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FSA PROJECT A03057

Nonylphenol in food contact plastics and migration into foods

PROJECT INFORMATION

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SUMMARY

Nonylphenol (NP) is a generic term used to describe a complex commercial mixture of mainly nonyl-substituted phenol. The complexity arises from the production of the compound from commercial nonene that is used to alkylate the phenol. Commercial nonene is a complex mixture of C9 alkenes and the resultant product, although predominantly 4-nonylphenol (>90%, because alkylation favours the para position) also contains small quantities of 2-nonylphenol and decylphenol. Most NP produced commercially is 4-nonylphenol with varied alkyl chain branching.

The presence of NP in certain food contact materials has been reported. Although NP itself is not authorised for use in food contact plastics it may be present as an impurity or a breakdown product of alkylphenol ethoxylates or tris(nonylphenyl)phosphite. Alkylphenol ethoxylates are used as dispersing or stabilising agents in some food packaging polymers (plastics and rubber) and papers. Tris(nonylphenyl)phosphite is an antioxidant used to stabilise some food contact plastics.

Methodology for the determination of NP in food contact plastics was developed in an earlier Food Standards Agency project (A03047). The method developed in that project used either high temperature soxhlet extraction or dissolution followed by solvent extraction depending on the material type, followed by purification via chromatography on deactivated alumina. The procedures were internally standardised by ^{13}C-labelling and the analysis was carried out by gas chromatography-mass spectrometry (GC-MS). In-house validation parameters were derived for this method which was written as a Standard Operating Procedure.

In the present project, between-laboratory validation studies with two additional laboratories highlighted a number of critical steps in the method that must be controlled to ensure satisfactory performance. The Standard Operating Procedure (SOP) was revised accordingly although performance of the method in the other laboratories was still not satisfactory. It is concluded that the method SOP for packaging analysis is suitable as a general screening method giving semi-quantitative results, but that fully quantitative analysis remains the preserve of laboratories experienced in NP analysis, evidenced for example by accreditation for this or similar contaminants.

Ten samples of PVC (polyvinyl chloride) cling films and ten samples of HIPS (high-impact polystyrene), taken from pre-packaged foods) were tested since earlier work indicated that these plastics may contain NP. Two of the ten PVC samples contained NP at 2590 and 4150 mg/kg and three of the ten HIPS samples contained NP at 28, 925 and 1760 mg/kg. It was demonstrated by using control experiments that these findings of NP were not a result of decomposition of any alkylphenol ethoxylates or tris(nonylphenyl)phosphite, that the samples may have contained, during the analytical procedure.

The two positive PVC films were tested for migration in contact with cheese and cake for 4 days at 20^{o}C. For the conventional packaging ratio of 1 kg food in contact with 6 dm^2 the results obtained correspond to a NP migration of about 0.2 to 0.8 mg/kg into cheese and 0.3 to 0.6 mg/kg into cake.

The foods packaged in the three positive HIPS samples were tested for NP. The surface layer of the solid foods was tested, since this could be expected to be where any migration would be located and concentrated. However, any migration was below the detection limit which was estimated to be about 0.2 mg/kg. These experimental findings were supported by migration modelling results which indicated a low migration potential, given the concentrations of NP determined in the HIPS itself and the fact that HIPS is a low diffusivity plastic.

Since it is an impurity or a degradation product and not an authorized substance, no EU specific migration limit has been assigned to NP. A Tolerable Daily Intake (TDI) of

5 µg/kg body weight has been proposed by the Danish Institute of Safety and Toxicology. Migration from PVC cling film has the potential to result in exposures approaching or exceeding this proposed TDI.

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ABBREVIATIONS

CEN	European Committee for standardisation
CSL	Central Science Laboratory
EI	Electron impact
Fera	The Food and Environment Research Agency
FSA	Food Standards Agency
GC-MS	Gas chromatography-mass spectrometry
HDPE	High density polyethylene
HIPS	High impact polystyrene
IS	Internal standard
LOD	Limit of detection
LOQ	Limit of quantification
NIAS	Non-intentionally added substances
NP	Nonylphenol
PAR	Peak area ratio
PET	Polyethylene terephthalate
PS	Polystyrene
PTV	Programmable temperature vapourisation
PVC	Polyvinyl chloride
QM	Quantity permitted in the final material or article
RRF	Relative response factor
SIM	Selected ion monitoring
SOP	Standard operating procedure
TNPP	Tris(nonylphenyl)phosphite
IUPAC	International Union of Pure and Applied Chemistry

1. INTRODUCTION

1.1 Background

Nonylphenol (NP), a recognised environmental contaminant (1), is a generic term commonly used to describe a complex commercial mixture of mainly nonyl-substituted phenol. The complexity arises from the production of the compound from commercial nonene that is used to alkylate the phenol. Commercial nonene is a complex mixture of C9 alkenes and the resultant product, although predominantly 4-nonylphenol (>90%, because alkylation favours the para position) also contains small quantities of 2-nonylphenol and decylphenol. Most NP produced commercially is 4-nonylphenol with varied alkyl chain branching. As an environmental contaminant, NP is derived mainly from the hydrolytic degradation of its parent compounds - the alkylphenol ethoxylates. Alkylphenol ethoxylates are widely used as non-ionic surfactants with a range of industrial agricultural, institutional and domestic applications, principally as emulsifying, wetting, dispersing or stabilising agents. They are used as dispersing or stabilising agents in food packaging polymers (plastics and rubber) and papers. Another source of NP is as an impurity and hydrolysis product of tris(nonylphenyl)phosphite (TNPP). TNPP is used as an antioxidant and heat stabiliser in several polymers used as food packaging materials, for example rubber, styrenes, vinyl polymers, and polyolefins (2). This widespread utility (3, 4) combined with non-bonded incorporation in materials such as plastics, rubbers and other polymers results in widespread environmental distribution resulting ultimately in occurrence in foods. TNPP can also migrate to food and its hydrolysis in ingested food can release NP (5).

The presence of NP in food contact materials has been reported (9, 10). To confirm these findings the UK Food Standards Agency (FSA) commissioned work to develop a method of analysis for NP in different types of food packaging (11). The method developed in that study uses either high temperature soxhlet extraction or dissolution followed by solvent extraction depending on the material type, followed by purification via chromatography on deactivated alumina. The procedures were internally standardised by ^{13}C-labelling and the analysis carried out by GC-MS. The method developed was validated in-house and was used to analyse duplicate specimens of 25 packaging materials. NP was detected at parts per million levels in examples of polystyrene (PS) and polyvinyl chloride (PVC), but also in ethylene vinyl acetate, polycarbonate and rubber at much lower levels. Following that study, NP was reported by other workers as being present in PVC gaskets on metal lids for glass jars (12).

Although NP is not permitted for use in the manufacture of food contact plastics it may be present as an impurity or a degradation product formed from permitted plastics additives. As such it is a non-intentionally added substance (NIAS). The compliance of food contact plastics with existing legislation should take into account both (a) known ingredients and (b) their impurities, reaction products and breakdown products. To date most studies have concentrated on the former and have largely neglected the latter. A limited number of migration studies have been carried out on NP. The migration of NP from high density polyethylene (HDPE) and PVC bottles into drinking water and a milk surrogate (13), from PVC packaging films into food simulants and foods (14), from PVC gloves into food simulants (15), from containers into food simulants (16) and from rubber into *n*-heptane, water and 20% ethanol (10) have been reported.

2. AIMS AND OBJECTIVES

2.1 Project aims

The project aimed to provide additional between laboratory validation data for the method of analysis developed in Food Standards Agency project A03047 - Method Development for the Analysis of Nonylphenol in Different Types of Food Packaging. In addition the project aimed to derive information on the source of NP in foods, i.e. migration from food contact materials or from environmental contamination.

2.2 Project objectives

Objective 01. Prepare an SOP and method validation report for the determination of NP in food contact materials

Objective 02. Obtain samples (2 HIPS, 2 PVC, 1 PET) and confirm the presence/absence of NP

Objective 03. Between laboratory validation of the method of analysis

Objective 04. Obtain examples of PVC cling film and foods packaged in HIPS and determine the NP concentrations in the plastics

Objective 05. Determine the concentration of NP in the foods packaged in the HIPS samples obtained in Objective 04

Objective 06. Determine the concentration-depth profile of NP in the foods packaged in the HIPS samples obtained in Objective 04

Objective 07. Determine the migration of NP into foods placed in contact with plastics obtained in Objective 04

Objective 08. Evaluate food concentration data and determine the relative contribution of migration compared to NP occurrence from other sources

Objective 09. Prepare final project report

3. MATERIALS

For the method validation studies, two high impact polystyrene (HIPS) samples containing NP, two polyvinyl chloride (PVC) cling film samples containing NP and one polyethylene terephthalate (PET) sample with no detectable NP, were obtained from retail outlets in the North of England.

For the migration studies 10 PVC cling films and 10 foodstuffs packaged in HIPS were purchased from retail outlets in the North of England. Processed cheese and cake (packaged in materials that did not contain NP) were purchased for use in the PVC cling film migration studies. Three of the HIPS packages were found to contain NP and so each of the three packaged foods was then purchased in triplicate. They were chilled fish products, being fishcakes, lemon sole and prawns.

4. CHEMICALS

4-Nonylphenol technical mixture was obtained from ChemService (Pa. USA). $^{13}C_6$-4-n-Nonylphenol (100 µg/ml in nonane) and $^{13}C_{12}$-PCB 52 (40 µg/ml in nonane) were purchased from Cambridge Isotope Laboratories (CIL) Mass, USA). Nonane,

methanol, cyclohexane, dichloromethane, petroleum ether was obtained from Rathburn Chemicals Ltd, Walkerburn, Scotland, UK. Alumina Brockmann Grade 1 was purchased from, Fluka and Sigma Aldrich, sulphuric acid – Reagent Grade and sodium sulphate anhydrous – Laboratory reagent grade, were from Fisher Scientific. Deionised water was generated within the laboratory.

5. STANDARD OPERATING PROCEDURE AND METHOD VALIDATION PARAMETERS FROM FSA PROJECT A03047

The method of analysis developed in FSA project A03047 was written as a standard operating procedure (SOP) in CEN format and the method validation parameters derived in this project were reported in accordance with the IUPAC harmonised guidelines for single laboratory validation and the CEN report on the validation and interpretation of analytical methods, migration testing and analytical data for materials and articles in contact with food. The SOP and the validation report are provided as Annexes 1 and 2.

6. RE-ESTABLISHING THE METHOD OF ANALYSIS IN-HOUSE

Changes in solvents, instrumentation and personnel meant that the method of analysis needed to be re-established in-house prior to embarking on the between-laboratory validation exercise.

6.1 Analysis of solvents

The analytical method as described in the SOP (Annex 1) involves the use of several solvents for extraction and clean-up. All of these have the potential to contain nonylphenol and thereby contaminate the sample. To ensure that current batches of solvent available in-house did not contain NP, 50 ml portions were evaporated to dryness, any residue was reconstituted in nonane (200 µl) and analysed by GC-MS according to the conditions described in the SOP in Annex 1. NP-free solvents were selected in this way and were set-aside for exclusive use in this study.

6.2 GC-MS instrumentation

The SOP provides details of the gas chromatographic parameters that were found to be suitable in project A03047 but also states that “appropriate operating conditions have to be established for the specific equipment used for the determination”. The GC column and instrument conditions described below were found to be suitable for use with the Agilent 5973inert GC-MS instrumentation used in this project.

Column: ZB-5ms	(Phenomenex, 5% phenyl and 95% dimethylpolysiloxane) 30 m x 0.25 mm id x 0.25 µm film thickness
GC parameters:	
Column oven	isothermal 3 minutes at 60℃, then rai sed at 25℃/minute to 160℃ and held for 2 minutes, then at 0.8℃/minu te to

	165℃, then at 30℃/minute to 300℃ and held for 5 minutes
Injector	Splitless, 1 minute
Injection volume	1 µl
Inlet temperature	250℃
Carrier gas	helium at 1 ml/minute
Transfer line temp.	280^{o}C
MS detection:	
Mode	selected ion monitoring (SIM) EI mode
SIM ions recorded	107, 121, 135, 149, 163, 220 for 4-nonylphenol
	113, 226 for $^{13}C_6$-4-n-nonylphenol
	302, 304 for $^{13}C_{12}$-PCB 52

The GC-MS chromatogram obtained from the analysis of the RRF (relative response factor) standard (Annex 1 / 2) are shown in Figure 1.

6.3 In-house validation

6.3.1 Linearity

Solvent standards were prepared as described in the SOP in Annex 1. The resulting calibration graph plotting the peak area ratio (PAR) of the quantitation ion for NP (m/z 220) against the equivalent ion in the labelled internal standard (m/z 226) is shown in Figure 2.

6.3.2 Limit of detection and limit of quantification

The limit of detection (LOD) for the determination of NP in nonane (the final solvent) was determined by analysis of standard solutions of the nonylphenol technical mix. The limit of detection (1.5 µg/ml in nonane) was calculated as 3x signal:noise ratio.

Although the solvents were confirmed as being NP-free adventitious pick-up of NP during the procedure did occur therefore the limit of quantification (LOQ) could not be calculated using the solvent standards alone. Following the procedure described in the SOP for the preparation of blank samples an average background of NP equivalent to 0.017 µg/g and a standard deviation equivalent to 0.003 µg/g was determined. From this the LOQ was calculated to be 0.047 µg/g, i.e. the mean background concentration + (10 x standard deviation).

6.3.3 Recovery

The SOP is written such that analytical recovery is assessed for each sample based on the internal standard used. The recovery is calculated relative to the internal sensitivity standard (^{13}Carbon labelled PCB 52) that is added to the sample extract just prior to GC-MS measurement. This is calculated as a percentage by comparing ratio of the internal standard (taken through the whole analytical procedure) to the internal standard sensitivity standard (added just prior to the GC-MS measurement) with the ratio of the two in the RRF standard. It should be noted that as the methodology uses

internal standardisation, concentration values reported for the materials are automatically corrected for recovery losses.

6.3.4 Repeatability

Rather than overspike a sample with the NP technical mix the repeatability was determined by the analysis of replicate specimens of a polymer found to contain high levels of NP. Analysis of eight replicate specimens from a HIPS sample was carried out according to the SOP in Annex 1. The results obtained are shown in Table 1.

6.3.5 Confirmation

The presence of nonylphenol in the samples is confirmed by comparison of the ion ratios (220/163, 220/149 and 220/135) of the nonylphenol extracted from the samples with those obtained from the analysis of the RRF standard analysed at the same time. The ion ratios of the samples extracted in the repeatability exercise and the average RRF ion ratios analysed at the same time are given in Table 2. These agree within ± 20% and therefore the confirmation criteria defined in the SOP was met.

6.3.6 Stability of nonylphenol polyethoxylates and tris(nonylphenyl) phosphite

As mentioned in the introduction nonylphenol polyethoxylates and TNPP may be used as additives in food contact materials. Work was carried out to confirm that the NP detected in the samples was not present as a consequence of the breakdown of either of these substances during the extraction and purification steps. This was investigated by fortifying a material with native nonylphenol polyethoxylates and TNPP, followed by analysis. PET was the material chosen because preliminary investigations showed no detectable levels of NP in it. Aliquots of the material were fortified with TNPP at concentrations of 100 and 1000 µg/kg and analysed as normal. Additional aliquots were fortified with nonylphenol polyethoxylates at concentrations of 100 and 1000 µg/kg and analysed as normal. No NP was detected in the resulting test solutions which demonstrates that the NP detected in the samples was not formed by this route. SIM chromatograms obtained from the analysis of the PET, PET spiked with TNPP at 1000 µg/kg, PET spiked with nonylphenol polyethoxylates at 1000 µg/kg and PET spiked with NP at 1000 µg/kg are shown in Figure 3.

7. SELECTION OF SAMPLES FOR THE BETWEEN LABORATORY VALIDATION EXERCISE

Two high impact polystyrene (HIPS) samples, two polyvinyl chloride (PVC) cling film samples and one polyethylene terephthalate (PET) sample were obtained from retail outlets in the North of England. Each plastic was, separately, cut into small pieces, mixed and equal portions (2 g) were weighed into thirty glass vials.

7.1 Homogeneity determination

Homogeneity testing was carried out by the duplicate analysis of 10 randomly selected specimens. This is the practice routinely used to test the homogeneity of candidate materials intended for FAPAS. The two PVC and two PS samples were analysed in this way. The PET sample was not tested in this way as it had already been established that PET was NP-free. The relative standard deviation (RSD, %) of the

analyses were in the range 9-17% and therefore the samples were considered to be homogeneous and suitable for the between-laboratory validation exercise. The NP concentrations measured in the homogeneity study are given in Tables 3 to 6 along with the recovery values calculated as described above and provided for information. NOTE: the data presented in Tables 3-6 was derived using the revised SOP given in Annex 3 (see later). The differences in the recoveries within the PVC 1 homogeneity samples are that the second half of the samples (6a-10b in Table 5) did not have the upper sodium sulphate plug added to the top of the alumina columns (this was omitted by mistake). The additional water on the alumina columns is believed to have resulted in further, undesired, deactivation and to have caused the reduced recovery values. Although the recoveries were much lower the method corrects for this and the absolute values were of the same order of magnitude (as shown in the concentrations column). The PVC 1 and the Blue PS samples were analysed with the same batch of dichloromethane rinsed sodium sulphate used to plug the columns. Analysis of the Clear PS and PVC 2 samples used all the same solvents and batch of alumina. The only difference was the batch of dichloromethane rinsed sodium sulphate used. Again this difference resulted in lower recovery values.

8. BETWEEN LABORATORY VALIDATION EXERCISE

When the method of analysis described in the SOP in Annex 1 had been re-established in house a second laboratory (Laboratory 2) was provided with the SOP and five polymer samples (2 x PVC, 2 x HIPS and 1 x PET). Two analytical chemists from this laboratory visited Fera (previously CSL) for training in the use of the method. Following this training exercise, in which the method was demonstrated in full, they were asked to analyse the five polymers following the SOP and to report the concentrations found in the polymers as well as the method performance characteristics.

8.1 Results Laboratory 2 – Phase 1

8.1.1 Chromatography

The EI chromatograms obtained from the analysis of the RRF standard and a PVC extract are shown in Figures 4 and 5 respectively.

8.1.2 Linearity

The linearity of the nonylphenol response was determined by analysing a series of ten standards in the 0 - 1000 µg/ml range. The peak area ratio of the analyte and internal standard (C^{13} labelled nonylphenol) was plotted against the concentration range of the standards, which demonstrated a linear relationship with a correlation of 0.9995 (Figure 6).

8.1.3 Limit of quantification

As described in Section 6.3.2 of this report some degree of NP contamination is expected during the procedure therefore the limit of quantification (LOQ) cannot be calculated using solvent standards alone. As above the data generated from the analysis of the procedural blank samples (Table 7) was used to calculate the LOQ. The LOQ was calculated to be 0.135 µg/g, the mean background concentration + (3 x standard deviation). The LOQ was higher than expected if clean (i.e. NP-free) solvents

were used for the extraction and clean-up steps. Solvents were not checked to confirm that they were NP free (as this was not specified in the SOP). The use of clean solvents is expected to reduce this value. The SOP was subsequently modified to highlight the importance of using clean, NP free, solvents.

8.1.4 Recovery and repeatability

The recovery and repeatability were determined by the triplicate (labelled rep-1, rep-2 and rep-3) analysis of two of the polymer samples provided by Fera. The samples were analysed on two separate occasions (labelled analysis 1 and analysis 2). The results obtained are shown in Table 8.

8.1.5 Confirmation

The presence of nonylphenol was confirmed by comparison of the ion ratios detected in the sample, to the ion ratios detected in the relative response factor standard (RRF). The criterion for confirmation is an agreement of ± 20%. Table 9 illustrates this confirmation.

8.1.6 Discussion

The results of the analysis of two of the test materials revealed that both the within- and between- batch repeatability data was not satisfactory. Low recovery values were also obtained. Comments received on the method were:

> "Section 4.3.1 (*of the SOP)* describes an initial three-stage liquid/liquid extraction between the cyclohexane sample extract, and acidified methanol. During this extraction it was noted that most, if not all of the cyclohexane was miscible with the methanol and as a consequence of this a consistent separation was very difficult to achieve. The second extraction stage using approximately three times more methanol appeared to be more effective, and a more efficient and seemingly more reproducible separation was possible.
>
> The drying down of the above extract, followed by chromatographic purification (section 4.3.3 *of the SOP*), and then further drying down of the eluent was thought to introduce additional errors and inconsistencies to the replicates. Whether in the absence of foodstuff contamination (*the method was originally adapted from one used to measure NP in foods*) this step is appropriate is debatable and could well be removed to simplify the method.
>
> It appeared that the chromatography degraded and sensitivity dropped significantly during a sequence of samples. A possible explanation for this effect could be the deposit of an amount of non-volatile material (possibly polymer) present in the extracts on the front of the analytical column. This effect may have been exaggerated with the use of cool-on-column injection as opposed to programmable temperature vaporisation (PTV) injection as specified in the method. The problem was somewhat resolved by the injection of dichloromethane blanks between samples, which appeared to 'clean-up' the column, in preparation for the next injection of sample.
>
> Upon completion of the second analysis of SRL10009/2 (*sample code for one of the PVC test materials*) a consistent result and percent recovery was obtained, though it still disagreed massively with the previously acquired

result for the same sample. This further inconsistency was an obvious concern with this method. It may be a homogeneity issue, however as the sample was rigorously prepared to ensure overall homogeneity it could well be highlighting other problems with the method."

In conclusion, poor agreement in between laboratory exercises involving a small number of laboratories to produce precision data may be due to a number of reasons:

- the laboratory lacked competence in performing the test·
- the test method is inadequately described
- the reagents are not of suitable quality
- the test method lacks adequate 'ruggedness' and requires further development.

8.1.7 Follow-up activities

Each of the potential sources of poor performance listed above were considered and follow-up actions were carried out.

The laboratory lacked competence in performing the test.· A training exercise was carried out prior to the analysis of any of the samples by the second laboratory. Having observed the analysis the laboratory staff confirmed that they had no further questions and that they were happy to proceed with the analysis. To ensure that the method was being correctly implemented a member of Fera staff visited the laboratory to observe the analytical procedure. During this visit it was apparent that several steps specified in the method were not being carried out as described.

(i) The glass columns used for the clean-up were being re-used. Although these were washed between use, this was ineffective since residue was apparent in the columns which may have been contaminated with NP.

(ii) Solvents were not supplied by Rathburn as described in the SOP and the freedom from NP background contamination was not checked.

(iii) The addition of 5.6 g of alumina was carried out using visual estimation and not accurately measured as the SOP describes.

(iv) The internal standard was introduced after the extraction solvent had been added to the vial. The SOP states that the internal standard should be added onto the plastic before the extraction solvent.

Differences in the approaches by the two laboratories were highlighted and it was agreed that additional detail should be added to the SOP such that these steps could not be mis-interpreted.

The test method is inadequately described. We were advised that the use of deactivated alumina as a clean-up tool needs to be well controlled to avoid failings in method repeatability (18). This was investigated.

Alumina was prepared and deactivated as described in the SOP in Annex 1 (at 450℃ for 4 hours). After preparation and deactivation the alumina was left to stand at room temperature for several days prior to use. Results obtained from the analysis of standard solutions containing the NP technical mix and the internal standard revealed

that the order in which the columns were prepared influenced the recovery data generated, i.e. those columns prepared using the alumina on the surface and in contact with the air behaved differently from those prepared from within the bulk.

A second batch of alumina was prepared at an elevated oven temperature (600℃) for 12 hours and was then deactivated for 24 hours. Columns were prepared as soon as the alumina was removed from the roller mixer. Standard solutions containing the NP technical mix and the internal standard were passed down the columns and recoveries were all acceptable, in the range 89-109%. No differences were observed in the order that the columns were prepared.

Therefore the preparation of the alumina columns was identified as a critical step in the methodology and the SOP was revised to highlight these findings.

The reagents are not of suitable quality. The relatively high background levels observed in the analysis of the procedural blank samples suggests that the solvents used may be of unsuitable quality. Ensuring the quality of the solvents was highlighted in the revised SOP.

The test method lacks adequate 'ruggedness' and requires further development. The comments received on the method suggest that this is a likely source of the error. Problems were reported with the partitioning in the liquid:liquid extraction phase and questions were raised with respect to the need to perform the clean-up steps using the alumina columns.

The problems associated with the phase separation in the liquid:liquid extraction were noted. Differences in miscibility observed by the two laboratories in the initial extraction step may be due to the presence of water in the solvents. As mentioned above the solvents used by the second laboratory were not the same as those used by Fera.

The method of analysis described in the SOP in Annex 1 is a modified version of that developed for the analysis of NP in foods and therefore the extraction and clean-up procedures were not originally developed with plastics in mind. Typically dichloromethane is used as an exhaustive extraction solvent for PS and PVC polymers with subsequent precipitation of the dissolved polymer with methanol. Replacing 4.3.1 in the SOP in Annex 1 with this procedure was investigated with and without the clean-up steps. The protocol followed was:

For each sample (2 x HIPS and 2 x PVC) polymer (0.10 g ± 0.001 g) was weighed into six 40 ml vials. Using a glass syringe 50 µl of the 10 µg/ml solution of $^{13}C_6$-4-n-nonylphenol was added to each vial. Dichloromethane (10 ml) was added and the vials were sonicated to dissolve the polymer. Methanol (5 ml) was added to each vial to precipitate the polymer. The supernatant was transferred into clean 40 ml glass vials. Another 5 ml methanol was added to each vial to ensure that the precipitation was complete. The supernatant was combined with the first fraction. Six blanks were prepared in the same way but without the addition of the polymer.

For three vials from each polymer and three of the blanks the supernatant was evaporated to 0.5 ml under a gentle stream of nitrogen and was transferred quantitatively to a GC sample vial. This was evaporated to dryness under a gentle stream of nitrogen, the internal sensitivity standard (25 µl of a 1 µg/ml solution of ^{13}C PCB 52) and nonane 175 µl were added, the contents mixed and analysed by GC-MS (as described in the SOP).

For the other three vials from each polymer and the other three blanks the solvent was evaporated just to dryness and the residue was reconstituted in cyclohexane (0.5 ml). The extract was then subjected to the chromatographic purification step as described in 4.3.3 in the SOP in Annex 1. The alumina was prepared as described above (under ACTION 2).

The extracts were analysed alongside an RRF standard prepared as described in the SOP. The results are given in Table 10. Recoveries and repeatability of the analysis for three of the four samples that were not subjected to the chromatographic purification step analysed were acceptable. However for the fourth sample, one of the PVC's, was not. Recoveries were ~ 300% for this sample. Following the clean-up procedure the recoveries dropped but were more consistent. The absence of the plugs of the dichloromethane washed anhydrous sodium sulphate (present to absorb any water in the solvents) also resulted in a lowering of the recovery of the NP. This highlighted the need to control the water applied to the alumina columns. It was concluded that for the method to be applicable for all sample types the clean-up procedure is required.

A further improvement of the method was made by the inclusion of a centrifugation step following polymer precipitation. These findings were included in the revised SOP.

8.1.8 Revised SOP

The revised SOP, including all of the critical points highlighted above, is given in Annex 3. The analysis of the four polymers was carried out in duplicate according to this revised SOP. The NP concentrations detected in the polymers are given in Table 11.

8.2 Familiarisation study – Laboratory 2

Following the method modification/clarification work carried out by Fera a second phase of testing was carried out. This testing was included to provide the laboratory with experience of the revised extraction procedure and to check that the analysis of standard solutions of NP passed through the columns resulted in satisfactory recovery values. The protocol followed is given in Annex 4. The nonylphenol concentrations detected in the blank solvents, in the solvent extracts and the purified standards are given in Tables 12, 13 and 14 respectively.

Comments made by the second laboratory following the delivery of this work were:

> "Upon concentration of the sample extracts after precipitation, some further polymeric material was observed to precipitate from the extraction solvent as the volume was reduced. It was also observed that this precipitated material did not pass back into solution when re-constituted in the injection solvent (nonane) and therefore could potentially influence the chromatography. This effect was still observed with the addition of centrifugation into the methodology.
>
> The resolution and overall peak shapes were affected for all analytes and standards by co-extractives from the samples interfering with the chromatography. An obvious contamination and discolouration was observed on removal of inlet liners after analysis which suggests the contamination to be non-volatile material, probably polymer. It was noted that the peak shapes for the PVC 1 were worst affected, with the Blue PS samples being least affected. It must also be noted that the chromatography and peak shapes in general were not considered to be

ideal even without interferences from the samples, and therefore some GC method optimisation would be recommended.

The chromatographic purification step resulted in varied recoveries for different concentrations of standards, approximately 50% for the 10 µg/ml and 75% for the 100 µg/ml standards. The low level and blank determination were calculated to have a higher level of NP than was expected. It is possible that this could have been introduced by the petroleum ether used to elute the alumina columns as NP was detected at an average concentration of 0.06 µg/ml in the background determination of this solvent."

The final analysis conducted at the second laboratory was carried out under the supervision of Fera staff. A number of amendments to the SOP and NP plan were suggested. These included the addition of the centrifugation steps between precipitation of the dissolved polymer and concentration of the extract, and also the interspersion of dichloromethane blank injections between sample runs to help reduce chromatographic problems. The result of these amendments gave slightly improved chromatography, but with the observed poor repeatability remaining.

It was noted that this phase of testing was included to familiarise the laboratory with the revised extraction procedure and to highlight the importance of the use of NP free solvents. Problems with chromatography may be laboratory specific and as with any CEN standard the phrases:

'NOTE: The following column and parameters have been found to be suitable'

and

'GC and MS apparatus should be optimised according to manufacturer's instruction.'

This translates as any instrument, column and parameters can be used and that the laboratory should optimise conditions to suit their apparatus.

Concern remained that the application of the clean-up procedure resulted in relatively high losses of NP. This was proposed to be due to problems with the water activity of the columns. The precise method of preparation is well defined in the revised SOP given in Annex 3.

8.3 Results Laboratory 2– Phase 2

Following on from the second phase of testing the second laboratory was asked to analyse the two PS and two PVC polymers according to the final SOP given in Annex 3. This SOP contains all of the critical points identified in the other phases of testing and in the Fera method simplification/improvement work. The results of the analysis of the four polymers are given in Table 15. The confirmation data is provided in Table 16. The differences in the absolute NP concentrations measured by the two laboratories are shown in Table 17.

The ratio (calculated as the ratio between the Laboratory 2 and Fera derived NP concentrations expressed as a percentage) is consistent with the losses in NP measured when the standard solutions were passed down the alumina columns. Therefore this problem was not resolved with the highlighting of the critical points in the SOP. As mentioned previously (Section 7.1) additional water deactivation of the alumina columns is believed to result in lower recovery values.

8.4 Between laboratory validation exercise – Laboratory 3

As the problems were not resolved even with the revision of the SOP a third laboratory was contracted to test the polymers according to the SOP given in Annex 3. Following a period of training which highlighted the critical points in the SOP Laboratory 3 was asked to analyse the same PS and PVC samples.

Laboratory 3 staff reported problems eluting the NP from the alumina columns. Using the same grade of alumina as that used by Fera the labelled-NP internal standard could not be detected and only the early eluting NPs (on the GC column) were present in the extract. This suggests that the other isomers and the internal standard remain on the alumina column and that they are not eluted using the dichloromethane solvent. By including methanol in the elution solvent then a recovery of about 60% was obtained. It was recommended that the SOP should be modified to include text stating that the elution solvent should be optimised prior to analysis of the test samples.

Laboratory 3 staff also reported losses (up to 50%) of NP in the evaporation/concentration step if it was not properly controlled. Given that the internal standard elutes later in the GC analysis than the NP technical mix it is possible that the more volatile NP isomers are being lost resulting in lower concentrations being reported. Therefore it was recommended that text should be added to the SOP to ensure that this is controlled.

The results obtained are compared with those of Fera and Laboratory 2 in Table 18. It should be noted that the Laboratory 3 results were obtained using the methanol:dichloromethane elution solvent and therefore the methods used were not directly comparable.

8.5 Follow-up activities

8.5.1 Analyte stability

As the results obtained by the three laboratories decreased in concentration according to the date on which they were analysed (Fera tested the polymers first, then Laboratory 2 and finally Laboratory 3) the stability of the analyte in the plastic was determined. All four polymers were re-tested by Fera following the SOP given in Annex 3. The results obtained are shown in Table 19. The results obtained (April 2009) were between 78 and 106% of the concentrations measured when the same polymers were tested in November 2007. In the interim the polymers had been stored in glass vials at ambient temperature. The polymers were therefore considered to be stable throughout this study.

8.5.2 Effect of the alumina on the measured NP concentrations

From the findings reported by both Laboratories 2 and 3 it was proposed that the problems with the method were related to the clean-up step using the alumina column. If this step is not properly controlled then differences in the recoveries and relative amounts of the different NPs eluting from the column are observed. As a result the absolute concentrations of the NP in the extracts also differ. Therefore further studies were carried out to identify the effect of different batches of alumina and the preparation of the alumina in different laboratories. The NP concentrations in one of the four polymers (Blue PS) was obtained following the SOP given in Annex 3 and using:

I. The alumina used by Fera in the stability studies – prepared at Fera

II. The alumina used by Laboratory 3 in the between laboratory validation exercise – prepared at Fera

III. The alumina used by Laboratory 3 in the between laboratory validation exercise – prepared at Laboratory 3

The results obtained are shown in Table 20. These values are lower than previously reported due to an old RRF standard being analysed alongside the samples. Applying the RRF value obtained using a fresh standard then the average concentrations were 417 mg/kg (Fera alumina) and 367 mg/kg (Laboratory 3 alumina).

8.5.3 Discussion

There was no difference between the Fera alumina and Laboratory 3 alumina when both were deactivated by Fera. The Laboratory 3 ready deactivated alumina did not recover any native NP or internal standard (IS). The alumina was rolled overnight prior to being used on the columns. Given that the same results were obtained for both samples tested using the alumina deactivated at Fera this confirms that this is the critical step in the SOP that is not under control. It could not be confirmed if the alumina was incorrectly deactivated or if it had become further deactivated during transit.

8.6 Between laboratory validation - conclusions

Despite the efforts of all three laboratories the methodology cannot be considered to be sufficiently robust to transfer to another laboratory. Fera has a lot of experience in the analysis of trace levels of environmental contaminants in various matrices which routinely use this type of methodology including alumina clean-up. This was not the case for the other two laboratories involved in this exercise.

However the method is clearly capable of detecting NP in polymeric food contact materials and articles. No QM (quantity permitted in the final material or article) restrictions exist for NP in food contact materials and therefore any test to detect NP would be required to establish its presence (or absence) in the material prior to the analysis of any foodstuff with which it comes into contact, i.e. in a screening test. All three laboratories have demonstrated that this is within the methods capabilities as they all detected NP in the positive samples.

Based on all discussions with the two laboratories and on the outcome of the all of the analyses performed a third version of the SOP was written (Annex 5). This contains additional clarification of the points highlighted following the external review process.

9. DETERMINATION OF NP IN FOODS

9.1 Selection of materials for the migration studies

Ten PVC cling films and 10 foodstuffs packaged in HIPS materials were purchased from retail outlets in the north of England and the cling film and HIPS packaging samples were tested for the presence of NP by extracting with isooctane and analysing by GC-MS. Attention was focussed on these two types of plastic since earlier work indicated that they may contain NP. Those samples in which NP was detected were then analysed quantitatively according to the SOP given in Annex 3. The NP concentrations in these samples are given in Table 21.

9.2 NP migration from cling film

Only two of the ten samples tested contained measurable levels of NP and therefore only these two samples were used in the migration studies.

Cheddar cheese slices and all-butter Madeira cake were selected as high fat foodstuffs that may be expected to be overwrapped in cling film. The presence of the fat may be expected to result in high migration of NP.

9.2.1 Exposure

Cheese slices were sandwiched between two layers of the cling film (2 x 1 dm^2 – total mass of film = 0.26 g). The average mass of the cheese slices was 24.3 g.

The Madeira cake was prepared by cutting off the upper and lower crusts. A 10 cm x 5 cm block was prepared and ~ 0.5 cm slices were cut off the block. A sample was comprised of 2 slices sat side by side to make a square ~ 10 cm x 10 cm square. The average mass of the cake samples was 20.5 g. The samples were sandwiched between two layers of the cling film (2 x 1 dm^2 – total mass of film = 0.26 g).

Both of the cling films A and B described above were tested with each food, in triplicate.

The sample and film were overwrapped in aluminium foil before exposure for 94 hours. at 20^oC. Duplicate blank samples were prepared in the same way but in the absence of the film.

9.2.2 Extraction and analysis

9.2.2.1 Sample preparation

Following exposure samples were homogenised and a representative portion (15 g) was weighed into a 40 ml glass vial. Internal standard ($^{13}C_6$ 4-n-nonylphenol, 50 µl of a 10 µg/ml solution) was added to all vials and allowed to equilibrate (2 minutes). One of the two blank samples (exposed in the same way but in the absence of the film) was treated in the same way. The second was overspiked with the 4-NP Technical Mix (75 µl of a 100 µg/ml solution) and allowed to equilibrate (2 minutes) before being treated as described above. A procedural blank (no food) and a procedural overspiked sample were prepared in the same way.

9.2.2.2 Sample extraction

The samples, prepared as described above, were extracted with methanol (20 ml) using an orbital shaker (30 minutes). After this time had elapsed the samples were centrifuged (2500 rpm, 3 minutes) and the supernatant was poured off into a separating funnel (250 ml). This procedure was repeated with a second aliquot of methanol (20 ml for cheese, 15 ml for cake), shaken for 15 minutes, centrifuged and the supernatant combined with the first extract. 25 ml of acidified water (sulphuric acid, S.G 1.84, 1.7 g in 500 ml water) was added to the methanol extracts in the separating funnels and shaken (1 minute). Cyclohexane (70 ml) was added and shaken (1 minute). The methanol/water was run off into a beaker and retained, the cyclohexane collected in a round bottom flask and the methanol/water was returned to the separating funnel for two further extractions with cyclohexane (70 ml and 60 ml, each shaken for 30 seconds). The cyclohexane (~ 200 ml) was rotary evaporated (45^oC) to a few ml and quantitatively transferred to a 10 ml glass vial. The volume was reduced to 0.5 ml at 40℃ under a gentle stream of nitrogen.

9.2.2.3 Sample clean-up

Sample clean-up was carried out using alumina. The alumina was deactivated and the columns were prepared as described in the SOP for plastics (Annex 3). The alumina was conditioned with cyclohexane (10 ml), the extracts were loaded onto the column and cleaned up with petroleum ether (40/60, 50 ml) and the NP was eluted with dichloromethane (50 ml). The dichloromethane eluate was reduced in volume (to ~ 3 ml) under a gentle stream of nitrogen (40℃) before being quantitatively transferred to a 10 ml glass vial. The solvent was removed under a gentle stream of nitrogen (40℃, until it just reached dryness) and nonane (1 75 µl) and the sensitivity standard (^{13}C12 PCB52, 25 µl of a 0.8 µg/ml solution) were added. The vials were vortex mixed and the extract was transferred to a glass vial for analysis by GC-MS.

9.2.2.4 Analysis

The sample extracts were analysed by GC-MS using an Agilent 6890 gas chromatograph (Agilent, Palo Alto, CA, USA) coupled with an Agilent 5973 inert mass selective detector. Splitless injection (splitless time 1 minute) of 1 µl of extract was carried out into a ZB-5MS capillary column (5% phenyl and 95% dimethylpolysiloxane; 30 m x 250 µm i.d., 0.25 µm film thickness; Phenomenex, USA). Following injection the oven was held at 60℃ for 3 minutes and then ra ised at 25℃/minute to 160℃, held for 2 minutes, raised at 0.8℃/minute to 165℃, the n at 30℃/minutes to 300℃ and held for 5 minutes. The injector was held at 250℃. Helium (1 mL/min constant flow) was employed as the carrier gas. The MS was operated in electron impact mode with selected ion monitoring:

Analyte	Quantifying ion (m/z)	Qualifying ions(s) (m/z)
4-n-nonylphenol	220	163, 149, 135
$^{13}C_6$ 4-n-nonylphenol (IS)	226	113
$^{13}C_{12}$ PCB52 (SS)	304	302

9.2.3 Worst case migration (total mass transfer calculation)

Taking into account the mass of the film exposed and the mass of the foodstuff the worst case migration was calculated assuming total mass transfer.

Cling film A = 4153 mg NP /kg film. 0.26 g of film was used in contact with 24.3 g of cheese or 20.5 g of cake. Assuming 100% transfer the worst case migration was calculated to be 44.4 mg NP/kg cheese and 52.7 mg NP/kg cake.

Cling film B = 2588 mg NP /kg film. 0.26 g of film was used in contact with 24.3 g of cheese or 20.5 g of cake. Assuming 100% transfer the worst case migration was calculated to be 27.7 mg NP/kg cheese and 32.8 mg NP/kg cake.

9.2.4 Measured migration

The concentrations of NP measured in the cheese and cake samples are shown in Table 22. In all cases the migration was measurable. Allowing for the background levels in the procedural blanks and the blank foods, migration into cheese was about 3 and 12 mg//kg and migration into cake was about 5 and 9 mg/kg, for the films A and B respectively. Recovery of the overspiked analyte was acceptable in the range 87 –

109%. Chromatograms of the RRF, the overspiked and exposed foods are shown in Figures 7 and 8. From these NP profiles it can be seen that all isomers migrate from the cling film to the same extent (the profiles for the RRF, the overspiked foods and the exposed foods are the same) and therefore if NP is detected in packaged foods and the source of the NP is the HIPS packaging materials then the NP profiles would be expected to be the same.

In these tests the surface area to food mass ratio was exaggerated, being 21-24g of food placed in contact with 2 dm^2 of film. For the conventional packaging ratio of 1 kg food in contact with 6 dm^2 these results would correspond to a NP migration of about 0.2 to 0.8 mg/kg into cheese and 0.3 to 0.6 mg/kg into cake.

9.3 NP migration from HIPS

For these pre-packaged food samples, If any NP in the foodstuffs is present as a consequence of migration from the packaging then the concentration would be expected to be highest in the external layer of the foodstuff (i.e. that closest to the packaging). For each of the three foods tested the outer 0.5 – 1 cm was removed, homogenised and extracted and analysed as described in 9.2.2. This approach was taken following a consideration of the low diffusivity of the HIPS polymer, the levels present compared to the mass of the foodstuff, and the sensitivity of the method of analysis.

9.3.1 Worst case migration (total mass transfer calculation)

Taking into account the mass of the HIPS packaging and the mass of the foodstuff the worst case migration was calculated assuming total mass transfer.

HIPS/river cobbler = 28 mg NP/kg HIPS. 14.5 g of the packaging was in contact with 265 g river cobbler. Assuming 100% transfer the worst case migration was calculated to be 1.5 mg NP/kg river cobbler.

HIPS/lemon sole fillets = 1761 mg NP/kg HIPS. 15.6 g of the packaging was in contact with 250 g lemon sole fillets. Assuming 100% transfer the worst case migration was calculated to be 109 mg NP/kg lemon sole fillets.

HIPS/fishcakes = 924 mg NP/kg HIPS. 12.4 g of the packaging was in contact with 230 g fishcakes. Assuming 100% transfer the worst case migration was calculated to be 50 mg NP/kg fishcakes.

9.3.2 Measured migration

Chromatograms of the RRF, the packaged food extracts and the overspiked foodstuffs are shown in Figures 9 – 11. Although a response was observed in the quantifying ion chromatogram (m/z 220) the profile was not consistent with that of NP (as seen in the overspiked chromatograms). In all cases ion ratios failed (they did not agree with the ion ratios calculated for the RRF samples analysed at the same time) and therefore the response could not be confirmed as being due to NP. Given that no NP was detected in the food contact layer then no NP depth profile studies were carried out. The sensitivity of the method would have been sufficient to determine NP migration if the worst case 100% transfer had occurred (9.3.1) however HIPS is a low diffusivity polymer, the foodstuff is packaged chilled and only has a limited shelf-life and therefore NP migration is only likely to have occurred from the area of the polymer in direct contact with the foodstuff. Taking this into account the sensitivity of the method (LOD ~ 200 µg/kg) may not be sufficient to determine NP migration from such a polymer. Migration modelling predicts that under exposure conditions of 7 days at 5℃ < 0.3% of

the NP in the HIPS will migrate into the foodstuff. Therefore using the lemon sole fillets as an example the maximum concentration of NP that may be derived from migration from the HIPS tray is 327 µg/kg (0.3% of the calculated total transfer – see above), i.e. close to the detection limit of the method. As a result no further testing was carried out.

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Figure 1. GC-MS chromatogram of the RRF standard

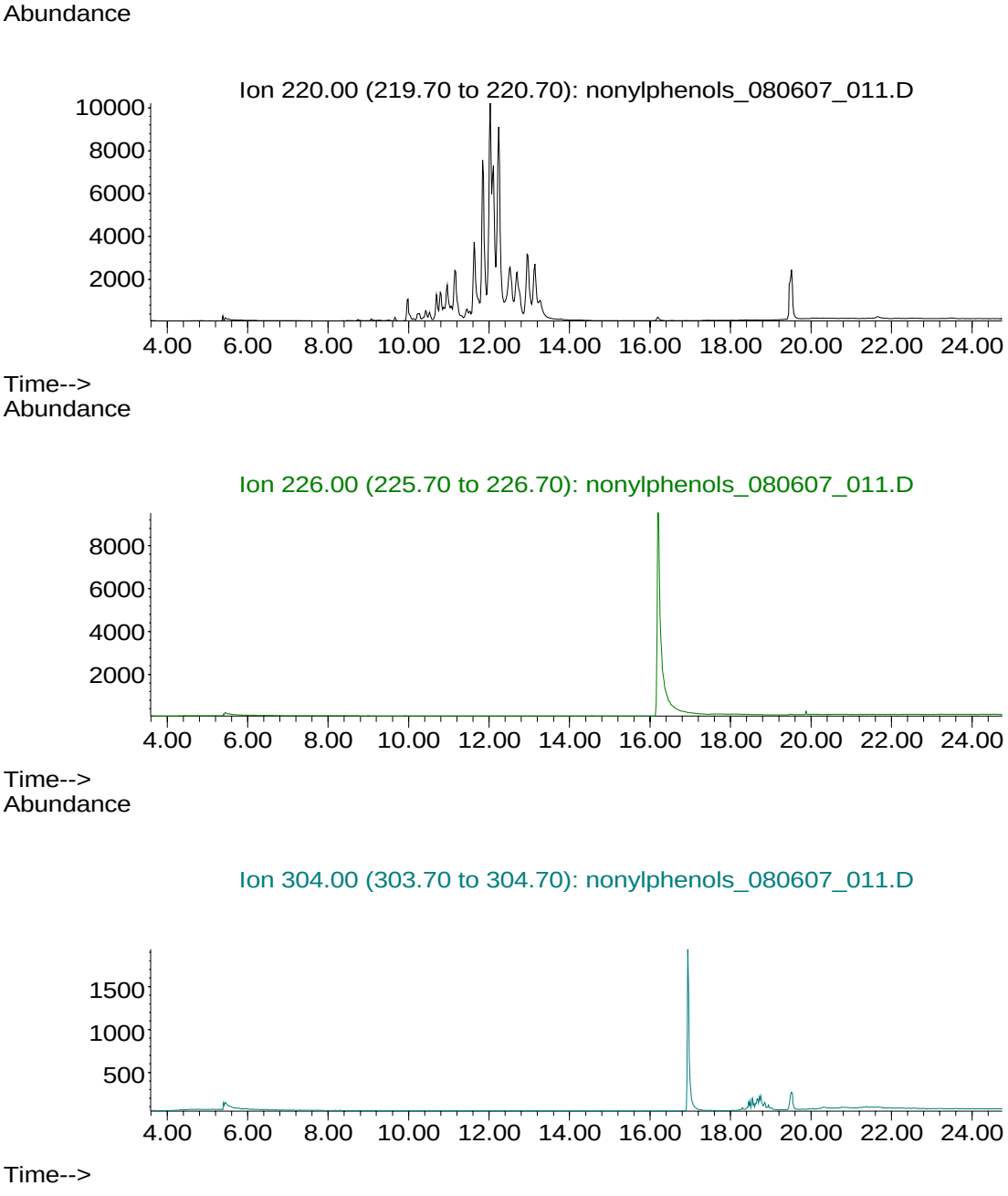


Figure 2. Calibration graph for the NP technical mix

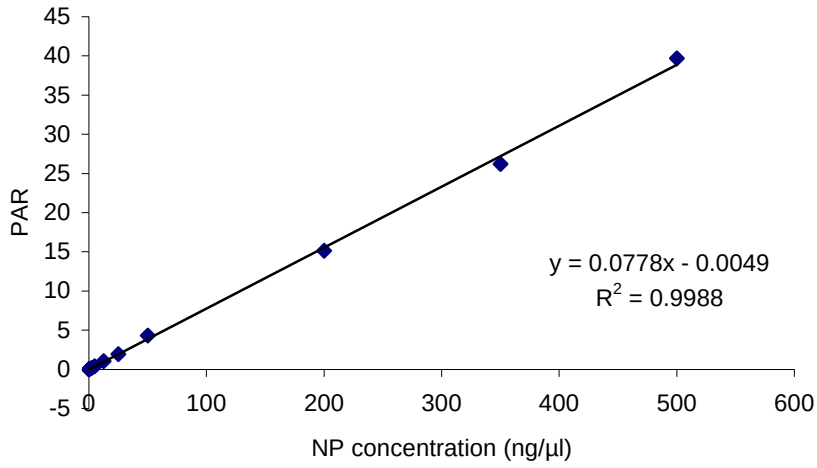
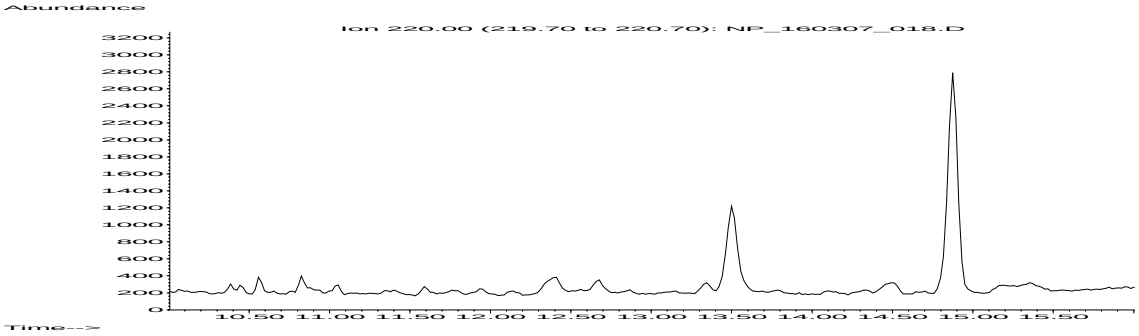
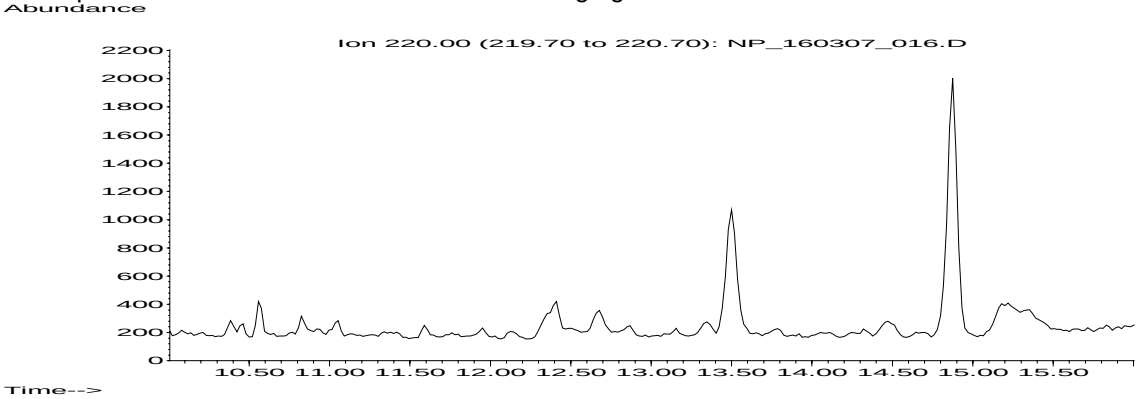


Figure 3. SIM (m/z 220) chromatogram obtained from the analysis of PET, PET spiked with TNPP at 1000 µg/kg, PET spiked with nonylphenol polyethoxylates at 1000 µg/kg and PET spiked with NP at 1000 µg/kg.

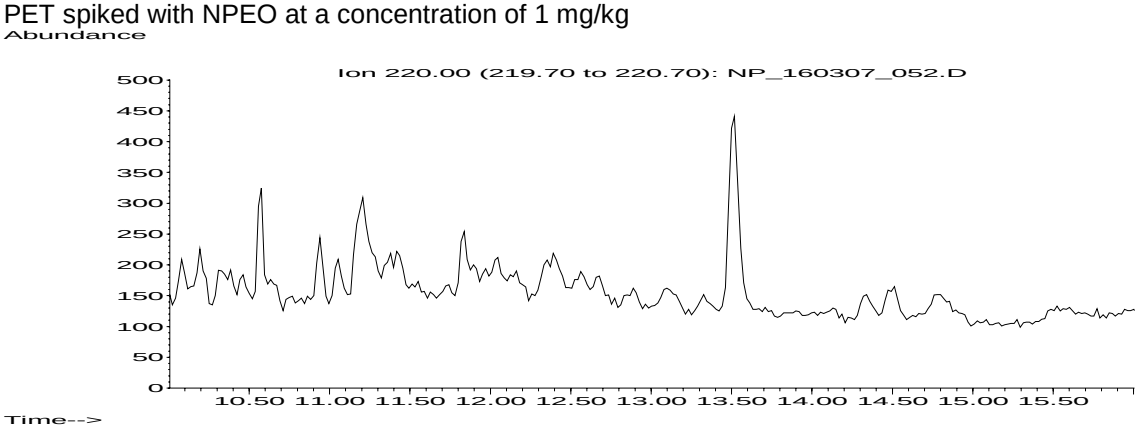
PET



PET spiked with TNPP at a concentration of 1 mg/kg



PET spiked with NPEO at a concentration of 1 mg/kg



PET spiked with NP at a concentration of 1 mg/kg

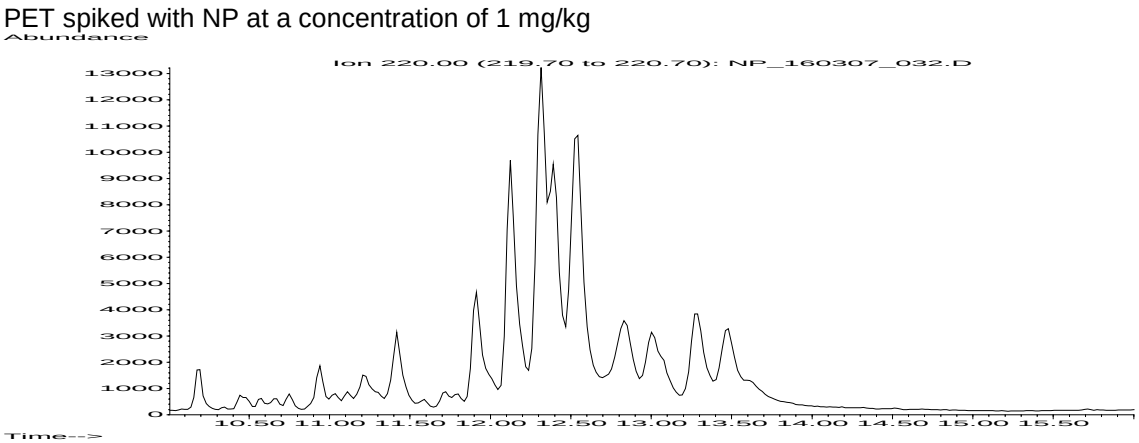


Figure 4. GC-MS chromatogram of the RRF standard as analysed by Laboratory 2.

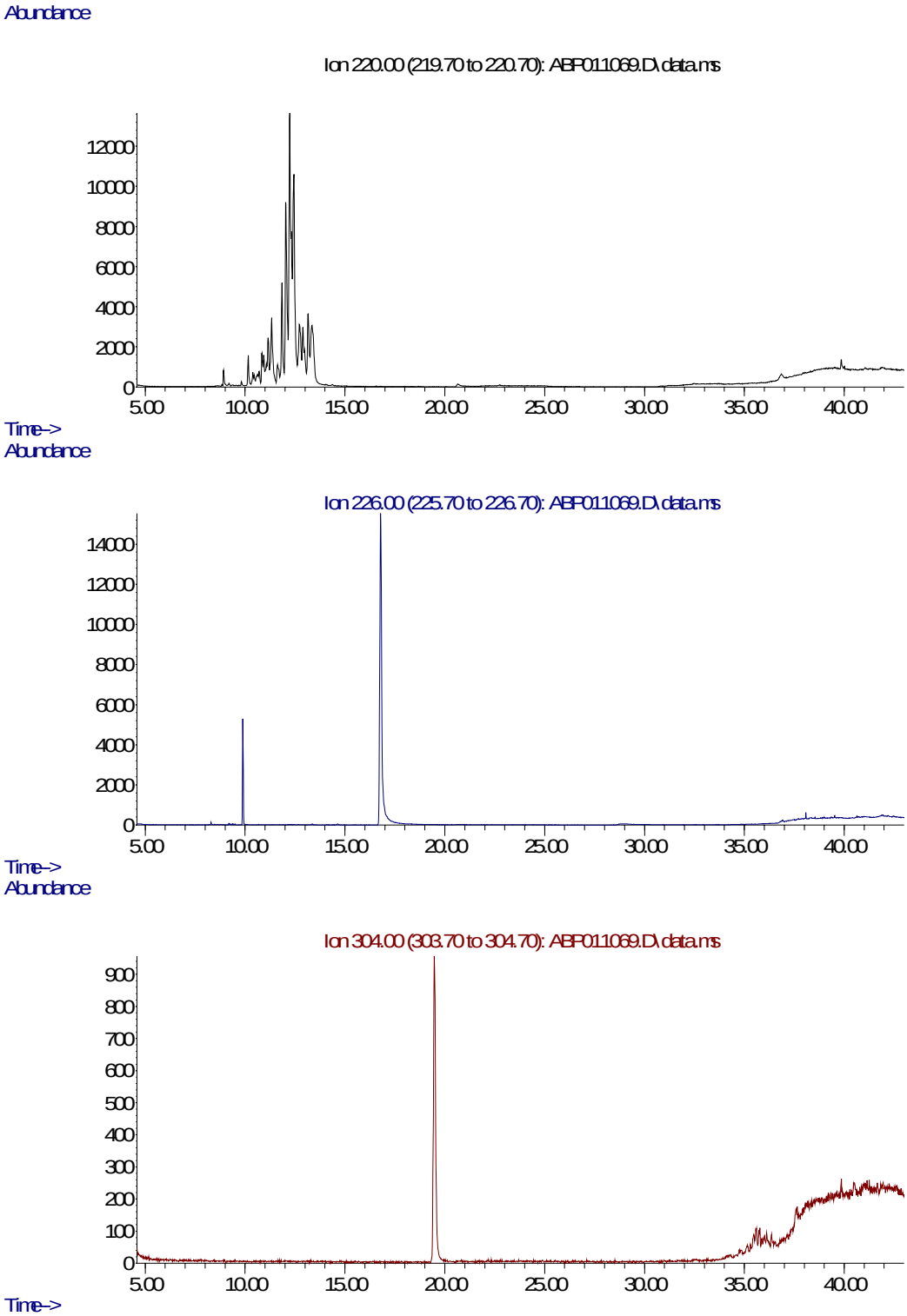


Figure 5. GC-MS chromatogram of a PVC extract as analysed by Laboratory 2

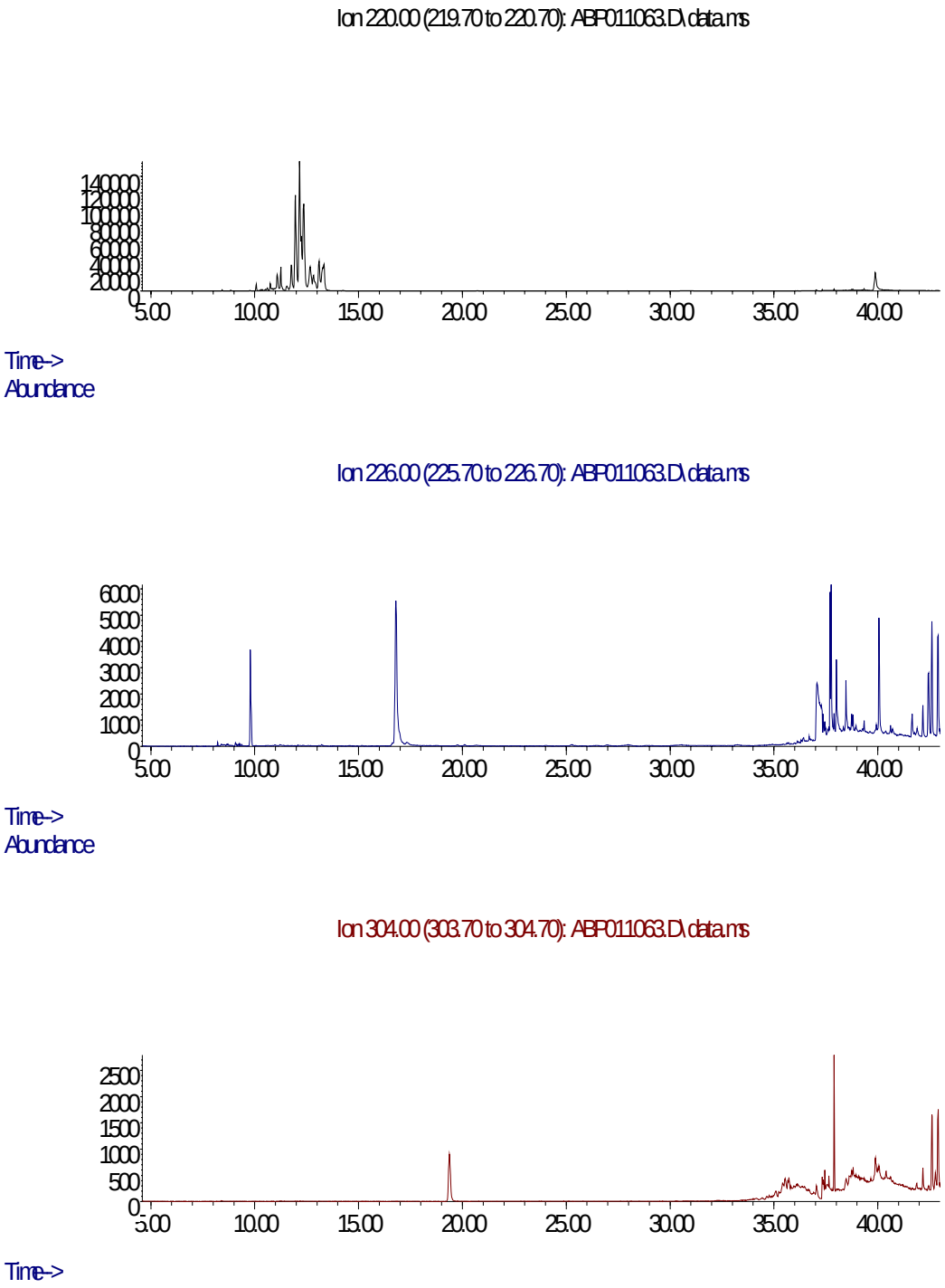


Figure 6. Laboratory 2 linearity assessment

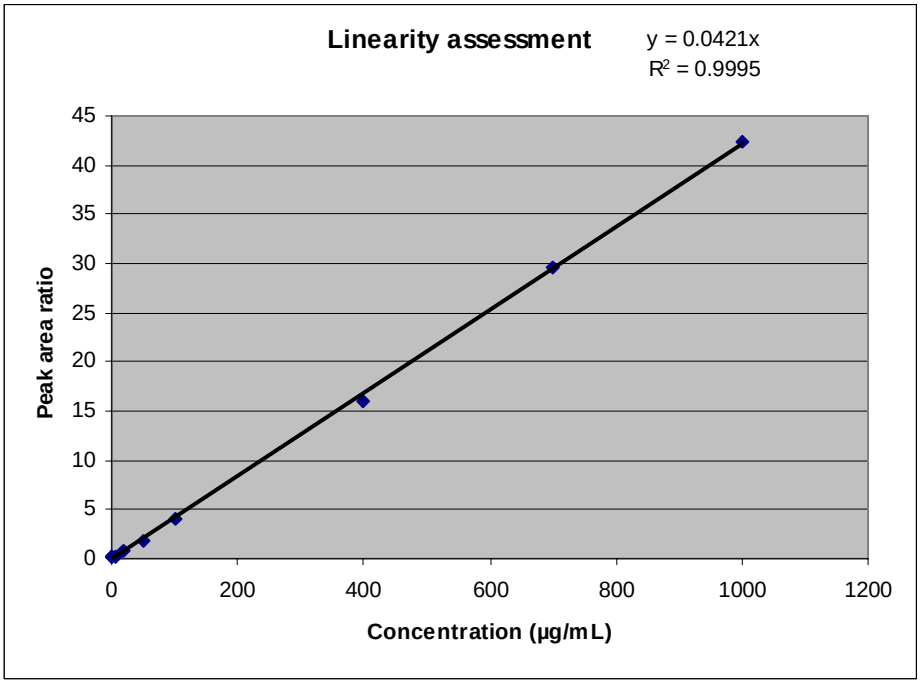
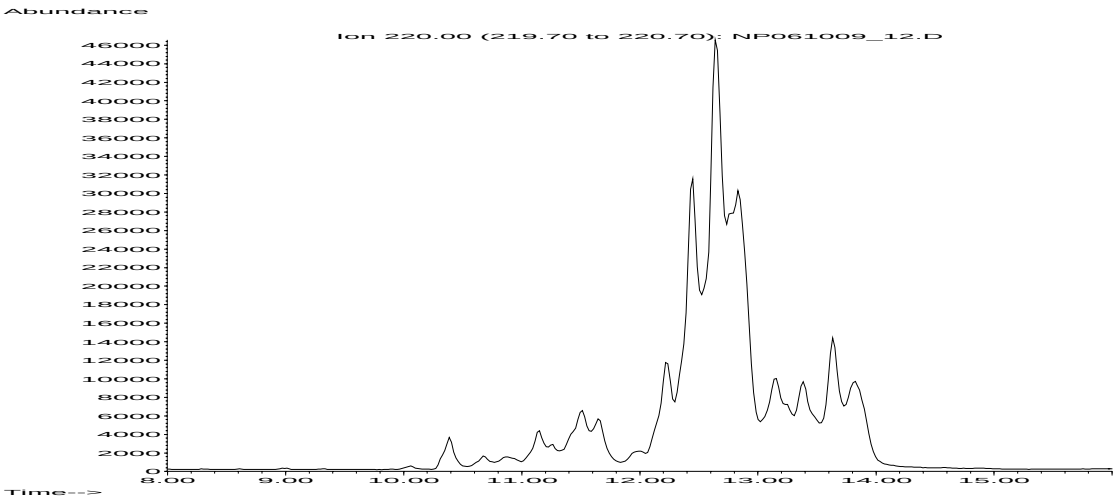
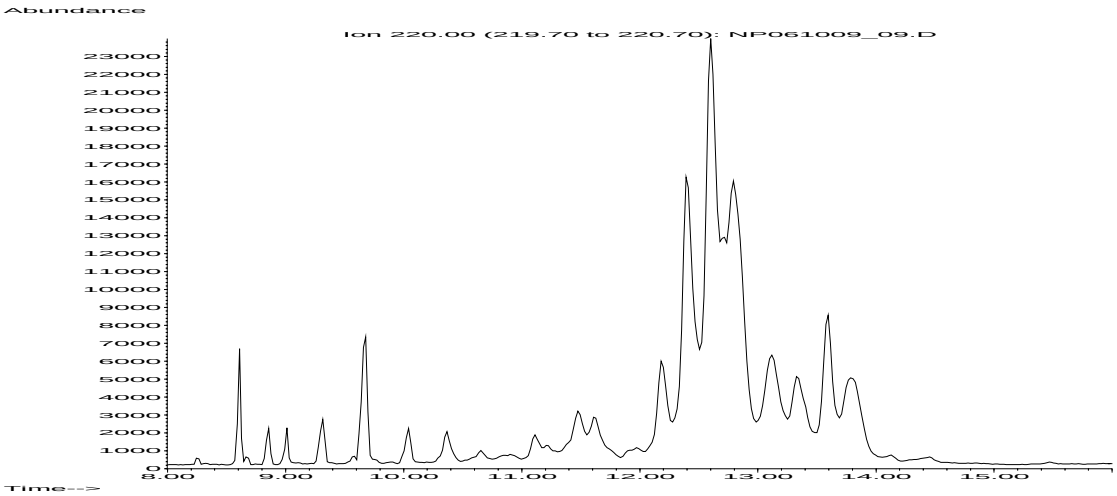


Figure 7. Selected ion (m/z 220) chromatograms of the RRF, NP overspiked cheese and cheese exposed to cling film A

RRF



Cheese overspiked with NP



Cheese exposed to cling film A

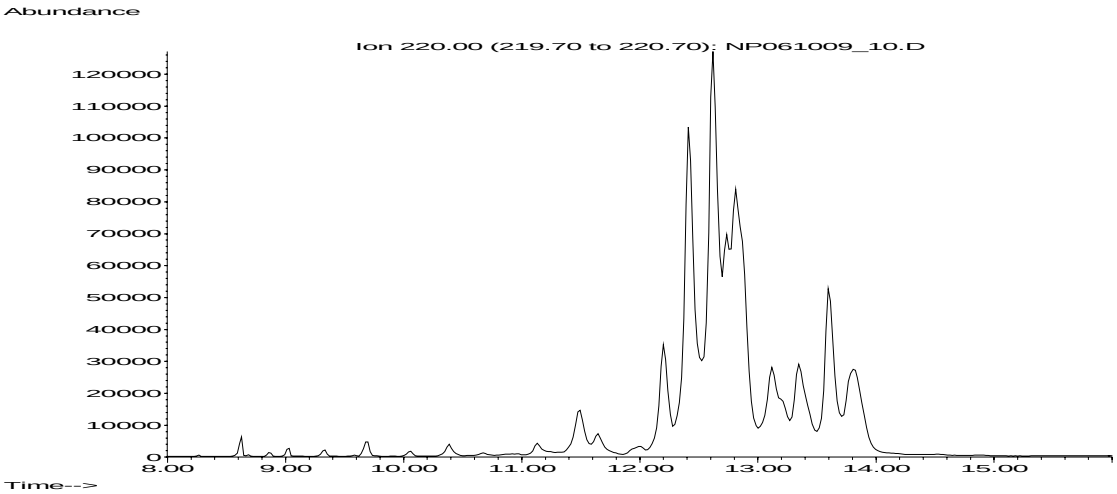
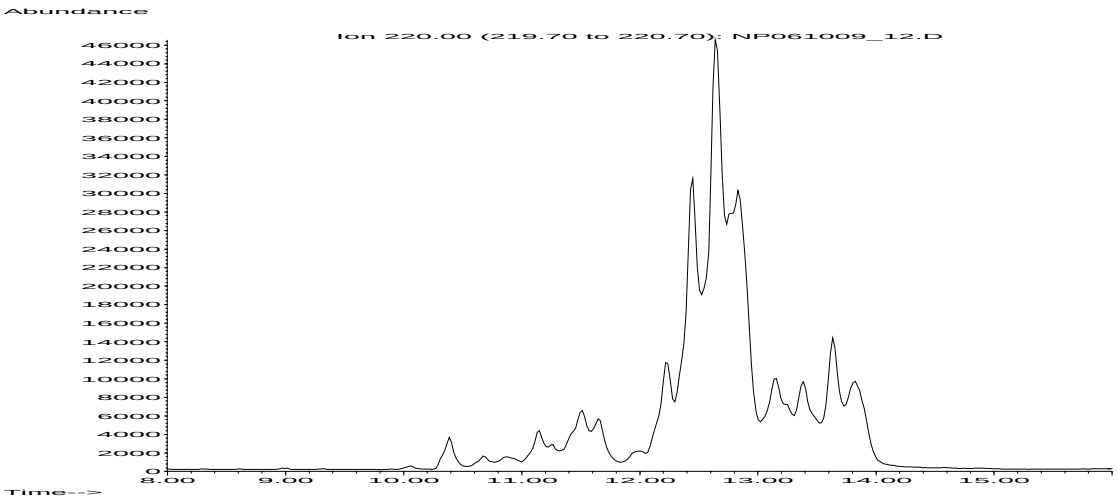
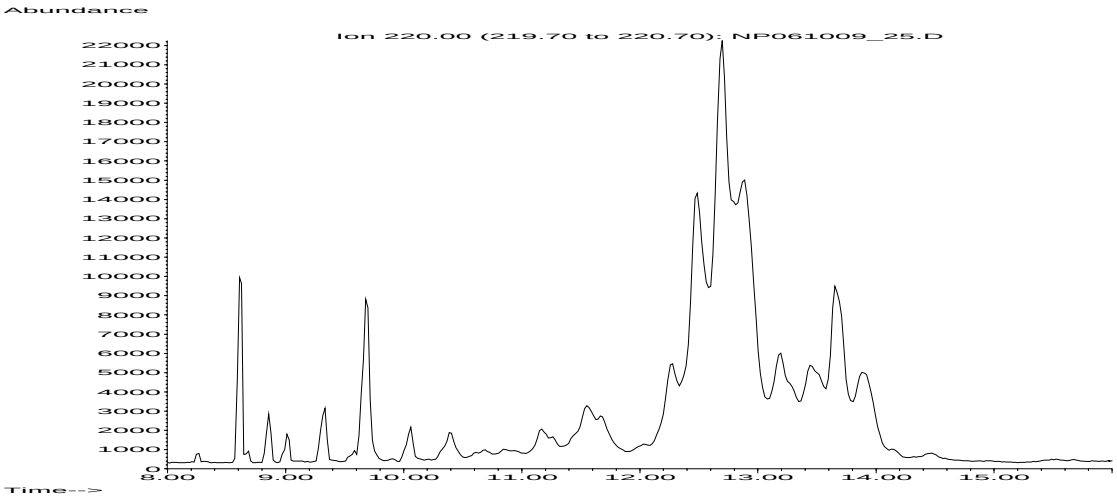


Figure 8. Selected ion (m/z 220) chromatograms of the RRF, NP overspiked cake and cake exposed to cling film A

RRF



Cake overspiked with NP



Cake exposed to cling film A

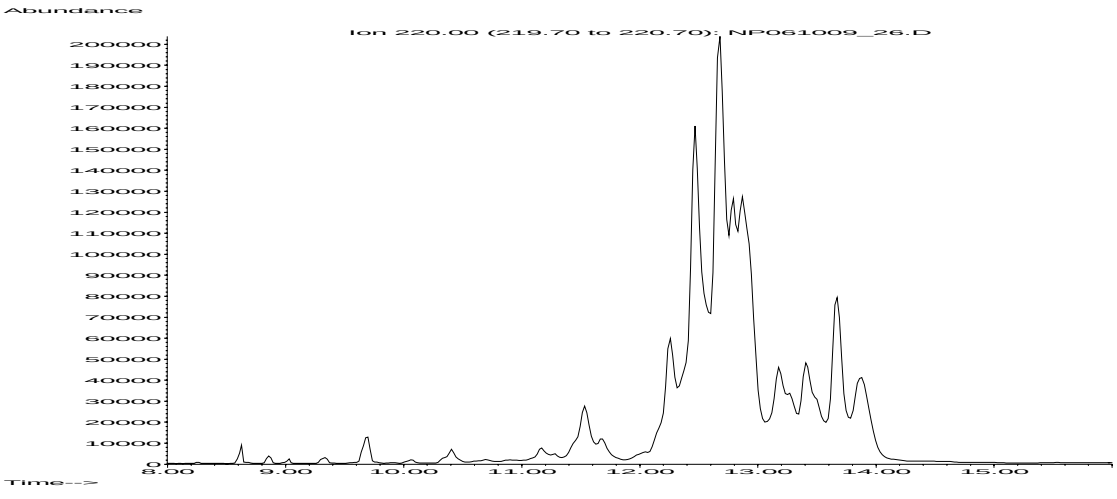
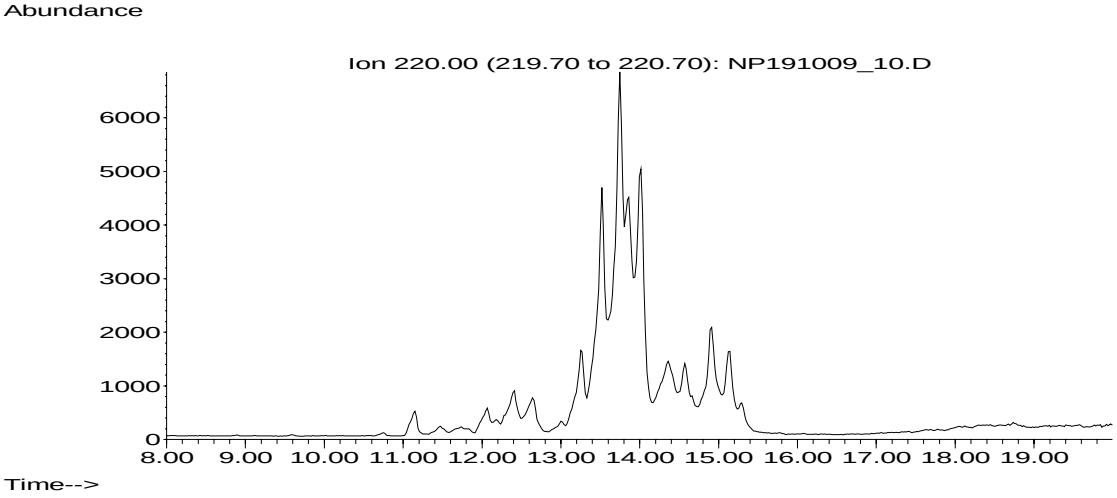
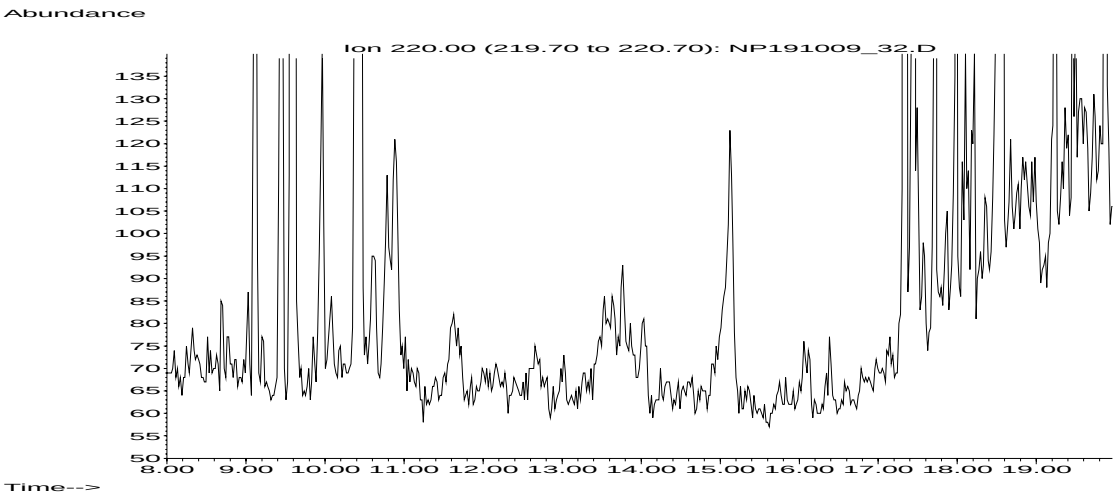


Figure 9. Selected ion (m/z 220) chromatograms of the RRF, river cobbler and NP overspiked river cobbler

RRF



River cobbler (food contact layer)



NP overspiked river cobbler (food contact layer)

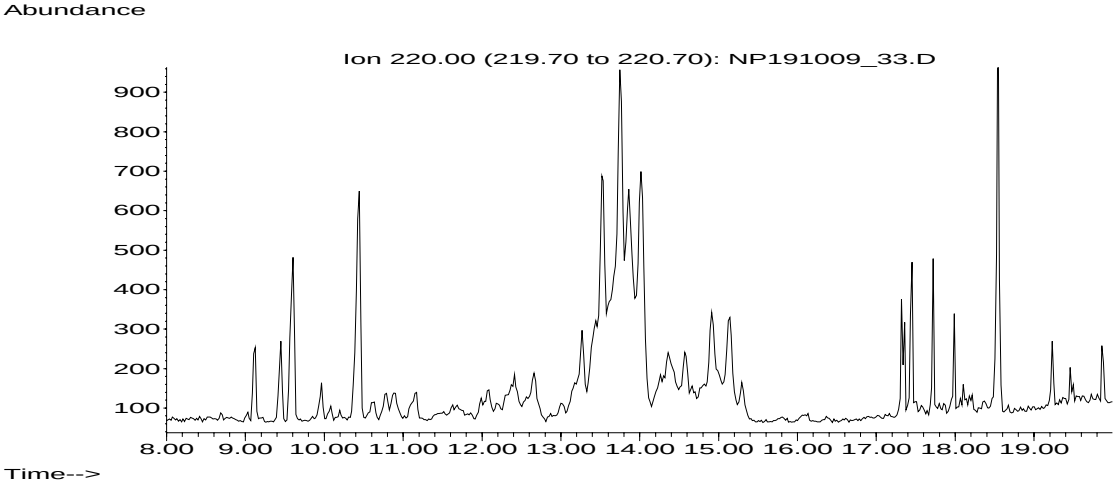
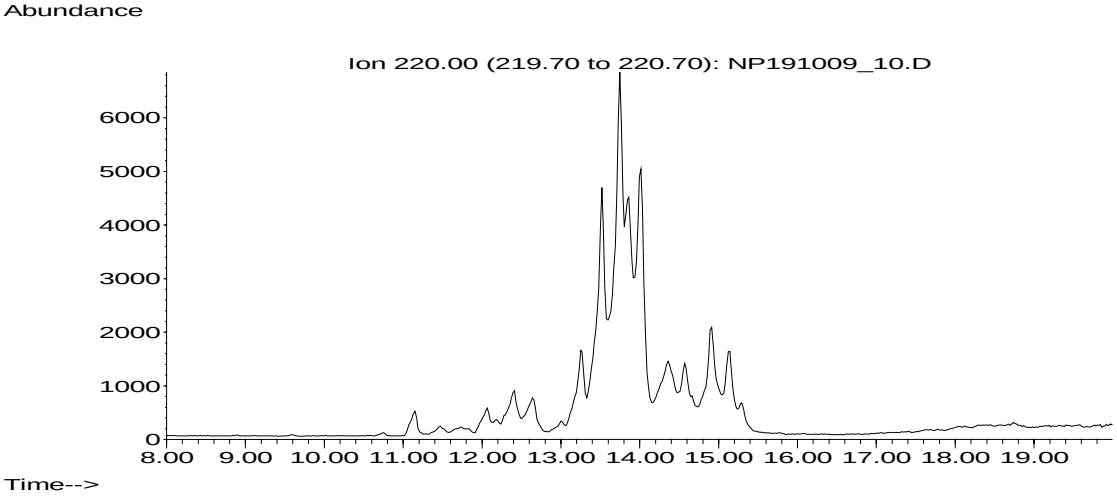
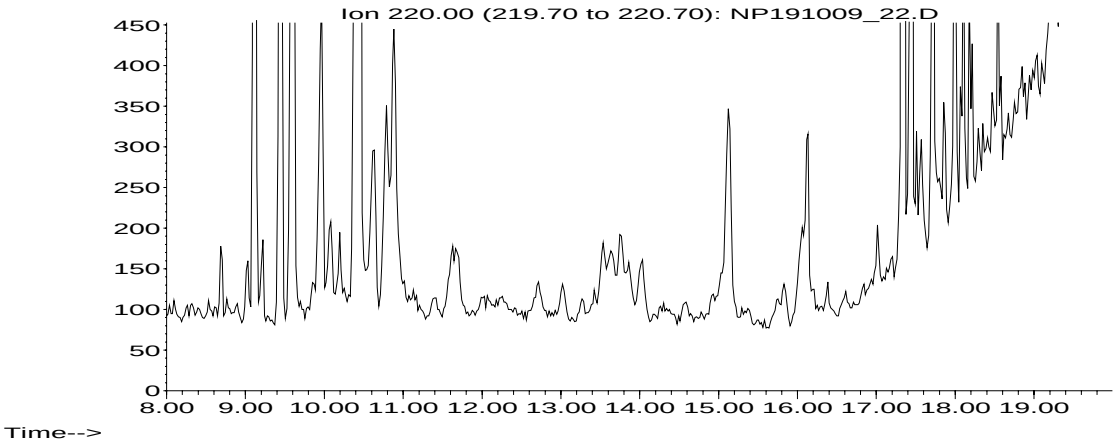


Figure 10. Selected ion (m/z 220) chromatograms of the RRF, lemon sole fillets and NP overspiked lemon sole fillets

RRF



Lemon sole fillets (food contact layer)



NP overspiked lemon sole fillets (food contact layer)

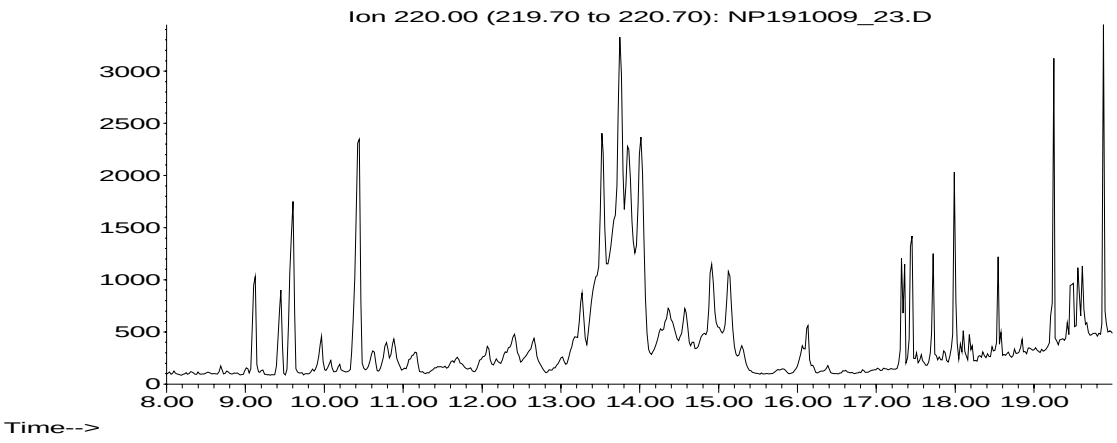
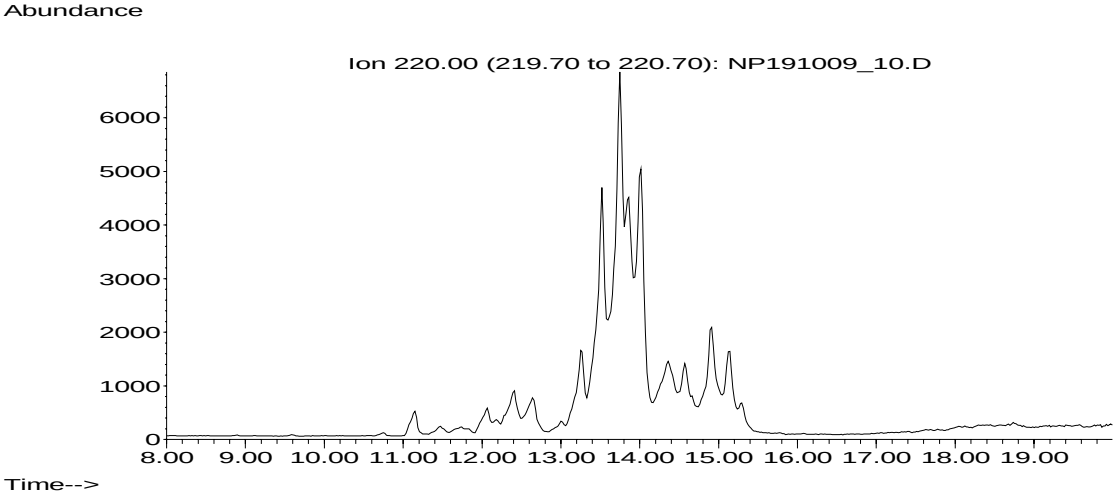
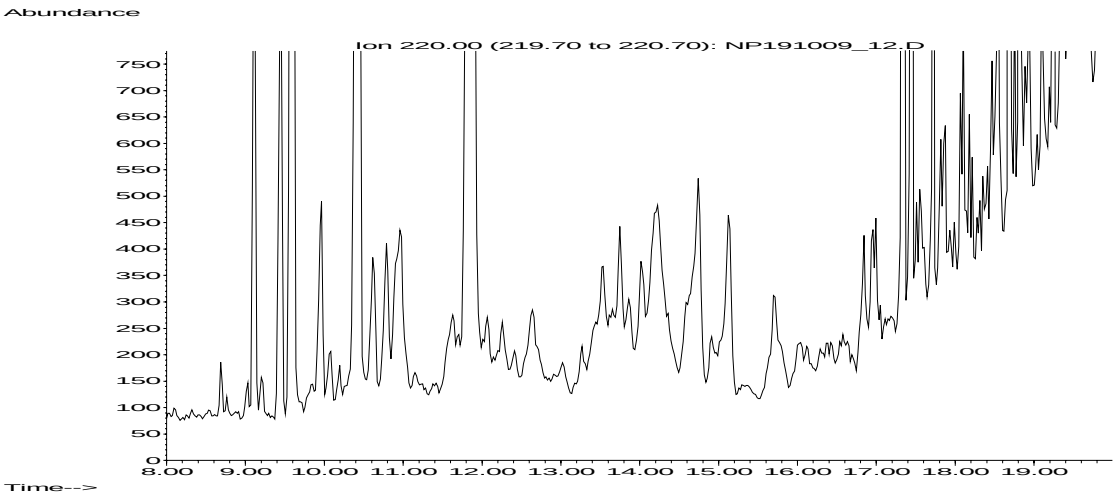


Figure 11. Selected ion (m/z 220) chromatograms of the RRF, fishcake and NP overspiked fishcake

RRF



Fishcake (food contact layer)



NP overspiked fishcake (food contact layer)

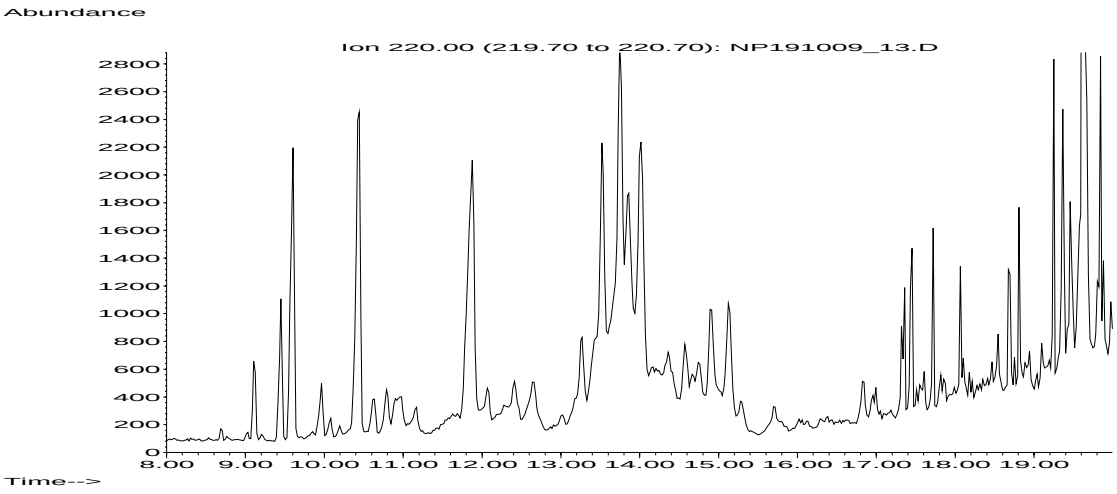


Table 1. Repeatability data

Sample	NP concentration (mg/kg) *
Specimen 1	2410
Specimen 2	2433
Specimen 3	2137
Specimen 4	2168
Specimen 5	2454
Specimen 6	2610
Specimen 7	1934
Specimen 8	2104
Average	**2281**
Standard deviation	**228**
Relative standard deviation (%)	**10**

* The reported concentrations have been corrected for recovery.

Table 2. Ion ratios

	Ion ratio		
Sample	**220/163**	**220/149**	**220/135**
Average RRF	0.629	0.183	0.076
Acceptable range (± 20%)	0.504 – 0.755	0.146 – 0.219	0.061 – 0.092
Specimen 1	0.721	0.199	0.078
Specimen 2	0.723	0.198	0.076
Specimen 3	0.723	0.188	0.073
Specimen 4	0.733	0.191	0.074
Specimen 5	0.751	0.202	0.079
Specimen 6	0.725	0.188	0.074
Specimen 7	0.729	0.190	0.074
Specimen 8	0.724	0.188	0.073

Table 3. Clear PS homogeneity data analysed according to the SOP provided in Annex 3

Sample	NP concentration (mg/kg) *	Recovery (%)
1a	693	53
1b	685	54
2a	477	36
2b	711	47
3a	792	42
3b	623	68
4a	579	43
4b	580	66
5a	617	51
5b	623	62
6a	751	35
6b	524	34
7a	648	41
7b	602	73
8a	644	55
8b	585	39
9a	554	44
9b	483	47
10a	644	50
10b	472	52
Average	**595**	
Standard deviation	**79**	
RSD (%)	**13**	

* The reported concentrations have been corrected for recovery.

Table 4. Blue PS homogeneity data analysed according to the SOP provided in Annex 3

Sample	NP concentration (mg/kg) *	Recovery (%)
1a	610	87
1b	527	70
2a	503	79
2b	502	70
3a	494	80
3b	561	82
4a	494	83
4b	581	81
5a	512	79
5b	525	94
6a	594	88
6b	503	82
7a	653	99
7b	539	80
8a	487	92
8b	634	83
9a	592	86
9b	499	71
10a	521	79
10b	565	96
Average	**545**	
Standard deviation	**51**	
RSD (%)	**9**	

* The reported concentrations have been corrected for recovery.

Table 5. PVC 1 homogeneity data analysed according to the SOP provided in Annex 3

Sample	NP concentration (mg/kg) *	Recovery (%)
1a	1687	94
1b	1426	95
2a	1785	90
2b	2328	114
3a	2010	83
3b	2117	92
4a	1800	102
4b	1877	74
5a	2088	90
5b	2077	84
6a	2469	23 #
6b	2287	29 #
7a	2043	34 #
7b	2006	38 #
8a	2459	32 #
8b	2319	29 #
9a	1608	33 #
9b	1472	36 #
10a	1469	31 #
10b	1714	27 #
Average	**1952**	
Standard deviation	**237**	
RSD (%)	**17**	

* The reported concentrations have been corrected for recovery.

Alumina clean-up columns were prepared without the anhydrous sodium sulphate plug. This allowed water present in the sample to affect the columns resulting in these low recovery values.

Table 6. PVC 2 homogeneity data analysed according to the SOP provided in Annex 3

Sample	NP concentration (mg/kg) *	Recovery (%)
1a	2162	48
1b	2069	57
2a	2207	52
2b	1823	60
3a	1888	40
3b	1832	57
4a	2011	49
4b	1836	45
5a	2655	40
5b	2067	52
6a	2330	52
6b	1922	47
7a	1809	44
7b	2002	69
8a	2027	57
8b	1576	45
9a	2085	39
9b	1847	49
10a	2017	52
10b	1625	41
Average	**1937**	
Standard deviation	**215**	
RSD (%)	**11**	

* The reported concentrations have been corrected for recovery.

Table 7. NP concentrations measured in the procedural blank samples

Sample	NP concentration (µg/kg)
Procedural blank (analysis 1)	105.76
Procedural blank (analysis 2)	108.45
Procedural blank (analysis 3)	107.41
Procedural blank (analysis 4)	85.32
Average	**101.74**
Standard deviation	**11.00**
LOQ = average + (3 x standard deviation)	**134.73**

Table 8. NP concentrations detected and the recoveries obtained in the analysis of the two PVC samples provided by Fera

Sample	NP concentration (mg/kg)	Recovery (%)
PVC 1 (analysis 1, rep-1)	1264.77	43.79
PVC 1 (analysis 1, rep-2)	2150.70	36.69
PVC 1 (analysis 1, rep-3)	2373.46	3.21
PVC 1 (analysis 2, rep-1)	1033.09	43.63
PVC 1 (analysis 2, rep-2)	1079.03	44.13
PVC 1 (analysis 2, rep-3)	779.59	44.14
PVC 2 (analysis 1, rep-1)	2898.22	31.50
PVC 2 (analysis 1, rep-2)	#	#
PVC 2 (analysis 1, rep-3)	2962.59	48.83
PVC 2 (analysis 2, rep-1)	1645.15	61.54
PVC 2 (analysis 2, rep-2)	1418.24	62.57
PVC 2 (analysis 2, rep-3)	1539.52	62.26

* The reported concentrations have been corrected for recovery.

Sample lost during preparation.

Table 9. Confirmation of the presence of nonylphenol

	220/163	**220/149**	**220/135**
SRL10009/1	0.625	0.144	0.061
RRF STD.	0.584	0.161	0.068
Variance (%)	6.6	-11.9	-10.8

Table 10. Analysis of samples in the method improvement phase

Sample	NP concentration (mg/kg) *	Recovery (%)
PS blue – no clean-up (rep 1)	240	86
PS blue – no clean-up (rep 2)	225	64
PS blue – no clean-up (rep 2)	331	73
PS blue – with clean-up (rep 1)	504	29
PS blue – with clean-up (rep 2)	437	48
PS blue – with clean-up (rep 3)	392	63
PS clear – no clean-up (rep 1)	360	89
PS clear – no clean-up (rep 2)	336	112
PS clear – no clean-up (rep 2)	319	80
PS clear – with clean-up (rep 1)	383	55
PS clear – with clean-up (rep 2)	388	54
PS clear – with clean-up (rep 3)	221	29
PVC 1 – no clean-up (rep 1)	576	84
PVC 1 – no clean-up (rep 2)	567	68
PVC 1 – no clean-up (rep 2)	571	91
PVC 1 – with clean-up (rep 1)	1657	84
PVC 1 – with clean-up (rep 2)	1751	83
PVC 1 – with clean-up (rep 3)	1748	85
PVC 2 – no clean-up (rep 1)	769	298
PVC 2 – no clean-up (rep 2)	#	#
PVC 2 – no clean-up (rep 2)	822	299
PVC 2 – with clean-up (rep 1)	2483	65
PVC 2 – with clean-up (rep 2)	2183	72
PVC 2 – with clean-up (rep 3)	2167	70

* The reported concentrations have been corrected for recovery.

no data

Table 11. NP concentrations detected in samples analysed according to the revised SOP (Annex 3)

Sample	NP concentration (mg/kg) *	Recovery (%)
PS blue	680	88
PS blue	613	85
PS clear	542	84
PS clear	529	95
PVC 1	2593	101
PVC 1	2975	102
PVC 2	2296	89
PVC 2	2081	105

* The reported concentrations have been corrected for recovery.

Table 12. Laboratory 2 NP concentrations detected in blank solvents

Sample	NP concentration (µg/ml)	Recovery (%)
Dichloromethane solvent check 1	0.00	70
Dichloromethane solvent check 2	0.00	69
Methanol solvent check 1	0.00	36
Methanol solvent check 2	0.00	31
Petroleum ether solvent check 1	0.05	78
Petroleum ether solvent check 2	0.06	72

Table 13. Laboratory 2 NP concentrations detected in extracted polymers (no clean-up)

Sample	NP concentration (mg/kg) *	Recovery (%)
PVC 1 (Rep-1)	4721.55	21
PVC 1 (Rep-2)	4449.85	15
PVC 1 (Rep-3)	4240.18	23
PVC 2 (Rep-1)	1592.53	90
PVC 2 (Rep-2)	2494.55	44
PVC 2 (Rep-3)	1605.61	40
Blue PS (Rep-1)	437.52	34
Blue PS (Rep-2)	624.46	37
Blue PS (Rep-3)	629.64	39

* The reported concentrations have been corrected for recovery.

Table 14. Laboratory 2 NP concentrations detected in standard NP solutions passed through the alumina columns (according to Annex 4)

Sample	NP concentration (µg/ml) *	Recovery (%)
0 µg/ml clean-up recovery check	1.37	27
0.12 µg/ml clean-up recovery check	1.23	29
1.25 µg/ml clean-up recovery check	1.79	30
12.46 µg/ml clean-up recovery check	6.60	38
124.60 µg/ml clean-up recovery check	95.44	66

* The reported concentrations have been corrected for recovery.

Table 15. Laboratory 2 NP concentrations detected in the four polymers analysed according to the SOP provided in Annex 3

Sample	NP concentration (mg/kg) *	Recovery (%)
Procedural blank Rep-1	ND	35
Procedural blank Rep-2	ND	45
Procedural blank Rep-3	ND	35
Clear PS Rep-1	253	58
Clear PS Rep-2	238	54
Clear PS Rep-3	239	50
Blue PS Rep-1	419	10
Blue PS Rep-2	404	9
Blue PS Rep-3	391	17
PVC 1 Rep-1	1,760	39
PVC 1 Rep-2	1,381	48
PVC 1 Rep-3	1,307	No data provided
PVC 2 Rep-1	1,874	43
PVC 2 Rep-2	1,851	43
PVC 2 Rep-3	1,943	35

* The reported concentrations have been corrected for recovery.

Table 16. Confirmation of the presence of NP by ion ratios

Designation	220/163	220/149	220/135
Clear PS	80%	139%	110%
Blue PS	87%	105%	112%
PVC 1	92%	98%	109%
PVC 2	84%	124%	111%

Table 17. Comparing the NP concentrations derived by Fera with those reported by the second laboratory involved in the between laboratory validation exercise

Designation	Fera *	Laboratory 2	220/135
Clear PS	595 mg/kg	243 mg/kg	41
Blue PS	545 mg/kg	404 mg/kg	74
PVC 1	1952 mg/kg	1483 mg/kg	76
PVC 2	1937 mg/kg	1889 mg/kg	98

* average concentration measured in the homogeneity testing.

Table 18. Comparison of the NP concentrations in two polystyrene and two polyvinyl chloride as measured by the three laboratories

	NP concentration in the polymer (mg/kg) *		
Sample	**Fera**	**Laboratory 2**	**Laboratory 3** [#]
Clear PS a	542	253	195
Clear PS b	529	238	153
Clear PS c	not tested	239	215
Average	**536**	**243**	**188**
Blue PS a	680	419	169
Blue PS b	613	404	92
Blue PS c	not tested	391	151
Average	**647**	**405**	**137**
PVC 1 a	2593	1760	632
PVC 1 b	2975	1381	806
PVC 1 c	not tested	1307	433
Average	**2784**	**1483**	**624**
PVC 2 a	2296	1874	537
PVC 2 b	2081	1851	575
PVC 2 c	not tested	1943	557
Average	**2189**	**1889**	**556**

* The reported concentrations have been corrected for recovery.

[#] Following the SOP Laboratory 3 reported problems eluting the NP from the alumina columns therefore these results were obtained using a modified method using methanol in the elution solvent.

Table 19. NP concentrations detected in the polymer

Sample	NP concentration in the polymer (mg/kg) *
Clear PS a	514
Clear PS b	428
Clear PS c	457
Average	**466**
Blue PS a	467
Blue PS b	513
Blue PS c	520
Average	**500**
PVC 1 a	2276
PVC 1 b	2023
PVC 1 c	2484
Average	**2261**
PVC 2 a	2332
PVC 2 b	2305
PVC 2 c	2296
Average	**2311**

* The reported concentrations have been corrected for recovery.

Table 20. NP concentrations detected using the different alumina samples

Sample	NP concentration in the polymer (mg/kg) *		
	Fera alumina	Laboratory 3 alumina deactivated by Fera	Laboratory 3 alumina deactivated by Laboratory 3
Blue PS a	345	407	Nd
Blue PS b	358	359	Nd
Blue PS c	324	326	Nd
Average	**342**	**364**	**Nd**

* The reported concentrations have been adjusted for recovery.

Nd = not detected.

Table 21. NP concentrations in the HIPS and cling film samples

Sample	NP concentration (mg/kg)	Recovery (%)
HIPS – river cobbler	28	56
HIPS – lemon sole fillets	1761	57
HIPS – fishcakes	924	21
Cling film A	4153	74
Cling film B	2588	66

Table 22. Measured NP concentration in foods exposed to cling film

Sample	Film	NP concentration (mg/kg)	Recovery (%)
Procedural blank (no foodstuff)	---	0.18	33
Procedural overspike (no foodstuff)*	---	0.73	39
Blank cheese	---	0.34	74
Overspiked blank cheese *	---	0.78	57
Cheese – sample i	A	3.28	66
Cheese – sample ii	A	2.99	61
Cheese – sample iii	A	3.12	63
Average		**3.13**	
Cheese – sample i	B	10.61	37
Cheese – sample ii	B	13.71	41
Cheese – sample iii	B	12.00	56
Average		**12.11**	
Blank cake	---	0.18	59
Overspiked blank cake *	---	0.67	51
Cake – sample i	A	4.48	52
Cake – sample ii	A	4.80	41
Cake – sample iii	A	5.02	41
Average		**4.77**	
Cake – sample i	B	10.32	48
Cake – sample ii	B	7.92	45
Cake – sample iii	B	10.70	41
Average		**9.65**	

* NP was overspiked at a concentration equivalent to 0.5 mg/kg.

ANNEX 1 – ORIGINAL SOP

The Food and Environment Research Agency

Sand Hutton

York

YO41 1LZ

STANDARD OPERATING PROCEDURE FOR DETERMINATION OF NONYLPHENOL IN PLASTIC FOOD CONTACT MATERIALS AND ARTICLES

0 INTRODUCTION

Nonylphenol (NP) is a generic term commonly used to describe a complex commercial mixture of mainly nonyl-substituted phenol. The complexity arises from the production of the compound from commercial nonene that is used to alkylate the phenol. Commercial nonene is a complex mixture of C9 alkenes and the resultant product, although predominantly 4-nonylphenol (>90%, because alkylation favours the para position) also contains small quantities of 2-nonylphenol and decylphenol. Most NP produced commercially is 4-nonylphenol with varied alkyl chain branching.

The analytical method described allows for the determination of NP in a range of food contact plastic materials and articles.

1 SCOPE

This document describes a method for the determination of NP in a range of food contact plastic materials and articles. The method is appropriate for the quantitative determination in the analyte concentration range of 0.5 – 1000 mg/kg for polyvinyl chloride and polystyrene samples and 0.025 – 50 mg/kg for other plastic samples.

2 REAGENTS

2.1 Analytes

2.1.1 4-Nonylphenol technical mixture (ChemService, Pa. USA)

2.1.2 $^{13}C_6$-4-n-Nonylphenol (100 µg/ml in nonane, Cambridge Isotope Laboratories (CIL) Mass, USA (purity - 99%))

2.1.3 $^{13}C_{12}$-PCB 52 (50 µg/ml in nonane, CIL, Mass, USA)

2.2 Chemicals

2.2.1 Nonane

2.2.2 Methanol

2.2.3 Cyclohexane

2.2.4 Sulphuric acid

2.2.5 Deactivated alumina

Place the alumina in a glass dish and activate in a muffle furnace at 450℃ for a minimum of 4 hours. Allow the alumina to cool and weigh into a glass bottle. Deactivate the alumina with 15% (w/w) water. Seal the bottle and mix using a roller mixer for a minimum of 4 hours.

2.2.6 Sodium sulphate, rinsed with dichloromethane

2.2.7 Petroleum ether (boiling point range 40℃ to 60℃)

2.2.8 Dichloromethane

2.3 Solutions

2.3.1 4-Nonylphenol technical mixture, 1 mg/ml stock solution

Weigh to the nearest 0.1 mg approximately 10 mg of 4-nonylphenol technical mixture (2.1.1) in a 10 ml volumetric flask and make up to the mark with nonane (2.2.1).

Calculate the actual concentration in milligrams of 4-nonylphenol per millilitre of solution.

2.3.2 4-Nonylphenol technical mixture, 1 µg/ml dilute solution

Pipette 50 µl of the 1 mg/ml 4-nonylphenol technical mixture stock solution (2.3.1) into a 50 ml volumetric flask. Make up to the mark with nonane (2.2.1). Cap and mix thoroughly.

Calculate the actual concentration in milligrams of 4-nonylphenol per millilitre of solution.

2.3.3 $^{13}C_6$-4-n-Nonylphenol, 10 µg/ml working internal solution

Pipette 200 µl of the $^{13}C_6$-4-n-nonylphenol solution (2.1.2) into a 2 ml volumetric flask and make up to the mark with nonane (2.2.1).

2.3.4 $^{13}C_{12}$-PCB 52, 1 µg/ml sensitivity standard solution

Pipette 40 µl of the $^{13}C_{12}$-PCB 52 solution (2.1.3) into a 2 ml volumetric flask and make up to the mark with nonane (2.2.1).

3 APPARATUS

NOTE: An instrument or item of apparatus is listed only where it is special or

made to a particular specification, the usual laboratory glassware and equipment being assumed to be available.

3.1 Gas chromatograph, equipped with an automatic sampler and a mass spectrometric detector.

Appropriate operating conditions have to be established for the specific equipment used for the determination.

3.2 Gas chromatographic parameters

NOTE: The following column and parameters have been found to be suitable:

Column:	DB-5 (J & W Scientific, 5% phenyl and 95% dimethylpolysiloxane) 30 m x 0.25 mm id x 0.25 µm film thickness
GC parameters:	
Column oven	isothermal 3 minutes at 60℃, then rai sed at 25℃/minute to 160℃ and held for 2 minutes, then at 0.8℃/minu te to 180℃, then at 30℃/minute to 300℃ and held for 5 minutes
Injection	programmed temperature vapourisation (PTV) concentrator injection of 2 µl at 60℃ for 2 minute s then raised to 300℃ and held for 5 minutes then raised to reach a final temperature of 320℃
Carrier gas	helium
Inlet pressure	32 psi
Transfer line temp.	260^{o}C
MS detection:	
Mode	selected ion monitoring (SIM) EI mode
SIM ions recorded	107, 121, 135, 149, 163, 220 for 4-nonylphenol
	113, 226 for $^{13}C_6$-4-n-nonylphenol
	302, 304 for $^{13}C_{12}$-PCB 52

GC and MS apparatus should be optimised according to manufacturer's instruction.

3.3 Micro syringes, 10, 50, 100 and 500 µl

3.4 Pre-cleaned Soxhlet apparatus

Extract the Soxhlet apparatus with methanol (2.2.2) prior to the introduction of the plastic sample.

3.5 Glass columns (300 x 8 mm i.d.)

Soak the glass columns in methanol (2.2.2) for 3 - 4 hours and dry in an oven at 105℃. Plug the washed and dried columns with sila nised glass wool until required.

4 SAMPLES

4.1 Linearity standards

Prior to analysing any samples the linearity of the 4-nonylphenol response should be checked. This is achieved through the analysis of standard solutions.

To a series of three 1 ml volumetric flasks add 0, 500 and 1000 µl of the dilute (1 µg/ml) solution of 4-n-nonylphenol (2.3.2).

To a further seven 1 ml volumetric flasks add 5, 20, 50, 100, 400, 700 and 1000 µl of the stock (1 mg/ml) solution of 4-n-nonylphenol mix (2.3.1).

Make each up to the mark with nonane. The concentrations of the standard solutions prepared in this way are thus: 0, 0.5, 1, 5, 20, 50, 100, 400, 700 and 1000 µg/ml.

Transfer 100 µl of each of the standard solutions to a glass GC-MS vial. To each GC-MS vial add 50 µl of the 10 µg/ml $^{13}C_6$-4-n-nonylphenol working solution, 25 µl of the 1 µg/ml $^{13}C_{12}$-PCB 52 sensitivity standard solution and 25 µl of nonane (i.e. a total volume of 200 µl).

Analyse the linearity standards prepared in this way by GC-MS.

4.2 Sample receipt

Assign each sample a unique number using the Fera Nautilus LIMS system.

Confirm the plastic type by Fourier transform-infra red spectroscopy.

4.3 Initial screening

4.3.1 Polyvinyl chloride and polystyrene samples

Cut the whole sample into 1-3 mm^2 pieces using dichloromethane-rinsed scissors or metal cutters and mix the pieces.

Weigh to the nearest 0.001 g approximately 0.10 g of the test sample into a 40 ml glass vial. Record the accurate mass of the sample. Using a glass syringe add 50 µl of the 10 µg/ml solution of $^{13}C_6$-4-n-nonylphenol (2.3.3). Add 20 ml of cyclohexane (2.2.3) and sonicate for 30 minutes at 40℃. Transfer the extract to a 250 ml separating funnel. Rinse the 40 ml glass vial with 5 ml of cyclohexane and transfer the rinsings to the separating funnel. Extract the cyclohexane with 25 ml of methanol acidified with 4 drops of sulphuric acid (2.2.4). Repeat using two further portions of acidified methanol. Combine the methanol extracts and transfer to a clean separating funnel. Extract the methanol with 3 x 65 ml portions of cyclohexane (2.2.3) by shaking for 3 minutes. Combine the cyclohexane extracts and reduce in volume to ~ 0.5 ml.

4.3.2 Other plastic samples

Cut the whole sample into 1-3 mm^2 pieces using dichloromethane-rinsed scissors or metal cutters and mix the pieces.

Weigh to the nearest 0.01 g approximately 2 g of the test sample into a 40 ml glass vial. Record the accurate mass of the sample. Using a glass syringe add 50 µl of the 10 µg/ml solution of $^{13}C_6$-4-n-nonylphenol (2.3.3). Extract the sample with 100 ml of methanol (2.2.2) using a pre-cleaned Soxhlet apparatus (3.4) for approximately 20 hours. Concentrate the sample extract to a volume of approximately 50 ml using a Turbo VAP II™ (Zymark Corporation) apparatus. Acidify the concentrated methanolic extract with four drops of sulphuric acid (2.2.4) and extract with 3 x 65 ml portions of cyclohexane (2.2.3) by shaking for 3 minutes. Combine the cyclohexane extracts and reduce in volume to ~ 0.5 ml.

4.3.3 Chromatographic purification

When required pack the columns with 5.6 g of deactivated alumina (2.2.5). Plug the column with glass wool, place a 5 mm layer of dichloromethane-rinsed sodium sulphate above (2.2.6) and below the alumina (5.6 g) to prevent any further deactivation by water present in the extracts. Condition the columns with cyclohexane (10 ml).

Transfer the concentrated extracts prepared in 4.3.1 and 4.3.2 onto the conditioned columns and elute with successive 50 ml portions of petroleum ether (2.2.7) followed by dichloromethane (2.2.8). Discard the petroleum ether layer. Evaporate the dichloromethane fraction to 0.5 ml in a sample concentrator and transfer quantitatively to a GC sample vial. Evaporate to dryness under a gentle stream of nitrogen. Add the internal sensitivity standard (25 µl of a 1 µg/ml solution of ^{13}C PCB 52, 2.3.4) to the GC vial and nonane 175 µl. Analyse by GC-MS.

4.4 Test samples

Calculate, according to clause 5.3.1, the approximate 4-nonylphenol concentration in the initial screening sample(s) prepared in clause 4.3 and analysed in clause 5.2.

4.4.1 Samples

Prepare test samples in triplicate as described in 4.3 (if appropriate alter the mass of the sample to fall within the concentration range for which the linearity has been established).

4.4.2 Blank samples

Prepare a blank sample as described in 4.3 but in the absence of the plastic test sample.

4.4.3 Relative response factor (RRF) standard

To a 1 ml volumetric flasks add 100 µl of the stock (1 mg/ml) solution of 4-n-nonylphenol mix (2.3.1).

Make up to the mark with nonane. The concentration of this standard solution is thus: 100 µg/ml.

Transfer 100 µl of this standard solution to a glass GC-MS vial. Add 50 µl of the 10 µg/ml $^{13}C_6$-4-n-nonylphenol working solution, 25 µl of the 1 µg/ml $^{13}C_{12}$-PCB 52 sensitivity standard solution and 25 µl of nonane (i.e. a total volume of 200 µl).

5 PROCEDURE

5.1 GC-MS analysis

The same operating conditions for the GC-MS system should be maintained throughout the analysis of all samples in a batch, i.e. spiked samples, blank samples and relative response factor (RRF) standards.

Inject in the following order: Relative response factor (RRF) standard, blank, samples, RRF. If the number of samples exceeds 5, run samples 6-12 after a second RRF standard and blank, then run a final RRF at end of run.

5.2 Sample treatment and execution of the determination

Place the vials with the samples prepared in 4.3 or 4.4 in the autosampler and analyse using the conditions given in clause 3.2.

Identify the peaks for the analyte, internal standard and sensitivity standard. Measure the respective peak areas: 4-nonylphenol (m/z 220), $^{13}C_6$-4-n-nonylphenol (m/z 226) and $^{13}C_{12}$PCB52 (304). A typical chromatogram for the analyte is shown in Figure 1.

5.3 Evaluation of data

5.3.1 Calculation of analyte levels in the plastic samples

a. Calculate the average RRF from the RRF standards

   = response (native) / amount (native) divided by response (IS) / amount (IS)

b. Calculate unknown amount

   = [amount (IS) / response (IS)] x [1 / RRF] x response unknown

c. Calculate concentration

   = unknown amount / sample weight

d. Calculate recovery (for QC purpose only samples are not recovery corrected)

= response (sample IS) / response (sample SS) divided by response (RRF standard IS) / response (RRF standard SS)

IS = internal standard

SS = sensitivity standard ($^{13}C_{12}$-PCB 52)

6 CONFIRMATION

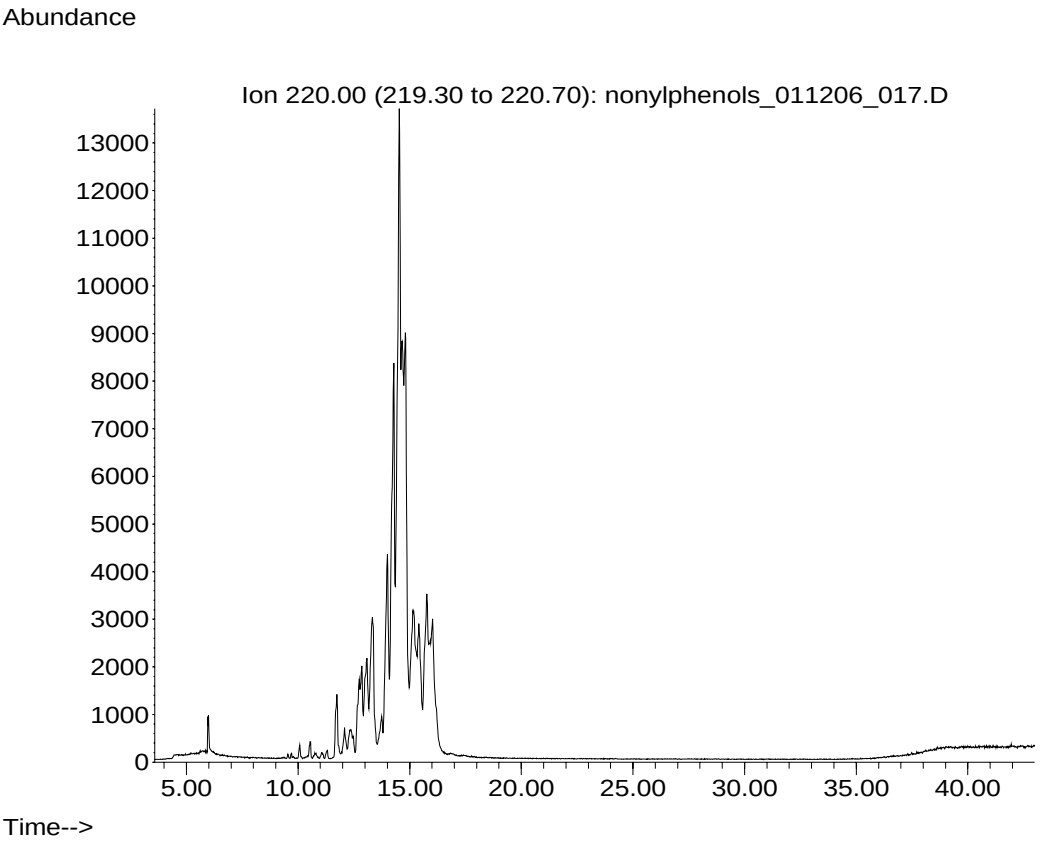
The presence of nonylphenol is confirmed by applying the criteria:

(i) The ion ratios are calculated using the following ions:

220/163, 220/149 and 220/135

The criteria is that the ratios of the nonylphenol extracted from the samples agree to ± 20% of those obtained from the RRF standard analysed at the same time.

Figure 1. Nonylphenol (m/z 220) selected ion chromatogram



ANNEX 2 – ORIGINAL METHOD – IN-HOUSE VALIDATION REPORT

The Food and Environment Research Agency

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METHOD OF ANALYSIS FOR NONYLPHENOL IN FOOD CONTACT MATERIALS AND ARTICLES

0. Summary

This report describes the procedure and the performance characteristics of a test method for the analysis of nonylphenol in food contact materials and articles.

The analysis is performed utilising GC-MS.

Performance of the method has been assessed by the analysis of twenty seven food contact materials/articles. The repeatability was calculated from the analysis of replicate specimens (n = 6) of two of the high positive samples. Relative standard deviations of 7.9 and 10.3% were obtained. Duplicate specimens of a further four samples were analysed and the agreement was good. The results showed that the analysis was linear (regression analysis value of 0.9995) over the concentration range investigated for NP normalised to the ^{13}Carbon labelled internal standard. The limit of detection (defined as mean + 3 times SD of the blank) was calculated to be 0.026 mg/kg. The limit of quantification (defined as mean + 10 times SD of the blank) was calculated to be 0.047 mg/kg. Analytical recovery was assessed for each sample based on the internal standard used. The average recovery was of the order of 75% and typically ranged from ~ 50 to 100%. One sample was fortified with NP at a concentration of 0.05 mg/kg and a recovery of 84% was obtained.

1. Background

Nonylphenol (NP), a recognised environmental contaminant, has been reported to be present in food and in several classes of food contact materials and articles. A method of analysis was required to confirm the presence of NP in these materials and articles and their concentrations. This method was developed in FSA project A 03047.

2. Experimental

2.1 Chemicals

4-Nonylphenol technical mixture was obtained from ChemService, Pa. USA, $^{13}C_6$ labelled 4-n-NP was obtained from Cambridge Isotope Laboratories (CIL) Mass, USA (purity - 99%); $^{13}C_{12}$ labelled PCB 52 was obtained from CIL, Mass, USA. Nonane, methanol, cyclohexane, petroleum ether (boiling point range 40℃ to 60℃) and dichloromethane were all ob tained from Rathburn Chemicals Ltd, Walkerburn, Scotland, UK. Alumina (Brockmann Grade 1) was purchased from Fluka. Reagent grade sulphuric acid and laboratory reagent grade sodium sulphate anhydrous were purchased from Fisher Scientific.

2.2 Standards

A stock solution containing 4-nonylphenol technical mix was prepared in nonane at a concentration of 1 mg/ml. A dilute working standard solution was prepared from this stock solution containing 4-nonylphenol technical mix at a concentration of 1 µg/ml by dilution with nonane.

A stock solution containing $^{13}C_6$-4-n-nonylphenol (internal standard) was prepared in nonane at a concentration of 1 µg/ml.

A stock solution containing $^{13}C_{12}$-PCB 52 (sensitivity standard) was prepared in nonane at a concentration of 1 µg/ml.

2.2.1 Preparation of relative response factor standards

A series of relative response factor standards in nonane was prepared from the 1 µg/ml dilute working standard solution to achieve concentrations of 0, 0.5, 5, 50, 100, 500 and 1000 µg/ml. These concentrations equate to 1 ng – 2 µg on-column which is equivalent to a range of ~ 10 µg/kg to 1000 mg/kg depending on the sample mass extracted.

Each standard solution also contained 0.5 µg/ml of the internal standard and sensitivity standard. The linearity of the method for NP was confirmed by the analysis of these standards by GC-MS.

2.3 Sample extraction

Samples were cut into 1-3 mm^2 particles using dichloromethane-rinsed scissors or metal cutters and the pieces were mixed.

For PVC and PS food contact materials/articles, between 0.1 and 0.5 g of the test sample, to which internal standard was added (50 µl of the 1 µg/ml solution in nonane), was sonicated with cyclohexane. The cyclohexane was extracted with acidified methanol and the methanol extract was then extracted three times with cyclohexane. The cyclohexane extracts were combined and reduced to a volume of ~ 0.5 ml. Replicate (n = 6) specimens of a PVC and a PS sample were extracted to allow the repeatability of the extraction and analysis to be determined.

For other (non-PVC/PS) food contact materials/articles, approximately 2 g of the test sample, to which internal standard was added (50 µl of the 1 µg/ml solution in nonane) was soxhlet extracted with methanol. The sample extract

was concentrated, acidified and extracted three times with cyclohexane. The cyclohexane extracts were combined and reduced to a volume of ~ 0.5 ml.

The concentrated extracts prepared were purified on a column of deactivated alumina, which was washed with petroleum ether and dichloromethane. The dichloromethane fraction was evaporated to 0.5 ml in a sample concentrator and transferred quantitatively to a GC sample vial. The internal sensitivity standard (25 µl of a 1 µg/ml solution of $^{13}C_{12}$ PCB 52) was added to the GC vial containing the extract and the extract was concentrate to a final volume of approximately 100 µl for analysis by GC-MS.

2.4 Spiked samples and blanks

Fortified samples were prepared by spiking a 'blank' plastic (polyethylene terephthalate) with NP at a concentration of 0.05 mg/kg. These samples were prepared to determine the analytical recovery.

Procedural blanks were prepared for all matrices.

2.5 Gas chromatography-mass spectrometry (GC-MS)

Samples were analysed by GC-MS using a Fisons GC800/MD800 operated in selected ion monitoring (SIM) mode with electron impact ionisation. The ions monitored were:

107, 121, 135, 149, 163, 220 for 4-nonylphenol

113, 226 for $^{13}C_6$-4-n-nonylphenol

302, 304 for $^{13}C_{12}$-PCB 52

Separation was carried out using a DB-5 column (J & W Scientific, 5% phenyl and 95% dimethylpolysiloxane) 30 m x 0.25 mm id x 0.25 µm film thickness. Sample introduction was using a programmed temperature vapourisation (PTV) concentrator injection of 2 µl at 60℃ for 2 minutes then raised to 300℃ and held for 5 minutes then raised to reach a final temperature of 320℃. Following injection the oven was held at an initial temperature of 60℃ for 3 minutes, the column was programmed at 25℃/minute to 160℃ and held for 2 minutes, then at 0.8℃/minute to 180℃ and then a t 30℃/minute to 300℃ and held for 5 minutes. Helium was used as the carrier gas, the inlet pressure was 32 psi. The GC-MS interface transfer line was held at 260℃.

2.6 Quantification

Quantification was based on the molecular mass ions m/z 220 for NP, and m/z 226 for the ^{13}Carbon labelled internal standard. Although these ions give a lower response than some of the fragment ions, the higher mass was more selective and thus less prone to interference. Ions corresponding to $^{13}C_{12}$ labelled PCB52 - m/z 302 and m/z 304 as internal sensitivity standard were also monitored. NP levels were determined relative to the internal standard response.

2.7 Method performance

The limit of detection, linearity, repeatability and recovery were determined and are reported in Section 3.

3. Results and discussion

3.1 Experimental design

The method of analysis was developed in an earlier FSA project to establish the levels of NP in food contact materials and articles. The validation parameters derived in this study are described in this report.

3.2 Limit of detection

The analytical methodology has considerable potential to be compromised due to method blank levels, as the analyte may be commonly used as a stabiliser in standard laboratory equipment such as tubing, plastic shelving, detergents, etc. Careful selection of solvents and conditioning of the reagents together with scrupulously clean glassware resulted in low blank levels (0.014 – 0.02 mg/kg). The average LOD (calculated as mean + 3 times the standard deviation of the blank determinations) thus achieved was 0.026 mg/kg. The average limit of quantification (calculated as mean + 10 times the standard deviation of the blank determinations) thus achieved was 0.047 mg/kg.

3.3 Linearity of the method

The linearity of NP response to the SIM GC-MS conditions was investigated with respect to the ions used for quantification (molecular ions for NP). The results showed that the GC-MS measurement process was linear (regression analysis value of 0.9995) over the concentration range investigated for NP normalised to the ^{13}Carbon labelled internal standard.

3.4 Repeatability

The repeatability of the analysis of was determined by the analysis of replicate specimens taken from two positive samples, one a PVC and the other a high impact polystyrene (HIPS). The results obtained are shown below.

	NP concentration (mg/kg)	
Replicate	**PVC**	**HIPS**
1	312	251
2	307	285
3	269	278
4	276	285
5	316	260
6	242	233
Mean	**287**	**265**
Standard deviation	**29.6**	**21.0**
RSD (%)	**10.3**	**7.9**

As the replicate analyses described above were carried out on materials containing relatively high NP concentrations, materials that showed lower

levels were also investigated by the analysis of duplicate specimens. The results obtained are shown below:

Sample description	Specimen	NP concentration (mg/kg)
High density polyethylene – dairy drink container	a	< 0.026
High density polyethylene – dairy drink container	b	< 0.026
Clear polypropylene – snack pots	a	0.049
Clear polypropylene – snack pots	b	0.041
Polycarbonate – baby feeding bottle	a	0.311
Polycarbonate – baby feeding bottle	b	0.319
Rubber latex – baby natural latex teats	a	0.394
Rubber latex – baby natural latex teats	b	0.386

These results demonstrate that even at low concentrations the repeatability of the method is acceptable.

3.5 Recovery

Analytical recovery was assessed for each sample based on the internal standard used. This parameter is a measure of the efficacy of the methodology used and gives an indication of the processing losses during the extraction, purification and measurement stages. The recovery was calculated relative to the internal sensitivity standard (^{13}Carbon labelled PCB 52) that is added to the sample extract just prior to measurement. The average recovery was of the order of 75% and typically ranged from ~ 50 to 100%. It should be noted that as the methodology uses internal standardisation, concentration values reported for the materials are corrected for recovery losses.

Analytical recovery was confirmed by fortifying a material with NP, followed by analysis. The choice of the material was based on low NP concentration. PET was chosen as preliminary investigations showed no detectable levels of NP. A portion of the material was fortified with NP at the 0.05 mg/kg level and analysed as normal. Measurement for this analysis gave a concentration of 0.047 mg/kg with an internal standard recovery of 84%. This also confirms that the analytes are not degraded during the extraction and purification process.

4. Conclusion

The GC-MS analysis applied performs to the required sensitivity and is capable of measuring NP in food contact materials and articles.

ANNEX 3 – REVISED SOP

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STANDARD OPERATING PROCEDURE FOR DETERMINATION OF NONYLPHENOL IN PLASTIC FOOD CONTACT MATERIALS AND ARTICLES

0 INTRODUCTION

Nonylphenol (NP) is a generic term commonly used to describe a complex commercial mixture of mainly nonyl-substituted phenol. The complexity arises from the production of the compound from commercial nonene that is used to alkylate the phenol. Commercial nonene is a complex mixture of C9 alkenes and the resultant product, although predominantly 4-nonylphenol (>90%, because alkylation favours the para position) also contains small quantities of 2-nonylphenol and decylphenol. Most NP produced commercially is 4-nonylphenol with varied alkyl chain branching.

The analytical method described allows for the determination of NP in a range of food contact plastic materials and articles.

1 SCOPE

This document describes a method for the determination of NP in a range of food contact plastic materials and articles. The method is appropriate for the quantitative determination in the analyte concentration range of 0.5 – 1000 mg/kg for polyvinyl chloride and polystyrene samples and 0.025 – 50 mg/kg for other plastic samples.

2 REAGENTS

2.1 Analytes

2.1.1 4-Nonylphenol technical mixture (ChemService, Pa. USA)

2.1.2 $^{13}C_6$-4-n-Nonylphenol (100 µg/ml in nonane, Cambridge Isotope Laboratories (CIL) Mass, USA (purity - 99%))

2.1.3 $^{13}C_{12}$-PCB 52 (40 µg/ml in nonane, CIL, Mass, USA)

2.2 Chemicals

2.2.1 Nonane

2.2.2 Methanol

2.2.3 Cyclohexane

2.2.4 Sulphuric acid

2.2.5 Deactivated alumina

Place the alumina in a ceramic dish and heat in a muffle furnace at 600℃ for 12 hours. Allow the alumina to cool and transfer into a glass bottle. Deactivate the alumina with 15% (w/w) water. Seal the bottle and mix using a roller mixer for 24 hours. Prepare the columns and purify the extracts straight away. Do not leave the alumina to stand.

NOTE: This procedure must be followed without deviation.

2.2.6 Sodium sulphate, rinsed with dichloromethane

2.2.7 Petroleum ether (boiling point range 40℃ to 60℃)

2.2.8 Dichloromethane

NOTE: The purity of the methanol, petroleum ether, cyclohexane and dichloromethane solvents should be ascertained before use.

2.3 Solutions

2.3.1 4-Nonylphenol technical mixture, 1 mg/ml stock solution

Weigh to the nearest 0.1 mg approximately 10 mg of 4-nonylphenol technical mixture (2.1.1) in a 10 ml volumetric flask and make up to the mark with nonane (2.2.1).

Calculate the actual concentration in milligrams of 4-nonylphenol per millilitre of solution.

2.3.2 4-Nonylphenol technical mixture, 1 µg/ml dilute solution

Pipette 50 µl of the 1 mg/ml 4-nonylphenol technical mixture stock solution (2.3.1) into a 50 ml volumetric flask. Make up to the mark with nonane (2.2.1). Cap and mix thoroughly.

Calculate the actual concentration in milligrams of 4-nonylphenol per millilitre of solution.

2.3.3 $^{13}C_6$-4-n-Nonylphenol, 10 µg/ml working internal solution

Pipette 200 µl of the $^{13}C_6$-4-n-nonylphenol solution (2.1.2) into a 2 ml volumetric flask and make up to the mark with nonane (2.2.1).

2.3.4 $^{13}C_{12}$-PCB 52, 0.8 µg/ml sensitivity standard solution

Pipette 40 µl of the $^{13}C_{12}$-PCB 52 solution (2.1.3) into a 2 ml volumetric flask and make up to the mark with nonane (2.2.1).

3 APPARATUS

NOTE: An instrument or item of apparatus is listed only where it is special or made to a particular specification, the usual laboratory glassware and equipment being assumed to be available.

3.1 Gas chromatograph, equipped with an automatic sampler and a mass spectrometric detector.

Appropriate operating conditions have to be established for the specific equipment used for the determination.

3.2 Gas chromatographic parameters

NOTE: The following column and parameters have been found to be suitable:

Column:	DB-5 (J & W Scientific, 5% phenyl and 95% dimethylpolysiloxane) 30 m x 0.25 mm id x 0.25 µm film thickness
GC parameters:	
Column oven	isothermal 3 minutes at 60℃, then rai sed at 25℃/minute to 160℃ and held for 2 minutes, then at 0.8℃/minu te to 180℃, then at 30℃/minute to 300℃ and held for 5 minutes
Injection	programmed temperature vapourisation (PTV) concentrator injection of 2 µl at 60℃ for 2 minute s then raised to 300℃ and held for 5 minutes then raised to reach a final temperature of 320℃
Carrier gas	helium
Inlet pressure	32 psi
Transfer line temp.	260^{o}C
MS detection:	
Mode	selected ion monitoring (SIM) EI mode
SIM ions recorded	107, 121, 135, 149, 163, 220 for 4-nonylphenol
	113, 226 for $^{13}C_6$-4-n-nonylphenol
	302, 304 for $^{13}C_{12}$-PCB 52

GC and MS apparatus should be optimised according to manufacturer's instruction.

3.3 Micro syringes, 10, 50, 100 and 500 µl

3.4 Pre-cleaned Soxhlet apparatus

Extract the Soxhlet apparatus with methanol (2.2.2) prior to the introduction of the plastic sample.

3.5 Glass columns (300 x 8 mm i.d.)

Soak the glass columns in methanol (2.2.2) for 3 - 4 hours and dry in an oven at 105℃. Plug the washed and dried columns with sila nised glass wool until required.

4 SAMPLES

4.1 Linearity standards

Prior to analysing any samples the linearity of the 4-nonylphenol response should be checked. This is achieved through the analysis of standard solutions.

To a series of three 1 ml volumetric flasks add 0, 500 and 1000 µl of the dilute (1 µg/ml) solution of 4-n-nonylphenol (2.3.2).

To a further seven 1 ml volumetric flasks add 5, 20, 50, 100, 400, 700 and 1000 µl of the stock (1 mg/ml) solution of 4-n-nonylphenol mix (2.3.1).

Make each up to the mark with nonane. The concentrations of the standard solutions prepared in this way are thus: 0, 0.5, 1, 5, 20, 50, 100, 400, 700 and 1000 µg/ml.

Transfer 100 µl of each of the standard solutions to a glass GC-MS vial. To each GC-MS vial add 50 µl of the 10 µg/ml $^{13}C_6$-4-n-nonylphenol working solution, 25 µl of the 0.8 µg/ml $^{13}C_{12}$-PCB 52 sensitivity standard solution and 25 µl of nonane (i.e. a total volume of 200 µl).

Analyse the linearity standards prepared in this way by GC-MS.

4.2 Sample receipt

Assign each sample a unique number using the Fera Nautilus LIMS system.

Confirm the plastic type by Fourier transform-infra red spectroscopy.

4.3 Initial screening

4.3.1 Polyvinyl chloride and polystyrene samples

Cut the whole sample into 1-3 mm^2 pieces using dichloromethane-rinsed scissors or metal cutters and mix the pieces.

Weigh to the nearest 0.001 g approximately 0.10 g of the test sample into a 40 ml glass vial. Record the accurate mass of the sample. Using a glass syringe add 50 µl of the 10 µg/ml solution of $^{13}C_6$-4-n-nonylphenol (2.3.3). Add 10 ml of dichloromethane (2.2.8) and sonicate for 15 minutes at 20℃. Pipette 5 ml methanol (2.2.2) into the vial to precipitate the polymer. Centrifuge the vial for 5 minutes at 3000 rpm. Transfer the supernatant into a clean 40 ml vial. Add another 5 ml methanol to the dichloromethane extract. Again centrifuge for 5 minutes at 3000 rpm and transfer the supernatant into a clean 40 ml vial.

Evaporate the extract to dryness and reconstitute in 0.5 ml cyclohexane. Carry out the purification step described in 4.3.3.

4.3.2 Other plastic samples

Cut the whole sample into 1-3 mm^2 pieces using dichloromethane-rinsed scissors or metal cutters and mix the pieces.

Weigh to the nearest 0.01 g approximately 2 g of the test sample into a 40 ml glass vial. Record the accurate mass of the sample. Using a glass syringe add 50 µl of the 10 µg/ml solution of $^{13}C_6$-4-n-nonylphenol (2.3.3). Extract the sample with 100 ml of methanol (2.2.2) using a pre-cleaned Soxhlet apparatus (3.4) for approximately 20 hours. Concentrate the sample extract to a volume of approximately 50 ml using a Turbo VAP II™ (Zymark Corporation) apparatus. Acidify the concentrated methanolic extract with four drops of sulphuric acid (2.2.4) and extract with 3 x 65 ml portions of cyclohexane (2.2.3) by shaking for 3 minutes. Combine the cyclohexane extracts and reduce in volume to ~ 0.5 ml.

4.3.3 Chromatographic purification

Pack the columns with 5.6 g of deactivated alumina (2.2.5) immediately taken from the roller or shaker. Plug the column with glass wool, place a 5 mm layer of dichloromethane-rinsed sodium sulphate above (2.2.6) and below the alumina (5.6 g) to prevent any further deactivation by water present in the extracts. Condition the columns with cyclohexane (10 ml).

Transfer the concentrated extracts prepared in 4.3.1 and 4.3.2 onto the conditioned columns and elute with successive 50 ml portions of petroleum ether (2.2.7) followed by the NP elution solvent dichloromethane (2.2.8). NOTE: The NP elution solvent should be optimised prior to analysis of the test samples. Discard the petroleum ether layer. Evaporate the dichloromethane fraction to 0.5 ml in a sample concentrator and transfer quantitatively to a GC sample vial. Evaporate to dryness under a gentle stream of nitrogen. Add the internal sensitivity standard (25 µl of a 0.8 µg/ml solution of ^{13}C PCB 52, 2.3.4) to the GC vial and nonane 175 µl. Analyse by GC-MS.

4.4 Test samples

Calculate, according to clause 5.3.1, the approximate 4-nonylphenol concentration in the initial screening sample(s) prepared in clause 4.3 and analysed in clause 5.2.

4.4.1 Samples

Prepare test samples in triplicate as described in 4.3 (if appropriate alter the mass of the sample to fall within the concentration range for which the linearity has been established).

4.4.2 Blank samples

Prepare a blank sample as described in 4.3 but in the absence of the plastic test sample.

4.4.3 Relative response factor (RRF) standard

To a 1 ml volumetric flasks add 100 µl of the stock (1 mg/ml) solution of 4-n-nonylphenol mix (2.3.1).

Make up to the mark with nonane. The concentration of this standard solution is thus: 100 µg/ml.

Transfer 100 µl of this standard solution to a glass GC-MS vial. Add 50 µl of the 10 µg/ml $^{13}C_6$-4-n-nonylphenol working solution, 25 µl of the 0.8 µg/ml $^{13}C_{12}$-PCB 52 sensitivity standard solution and 25 µl of nonane (i.e. a total volume of 200 µl).

5 PROCEDURE

5.1 GC-MS analysis

The same operating conditions for the GC-MS system should be maintained throughout the analysis of all samples in a batch, i.e. spiked samples, blank samples and relative response factor (RRF) standards.

Inject in the following order: Relative response factor (RRF) standard, blank, samples, RRF. If the number of samples exceeds 5, run samples 6-12 after a second RRF standard and blank, then run a final RRF at end of run.

5.2 Sample treatment and execution of the determination

Place the vials with the samples prepared in 4.3 or 4.4 in the autosampler and analyse using the conditions given in clause 3.2.

Identify the peaks for the analyte, internal standard and sensitivity standard. Measure the respective peak areas: 4-nonylphenol (m/z 220), $^{13}C_6$-4-n-nonylphenol (m/z 226) and $^{13}C_{12}$PCB52 (304). A typical chromatogram for the analyte is shown in Figure 1.

5.3 Evaluation of data

5.3.1 Calculation of analyte levels in the plastic samples

e. Calculate the average RRF from the RRF standards

= response (native) / amount (native) divided by response (IS) / amount (IS)

f. Calculate unknown amount

= [amount (IS) / response (IS)] x [1 / RRF] x response unknown

g. Calculate concentration

= unknown amount / sample weight

h. Calculate recovery (for QC purpose only samples are not recovery corrected)

= response (sample IS) / response (sample SS) divided by response (RRF standard IS) / response (RRF standard SS)

IS = internal standard

SS = sensitivity standard ($^{13}C_{12}$-PCB 52)

6 CONFIRMATION

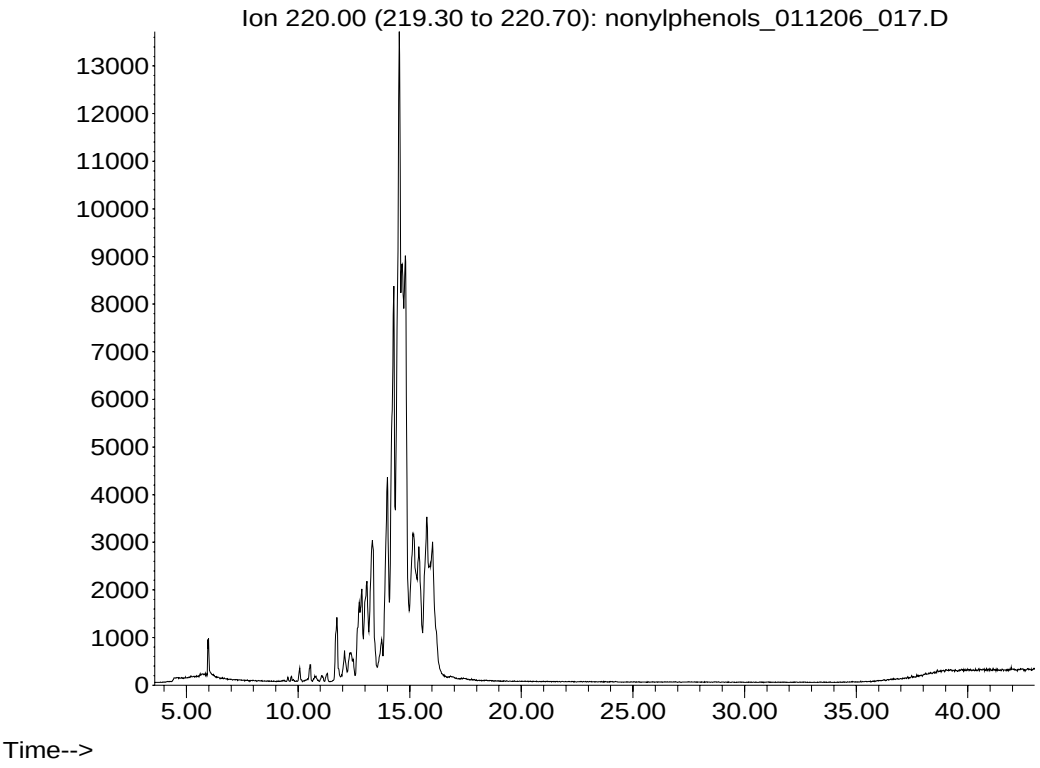
The presence of nonylphenol is confirmed by applying the criteria:

(i) The ion ratios are calculated using the following ions:

220/163, 220/149 and 220/135

The criteria is that the ratios of the nonylphenol extracted from the samples agree to ± 20% of those obtained from the RRF standard analysed at the same time.

Figure 1. Nonylphenol (m/z 220) selected ion chromatogram



ANNEX 4 – LABORATORY FAMILIARISATION PHASE TESTING PROTOCOL

1) Calibration standards

Prepare a set of calibration standards according to the original SOP (Annex1).

2) Establishing background levels in solvents

Add 50 µl of the 10 µg/ml internal standard solution to 50 ml of each of:

Dichloromethane

Methanol

Petroleum ether

Evaporate to dryness.

Add the internal sensitivity standard (25 µl of a 1 µg/ml solution of ^{13}C PCB 52) to the GC vial and nonane 175 µl. Analyse by GC-MS.

Calculate the background levels of NP in the solvents.

This should be carried out in duplicate

NOTE: We have used Rathburn solvents and found these to be NP free.

3) PVC extraction step

For one vial of each sample weigh 0.10 g (to the nearest 0.001 g) into three 40 ml vials.

Using a glass syringe add 50 µl of the 10 µg/ml solution of $^{13}C_6$-4-n-nonylphenol

Add 10 ml dichloromethane to each vial and sonicate (to dissolve the polymer).

Add 5 ml methanol to each vial (to precipitate the polymer).

Transfer the supernatant into clean 40 ml glass vials.

Add another 5 ml methanol to each vial.

Transfer the supernatant into clean 40 ml glass vials.

Prepare three blanks in the same way but without the addition of the polymer.

Evaporate the supernatant to 0.5 ml under a gentle stream of nitrogen and transfer quantitatively to a GC sample vial. Evaporate to dryness under a gentle stream of nitrogen. Add the internal sensitivity standard (25 µl of a 1 µg/ml solution of ^{13}C PCB 52, 2.3.4) to the GC vial and nonane 175 µl. Analyse by GC-MS.

Calculate the NP concentrations in the polymers.

4) Effect of the purification step on recovery (without interferences from the plastic)

Prepare standard solutions of the nonylphenol technical mix at concentrations of 0, 0.1, 1, 10 and 100 µg/ml methanol:dichloromethane 1:1 in a series of 20 ml volumetric flasks each containing 50 µl of the 10 µg/ml internal standard solution.

Evaporate each just to dryness and reconstitute in cyclohexane.

Preparation of the alumina:

Place the alumina in a glass dish and activate in a muffle furnace at 600℃ for 12 hours. Allow the alumina to cool and weigh into a glass bottle. Deactivate the alumina with 15% (w/w) water. Seal the bottle and mix using a roller mixer for 24 hours. Prepare the columns and purify the extracts straight away. Do not leave the alumina to stand. Pack the columns with 5.6 g of deactivated alumina immediately taken from the roller. Plug the column with glass wool, place a 5 mm layer of dichloromethane-rinsed sodium sulphate above and below the alumina (5.6 g) to prevent any further deactivation by water present in the extracts. Condition the columns with cyclohexane (10 ml).

Transfer the concentrated standard solutions in cyclohexane (from above) onto the conditioned columns and elute with successive 50 ml portions of petroleum ether followed by dichloromethane. Discard the petroleum ether layer. Evaporate the dichloromethane fraction to 0.5 ml in a sample concentrator and transfer quantitatively to a GC sample vial. Evaporate to dryness under a gentle stream of nitrogen. Add the internal sensitivity standard (25 µl of a 1 µg/ml solution of ^{13}C PCB 52, 2.3.4) to the GC vial and nonane 175 µl. Analyse by GC-MS.

Calculate the NP concentrations.

ANNEX 5 – FINAL SOP

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STANDARD OPERATING PROCEDURE FOR DETERMINATION OF NONYLPHENOL IN PLASTIC FOOD CONTACT MATERIALS AND ARTICLES

0 INTRODUCTION

Nonylphenol (NP) is a generic term commonly used to describe a complex commercial mixture of mainly nonyl-substituted phenol. The complexity arises from the production of the compound from commercial nonene that is used to alkylate the phenol. Commercial nonene is a complex mixture of C9 alkenes and the resultant product, although predominantly 4-nonylphenol (>90%, because alkylation favours the para position) also contains small quantities of 2-nonylphenol and decylphenol. Most NP produced commercially is 4-nonylphenol with varied alkyl chain branching.

The analytical method described allows for the determination of NP in a range of food contact plastic materials and articles.

1 SCOPE

This document describes a method for the determination of NP in a range of food contact plastic materials and articles. The method is appropriate for the quantitative determination in the analyte concentration range of 0.5 – 1000 mg/kg for polyvinyl chloride and polystyrene samples and 0.025 – 50 mg/kg for other plastic samples.

3 REAGENTS

2.1 Analytes

2.1.1 4-Nonylphenol technical mixture (ChemService, Pa. USA)

2.1.2 $^{13}C_6$-4-n-Nonylphenol (100 µg/ml in nonane, Cambridge Isotope Laboratories (CIL) Mass, USA (purity - 99%))

2.1.3 $^{13}C_{12}$-PCB 52 (40 µg/ml in nonane, CIL, Mass, USA)

2.2 Chemicals

2.2.1 Nonane

2.2.2 Methanol

2.2.3 Cyclohexane

2.2.4 Sulphuric acid

2.2.5 Deactivated alumina

Place the alumina (aluminium oxide, activated, neutral, Brockmann I) in a ceramic dish and heat in a muffle furnace at 600℃ for 12 hours. Allow the alumina to cool and transfer into a glass bottle. Deactivate the alumina with 15% (w/w) water (add 15 g water to 100 g alumina). Seal the bottle and mix using a roller mixer for 24 hours. Prepare the columns and purify the extracts straight away.

NOTE: Do not leave the prepared alumina columns to stand.

NOTE: This procedure must be followed without deviation.

NOTE: Do not store the alumina for future use.

2.2.6 Sodium sulphate, rinsed with dichloromethane

Rinse 50 g of anhydrous sodium sulphate with dichloromethane (200 mL) by weighing the anhydrous sodium sulphate into a glass funnel (with a frit) and pouring the dichloromethane through. Transfer the dichloromethane-rinsed sodium sulphate to an evaporating basin and allow the solvent to evaporate in air. Once the dichloromethane has evaporated complete the drying procedure in an oven overnight at 105°. Store the dichlorome thane-rinsed sodium sulphate in an air tight Duran bottle prior to use.

2.2.7 Petroleum ether (boiling point range 40℃ to 60℃)

2.2.8 Dichloromethane

NOTE: The purity of the methanol, petroleum ether, cyclohexane and dichloromethane solvents should be ascertained before use.

2.3 Solutions

2.3.1 4-Nonylphenol technical mixture, 1 mg/ml stock solution

Weigh to the nearest 0.1 mg approximately 10 mg of 4-nonylphenol technical mixture (2.1.1) in a 10 ml volumetric flask and make up to the mark with nonane (2.2.1).

Calculate the actual concentration in milligrams of 4-nonylphenol per millilitre of solution.

2.3.2 4-Nonylphenol technical mixture, 1 µg/ml dilute solution

Pipette 50 µl of the 1 mg/ml 4-nonylphenol technical mixture stock solution (2.3.1) into a 50 ml volumetric flask. Make up to the mark with nonane (2.2.1). Cap and mix thoroughly.

Calculate the actual concentration in milligrams of 4-nonylphenol per millilitre of solution.

2.3.3 $^{13}C_6$-4-n-Nonylphenol, 10 µg/ml working internal solution

Pipette 200 µl of the $^{13}C_6$-4-n-nonylphenol solution (2.1.2) into a 2 ml volumetric flask and make up to the mark with nonane (2.2.1).

2.3.4 $^{13}C_{12}$-PCB 52, 0.8 µg/ml sensitivity standard solution

Pipette 40 µl of the $^{13}C_{12}$-PCB 52 solution (2.1.3) into a 2 ml volumetric flask and make up to the mark with nonane (2.2.1).

3 APPARATUS

NOTE: An instrument or item of apparatus is listed only where it is special or made to a particular specification, the usual laboratory glassware and equipment being assumed to be available.

3.1 Gas chromatograph, equipped with an automatic sampler and a mass spectrometric detector.

Appropriate operating conditions have to be established for the specific equipment used for the determination.

3.2 Gas chromatographic parameters

NOTE: Two sets of conditions have been found to be suitable:

Column and conditions 1:

Column:	DB-5 (J & W Scientific, 5% phenyl and 95% dimethylpolysiloxane) 30 m x 0.25 mm id x 0.25 µm film thickness
GC parameters:	
Column oven	isothermal 3 minutes at 60℃, then raised at 25℃/minute to 160℃ and held for 2 minutes, then at 0.8℃/minute to 165℃, then at 30℃/minute to 300℃ and held for 5 minutes
Injection	programmed temperature vapourisation (PTV) concentrator injection of 2 µl at 60℃ for 2 minutes then raised to 300℃ and held for 5 minutes then raised to reach a final temperature of 320℃
Carrier gas	helium
Inlet pressure	32 psi
Transfer line temp.	280°C

Column and conditions 2:

Column: ZB-5ms	(Phenomenex, 5% phenyl and 95% dimethylpolysiloxane) 30 m x 0.25 mm id x 0.25 µm film thickness
GC parameters:	
Column oven	isothermal 3 minutes at 60℃, then rai sed at 25℃/minute to 160℃ and held for 2 minutes, then at 0.8℃/minu te to 165℃, then at 30℃/minute to 300℃ and held for 5 minutes
Injector	Splitless, 1 minute
Injection volume	1 µl
Inlet temperature	250℃
Carrier gas	helium at 1 ml/minute
Transfer line temp.	280^{o}C

MS detection:

Mode	selected ion monitoring (SIM) EI mode
SIM ions recorded	107, 121, 135, 149, 163, 220 for 4-nonylphenol
	113, 226 for $^{13}C_6$-4-n-nonylphenol
	302, 304 for $^{13}C_{12}$-PCB 52

GC and MS apparatus should be optimised according to manufacturer's instruction.

3.3 Micro syringes, 10, 50, 100 and 500 µl

3.4 Pre-cleaned Soxhlet apparatus

Extract the Soxhlet apparatus with methanol (2.2.2) prior to the introduction of the plastic sample.

3.5 Glass columns (300 x 8 mm i.d.)

Soak the glass columns (10 ml glass pipettes were used as the columns) in methanol (2.2.2) for 3 - 4 hours and dry in an oven at 105℃. Plug the washed and dried columns with silanised glass wool until required.

4 SAMPLES

4.1 Linearity standards

Prior to analysing any samples the linearity of the 4-nonylphenol response should be checked. This is achieved through the analysis of standard solutions.

To a series of three 1 ml volumetric flasks add 0, 500 and 1000 µl of the dilute (1 µg/ml) solution of 4-n-nonylphenol (2.3.2).

To a further seven 1 ml volumetric flasks add 5, 20, 50, 100, 400, 700 and 1000 µl of the stock (1 mg/ml) solution of 4-n-nonylphenol mix (2.3.1).

Make each up to the mark with nonane. The concentrations of the standard solutions prepared in this way are thus: 0, 0.5, 1, 5, 20, 50, 100, 400, 700 and 1000 µg/ml.

Transfer 100 µl of each of the standard solutions to a glass GC-MS vial. To each GC-MS vial add 50 µl of the 10 µg/ml $^{13}C_6$-4-n-nonylphenol working solution, 25 µl of the 0.8 µg/ml $^{13}C_{12}$-PCB 52 sensitivity standard solution and 25 µl of nonane (i.e. a total volume of 200 µl).

Analyse the linearity standards prepared in this way by GC-MS.

4.2 Sample receipt

Assign each sample a unique number using the Fera Nautilus LIMS system.

Confirm the plastic type by Fourier transform-infra red spectroscopy.

4.3 Initial screening

4.3.1 Polyvinyl chloride and polystyrene samples

Cut the whole sample into 1-3 mm^2 pieces using dichloromethane-rinsed scissors or metal cutters and mix the pieces.

Weigh to the nearest 0.001 g approximately 0.10 g of the test sample into a 40 ml glass vial. Record the accurate mass of the sample. Using a glass syringe add 50 µl of the 10 µg/ml solution of $^{13}C_6$-4-n-nonylphenol (2.3.3). Add 10 ml of dichloromethane (2.2.8) and sonicate for 15 minutes at 20℃. Pipette 5 ml methanol (2.2.2) into the vial to precipitate the polymer. Centrifuge the vial for 5 minutes at 3000 rpm. Transfer the supernatant into a clean 40 ml vial. Add another 5 ml methanol to the dichloromethane extract. Again centrifuge for 5 minutes at 3000 rpm and transfer the supernatant into a clean 40 ml vial. Evaporate the extract to dryness and reconstitute in 0.5 ml cyclohexane. Carry out the purification step described in 4.3.3.

4.3.2 Other plastic samples

Cut the whole sample into 1-3 mm^2 pieces using dichloromethane-rinsed scissors or metal cutters and mix the pieces.

Weigh to the nearest 0.01 g approximately 2 g of the test sample into a 200 ml round bottom flask. Record the accurate mass of the sample. Using a glass syringe add 50 µl of the 10 µg/ml solution of $^{13}C_6$-4-n-nonylphenol (2.3.3). Extract the sample with 100 ml of methanol (2.2.2) using a pre-cleaned Soxhlet apparatus (3.4) for approximately 20 hours. Concentrate the sample extract to a volume of approximately 50 ml using a Turbo VAP II™ (Zymark Corporation) apparatus. Acidify the concentrated methanolic extract with four drops of sulphuric acid (2.2.4) and extract with 3 x 65 ml portions of cyclohexane (2.2.3) by shaking for 3 minutes. Combine the cyclohexane extracts and reduce in volume to ~ 0.5 ml.

4.3.3 Chromatographic purification

Pack the columns with 5.6 g of deactivated alumina (2.2.5) immediately taken from the roller or shaker. Plug the column with glass wool, place a 5 mm layer of dichloromethane-rinsed sodium sulphate above (2.2.6) and below the alumina (5.6 g) to prevent any further deactivation by water present in the extracts. Condition the columns with cyclohexane (10 ml).

Using a clean glass pipette transfer the concentrated extracts prepared in 4.3.1 and 4.3.2 onto the conditioned columns and allow the extract to soak into the alumina. Wash the columns with 50 ml of petroleum ether (2.2.7) and elute the NP from the column with 50 ml of the NP elution solvent dichloromethane (2.2.8).

NOTE: The NP elution solvent should be optimised prior to analysis of the test samples. If the NP is not eluted in the dichloromethane fraction then a more polar solvent (e.g. methanol) should be used.

Discard the petroleum ether layer. Evaporate the dichloromethane fraction to 0.5 ml in a sample concentrator and transfer quantitatively to a GC sample vial (rinse the sample concentrator vial with 0.5 ml dichloromethane and combine the two fractions). Evaporate to dryness under a gentle stream of nitrogen. Add the internal sensitivity standard (25 µl of a 0.8 µg/ml solution of ^{13}C PCB 52, 2.3.4) to the GC vial and nonane 175 µl. Analyse by GC-MS.

4.4 Test samples

Calculate, according to clause 5.3.1, the approximate 4-nonylphenol concentration in the initial screening sample(s) prepared in clause 4.3 and analysed in clause 5.2.

4.4.1 Samples

Prepare test samples in triplicate as described in 4.3 (if appropriate alter the mass of the sample to fall within the concentration range for which the linearity has been established).

4.4.2 Blank samples

Prepare a blank sample as described in 4.3 but in the absence of the plastic test sample.

4.4.3 Relative response factor (RRF) standard

To a 1 ml volumetric flasks add 100 µl of the stock (1 mg/ml) solution of 4-n-nonylphenol mix (2.3.1).

Make up to the mark with nonane. The concentration of this standard solution is thus: 100 µg/ml.

Transfer 100 µl of this standard solution to a glass GC-MS vial. Add 50 µl of the 10 µg/ml $^{13}C_6$-4-n-nonylphenol working solution, 25 µl of the 0.8 µg/ml $^{13}C_{12}$-PCB 52 sensitivity standard solution and 25 µl of nonane (i.e. a total volume of 200 µl).

5 PROCEDURE

5.1 GC-MS analysis

The same operating conditions for the GC-MS system should be maintained throughout the analysis of all samples in a batch, i.e. spiked samples, blank samples and relative response factor (RRF) standards.

Inject in the following order: Relative response factor (RRF) standard, blank, samples, RRF. If the number of samples exceeds 5, run samples 6-12 after a second RRF standard and blank, then run a final RRF at end of run.

5.2 Sample treatment and execution of the determination

Place the vials with the samples prepared in 4.3 or 4.4 in the autosampler and analyse using the conditions given in clause 3.2.

Identify the peaks for the analyte, internal standard and sensitivity standard. Measure the respective peak areas: 4-nonylphenol (m/z 220), $^{13}C_6$-4-n-nonylphenol (m/z 226) and $^{13}C_{12}$PCB52 (304). A typical chromatogram for the analyte is shown in Figure 1.

5.3 Evaluation of data

5.3.1 Calculation of analyte levels in the plastic samples

i. Calculate the average RRF from the RRF standards

= response (native) / amount (native) divided by response (IS) / amount (IS)

ii. Calculate unknown amount

= [amount (IS) / response (IS)] x [1 / RRF] x response unknown

iii. Calculate concentration

= unknown amount / sample weight

iv. Calculate recovery (for QC purpose only samples are not recovery corrected)

= response (sample IS) / response (sample SS) divided by response (RRF standard IS) / response (RRF standard SS)

IS = internal standard

SS = sensitivity standard ($^{13}C_{12}$-PCB 52), the sensitivity standard is used for QCV purposes only to calculate the recovery.

6 CONFIRMATION

The presence of nonylphenol is confirmed by applying the criteria:

(i) The ion ratios are calculated using the following ions:

220/163, 220/149 and 220/135

The criteria is that the ratios of the nonylphenol extracted from the samples agree to ± 20% of those obtained from the RRF standard analysed at the same time.

Figure 1. Nonylphenol (m/z 220) selected ion chromatogram