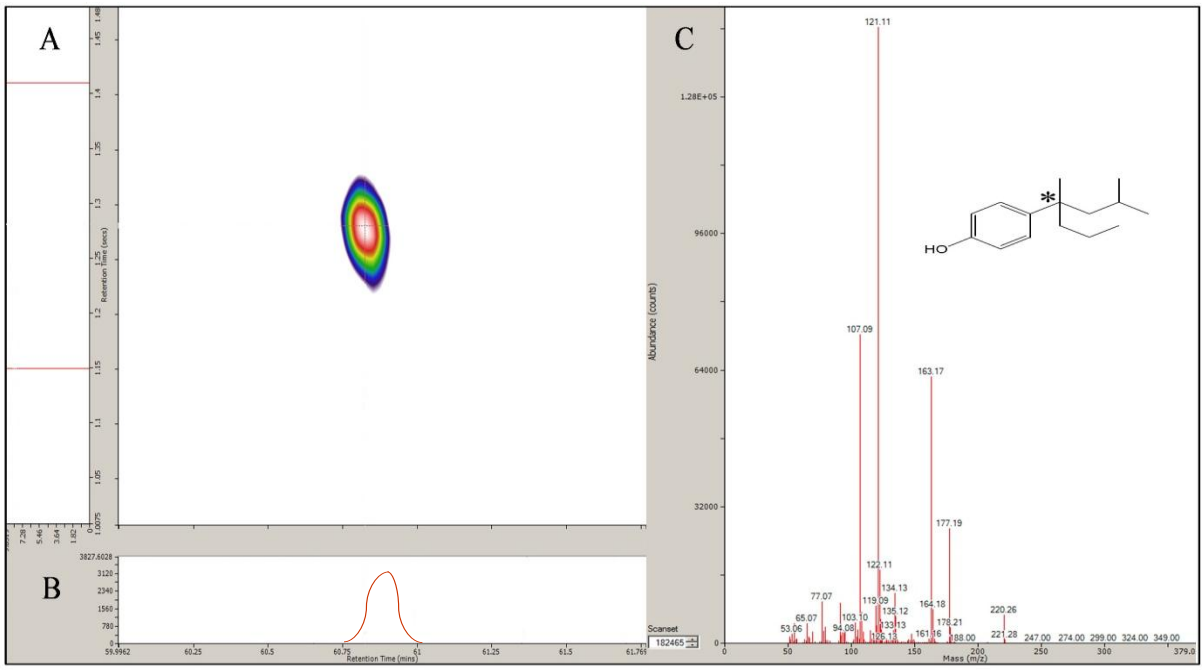


Figure 3.16: GC x GC-Tof-MS chromatogram of NP_{194} (concentration = 2 ng/mL^{-1}) separated using DB-WAX as the first dimension column (D1) and MEGA-17 MS FAST as the second dimension column (D2); (A) the retention time in the D2 column ; (B) the retention time in the D1 column; (C) The mass spectrum and chemical structure of NP_{194}.

3.3.4 Identification of Nonylphenols Isomers in Arabic Coffee:

Chromatograms of coffee samples showed six peaks, representing five isomers since one isomer had two chiral carbons. When the mass spectra of these isomers (Figures 3.17–3.22) were compared with those of the cross-reference isomers, it was observed that they had the same molecular ion (m/z 220) and characteristic fragment ions (m/z 107, 121, 135, 149, 163, 177, and 191), as shown in tables 2.3 and 3.3. Only two NP isomers, 4-[3-ethyl-1,1-dimethylpentyl] phenol or NP_{128} (peak 1) and 4-[1-ethyl-1,3-dimethylpentyl] phenol or $NP_{111a,b}$ (peaks 2 and 3), were identified by mass spectra, characteristic fragment ions, and retention times in the coffee sample.

Table 3.3: GC × GC/TOF-MS details of NP peaks from the coffee sample.

Peak No.	1st Retention time (min)	2nd Retention time (sec)	Group No.	Fragment ion *m/z*
Coffee Peak 1 (NP_{128})	62.27	1.27	1	135
Coffee Peak 2 (NP_{111a})	62.47	1.27	2	121, 149 and 191
Coffee Peak 3 (NP_{111b})	62.87	1.28	2	121, 149 and 191
Coffee Peak 4	63.72	1.30	1	135
Coffee Peak 5	64.02	1.31	5	121, 163 and 177
Coffee Peak 6	64.22	1.31	3	121 and 149

NP_{128}, with its α,α-dimethyl structure, belongs to *group 1* isomers, as illustrated in figure 3.13. Based on the mass spectra, $NP_{111a.b}$ belongs to *group 2* isomers with an α-ethyl-α-methyl configuration (Figure 3.11). $NP_{111a,b}$ exhibited a base peak at *m/z* 149, with *m/z* 121 and *m/z* 191 as specific fragment ions. Moreover, $NP_{111a,b}$ is a diastereomer and has demonstrated the highest estrogenic potency in a transcriptional activation cell assay.[9]

The remaining three significant components in the coffee sample, peaks 4, 5, and 6, have mass spectra and relative retention times that correspond to NPs first identified by Wheeler et al. (1997). Peak 4 had α,α-dimethyl structures (Figure 20) identical to NP_{128}, whereas peak 5 belonged to *group 5* (Figure 3.21). This isomer has an MS spectrum similar to the model compound NP_{194} 4-[1,3-dimethyl-1-propylbutyl]-phenol (Figure 16). Finally, the peak 6 NP isomer produced a base peak at *m/z* 149 and no significant fragments in the region *m/z* 151-219 (Figure 3.22). The presence of an *m/z* 149 base peak was like that of the α-methyl isomers and belonged to *Group 3* isomers. This NP isomer may have an α-methyl and α-ethyl substitution, branching at the *β*-carbon.[17]

$NP_{111a,b,}$ and NP_{128} are in t-NP.[119] Moreover, Zhang et al. (2012) found $NP_{111a,b}$ in landfill leachate and wastewater influent samples in Oklahoma City in the USA.[170] Although the study successfully identified two NP isomers in the coffee sample, further improvements in the methodology and technique are required to enhance NP isomer separation and identification in the future.

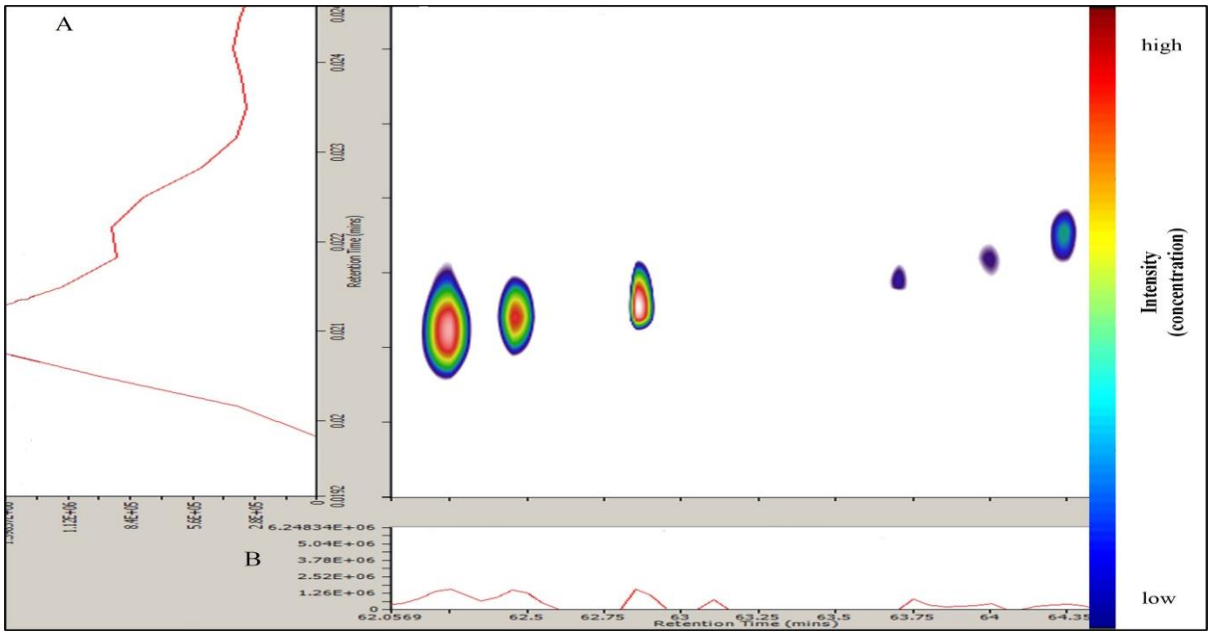


Figure 3.17: GC x GC-Tof-MS chromatogram of coffee sample separated using DB-WAX as the first-dimension column (D1) and MEGA-17 MS FAST as the second-dimension column (D2); (A) the retention time in the D2 column; (B) the retention time in the D1 column.

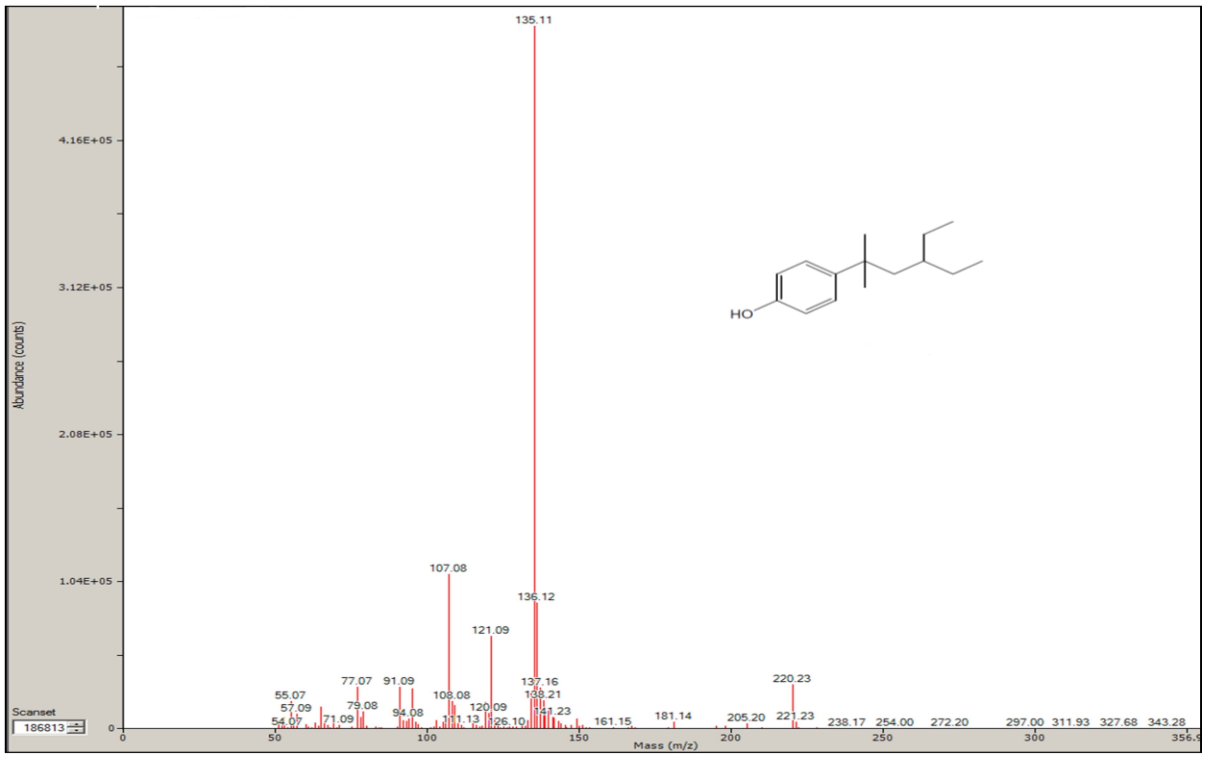


Figure 3.18: Mass spectrum and chemical structure: Nonylphenol isomer $NP_{128,}$ the first bulb in the coffee sample.

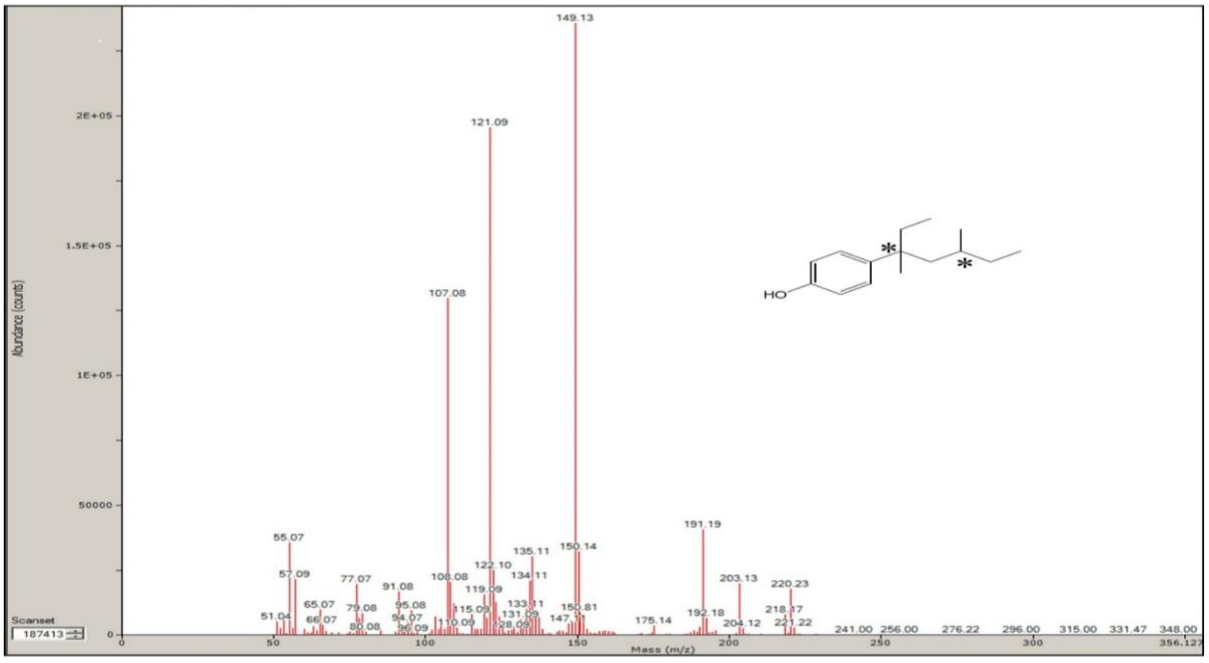


Figure 3.19: Mass spectrum and chemical structure of Nonylphenol isomer $NP_{111a,b}$ the second and third bulbs in the coffee sample.

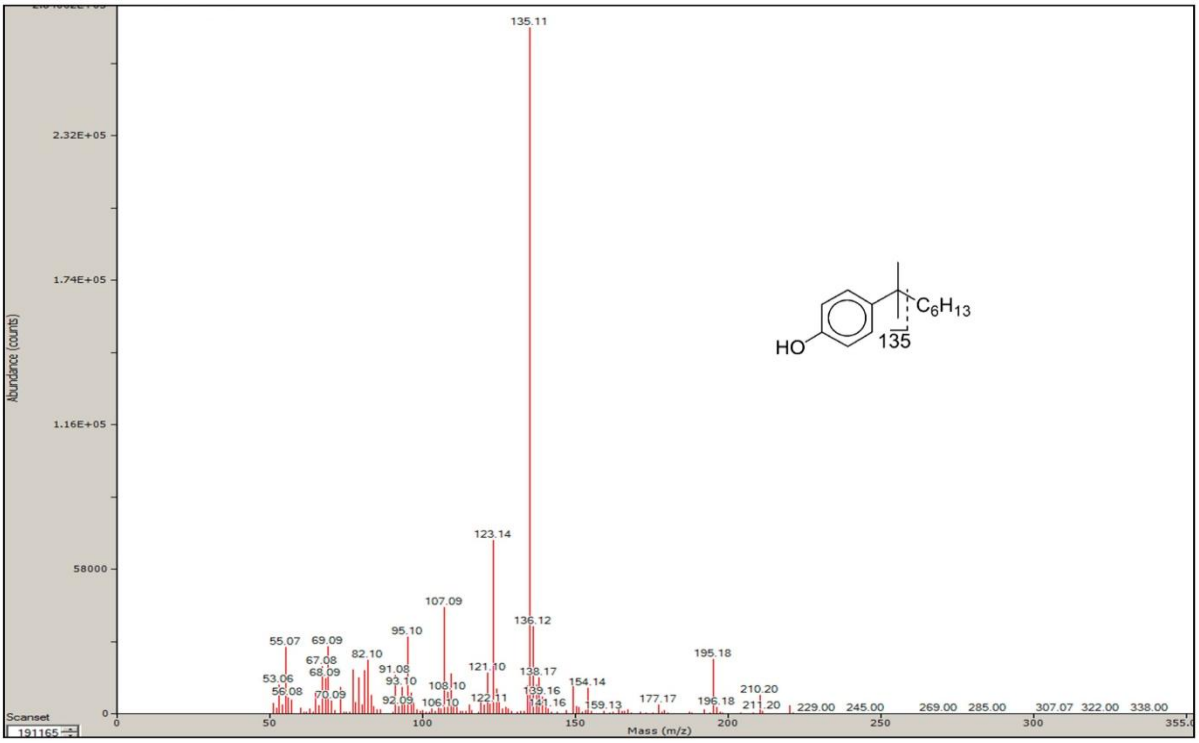


Figure 3.20: The mass spectrum of the Nonylphenol isomer in the fourth bulb in the coffee sample belongs to *group 1*.

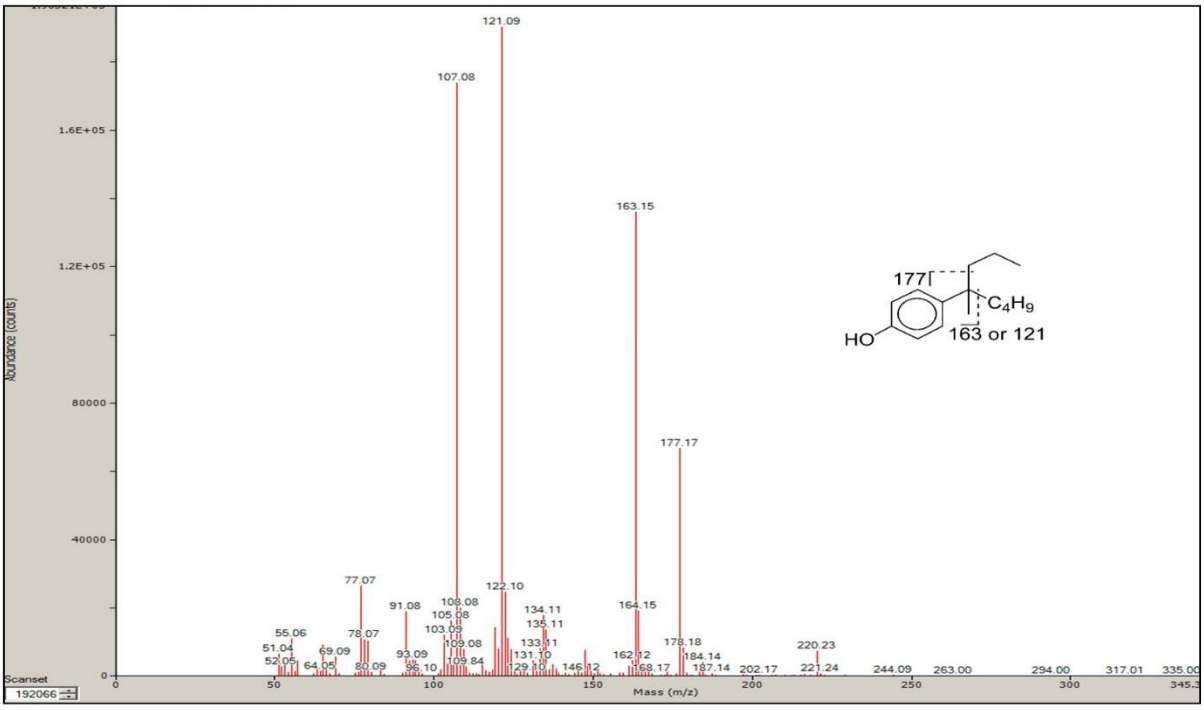


Figure 3.21: The mass spectrum of the Nonylphenol isomer in the fifth bulb in the coffee sample belongs to *group 5*.

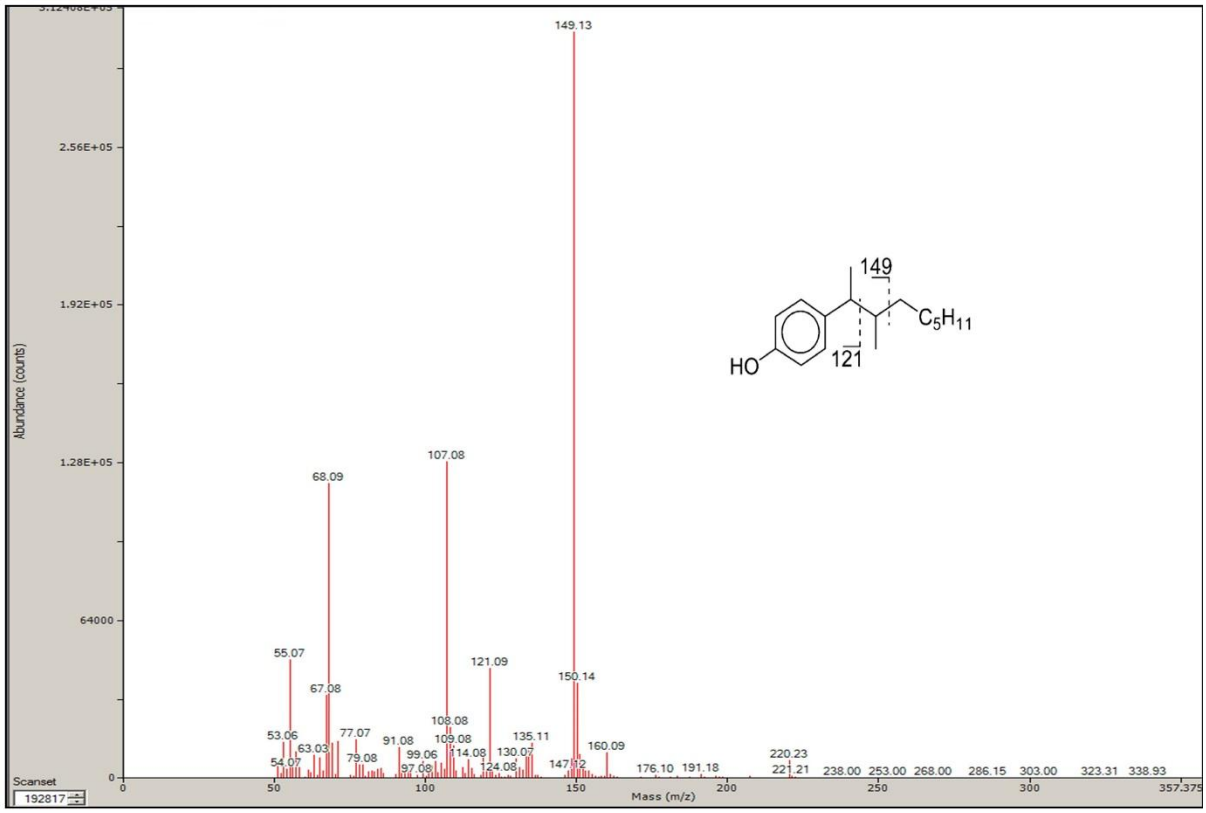


Figure 3.22: The mass spectrum of the Nonylphenol isomer in the sixth bulb in the coffee sample belongs to *group 3.*

3.4 Conclusion:

This study used LC/MS-MS to quantify NPs in selected foodstuffs from different countries. Furthermore, GC × GC–TOF–MS was used to detect and categorize NP isomers in coffee samples. The study established that eight out of the twelve screened samples contained NPs with concentrations of 0.5 to 18.9 µg kg^{-1} based on fresh weight. Additionally, the NP content in apples was observed to vary by country. Thus, the findings demonstrate the effect of the legislature on the use of NPEO products in regions globally. Thirteen cross-reference NP isomers were used to identify NP isomers in coffee samples. Based on the characteristic fragment ions and retention times, this study confirmed that $NP_{111a,b,}$ and NP_{128} were found in coffee samples. The applied GC x GC-TOF system with a liquid nitrogen cryogenic modulator is suitable for the crucial isomer-specific analysis of NPs. Based on these findings, further studies are needed to improve this method and technique for the isomer-specific examination of NPs in food control laboratories. Moreover, more studies should be conducted to identify the specific health risks associated with humans.

Acknowledgments: The German Research Society INST 217/783-1 FUGG supported the project.

3.5 Authors Contribution Statement:

This chapter is adapted from the research article N. Al Rashed, Dr. C. Gerlach and K. Günther, Determination of Nonylphenol in Selected Foods and Identification of Single Isomers in a Coffee Sample by Comprehensive Two-Dimensional Gas Chromatography-Time of Flight Mass Spectrometry, Analytical Letters, DOI.org/10.1080/00032719.2023.2180018. For this research article, the following paragraphs specify the individual contributions of each author.

Author 1; N. Al Rashed: Conceptualization, conceived and designed the experiments, performed the experiments, analysis, and interpretation of data, performed the calculations, writing (original draft preparation), writing (review and editing), designing the figures, and literature review.

Author 2: Dr. C. Gerlach: GC x GC-TOF-MS method development, writing (review and editing).

Author 3: Prof. Dr. K. Günther: writing (review and editing), supervision.

4. Conclusion and future studies

In the present thesis, endocrine-disrupting NPs and NPECs were analyzed in food samples; their daily intake was determined for German adults after Directive 2003/53/EC implementation, and two NPs isomers were identified in coffee samples.[126]

For the conducted studies, three questions were taken into consideration: First, are NPs still present in food samples, and has the daily intake of NPs for German adults decreased following the implementation of Directive 2003/53/EC? The second question is whether NPECs are present in food samples or not. Finally, would GC x GC Tof-Ms provide sufficient separation of a complex mixture of food samples to identify NPs isomers within the food matrix?

One of the main objectives of the present study was to reinvestigate the presence of NPs in the foods examined by Guenther et al. (2002) to determine the daily intake of NPs for German adults after the implementation of Directive 2003/53/EC.[126] 22 out of the 24 categories of food analyzed contained NPs, ranging from 0.1 to 6.3 g kg^{-1} on a fresh weight basis.[36]

Despite the lower concentrations reported here than those reported by Guenther et al. (2002), these results demonstrate that NPs have been present in most food samples since Directive 2003/53/EC was implemented in 2005. Based on the various samples examined, mayonnaise, liver sausages, and spinach showed the highest concentrations of NPs, 6.3, 5.4, and 4.4, respectively, while pasta and apples showed levels below the levels of LOD.

Compared with previous findings, the present study indicates that NP levels in apples decreased from 19.4 grams per kilogram to below the LOD. Other foodstuffs also showed a decrease in NP levels, including butter (from 14.4 to 2 g kg^{-1}), bacon (from 10.2 to 0.8 g kg^{-1}), milk chocolate (from 14.1 to 1.2 g kg^{-1}), and tomatoes (from 18.5 to 0.2 g kg^{-1}).

However, the concentration of NPs in spinach was nearly three times higher than previously reported. The difference in the NP levels between Guenther et al. (2002) (paper packaging) and this study (plastic bags) may be due to differences in the packaging materials.[36] Food packaging materials are a significant source of NPs, produced by the decomposition of tris(nonylphenol) phosphite (TNPP).

Based on the average food composition consumed by German adults. It was estimated that German adults' average daily intake of NPs was 2.7 7 µg day^{-1}. An important point to note is that the daily intake of NPs has decreased by more than half compared to what was reported by Guenther et al. (2002), where a daily intake of 7.5 7 µg was determined.

The second research question focused on the presence or absence of NPECs in food samples. It was, therefore, necessary to develop a method for extracting and measuring NPEC levels in food samples. An IS, $^{13}C_1$-$NPEC_{112}EC$ (10 µL), was added to each food sample (duplicate extraction) along with the appropriate procedural blank for testing the analytical procedure. 90 to 100% of the procedural blanks yielded efficiencies with a relative standard deviation of ≤ 4.2%. Consequently, the NPEC concentration calculations were corrected using the IS recoveries. The LOD was determined to be 0.08 ng, and LOQ was 0.12 ng. Throughout the investigation, all samples were below the LOD.

The presence of NPs and NPECs in the environment is ubiquitous and recalcitrant compounds. In wastewater treatment plants (WWTPs), NPECs are formed when short-chain NPEOs undergo biodegradation due to carboxylation of the terminal alcohol groups in the presence of oxygen.

There is, however, a more accurate indicator of the efficacy of a WWTP when NPs are measured in the final effluents obtained from the plant since NPs are the most abundant NPEO derivative in the effluent, and concentrations reaching up to 343 µg L^{-1} have been reported. To date, several studies have developed methods for determining the concentrations of NPECs in environmental samples.

For instance, Petrovic et al. (2003) determined that the water at the outlet of a treatment plant in Spain contained NPs and NPECs at levels of 85 and 12 ng L^{-1}, respectively.[151] In all foods examined in the present study, NPEC concentrations were below the LOD established by the method developed here. Therefore, it is evident that these compounds have no role to play in the food basket reviewed.

The final part of the study examined whether NPs isomers could be identified in food samples. To quantify NPs in selected foodstuffs from different countries, LC-MS/MS was used. According to the study results, eight of the 12 screened samples contained NPs with concentrations between 0.5 and 18.9 µg kg^{-1} based on the fresh weight of each sample.

Among the various samples analyzed, Arabic coffee, Chilean apples, Saudi dates, and Italian apples had the highest concentrations of NPs at 18.9, 8.3, 1.6, and 1.0 µg kg^{-1}, respectively. Low concentrations of NPs at 0.72, 0.68, 0.67, and 0.55 µg kg^{-1} were detected in fava beans, chocolate, tea, and wheat flour, respectively, while concentrations in cheese, milk, fruit juice, and German apple were below the LOD.

In contrast to previous research, our results demonstrate that foods grown in different regions can reflect the levels of NP in those regions and be an indicator of the laws of that region concerning the use of NPEOs in commercial/consumer products.

For instance, Arabic coffee is typically grown in the Middle East or on the African continent (in Ethiopia). We understand that most African countries, except South Africa, do not have regulations for NPEOs or NPs. Thus, it was highly likely that NPs would be detected at high concentrations.

Differences in NP levels among apples from different regions (Germany, Chile, and Italy) indicate the extent to which NPEO laws are enforced globally. German and Italian apples show low NP concentrations of (<=1 µg kg^{-1}), while apples from Chile show a considerably high concentration (>8 µg kg^{-1}). This can be attributed to Germany and Italy being member states of the EU, which instated a directive in June 2003 to restrict the use and marketing of toxic substances, particularly NPEOs and their toxic derivatives.

As a result of this directive, all EU member states have implemented it, with Germany going one step further and banning the industrial use of NPEOs in 2005. According to the NP values of apples from Germany, which fell below the LOD values of the test, this ban has had an adverse effect. Although Italian apples have lower levels of NP than Chilean apples, their levels of NP are higher than those of German apples since the textile industry in Italy still actively uses NPEO products.

NPs were found in the highest concentration in coffee samples, indicating that they are isomer-specific. The 13 cross-reference NP isomers were used to identify NP isomers in coffee samples using GC×GC–TOF–MS.

Chromatograms of coffee samples revealed six peaks, representing five isomers since one isomer contains two chiral carbons. By comparing the mass spectra of isomers with those cross-references, it was observed that they had the same molecular ion (m/z 221) and characteristic fragment ions (m/z 107, 121, 135, 149, 163, 177, and 191).

In the coffee sample, only two NP isomers were identified by mass spectra, characteristic fragment ions, and retention times: 4-[3-ethyl-1,1-dimethylpentyl] phenol or NP_{128} and 4-[1-ethyl-1,3-dimethylpentyl] phenol or $NP_{111a,b}$.

NP_{128}, with its α, α-dimethyl structure, belongs to *group 1* isomers. Based on the mass spectra, $NP_{111a.b}$ belongs to *group 2* isomers with an α-ethyl-α-methyl configuration. $NP_{111a,b}$ exhibited a base peak at *m*/*z* 149, with *m*/*z* 121 and *m*/*z* 191 as specific fragment ions. Moreover, $NP_{111a,b}$ is a diastereomer and has demonstrated the highest estrogenic potency in a transcriptional activation cell assay.

The remaining three significant components of the coffee sample, peaks 4, 5, and 6, have mass spectra and relative retention times consistent with the NPs first identified by Wheeler et al. (1997).[17] There was an identical structure to NP_{128} in peak 4, whereas peak 5 belongs to *group 5*. This isomer has an MS spectrum similar to the model compound NP194 4-[1,3-dimethyl-1-propylbutyl]-phenol. Finally, the peak 6 NP isomer produced a base peak at m/z 149 and no significant fragments in the region m/z 151-219. The presence of an m/z 149 base peak was like that of the α-methyl isomers and belonged to *Group 3* isomers.

In the overall results of this thesis, the effect of the legislation on the use of NPEO products in different regions of the world is demonstrated. The GC x GC-TOF system with a liquid nitrogen cryogenic modulator is suitable for the isomer-specific analysis of NPs. It will be necessary to conduct further research to improve this method and technique for examining NPs in food control laboratories based on their isomer specificity.

Moreover, more studies should be conducted to identify the specific health risks of NPs in humans. In addition, future studies must examine other potential sources of NPs contamination to support additional regulation and remediation. Furthermore, isomer-specific analysis and evaluation of the NPs contents in foods will be crucial in the future since the estrogenicity of each isomer varies significantly.

It should also be noted that NPs have been found in relevant concentrations in human blood. Moreover, there are often few restrictions on using NPs and NP precursors outside Europe. Hence, the global food trade contributes to the continued problem with this endocrine disruptor in the food chain and the human organism. Therefore, further research in this area is necessary.

Although almost all commercial foods examined contain detectable levels of NPs, future studies should examine other potential sources of NP contamination to support additional regulation and remediation. In order to gain a comprehensive understanding of the fate and transport of NP isomers, more environmental processes need to be considered. A primary source of NP release into the open environment is wastewater treatment plants (WWTPs).

Intense biological activities during activated sludge treatment, digestion, and composting may significantly alter the NP isomer composition of discharged effluent and sludge. Aquatic systems affected by effluent are likely to demonstrate isomer selectivity due to NP bioaccumulation or biomagnification by aquatic species.

Furthermore, when sludge is applied as a soil amendment, soil biodegradation and the uptake of NP isomers in plants can have direct implications for food safety and human health, where isomer selectivity is of scientific interest as well.

Isomer-specific analysis and evaluation of the NP content in foods will play an essential role in the future because of the significant variation in the estrogenicity of each isomer. In addition, the GC×GC–TOF–MS system with a liquid nitrogen cryogenic modulator employed in this study can be optimized for future routine analyses in the food control industry.

Note that a standard method is yet to be established to analyze isomer-specific NPs in foods. Additionally, the isomeric composition of technical NPs varies depending on the source and batch. There are also very few commercially available NPs isomers for use as reference standards on the market. In addition, most studies have yet to study matrix effects. Hence, obtaining comparable results among various laboratories is almost impossible.

Chemical manufacturers are strongly advised to produce 4-NP isomers. To ensure the comparability of laboratory results, certified matrix reference material(s) should also be developed.

Currently, the most used columns for separating 4-NP isomers are slightly polar GC columns with stationary phases incorporating 5% phenyl groups in the dimethylpolysiloxane polymer. Even though a more extended column (100 m) can separate 4-NP isomers more effectively, peak heights become smaller, and peaks become broader. As a result, although selectivity is improved, sensitivity is decreased.

Monitoring of 4-NP isomers in foods may not be enhanced by this method. Even though 2-dimensional gas chromatography (2D GC) has been used to resolve 4-NP isomers into more than 100 peaks, the identities and estrogenic activities of most 4-NP isomers are yet to be determined.

Aside from this, 2D GC is still less widely available than GC–MS/MS. Hence, GC–MS/MS is the most appropriate detection method. For the isomer-specific analysis of 4-NPs, GC–MS/MS provides better sensitivity and selectivity than GC–MS.

Seafood is one of the foodstuffs most likely to contain 4-NP isomers. Infant formula, cereals, fruit, legumes, and nuts can also contain 4-NP levels exceeding 10 or even 100 μg kg^{-1}. As a result, these food groups should also be included in monitoring programs. Because there has only been one study on the analysis of 4-NPs in infant food on an isomer-specific basis, more studies should be conducted.

Moreover, isotopically labeled standards, such as $^{13}C_6$–NP_{112}, are strongly recommended since matrix effects cannot be determined for 4-NP. Minor impurities may significantly contribute to estrogenicity. Consequently, it is also recommended that further research be conducted in the field of estrogenicity. Dietary risk assessments for 4-NPs cannot be conducted based on their isomers. A risk-based international protocol must ensure comparative results and address the toxicological concerns associated with 4-NP in food.

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