Guidance Document on the Determination of Polychlorinated Naphthalenes

Version 1.0

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Analytical Parameters for food and feed analysis

EURL POPs

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EURL for halogenated POPs in Feed and Food
c/o State Institute for Chemical and Veterinary Analysis Freiburg
Bissierstraße 5
79114 Freiburg
D-Germany
contact@eurl-pops.eu
+49 761 8855 500
eurl-pops.eu

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Authorship

This guidance document was prepared following a series of discussions on the analytics and occurrence of PCNs in food and animal feed. The authorship includes members of the Core Working Group "Brominated Contaminants (BCons) and PCNs" of the network of the European Union Reference Laboratory (EURL) and National Reference Laboratories (NRLs) for halogenated POPs in Feed and Food and invited experts.

Editors

Alwyn Fernandes (Independent expert, UK)

Alexander Schächtele (EURL, Germany)

Theresa Zwickel (EURL, Germany)

Members of the Working Group for Brominated Contaminants and PCNs	
Thorsten Bernsmann	Chemisches und Veterinäruntersuchungsamt Münsterland-Emscher-Lippe, Münster, Germany
Tatiana Cantillana	Swedish food Agency, Sweden
Alwyn Fernandes (Chairman)	Independent expert, UK
Aasim Ali	The Institute of Marine Research (Norwegian NRL for Halogenated POPs in feed and food), Bergen, Norway.
Leondios Leondiadis	National Centre for Scientific Research (NCSR) "Demokritos", Athens, Greece
Philippe Marchand	Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (LABERCA), France
Sebastian Maszewski	Państwowy Instytut Weterynaryjny, Puławy, Poland
Wolfgang Moche	Environment Agency, Vienna, Austria
Panu Rantakokko	National Institute for Health and Welfare, Helsinki, Finland
Katharina Rund	European Union Reference Laboratory (EURL) for Halogenated POPs, Freiburg, Germany
Alexander Schächtele	European Union Reference Laboratory (EURL) for Halogenated POPs, Freiburg, Germany
Georges Scholl	Centre de Recherche Analytique et Technologique (CART), Belgium
Anaïs Vénisseau	Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (LABERCA), France
Mariko Yamamoto	Federal Institute for Risk Assessment (BfR), Berlin, Germany
Dzintars Zacs	Institute of Food Safety, Animal Health and Environment "BIOR", Latvia
Theresa Zwickel	European Union Reference Laboratory (EURL) for Halogenated POPs, Freiburg, Germany

Abbreviation	Definition
CWG	Core working group
DCM	Dichloromethane
DL-PCBs	Dioxin-like polychlorinated biphenyls
EFSA	European Food Safety Authority
EI	Electron ionization
EURL	European Union Reference Laboratory
GC	Gas chromatograph(y)
HRMS	High resolution mass spectrometry
iLOD	Instrument Limit of Detection
IS	Internal standard
ISO	International Organisation for Standardisation
IUPAC	International Union of Pure and Applied Chemistry
LOQ	Limit of quantification
LRMS	Low resolution mass spectrometry
MAE	Microwave assisted extraction
m/z	Mass-to-charge ratio
MS/MS	Tandem mass spectrometry
NCI	Negative chemical ionization
NDL-PCBs	Non-dioxin-like polychlorinated biphenyls
NRL	National Reference Laboratory
OFL	Official Laboratory
PBDEs	Polybrominated diphenylethers
PCNs	Polychlorinated naphthalenes
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-*p*-dioxins
PCDFs	Polychlorinated dibenzofurans
POP(s)	Persistent organic pollutant(s)
REP	Relative Effect Potency
RRF	Relative response factor
RS	Recovery standard
S/N	Signal-to-noise ratio
U	Expanded measurement uncertainty
TIC	Total ion chromatogram
TOF	Time-of-flight
w.w.	Wet weight

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1. General

1.1. Scope and Field of Application

This guidance document describing the determination of polychlorinated naphthalenes (PCNs), is intended for laboratories involved in the official control of contaminants in food and feed and focuses on the practical determination of PCNs. It was developed by a working group within the network of the European Union Reference Laboratory (EURL) for halogenated POPs in Feed and Food, the respective National Reference Laboratories (NRLs) of EU member states and international experts in the analysis of persistent organic pollutants (POPs). Provisionally, the methods are also generally applicable to animal feed, pending further validation of a potentially extended congener range including some mono- to tri-PCNs which occur in feed. In addition, the recommendations may also be applicable to the analysis of other matrices such as human tissues and environmental biota, but these are beyond the scope of this document. The document provides general guidance on analytical methodology for laboratories and particularly for those that do not have an existing method for PCNs. The practical determination is described in a modular format composed of different techniques for each stage of analysis which allows laboratories the flexibility of utilising existing equipment and expertise. The document aims to contribute to further harmonization and the assurance of reliable data as part of the EURL's official mandate and scope of work.

Note 1: All recommendations given in this document should be considered as "Guidance for reliable analyses" and are intended for food monitoring. There are currently no regulations for the control of PCNs in food and animal feed within the EU.

Note 2: Due to similar physico-chemical properties, PCN can be analysed simultaneously with PCDD/Fs, PCBs and/or PBDEs in multi-POP methods.

Information on sampling is not included. The user is referred to Commission Regulations (EC) No 152/2009 [1] and (EU) 2017/644 [2], on PCDD/Fs and PCBs as the direction given therein is expected to be satisfactory for these very similar organochlorine contaminants.

The recommendations are designed to allow the monitoring of PCN concentrations in food (and subsequently animal feed) as part of studies that will enable the determination of current occurrence levels for:

- the establishment/maintenance of databases that may be used to recommend action levels and/or maximum levels
- the assessment of human exposure through dietary intake and assessment of risk

The guidance could also be used for studies on time trends and patterns of PCN occurrence in order to identify possible source(s) of contamination, particularly during incidents involving such contamination.

1.2. Background

Polychlorinated naphthalenes (PCNs) are industrial chemicals that were manufactured in high volumes during the last century. They were used in numerous applications, mostly as temperature moderating fluids in electrical equipment and cable insulation, but also as flame retardants, plastic and rubber additives, fungicides, sealants, etc. However, accidental occupational exposure to industrial workers and the ingestion of contaminated feed by farm animals revealed serious biological effects, including fatal liver damage in humans at extreme exposure levels [3,4]. Subsequent studies on lower levels of exposure reported a range of more subtle long-term conditions/impacts such as pre-carcinogenic and peri-natal effects. The concern arising from these findings along with their environmental and biological stability and ubiquitous occurrence led to PCNs being listed as persistent organic pollutants (POPs) by the Stockholm Convention [5]; the listing was ratified by most countries at the end of 2016. The ultimate aim is to eliminate the use and production of PCNs and minimise unintentional formation which can arise from industrial combustion and other processes. The industrial production of PCNs ceased almost half a century ago, so current exposure arises from the ongoing legacy of production and use, particularly in Europe which was a major producer, and from combustion sources. Although there are theoretically 75 possible PCN congeners, a considerably smaller number are actually formed during commercial production.

The adverse biological effects reported for PCNs include the capacity for dioxin-like toxicity [4]. For this effect, the relative potencies of individual PCNs vary but some congeners show a similar potency range to the most toxic polychlorinated biphenyls (PCBs) [4,6]. Consequently, it was recommended by a WHO-panel that PCNs should be included in the toxic equivalency (TEQ) scheme that is used to express the toxic content of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) and PCBs [6].

1.3. Analytes of interest

PCNs are a class of chlorinated diaromatic compounds, manufactured by the catalytic chlorination of technical grade naphthalene. Their chemical and physical properties are very similar to those of PCDD/Fs and PCBs. PCNs constitute a group of 75 possible congeners (with the degree of chlorination ranging from mono- to octa-chlorinated; $[C_{10}H_{(8-x)}Cl_x$ (x = 1, 2, ..., 8; = m + n)] as shown in **Figure 1**. Congeners are classified according to the number of chlorine atoms in the molecule. Like other POPs they are hydrophobic and highly resistant to degradation processes, particularly biodegradation, although photo-degradation is known to occur [7]. Adverse health effects have been reported for humans, animals and soil organisms.

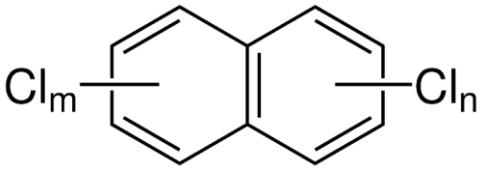

Figure 1: General PCN structural formula

Further information on PCNs, with respect to occurrence, toxicity and the estimation of risk through human exposure can be found in the following opinion by the European Food Safety Authority (EFSA) of 2024 [8]:

Risks for animal and human health related to the presence of polychlorinated naphthalenes (PCNs) in feed and food
https://www.efsa.europa.eu/en/efsajournal/pub/8640

1.4. Analyte Definition and Standards

The guidance given in this document applies to the analysis of food samples for the PCN congeners listed in **Table 1**. The procedures and analytical criteria described may also be applicable to the analysis of other matrices such as human tissues and environmental biota, but these are beyond the scope of this document. Laboratories may also apply similarly appropriate conditions to the measurement of other PCN congeners. Indeed, the investigation of the occurrence of other PCN congeners, particularly in animal feed, is encouraged as this information is currently scarce.

Table 1: Initial priority list for monitoring of PCN congeners in food

Homologue group	Configuration	Congener No.	CAS Number
Tetra	1,2,3,5 - TeCN	28	53555-63-8
	1,2,5,6 - TeCN	36	67922-22-9
	1,3,5,7 - TeCN	42	53555-64-9
	2,3,6,7 - TeCN	48	34588-40-4
Penta	1,2,3,5,7 – PeCN	52	53555-65-0
	1,2,3,5,8 – PeCN	53	150224-24-1
	1,2,4,5,8 – PeCN	59	150224-25-2
	1,2,4,6,7 – PeCN	60	150224-17-2
Hexa	1,2,3,4,5,6 – HxCN	63	58877-88-6
	1,2,3,4,5,7 – HxCN	64	67922-27-4
	1,2,3,4,6,7 – HxCN	66	103426-96-6
	1,2,3,5,6,7 – HxCN	67	103426-97-7
	1,2,3,5,6,8 – HxCN	68	103426-95-5
	1,2,3,5,7,8 – HxCN	69	103426-94-4
	1,2,3,6,7,8 – HxCN	70	17062-87-2
	1,2,4,5,6,8 – HxCN	71	90948-28-0
	1,2,4,5,7,8 – HxCN	72	103426-92-2
Hepta	1,2,3,4,5,6,7 – HpCN	73	58863-14-2
	1,2,3,4,5,6,8 – HpCN	74	58863-15-3
Octa	1,2,3,4,5,6,7,8 – OCN	75	2234-13-1

The rational basis for the selection of this set of PCN congeners to be prioritised for the analysis of food has been described in the EURL POPs Priority List of PCN congeners [9]. In brief, EURL's core working group "Brominated Contaminants (BCons) and PCNs" prioritised this initial group of 20 PCN congeners using the criteria of toxicological significance based on respective relative effect potencies (REPs) for dioxin-like activity combined with the patterns of occurrence in different food matrices and in human milk. In relevant matrices, the summed toxic equivalent (PCN-TEQ) of these congeners represented more than 98% of PCN toxicity using available REP data [10]. The prioritized congeners include four tetra-CNs (PCN-28, -36, -42, -48), four penta-CNs (PCN-52, -53, -59, -60), nine hexa-CNs (PCN-63, -64, -66, -67, -68, -69, -70, -71, -72), and hepta- and octa-CNs (PCN-73, -74, -75) – see **Table 1**.

As additional occurrence data becomes available, the list may be extended in the future in order to reflect the new findings.

Note: The limited amount of data that is available on PCN occurrence in animal feed does not allow a similar prioritisation for the monitoring of these matrices. Laboratories are encouraged to investigate the occurrence of a wide range of PCN congeners in animal feeds in order to assist the effort of congener prioritisation for these matrices.

1.5. Terms and Definitions

For purposes of this guidance document, the following terms and definitions apply.

Terms for analytical standards:

Internal standard (IS) also referred to as *'surrogate internal standard'*: a compound of known chemical purity that is added to calibration standards and every sample or procedural blank in a known amount prior to any analyte extraction and/or purification steps. It is used to monitor and compensate for analyte losses during the whole sample preparation procedure and variations in instrument sensitivity, occurring e.g. through matrix effects or variability of the volume of the extract injected into the GC. Isotope-labelled internal standards (surrogates of the analytes or closely related compounds) are used as the basis for quantification of the analytes using the isotope dilution analysis method [1,2].

Isotope dilution analysis method: Isotope-labelled analogues of the targeted analytes are added to the calibration standards and to each sample or procedural blank before extraction as internal standards in a known quantity; all analyte responses are normalised to that of the corresponding IS.

Recovery standard (RS) also referred to '*syringe/injection/volumetric standard'*: a compound of known chemical purity that is added to every sample, procedural blank or calibration standard at a known concentration, prior to measurement. Recovery standards are used as basis for quantification of the IS [1,2].

Definitions for analytical criteria:

Apparent recovery as defined by [11] means the recovery derived from an analytical procedure by means of a calibration graph. In the case of isotope dilution analysis, it is corrected for extraction/clean-up losses and matrix-effects and is about 100%.

Accuracy: Closeness of agreement between a test result or measurement result and the true value. It refers to a combination of trueness and precision [12,13].

Limit of quantification (LOQ): Lowest concentrations of the analyte which can be measured with reasonable statistical certainty [1,2].

Matrix effects (ME); interferences: The combined effects of all components of the extract other than the analyte, on the measurement of the quantity. If a specific component can be identified as causing an effect, then this is referred to as interference [14]. ME can occur at various steps in the analysis, including extraction and clean-up, chromatographic separation and during detection by GC-MS techniques due to adsorption originating from active spots in the injector, caused in part by the presence of residual matrix compounds.

Precision: Closeness of agreement between independent test/measurement results obtained under stipulated conditions [12,13].

- ***Repeatability*** or 'intra-day-precision' defined as precision under repeatability conditions. Repeatability conditions mean conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment [12,13].
- ***Reproducibility*** or 'inter-day-precision' defined as precision under reproducibility conditions. Reproducibility conditions mean conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment [12,13].
- ***Within-laboratory reproducibility:*** (intermediate precision) defined as precision under intermediate precision conditions. Intermediate precision conditions refer to conditions where measurement results are obtained with the same method, on identical test/measurement items in the same test or measurement facility, under some different operating conditions [12,13].

Recovery or Recovery Factor as defined by [11], also referred to as 'extraction recovery' or 'absolute recovery'. This is the yield of an analyte from the extraction/clean-up stage, e.g. the recovery of the IS calculated relative to the RS.

Selectivity: The ability of a method to distinguish between the analyte being measured and other substances [13].

Trueness: Closeness of agreement between the expectation of a test result or a measurement result and a true value. Trueness is usually expressed as bias [12,13].

Lower bound: Concept which requires using zero for the contribution of each non-quantified congener to a sum parameter [1,2].

Upper bound: Concept which requires using the limit of quantification for the contribution of each non-quantified congener to a sum parameter [1,2].

1.6. Guidance for Analytical Quality Assurance

Recommendations for Laboratories

- Laboratories should be accredited by a recognised body operating in accordance with EN ISO/IEC 17011 [15] to ensure that they are applying analytical quality assurance. The methods and scope of the laboratory should be accredited to the EN ISO/IEC 17025 standard [16].
- Laboratories should demonstrate proficiency in the analysis of PCNs at the concentration range of interest (i.e. range of 5 to 100 times the LOQ) by validation, ongoing internal quality control and continuous successful participation in inter-laboratory studies conducted by accredited bodies, e.g. the EURL.

General aspects regarding sample pre-treatment and storage

- Where required, laboratory samples should be thoroughly mixed by fine grinding, blending, etc. using a process that has been demonstrated to achieve complete homogenisation (e.g. ground to pass a 1 mm sieve for animal feed); samples may have to be dried before grinding if the moisture content is too high (the moisture content may optionally be determined for animal feed).
- The sample quantity used for the extraction should be sufficient to fulfil the requirements with respect to a sufficiently low working range including the concentrations at the suggested LOQs.
- The specific sample pre-treatment procedures used for the products under consideration shall follow European Regulations [1,2].
- Appropriate measures should be taken to ensure that the sample composition is representative of the consumed food or animal feed; e.g., removal of bones. In the case of fish, the skin has to be removed, as the contaminant content will relate to muscle meat without skin. However, it is necessary that all remaining muscle meat and fat tissue on the inner side of the skin is carefully and completely scraped off from the skin and added to the sample to be analysed.

General aspects to avoid contamination / high blank levels

- Measures must be taken to avoid cross-contamination at each stage of the sampling and analysis procedure in the laboratory.
- Glassware should be rinsed with solvents and/or heated at temperatures suitable to remove traces of PCNs from exposed surfaces and stored under conditions preventing re-contamination.
- Reagents, glassware and other equipment used for analysis and sampling should be controlled to avoid possible introduction or loss of PCNs.
- A procedural blank analysis should be performed by carrying out the entire analytical procedure omitting only the sample. The levels in the procedural blanks should be regularly monitored and maintained within an acceptable range.

1.7. Validation Parameters and General Quality Control Measures

Prior to the routine analysis of PCNs in food and feed samples, laboratories should demonstrate the performance of the methodology during the validation procedure and monitor this during routine analysis. Performance should be demonstrated in the relevant working range e.g. from < 5 x the LOQ to 100 x the LOQ with an acceptable coefficient of variation for **within-laboratory reproducibility** during the validation procedure and/or during routine analysis (see section 2.2).

Regular procedural blanks, and fortification experiments or analysis of control samples (preferably, using reference materials or in the absence of these, materials from successful and accredited PT exercises) should be performed as internal quality control measures. Quality control (QC) charts for procedural blanks, and fortification experiments or analysis of control samples should be recorded and checked to make sure that the analytical performance is in accordance with the requirements.

Table 2 provides recommendations of selected parameters for validation studies that should be carried out before routine analysis. Definitions can be found in **section 1.5**

Table 2: Recommendations for parameters for validation studies and routine quality control measures

Grouping of matrices	▪ Use of a single food or feed matrix to represent a matrix group if the matrices in the group share similar physico-chemical properties ▪ Example of a matrix group: meat, offal, fish, etc. ▪ Further sub-grouping might be necessary for analytes and/or methods if significant matrix effects are observed.
Selectivity of the analytical procedure	▪ Analytical methods should demonstrate the ability to reliably and consistently separate the analytes of interest from other co-extracted and possibly interfering compounds that may be present. ▪ Additionally, the chromatographic techniques that are used for clean-up, separation/measurement should be capable of differentiation between the congeners of interest and other occurring congeners.
Analytical working range	▪ Analytical methods should demonstrate the ability to reliably and consistently measure PCNs in the range of low to sub picograms (10^{-12} g) and above.
Trueness	▪ The measurement process used must provide a valid estimate of the true concentration in a sample. ▪ Trueness can be estimated from regular analysis of certified reference materials, fortification experiments or participation in successful inter-laboratory studies.
Precision	▪ Precision can be calculated from results for e.g. quality control samples generated under within-laboratory repeatability and reproducibility conditions. ▪ Laboratories are expected to progress to the determination of intermediate precision, following the setting-up and training phase.
Limit of quantification	▪ LOQs should be sufficiently low (see section 2.2) ▪ LOQs may be revised in the future according to evaluations resulting from new toxicological studies and hazard assessments

2. Specific Recommendations for PCN analysis

2.1. Extraction, Purification and Measurement

Extraction

Different extraction methods can be applied for the extraction of PCN congeners from respective matrices, following a suitable pre-treatment of the sample. These include cold, hot and pressurized extraction methods. Due to the relatively high volatility of PCNs, extraction and sample treatment procedures, in particular, processes applying elevated temperatures should be checked for losses of analytes of interest in these steps.

Exclusion of interfering substances

After complete extraction of the analytes of interest from the matrix, a suitable purification procedure should be applied to separate the PCN congeners from interfering matrix compounds and other halogenated substances. Suitable purification processes can be carried out using adsorption chromatography (recommended effective adsorbents are silica, alumina or Florisil™, as well as activated carbon).

Instrumentation

Gas Chromatography (GC) based analysis is recommended for PCNs. It should be noted that some of the PCN congeners listed in **Table 1** also co-elute with other PCN congeners. These co-elutions are reported for PCNs 28/36, PCNs 52/60, PCNs 64/68, PCNs 66/67 and PCNs 71/72 (**Chapter 3.4.4**) [18]. However, currently, these pairs cannot be fully separated using conventional GC-columns and should thus be reported as sum parameter of the co-eluting congener pairs. Suitable detection methods include GC-HRMS and GC-MS/MS. Other techniques may be used if the recommended performance parameters, e.g., LOQ, analyte identification, etc. can be achieved.

Analytical calibration range

The calibration curve that is used for GC-MS measurements and quantitation should cover the range of PCN concentrations that occur in food. Based on sum PCN data reported in the literature, concentrations in most European foods are in the range from 0.1 to 500 pg/g [19].

Quantification

It is recommended that internal standards (IS) are added at the very beginning of the analytical method e.g. prior to extraction, in order to validate the complete analytical procedure. If the IS is added after fat extraction (e.g. for foodstuffs of animal origin containing more than 10 % fat as done similarly for PCDD/F and PCB analysis [2]), an appropriate validation of the extraction efficiency should be carried out and verified by participation in relevant proficiency tests. At least one IS congener for each relevant homologue group for PCNs should be added. Although as congener responses vary, it is preferable that all available isotope-labelled IS of the

specified PCNs should be used as this would improve the reliability of the quantitation. If additional congeners (for which no isotope-labelled analogues are available) are being simultaneously determined, relative response factors shall be determined using appropriate isotope-labelled PCN congeners (e.g., from the same homologue group) and the validity of these can be confirmed either by using appropriate reference standards or by reference materials.

The recovery (syringe) standard (RS) should be added just prior to instrumental analysis. For MS detection, it is recommended that two isotopically labelled standards (for lower and higher measured masses) are added for PCN analysis in order to mitigate the variable response with increasing chlorination. As the number of commercially available isotope-labelled PCN congeners is limited, labelled congeners of other related compounds e.g., ^{13}C-PCBs, may also be used as recovery standards.

Table 3: Identification requirements for different MS techniques

GC-HRMS	
Accurate mass resolution	
Mass resolution	≥ 10 000 at 10 % valley (for the entire mass range)
Identification and confirmation of analyte signals	The retention time of the targeted analyte should be -1/+2 seconds relative to isotope-labelled standards
	2 ions should be monitored from the same isotopic cluster
Isotope ratio	± 20 % of the theoretical value, the average of the calibration standards or of the corresponding reference standard
GC-MS/MS	
Unit mass resolution	
Mass resolution	Unit mass (both quadrupoles) or wider resolution for Q1 and unit mass for Q3 as established during validation
Identification and confirmation of analyte signals	The retention time of the targeted analyte should be -1/+2 seconds relative to isotope-labelled standards
	2 transitions (including one precursor ion and one product ion each) e.g. from the same isotopic cluster should be monitored
Transition ratio[4]	± 20 % of the average of calibration standards from the same sequence or of the theoretical value or of the corresponding reference standard
Other identification requirements	
Target masses	See 3.4.4. Module 4
Chromatographic separation	See section 2.1 – Extraction, Purification and measurement (Instrumentation)

[4] Applying identical MS/MS conditions for calibration standard and samples, in particular collision energy and collision gas pressure, for each transition of an analyte.

2.2. Performance Parameters

Limit of quantification

The target LOQs for PCN congeners are based on recently reported levels of contamination in food and the understanding that better estimates of risk are facilitated by occurrence data that lies above the LOQ. Additionally, EFSA has also recommended [8] more sensitive measurements in future monitoring studies. The LOQ values may be revised in the future according to evaluations resulting from new toxicological studies and hazard assessments.

The recommended LOQ value for individual PCN congeners is 0.01 pg/g wet weight (w.w.). This value should allow sensitive reporting of occurrence concentrations as well as the toxic equivalence.

Since the approach chosen to estimate the LOQ has a great influence on the LOQ value, special attention should be paid to establishing this value. Approaches for the estimation of LOQs are described in the *"Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food"* [20] and the *"Guidance Document on the Estimation of LOQ for Measurements in the Field of Contaminants in Feed and Food"* [21].

Practically, the estimation of LOQs requires consideration of the procedural blanks as follows:

- Contribution of blank levels should be ≤ 20 % of the samples analysed in the accompanying batch
- Higher contribution requires the inclusion of blank levels in the estimation of LOQs
- Subtraction of blank concentrations may be performed, if blank levels remain relatively constant over time
- The variability of procedural blanks should be monitored and contributory factors (e.g. reagents, environmental conditions, equipment etc.) should be controlled in order to reduce it.

Analytical recovery

The analytical recovery of PCN internal standards as estimated using recovery standards (see section 2.1) should lie in the range of 40 - 120%. This range is based on the CWG experience of what is practically achievable on a routine basis, and the lower limit reflects the relatively high volatility of the lower chlorinated PCNs when compared to similarly chlorinated PCB or PCDD/F congeners. Lower recoveries (e.g. 30%) for individual congeners may be acceptable for lower chlorinated congeners (TeCN / PeCNs) due to their higher volatility.

Precision and trueness

As described in section 1.7, precision that is initially estimated from validation should be progressively confirmed as intermediate precision through results generated continuously under within-laboratory reproducibility conditions. Within-laboratory reproducibility should not be greater than 20 % for all listed PCN congeners.

Trueness can be estimated from regular analysis of certified reference materials, fortification experiments or by participation in inter-laboratory studies and should be between -25 % and +25 % (see **Table 4**).

Table 4: Typical performance characteristics for PCNs

Parameter	Characteristics
Trueness	- 25 % to + 25 %
Within-laboratory reproducibility	≤ 20 %
Difference between upper and lower bound calculation[1]	
Target LOQ (Food)	
Wet weight basis	0.01 pg/g
Fat basis	0.05 pg/g
Target LOQ (Feed)[1]	-
Recovery of internal standards[2]	40 – 120 %

[1] will be specified when regulations/decisions on elevated levels are established
[2] recoveries ≥ 30% are acceptable for lower chlorinated congeners

Measurement uncertainty

It is recommended that the uncertainty of measurement for individual congeners should be determined and reported with the results as an aid to the interpretation of the data. The recommended format is to report analytical results as x ± U where x is the analytical result and U is the expanded measurement uncertainty using a coverage factor of 2 (which gives a level of confidence of approximately 95 %). Additional estimation would be required for the reporting of sum parameters and any future comparison with legal limits.

As the measurement process for PCNs is very similar to PCDD/Fs and PCBs, guidance on how to estimate the measurement uncertainty can be found in the EURL POPs *'Guidance Document on Measurement Uncertainty for Laboratories performing PCDD/F and PCB Analysis using Isotope Dilution Mass Spectrometry'* [22].

3. Methodologies for PCN analysis in food

The methodologies detailed in this section describe the determination of PCNs in foods, but they can also be combined (as a multi-POP method) with methodologies currently used for the determination of PCDD/Fs, PCBs (and PBDEs) because of the similar physico-chemical properties. Generally, determination is based on the use of internal standardisation using ^{13}C-labelled surrogates that are commercially available and analyte separation using gas chromatography (GC) followed by measurement using high resolution mass spectrometry (GC-HRMS) or tandem mass spectrometry (GC-MS/MS). The targeted analytes are PCN congeners which may be reported as individual concentrations.

In order to allow flexibility of use and incorporation of individual laboratory practices, the methodology for sample extraction, purification and measurement by different GC-MS techniques is presented in modular form. Laboratories may choose modules based on available equipment. The description of analytical standards, quantitation and reporting format, is however, common, notwithstanding which modules are chosen.

Assuming competence in trace analysis and GC-MS, the first steps for a laboratory wishing to set up PCN determination would be procurement of the required standards and the use of these to establish the GC-MS conditions required to measure PCNs. Modules may then be chosen to complement the equipment available in individual laboratories. The modules are given as examples of working methodology that will allow users to meet the recommended method performance parameters. As the method itself is performance based, the procedures described here may be adapted to suit the prevailing circumstances / equipment in individual laboratories.

3.1. Analytical Standards that are used for PCN Determination

The analytical standards that are required for determination of the PCNs listed in **Table 5** are commercially available from current suppliers of POP standards, e.g. LGC Standards, Wellington Laboratories, Cambridge Isotope Laboratories, Inc. Restek Corporation, etc. An example of a full set of standards that could be used for routine determination is listed in **Table 5** below. Note that some standard mixtures, e.g. native PCNs, may be available as a solution that contains additional PCN congeners that are not listed in **Table 1**. These congeners may be helpful as indicators of wider PCN contamination in unknown samples. Mixed standard solutions may be prepared in nonane, toluene or another suitable solvent in the expected concentration range, depending on the matrix. Ready-to-use mixtures of standards may not contain all the relevant PCN congeners.

3.2. General Advice on Standards and Reagents

Recommendations for reliable analysis regarding analytical standards, reagents and procedures used during sample extraction, purification and measurement:

- Individual PCN congener standards or mixtures should be checked for impurities before use, with respect to other PCN congeners and also other halogenated POPs when applying multi-POP-methods. The resulting chromatograms should be clear of interferences, otherwise the standards may need to be purified before use.
- Reagents of recognized analytical grade and purity (free from PCNs and other contamination) should be used.
- Purity of the reagents and reference materials (e.g. standard solutions) should be verified and all possible contamination sources should be checked (all equipment used e.g. freeze dryer etc.) by analysing procedural blanks during method development and routinely, once the method is established using the validated methodology for PCN sample analysis. The resulting chromatograms should show no, or negligible amounts of PCNs, but also be clear of interferences at the retention time of targeted PCN congeners, otherwise the source of contamination needs to be identified and controlled.
- PCNs are relatively volatile, particularly the lower chlorinated congeners, so a check on the stability and integrity of the standards is advised. Practically, the volume of standard solutions should be verified by weighing before and after use.

Table 5: Analytical standards (commercially available native, isotopically ^{13}C-labelled internal (IS) and recovery standards (RS)) for the determination of PCNs listed in Table 1.

PCN Homologue Group	Configuration	Congener No.	Standard Availability, Use	CAS Number (native)
Tetra	1,2,3,5 - TeCN	**28**	Yes, Native	53555-63-8
	1,2,5,6 - TeCN	**36**	Yes, Native	67922-22-9
	1,3,5,7 - TeCN	**42**	Yes, Native & IS	53555-64-9
	2,3,6,7 - TeCN	**48**	Yes, Native & IS	34588-40-4
Penta	1,2,3,5,7 – PeCN	**52**	Yes, Native & IS	53555-65-0
	1,2,3,5,8 – PeCN	**53**	Yes, Native	150224-24-1
	1,2,4,5,8 – PeCN	**59**	Yes, Native & IS	150224-25-2
	1,2,4,6,7 – PeCN	**60**	Yes, Native	150224-17-2
Hexa	1,2,3,4,5,6 – HxCN	**63**	Yes, Native	58877-88-6
	1,2,3,4,5,7 – HxCN	**64**	Yes, Native & IS	67922-27-4
	1,2,3,4,6,7 – HxCN	**66**	Yes, Native	103426-96-6
	1,2,3,5,6,7 – HxCN	**67**	Yes, Native & IS	103426-97-7
	1,2,3,5,6,8 – HxCN	**68**	Yes, Native	103426-95-5
	1,2,3,5,7,8 – HxCN	**69**	Yes, Native	103426-94-4
	1,2,3,6,7,8 – HxCN	**70**	Yes, Native & IS	17062-87-2
	1,2,4,5,6,8 – HxCN	**71**	Yes, Native	90948-28-0
	1,2,4,5,7,8 – HxCN	**72**	Yes, Native & IS	103426-92-2
Hepta	1,2,3,4,5,6,7 – HpCN	**73**	Yes, Native & IS	58863-14-2
	1,2,3,4,5,6,8 – HpCN	**74**	Yes, Native & IS	58863-15-3
Octa	1,2,3,4,5,6,7,8 – OCN	**75**	Yes, Native & IS	2234-13-1
Suggested Congeners for QC - Recovery Standards (RS)				
	1,2,3,4 – TeCN	**27**	Yes, RS	20020-02-4
	1,2,3,6,7 – PeCN	**54**	Yes, RS	150224-16-1
	1,2,3,7,8 – PeCN	**56**	Yes, RS	150205-21-3
	1,2,3,4,5,8 – HxCN	**65**	Yes, RS	103426-93-3
	Alternatively, labelled PCBs, e.g., ^{13}C PCB-77, may also be used as RS			

3.3. General Aspects of Sample Preparation

A representative sample is required for reliable analytical data. For advice on representative sampling, practices/procedures such as those described in Commission Regulation (EC) No 152/2009 for PCDD/Fs and PCBs, Commission Regulation (EU) 2017/644 and EN ISO 6497 and EN ISO 6498 may be helpful [1,2,23,24].

Note: PCNs are relatively volatile, so care is advised, particularly with respect to sample preparation procedures which involve the use of elevated temperatures or extended periods of drying. It may be advisable to establish the extent of any losses of a particular sample preparation procedure, e.g. by standard addition to a blank matrix.

Representative samples of foods with a high moisture content may be initially blended, homogenised and then (freeze-) dried. After drying, the lyophilised or air-dried material may be re-homogenised to yield a dry and representative sample. Some laboratories may prefer to use a drying agent such as anhydrous Na_2SO_4 or polyacrylate when using small aliquots for analysis.

Representative samples of dry or low moisture products such as bread, nuts, cereals and cereal products, mixed feeds, and hay should be ground carefully so that the material can pass through a 1 mm mesh sieve. After grinding, the material should be homogenised to yield a representative ground sample.

High lipid content foods/feed such as butter, fish oils and vegetable oils, animal fats, etc. should be homogenised by blending when warm to ensure a representative sample. These matrices may not require further extraction and can be directly dissolved in suitable organic solvents.

Equipment (e.g. glassware, rotary evaporators, etc.) should be free from any PCN contamination. This may be achieved by carefully rinsing with e.g. dichloromethane, heptane, cyclohexane, acetone or mixtures of these solvents. Glassware may also be heated for 4 to 8 hours before use. This should be checked by procedural blank analysis and QC samples in each sample sequence if possible.

3.4. Independent Modules on sample extraction, purification and measurement

Depending on the equipment available, either manual or automated procedures may be used. Each module (1, 2, 3 and 4) describes a part of the whole PCN determination method. For sample preparation, each extraction method described in Module 1 can be combined with any clean-up method described in Module 2. Module 3 (section 3.4.3) describes a combined manual extraction and purification method. Each combination out of Module 1 and 2 or Module 3 independently, can be combined with the measurement procedure described in Module 4. All modular procedures described here are given as examples and could be adapted or substituted with alternative methods to match the equipment and expertise available in the laboratory.

3.4.1. Module 1 – Extraction

The procedures described in Module 1 are extraction methods for the isolation of PCNs from the sample. The extraction efficiency is affected by the properties of the chosen extraction solvent, the particle size of the raw material, the solvent-to-solid ratio as well as the extraction temperature, duration and number of extraction cycles. The procedures described are divided into cold, hot and hot pressurized extraction and can each be applied to effectively extract PCNs from any kind of food or feed sample matrix. Before extraction, the preparation recommendations for different types of samples should be followed as described above (section **3.3**).

Note: For those laboratories that intend to simultaneously determine PCDD/Fs and PCBs, the extraction procedures described in Module 1 may be used for multi-POP-analysis. See also standard EN 16215:2020 (Section 10.2 automated extraction procedure, 10.3 manual extraction procedure) [25].

For all procedures, representative sample aliquots are internally standardised by addition of isotopically labelled IS and extracted using mixed organic solvents or pressurised fluids, either at ambient temperature or higher temperatures depending on the equipment used.

Module 1A – Cold Extraction

Principle

Cold solvent extraction is an easy and relatively inexpensive procedure to isolate the analytes of interest from the raw material. The optimum solvent for this purpose penetrates into the matrix, dissolves the analytes and allows diffusion out of the matrix. Solvents with lower density separate at the top of the extraction mixture allowing the supernatant containing the analytes of interest, to be easily collected. Multiple different mechanical techniques e.g. shaking, stirring, open column extraction after mixing with drying agent or Ultra-Turrax® extraction of the fresh sample (according to [26]) are known and may be used if they allow the complete extraction of PCN congeners.

Extraction procedure using high-performance dispersion extraction

For high-performance dispersion extraction (using e.g. Ultra-Turrax®) isopropanol and cyclohexane are added to the homogenised sample. Immiscible liquid/liquid mixtures or raw powders can be homogenised, dispersed and extracted by high-speed stirring. The extraction efficiency is enhanced due to the high shear forces. After addition of water, the sample emulsion is extracted once again. The organic upper phase is collected and the extraction procedure is repeated twice. The combined organic extracts are subjected to further purification.

Ultrasonic extraction combined with H_2SO_4 treatment for preliminary purification

An appropriately sized (depending on fat content and iLOD) aliquot of a homogenised and freeze-dried sample is placed in a 250 mL glass bottle (e.g Fisherbrand™), spiked with ^{13}C-labelled internal standards, and equilibrated for at least 1 h. After equilibration, ~70 g of acid-modified silica gel (containing 44% of H_2SO_4) and 100 mL of n-hexane are added to the sample. The bottle is tightly closed with a screw cap and ultrasonicated for 20 min at 50°C. After sonication, the bottle is cooled to 10°C and centrifuged for 10 min at 1400 rpm. The upper organic layer is collected and the ultrasonication procedure is repeated two times using 50 mL portions of n-hexane. The combined organic extracts are subjected to further purification.

Module 1B – Hot Extraction

Principle

Continuous hot extraction procedures that operate at temperatures that are close to the boiling point of the extraction solvent or solvent mixture at atmospheric pressure are an efficient means of extracting PCN congeners from the matrix. The high temperature increases solubility and diffusion and therefore enhances the extraction efficiency of the analytes of interest. The extraction is most efficient when equilibrium of the solute is reached inside and outside the solid material.

Extraction procedures - manual

Before extraction the sample should be freeze-dried or mixed with a drying agent (e.g. polyacrylate or sodium sulphate). Depending on the composition of the sample, a mixture of cyclohexane and toluene (1/1, v/v) or ethanol and toluene (7/3, v/v) is used for e.g. in the Twisselmann extraction **(Figure 2).** The extraction at the boiling point of the solvent mixture is carried out for six hours. Permanent hot extraction of the sample takes place with solvent vapour and condensed solvent. A further cleaning of the raw extract with *tert*-butyl methyl ether for fat determination may be necessary depending on the sample matrix and co-extractives.

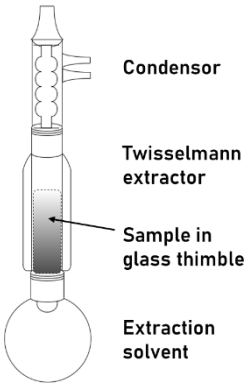

Figure 2: Twisselmann hot extractor with condenser, glass thimble and 500 – 1000 ml round bottom flask

A procedure for Soxhlet extraction as described in the standard EN 16215:2020 [25] (see section 10.3) for the extraction of PCDD/Fs and PCBs may also be used for the extraction of PCNs.

Extraction procedures – automated

The semi-automated Soxtec™ is a suitable hot extraction technique for POP analysis. Before extraction the sample aliquot should be freeze-dried or mixed with a drying agent. Samples are continuously extracted by an organic solvent mixture heated close to the boiling point at atmospheric pressure. Method parameters are summarized in **Table 6**.

Table 6: Example of a semi-automatic hot extraction instrumental method

Instrumentation	FOSS Soxtec™ 2055
Thimble volume	65 mL
Typical sample aliquot	5-10 g
Drying agent (if applicable)	sodium sulphate
Solvent, volume	DCM/hexane (1/1, v/v), 75 mL
Temperature	130 °C
Programme	1. immersion of the sample in refluxing solvent for 30 min; 2. rinsing of the thimble for 15 min; 3. careful solvent elimination for 15 min
Sample capacity	6 parallel samples

Extraction procedures – Microwave extraction

Principle

Microwave-assisted extraction (MAE) is an automated extraction technique that allows lower extraction times and reduced solvent consumption. During MAE, microwaves passing through the solvent rapidly heat the extraction mixture by direct interaction with the free water molecules present in the sample. This results in a drastic increase in the internal temperature (superheating), causing liquid vaporization within the cells. Rupture of the cell wall facilitates the release of fat and compounds of interest into the solvent.

Extraction procedure using ETHOS X (Milestone)

Before extraction, the sample should be freeze-dried or mixed with a drying agent (e.g. polyacrylate or sodium sulphate). The extraction solvents are similar to those used for Soxhlet or Twisselmann hot extraction or hot pressurized extraction. An example of extraction conditions is summarized in **Table 7**.

Table 7: Example of ETHOS X MAE extraction

Instrumentation	ETHOS X
Solvents	toluene/acetone (70/30, v/v)
Solvent volume	30 mL
Weight of sample	2-5 g
Temperature	110 °C
Static time	40 min
Cooling temperature	20 min

Module 1C – Hot Pressurized Extraction

Principle

The hot pressurized extraction process uses high temperature and high pressure, which decreases the extraction time and the quantity of solvent used. The high temperature increases solubility and diffusion and therefore enhances the extraction efficiency of the analytes of interest. The high pressure is used to keep the solvent in a liquid state as the temperature is increased above its boiling point, resulting in a higher solubility and higher diffusion rate of lipid solutes into the solvent, and higher penetration of the solvent into the matrix compared to other less drastic methods.

A procedure for automated pressurized fluid extraction as described in standard EN 16215:2020 [25] for the extraction of PCDD/Fs and PCBs may also be used for the extraction of PCNs.

Extraction procedure – ASE (ThermoFisher Scientific), SpeedExtractor (Büchi)

Pressurized liquid extraction requires freeze-drying or mixing of the sample with a drying agent. The type of drying agent that is used should be tested for each matrix in order to avoid clogging of cells. The extraction solvents are similar to those used with Soxhlet or Twisselmann hot extraction. Examples of suitable method conditions are summarized in **Table 8**.

Table 8: Examples of conditions for two hot pressurized extraction devices

	Dionex ASE 200 Food	Dionex ASE 200 Feed	SpeedExtractor E-914
Solvents	toluene/cyclohexane (50/50, v/v)	toluene/ethanol (80/20, v/v)	toluene/cyclohexane or toluene/ethanol
Cell volume	**33 mL**	**33 mL**	**120 mL**
Sample weight	10 – 17 g	10 – 17 g	10 – 30 g
Drying agent	5 – 10 g Hydromatrix 10 g Sodium acrylate/vinyl alcohol copolymer	5 – 10 g Hydromatrix 10 g Sodium acrylate/vinyl alcohol copolymer	20 – 50 g Hydromatrix 30 – 50 g Sodium acrylate/vinyl alcohol copolymer
Pressure	10 MPa	10 MPa	10 MPa
Temperature	120 °C	100 °C	100/120 °C
Static cycles	2	2	2
Heat time	6 min	5 min	5 min
Static time	5 min	10 min	5 min
N_2 Purge time	60 sec	20 sec	120 sec

3.4.2. Module 2 – Extract Purification

The procedures described in this module are manual, semi-automated or fully automated clean-up methods for the isolation of PCNs from other components and co-extractives present in a sample of food or animal feed. After extraction of the analytes of interest with an apolar solvent and evaporation to dryness, the extracted fat fraction is dissolved and an analyte specific purification procedure is carried out using firstly a multi-layer silica column followed by a second clean-up step using an alumina, Florisil® and/or carbon column.

Note: All clean-up procedures described in Module 2 may be combined with the fractionation and clean-up of PCDD/Fs, DL-PCBs and NDL-PCBs in a multi-method.

Module 2A - Manual purification method using two columns in series

The food matrix extract is applied to a two-column system, the first containing 25 g of acid-modified (44% H_2SO_4) silica gel which is connected directly to an activated carbon column containing 0.5 g of Supelclean ENVI-Carb™ mixed with 0.5 g of Celite-545. The maximum content of lipids in the applied extract should be considered in order to ensure effective destruction of matrix components. PCNs congeners are retained on the carbon while matrix residues and other non-planar compounds are flushed out of the two-column system with additional 50 mL of n-hexane. The upper column is disconnected and the carbon column is reverse-eluted with 100 mL of toluene containing ~40 mL of n-decane as a keeper solvent. The eluate is gently concentrated at <25°C to approx. 0.5 mL and treated with concentrated H_2SO_4. After intensive vortexing (several times), the extract is centrifuged repeatedly in order to obtain a clean and acid free extract. The extract is concentrated with the addition of the recovery standard and a keeper solvent (nonane, decane, etc.), to the required final volume under a gentle stream of nitrogen. Considering the volatility of some PCN congeners, evaporation to dryness should be avoided.

Module 2B – Automated Purification

This module describes procedures for the automated clean-up of PCNs from other components and co-extractives present in an extracted sample of food or animal feed. The procedures can be applied to extracts obtained using either a manual or automated extraction procedure as described in Module 1. This procedure is carried out using equipment that is commercially available. Descriptions of automated procedures using Miura™ and LC-Tech™ equipment are given below but other automated procedures may also be used.

Principle

Although differing in the details and order of columns used, the principle is essentially the same as that described in Module 2A. After manual or automated extraction (section 3.4.1) of PCNs the purification procedure is carried out by using automated adsorption chromatography with different kinds of clean-up columns (silica, sulphuric acid, carbon and alumina/Florisil® - These are available pre-packed from the equipment manufacturers/ suppliers).

For plant and feed matrices with low fat content, a high-capacity multi-layer silica column clean-up step should be integrated to hydrolyse interfering high-molecular substances (e.g. carotenoids) before automated purification. Waxes might be removed by using gel permeation chromatography (GPC) separation [25, section 11.5].

High-capacity multi-layer silica column clean-up (optional)

Preparation of adsorbents:

- Silica 60, 70-230 mesh: Baking over night at 600 °C
- Deactivation with water: Addition of 5 % of water
- Silica-sulphuric acid mixture: Addition of 32 % of conc. sulphuric acid (96 %)
- Silica-sodium hydroxide mixture: Addition of 23 % of 1 M NaOH solution
- Shaking of mixtures for several hours and equilibration over night

Preparation of chromatographic column:

Column: length 30 cm, inner diameter 40 mm

Packing of column (bottom to top):

- Glass wool plug
- Deactivated silica (5 g)
- Silica-sodium hydroxide mixture (10 g)
- Deactivated silica (5 g)
- Silica-sulphuric acid mixture (30 g)
- Deactivated silica (5 g)
- Silica-sodium hydroxide mixture (35 g)

Condition of column and elution of analytes of interest:

- Re-dissolving of dried extraction residue: 50 mL of cyclohexane/toluene (1/1, v/v) using ultrasonic bath
- Conditioning: 150 mL of cyclohexane/toluene (1/1, v/v)
- Elution: 150 mL of cyclohexane/toluene (1/1, v/v) in 500 mL round bottom flask
- Concentration of eluate to dryness using rotary evaporator and nitrogen

Purification procedure 1 – Miura™ GO-EHT (Miura Co. Ltd)

This procedure is carried out using the sample extract obtained from any of the extraction modules, and provides purified extracts for PCNs (as well as PCDD/Fs and PCBs). For the analysis of PCNs only, the carbon column may be omitted using a dummy column or a specific adapter.

The packed columns are provided ready-to-use by the supplier. The sample extract (or the equivalent of 2 to 3 g of the extracted fat) is dissolved in 10 mL of n-hexane and manually applied onto the top of the set of columns (column 1).

Purification is carried out using the automated programme described in Table 9: After clean-up on the sulphuric acid column (column 2) which is used for hydrolysing fats and other co-extracted material, and washing with 90 mL n-hexane, the PCNs are split in Fractions 1 and 2 after reverse elution of the alumina column with 1 mL toluene and reverse elution of the carbon column with 1.5 mL toluene (**Figure 3**).

In a multi-POP-method, mono- and di-ortho-PCBs also elute in Fraction 1. Non-ortho-PCBs and PCDD/Fs may be collected in Fraction 2 after reverse elution of the carbon column with 1.5 mL toluene. The conditioning, washing and elution steps are chosen according to the operating instruction from the manufacturer and were only slightly modified to optimise purification (**Table 9**).

Fraction 1 and 2 are combined and carefully evaporated to incipient dryness under a gentle stream of nitrogen and finally reconstituted with 20 µL toluene containing the recovery (syringe) standard.

Table 9: Programme for the PCN method using the Miura system; Fraction 1: mono- and di-ortho-PCBs, PCNs. Fraction 2: non-ortho-PCBs, PCDD/Fs, PCNs.

Step	Solvent	Volume [mL]	Flow [mL/min]	Time [min]	Analytes
Purification step					
Load. Hex	Hexane	90	2.5	2160	
Fractioning step					
Fraction 1	Toluene	1	0.5	168	**PCNs** (mono- and di-ortho-PCBs)
Fraction 2	Toluene	1.5	0.35	257	**PCNs** (non-ortho-PCBs, PCDD/Fs)

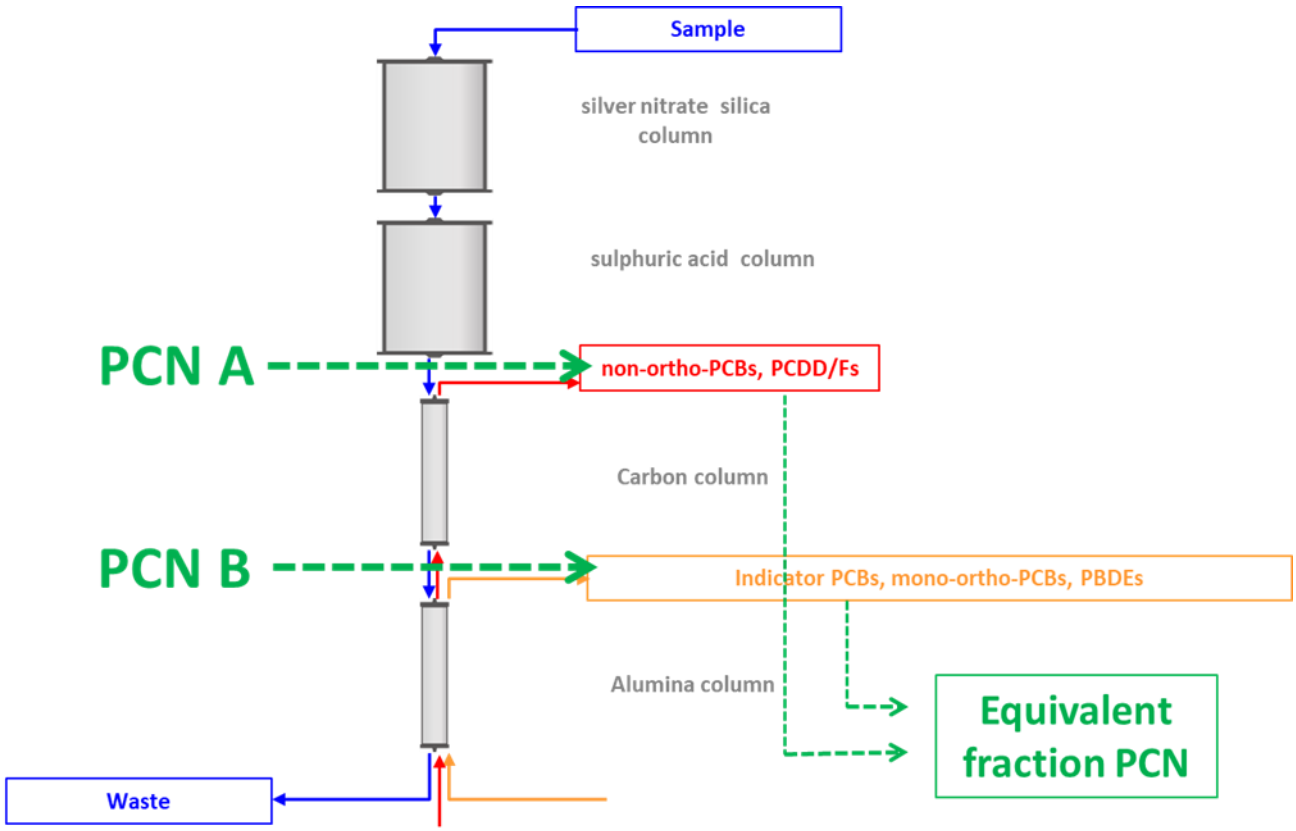

Figure 3: Schematic representation of automated purification using Miura GO-EHT equipment

Purification procedure 2 – DEXTech™ Plus/Pure (LCTech GmbH)

This procedure is carried out using the sample extract obtained from any of the extraction modules, and provides purified extracts for PCNs (as well as for PCDD/Fs, PCBs and PBDEs).

The sample (fat) extract is dissolved in 1 mL of acetone and 5 mL of cyclohexane. If there is incomplete dissolution, up to 1 mL of toluene can be added. As this solubilised extract will be automatically injected into the purification system, the final volume should not exceed 10 mL for a 15 mL sample loop.

Different sample types can be purified for the determination of PCNs (as well as PCDD/Fs and PCBs) using the steps described in **Table 10**, and as seen in the **Figure 4** below. This is a default method using a multi-layer silica sulphuric column (column 1) together with an alumina column (column 2) and a carbon column (column 3). The packed columns are provided ready-to-use by the supplier.

Table 10: Method parameters for purification using the DexTech system: Fraction 1: mono- and di-ortho-PCBs, PBDEs. Fraction 2: PCNs, non-ortho-PCBs, PCDD/Fs

Step	Solvent	Volume [mL]	Flow [mL/min]	Time [min]	Analytes
Purification					
Load	Hexane	182	7	26	
Fractioning					
Fraction 1	DCM/Hexane	36	3	12	NDL-PCBs, mono-ortho-PCBs, PBDEs
Fraction 2	Toluene	10	1	10	**PCNs,** non-ortho-PCBs, PCDD/Fs
N_2				3	
Rinse		1			
Sample		10			

The system starts with a column conditioning step, then injects the sample automatically and collects the individual fractions. Using this method, the purification uses 3 columns: an acidic silica-, an alumina- and a carbon-column. There are different kinds of acidic silica-columns available. The Universal and Standard-columns are used for fat samples with a maximum capacity of 5 g fat. The SMART-column is used for smaller amounts of fat with a maximum capacity of 1.5 g fat. Depending on the used acidic silica-column, the time taken and the amount of solvent consumed, will vary.

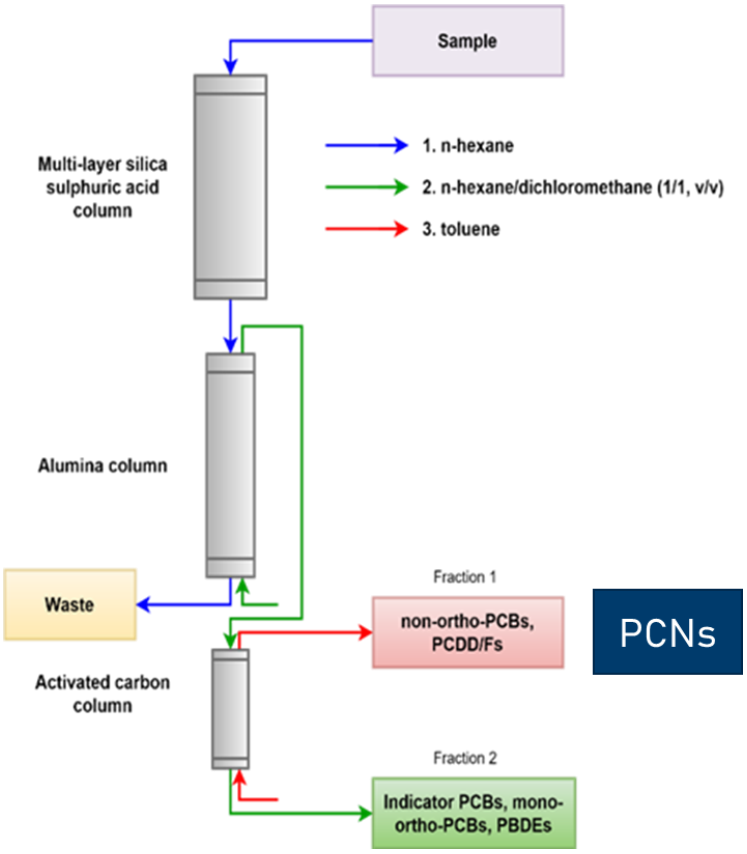

Figure 4: Schematic representation of automated PCN purification using DexTech equipment

3.4.3. Module 3 – Combined manual Extraction and Purification

This module combines sample extraction and purification in a single manual procedure. The internally standardised sample aliquot is extracted and purified by acid hydrolysis simultaneously on the same column. Further purification is carried out by adsorption chromatography using an alumina mini-column. The set-up consists of 12 columns for the analysis of 10 samples, a procedural blank and a reference material.

Principle

Aliquots of the test material are fortified with the internal standard, stabilised, comprehensively ground and dispersed in solution. The solution is passed through a multi-layer silica column where the PCNs are isolated from the matrix using cold solvent extraction with simultaneous preliminary purification through acid and base hydrolysis on the multi-layered silica. The eluate is concentrated and further purified using an activated basic alumina column. The PCN containing fraction from this column is reduced to the final extract volume with the introduction of the keeper solvent and the internal recovery standard.

Note: For those laboratories that are also required to determine PCDD/Fs and PCBs, this procedure may be combined for in-tandem fractionation of planar PCDD/Fs and dioxin-like PCBs (DL-PCBs), and non-dioxin-like PCBs (NDL-PCBs), by connecting the outflow of the multi-layered silica column directly to an activated carbon column, (alternatively, the concentrated outflow of the multi-layer column may be fractionated using a separate carbon column method). The fractions – mono-ortho-PCBs and NDL-PCBs (combined fraction) and planar PCDD/Fs and non-ortho-PCBs (combined fraction) may then be further purified on activated basic alumina. This procedure is described in detail in the EN 16215:2020 method – [25, section 11.3.4.]

Procedure

A representative sample aliquot, equivalent to 2 - 5 g of lipid is internally standardised using $^{13}C_{10}$-labelled PCN congeners. Following equilibration, the aliquot is blended with 75 g of H_2SO_4 modified silica gel (1:1) and 200 mL of n-hexane in a reagent bottle. The mixture is quantitatively transferred to a multilayer column (70 × 600 mm) packed from the top with 30 g of Na_2SO_4, 50 g of H_2SO_4 modified silica gel, 10 g of Na_2SO_4, and silanized glass wool. The column is connected sequentially to a carbon column (20 × 95 mm containing 0.1 g of activated carbon dispersed on 1 g of glass fibre). The columns are eluted with 400 mL of DCM/n-hexane (40/60, v/v)) The upper column is disconnected and 100 mL of DCM/toluene (2/8, v/v) is used to reversely elute the PCNs from the carbon column.

The eluate is concentrated gently at a temperature of <30 °C with solvent exchanged to ~0.5 mL of n-hexane. The concentrated extract is treated with 5 drops of concentrated H_2SO_4, using a vortex apparatus and allowed to separate into two layers. The aqueous layer is discarded and the process is repeated. The organic layer is chromatographed on two microcolumns (6 mm × 100 mm) in series, the upper column packed with acid modified silica gel is connected directly to the lower column containing approx. 7 cm of activated alumina. The columns are eluted with 15 mL of n-hexane to waste followed by disposal of the silica column and elution of the alumina column with 30 mL of DCM/n-hexane (30/70, v/v). This eluate is concentrated very gently to approximately 25 µL, (concentration to dryness must be avoided) with the addition of the $^{13}C_{10}$-labelled recovery standard contained in 25-50 µL of n-nonane as keeper.

3.4.4. Module 4 – Measurement of PCNs

Typical equipment set ups for the sensitive measurement of PCN congeners in food (and feed) extracts consist of:

- A gas chromatograph equipped with a splitless injector or with a large volume injector such as a programmable temperature vaporizer (PTV) inlet; a capillary GC column and a heated transfer line interfaced to the mass spectrometer.
- A high-resolution mass spectrometer (HRMS) using electron ionisation (EI) with magnetic and electrostatic sectors. Other high-resolution instruments e.g. Orbitrap, TOF may also be used.
- Alternatively, a tandem mass spectrometer (MS/MS) using electron ionisation (EI) may also be used.

General considerations for PCN congener separation by means of GC

A clean injector and liner as well as the use of a pre-column will help to maintain good GC separation and method sensitivity. If needed, removal of a small section of the column can help to remove matrix condensed at the injector end of the column. An injection liner without any filling material (such as glass wool) is preferable.

Examples for liners with good practical experiences are:

- PTV Liner, Single Baffle, Deactivated (e.g., Agilent, Restek, Thermo)
- Splitless liner, splitless single taper, deactivated (e.g., Agilent, Restek)

Most of the listed PCN congeners (**Table 1**) are routinely separable on commonly used GC columns (e.g. DB5-MS (Agilent), ZB-1701 (Phenomenex), Rtx-Dioxin2 (Restek); 30 or 60 m columns), except for the co-eluting pairs PCNs-28/36, PCNs-52/60, PCNs-64/68, PCNs-66/67 and PCNs-71/72 which require specialised stationary phases for separation. These pairs should be reported as the sum of both congeners (**Chapter 3.6**).

Note: Some of these congeners may be separable on specialised stationary phases (e.g., cyclodextrin incorporated into 14% cyanopropylphenyl/86% dimethyl polysiloxane Rt-b-DEXcst [27]), but these columns lack the robustness that would be required for routine food analysis.

In some cases, a 1-2 m long guard capillary (column without stationary phase) placed between the injector and GC column, may help to reduce contamination of the main analytical GC column with matrix components providing better peak shapes and responses.

General considerations for PCN congener detection by means of MS

After the sufficient performance of the GC-MS system has been verified, the extracts can be analysed according to good QC practice. Typically, the sequence of injections should commence and end with a set of PCN calibration standards, but other set-ups are also possible. The batch of samples including a procedural blank and reference or quality control material may be run in between these standards separated by solvent blanks to ensure that there is no carryover from the calibration standards or high contaminated samples.

Note: The physico-chemical properties of PCNs are very similar to PCBs and PCDD/Fs, and capillary gas chromatography (GC) coupled with high resolution mass spectrometry (HRMS sector field) detection is the most effective measurement technique as has been demonstrated in numerous studies on PCNs.

Module 4A –Gas Chromatography-High Resolution Mass Spectrometry (GC-EI-HRMS) – Sector field instrument

Principle

Sample extracts containing PCNs are introduced into the GC where they are volatilised and separated into congeners on the GC column, based on volatility and chromatographic affinity. The separated congeners are transferred sequentially into the MS where they are ionised (using electron ionisation - EI), separated by their mass-to-charge ratio (*m/z*) by the combined action of magnetic and electrostatic forces and recorded after amplification by a detection system. The acquired signals can then be processed to yield quantitative data.

Procedure - GC

The following volatilisation and temperature programmes may be used as initial guidance on conditions for PTV, SSL and GC oven programme for a 60 m x 0.25 mm i.d. J&W DB-5 MS GC column. Conditions may be modified depending on the length and type of GC column used.

Examples for PTV and SSL programmes:

PTV programme 1: 10 µL injections (solvent: nonane), 60°C (held for 3 min), ramped at 12°C/s to 320°C (held for 3 min), increased to 340°C at 12°C/s (held until end of run)

PTV programme 2: 5 µL injections (solvent: toluene), 100°C (0.17 min), ramped at 13.3°C/s to 340°C (held for 10 min)

SSL programme: 1 µL injection, splitless mode, injector temperature 300°C, purge time 2 min, purge flow 50 mL/min, gas saver at 5 min, 50 mL/min

Example of a GC oven programme (using a 60 m x 0.25 mm i.d. J&W DB-5 MS fused silica capillary column): 5-minute isothermal period at 60°C followed by heating at 24°C/min to 180°C for 2 min, then at 5°C/min to 250°C for 2 min, followed by 10°C/min to 300°C with a final isothermal period of 8 min. Other examples of GC oven programs are given with the chromatographic separations shown in Figure 5 and 6.

Procedure - MS

The mass spectrometer is operated in electron ionisation (EI) mode at a mass resolution of ≥10 000 (at 10% peak height). Selected ion monitoring (SIM) / multiple ion detection (MID) is used to record the two most intense ions in the molecular ion cluster for each monitored homologue group, for native and for ^{13}C-labelled internal and recovery standards (see **Table 12**). A suitable MS calibrant (e.g. FC43, FC5311, PFK) is used. Typical EI-HRMS instrument specific parameter ranges are summarized in **Table 11**.

Table 11: Typical EI-HRMS conditions used for PCN measurement using Autospec (Waters) or DFS (ThermoFisher Scientific) instruments.

Parameter	Specification
System used	GC-HRMS (Autospec, DFS)
Ionisation mode	EI +
Acquisition mode	Selected ions (MID)
Acquisition requirement	At least 10 ions at ≥10 000 within ~1s, cycle time e.g. 0.5 s
Source Ionisation energy	20 eV to 70 eV (depending on instrument)
Acceleration voltage	5 / 7 kV (depending on instrument)
Electron Trap current	450 to 600 µA (depending on instrument)

Table 12: *m/z* molecular ions for monitoring of unlabelled and ^{13}C-labelled PCN congeners

Homologue group	$M^+/[M+2]^+$ Unlabelled	$M^+/[M+2]^+$ ^{13}C-labelled
Tetra-CN	263.9067	273.9403
	265.9038	275.9373
Penta-CN	297.8677	307.9013
	299.8648	309.8983
Hexa-CN	333.8258	343.8594
	335.8229	345.8564
Hepta-CN	367.7868	377.8204
	369.7839	379.8174
Octa-CN	401.7479	411.7814
	403.7449	413.7785

Table 13 provides an example of the measured sections with exact masses of the molecular ions for quantifiers and qualifiers for native and ^{13}C-labelled PCN congeners and applicable lock masses using perfluorokerosene (PFK) as the mass calibration reference. An example chromatogram for the separation of all targeted PCN congeners is given in **Figure 5** (ZB-1701 column) and **Figure 6** (DB5 column).

Table 13: Exemplary PCN method set-up with time segments, molecular ions and lock masses (using PFK on a Autospec Waters instrument)

Function #1	**Retention time interval (min.)**			**12.0 - 17.05**		
Congener group	**m/z 1**	**m/z 2**	**Type**	**Channel time (ms)**	**Delay (ms)**	**Lock**
Tetra-CN	263.9067	265.9038	Native	100	10	
	273.9403	275.9373	^{13}C-labelled	50	10	
	280.98241		Lock mass	30	10	Yes
Function #2	**Retention time interval (min.)**			**17.05 - 18.5**		
Congener group	**m/z 1**	**m/z 2**	**Type**	**Channel time (ms)**	**Delay (ms)**	**Lock**
Tetra-CN	263.9067	265.9038	Native	100	10	
	280.98241		Lock mass	30	10	Yes
penta-CN	297.8677	299.8648	Nativ	10	10	
Function #3	**Retention time interval (min.)**			**18.5 - 22.6**		
Congener group	**m/z 1**	**m/z 2**	**Type**	**Channel time (ms)**	**Delay (ms)**	**Lock**
Penta-CN	297.8677	299.8648	Native	100	10	
	307.9013	309.8983	^{13}C-labelled			
	318.97922		Lock mass	30	10	Yes
Function #4	**Retention time interval (min.)**			**22.6 - 30.5**		
Congener group	**m/z 1**	**m/z 2**	**Type**	**Channel time (ms)**	**Delay (ms)**	**Lock**
Hexa-CN	333.8258	335.8229	Native	100	10	
	343.8594	345.8564	^{13}C-labelled	50	10	
	354.97922		Lock mass	30	10	Yes
Function #5	**Retention time interval (min.)**			**30.5 - 37**		
Congener group	**m/z 1**	**m/z 2**	**Type**	**Channel time (ms)**	**Delay (ms)**	**Lock**
Hepta-CN	367.7868	369.7839	Native	100	10	
	377.8204	379.8174	^{13}C-labelled	50	10	
	380.976		Lock mass	30	10	Yes
Function #6	**Retention time interval (min.)**			**37 - 50**		
Congener group	**m/z 1**	**m/z 2**	**Type**	**Channel time (ms)**	**Delay (ms)**	**Lock**
Octa-CN	401.7479	403.7449	Native	100	10	
	411.7814	413.7785	^{13}C-labelled	50	10	
	416.97603		Lock mass	30	10	Yes

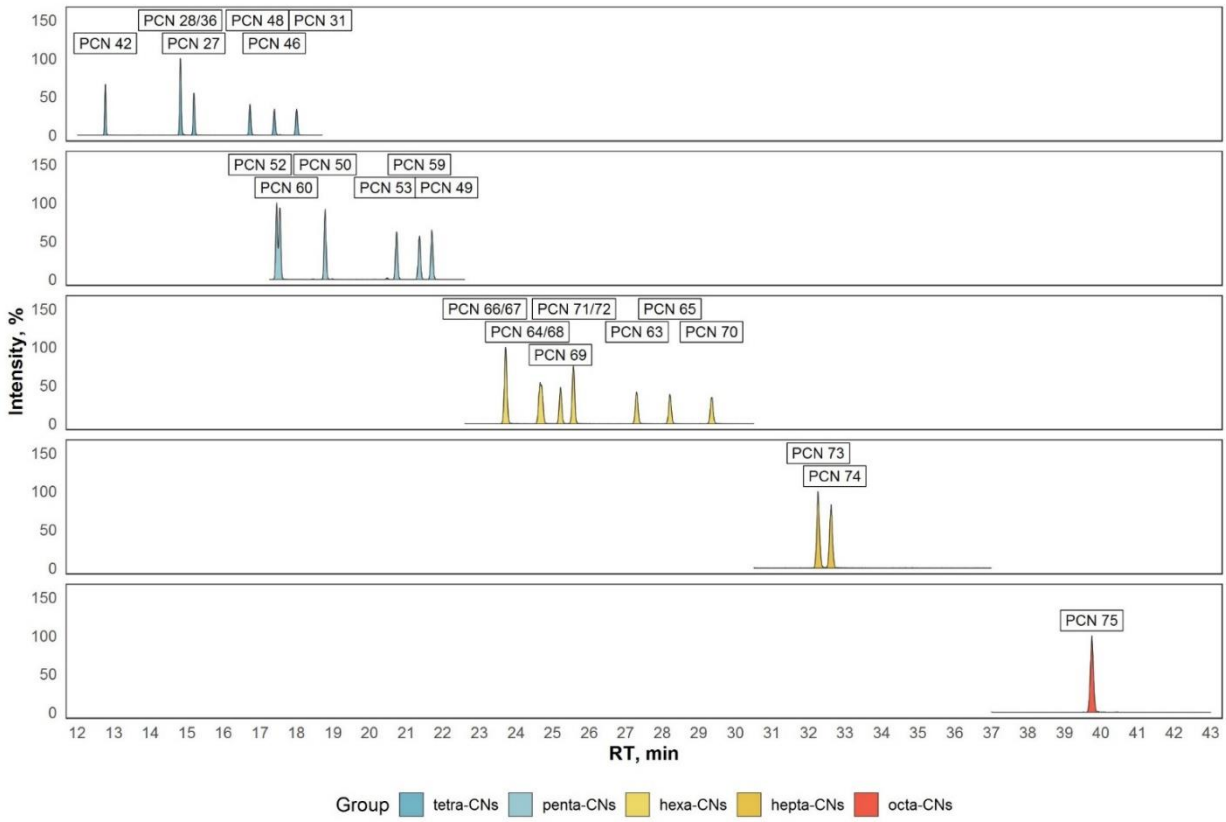

Figure 5: Chromatographic separation of a PCN mixture with all selected PCN congeners (the most intense ion trace is shown for each homologue group). Instrument: Autospec (Waters); PTV injector operated in pulsed splitless mode: Carrier gas helium, with a flow rate of 1.5 mL/min and pulsed pressure set at 150 kPa for 2 min; Injector temperature program 75 °C, ramp to 300 °C at 500 °C/min held for 2 min. GC-Column – Zebron ZB-1701P fused silica column (Phenomenex, Torrance, CA, USA) 30 m x 0.25 mm x 0.25 µm (2 m 0.25 mm guard). GC oven programme: 70 °C held for 1 min, ramp to 180 °C at 15 °C/min, ramp to 270 °C at 2 °C/min held for 10 min; The GC-MS transfer line and the ion source temperatures were kept at 280 °C.

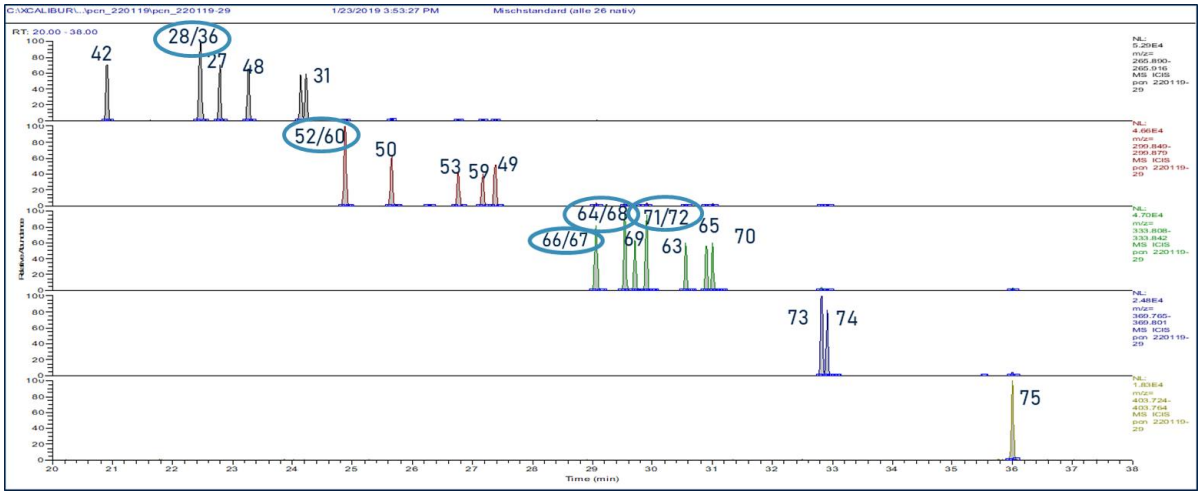

Figure 6: Chromatographic separation of a PCN mixture with all selected native PCN congeners (the most intense ion trace is shown for each homologue group). Instrument: DFS, Thermo; GC-Column:DB-5 60 m x 0.25 mm x 0.25 µm. GC oven programme: 80 °C (5 min) ramp with 24 °C/min to 180 °C (hold 2 min), ramp with 5 °C/min to 250 °C (hold 2 min) ramp with 10 °C/min to 300 °C (hold 7 min).

Note: Combined Measurement of PCNs and PCDD/Fs is possible in one single run. Therefore, all PCN and PCDD/F homologue groups can be measured in separate MID windows except for TCDD/F and Hexa-CN which might need to be combined in one window. The following GC programme might serve as initial guidance for the combined measurement on a DB5 column (60 m x 0.25 mm x 0.25 µm): 1.5-minute isothermal period at 90°C (depending on injection method and programme) followed by heating at 20 °C/min to 250 °C, then at 2.5 °C/min to 285 °C, followed by 10 °C/min to 300 °C with a final isothermal period of 20 min. Typical helium carrier gas flow rates are in the range of 1 mL/min or 1.5 mL/min.

3.5. Quantitation and Presentation of results

Similar to PCDD/Fs and PCBs, quantitation of PCNs is based on internal standardisation (or isotope dilution principle for those analytes with surrogate isotope-labelled internal standards). This approach automatically corrects for any extraction, purification or measurement losses and excludes the requirement for accurate volumetric treatment of the sample extract. The use of the labelled internal standards also allows for calculation of the recovery of the analytical process when measured against the recovery standard(s). For analytes without a surrogate labelled internal standard another closely eluting labelled internal standard with similar elution characteristics may be used.

Note: For analytes without a surrogate labelled internal standard the selection of the internal standard for quantification can have a significant influence on the calculated result, in particular if PCN congeners elute in different fractions during clean-up. Therefore, the selections need to be considered carefully when setting-up and validating the quantification method. Different fractions have to be checked for the presence / amount of PCN congener elution and surrogate labelled internal standards have to be chosen with regard to this. Fractions may need to be combined in order to obtain better recovery and quantitation. For example, PCN-42 may be eluted in two fractions when using the Miura System. If these fractions are not combined, $^{13}C_{10}$-PCN-42 should only be used for native PCN-42 and not for other tetra-chlorinated PCNs.

PCN congeners can be identified from the individual ion chromatograms based on the retention time of the native and corresponding labelled standard (if available), exact mass or transition and mass ratio (Table 3). These parameters and the identity of the targeted PCN congener should be established before quantitation. Congeners that are not separable by the GC method should be identified as a co-eluting pair e.g., PCN-52/60. Such co-eluting congeners should be quantified as a pair using the average relative response factor (RRF) derived from individual standards for the pair. The retention times of all PCNs should be checked and, if necessary, modified in the processing method that is usually provided with the instrument software. The updated method can then be used to integrate all ion chromatograms that were acquired during the analytical sequence. It is strongly recommended that the output is manually checked for correct integration.

It is recommended that a calibration curve covering the concentration range to be determined is prepared. This will verify the linearity of the PCN standard calibration for each compound. If the relative response for any congener is constant (less than 20 % coefficient of variation) over the calibration range, an averaged relative response factor (RRF) may be used for that congener. The RRF for a native PCN congener (i) in the analytical standard may be determined from the formula below. During calibration, the coefficient of variation for any ^{13}C-labelled PCN congener should be less than 30 %.

$$\mathrm{RRF}_i = \left(\frac{c_{iN}}{\mathrm{S}_{iN}}\right) \div \left(\frac{c_{iIS}}{\mathrm{S}_{iIS}}\right) \quad (1)$$

The amount m_i in [pg] (unit depending on the unit of the mass of the internal standard) of the congener (i) in the sample, may be determined from the derived formula:

$$\mathrm{m}_i = \frac{\mathrm{m}_{IS}}{\mathrm{S}_{iIS}} \times \frac{1}{\mathrm{RRF}_i} \times \mathrm{S}_{iN} \quad (2)$$

m_{IS}: amount (in pg) of the internal standard added to the test sample aliquot
c_{iN}: concentration of a native PCN congener$_{(i)}$ in the calibration level
c_{iIS}: concentration of the corresponding labelled PCN congener$_{(i)}$
S_{iN}: response (sum area of two m/z's) of the native PCN congener$_{(i)}$
S_{iIS}: response (sum area of two m/z's) of the corresponding labelled PCN congener$_{(i)}$

The concentration of a congener$_{(i)}$ in the sample may then be determined by dividing the determined amount m_i in the sample by the weight w of the aliquot taken for analysis.

$$c_i = \frac{m_i}{w} \quad (3)$$

The quantitation software on most GC-MS systems allows the above calculation process to be automated, so that a collated list of the PCN concentration values for the measured sample extract is obtained.

For quality control purposes, the analytical recovery of an internal standard can be calculated using equation 4 below:

$$\mathrm{R}_i[\%] = \left(\frac{\mathrm{S}_{iIS}}{\mathrm{m}_{IS}}\right) \div \left(\frac{S_{RS}}{m_{RS}}\right) \times \frac{1}{\mathrm{RRF}_{iIS}} \times 100 \quad (4)$$

m_{IS}: amount (in pg) of the internal standard$_{(i)}$ added to the test sample aliquot
m_{RS}: amount (in pg) of the recovery standard added to the test sample aliquot at the last step prior instrumental measurement
S_{iIS}: response (sum area of two m/z's) of the internal standard$_{(i)}$
S_{RS}: response (sum area of two m/z's) of the recovery standard
$RRF_{(iIS)}$: relative response factor of the internal standard$_{(i)}$

3.6. Reporting of Results – Format

PCNs are reported as individual congener concentrations. As noted in chapter 3.4 and 3.5, some PCN congeners are not separable by routine GC techniques and are quantified as a pair of congeners. The calculated concentrations of these congener pairs should be similarly reported e.g., ΣPCN-52/60. PCN concentrations are conventionally reported in pg/g and may be reported on a fat weight and wet weight (w.w.) basis in accordance with EU legislation for PCDD/Fs. An example of a reporting format for PCN concentrations in food and feed samples is given in Table 14

Table 14: Example of a reporting format for PCN samples

Sample ID	
Location	
Sample Type	
Year of sampling	
Routine or Incident related	

PCN congener/ PCN congener pair e.g. ΣPCN 52/60	**Content** [pg/g w.w.]	**Content** [pg/g fat]	**MU** [%]	**Recovery** [%]	**LOQ** [pg/g w.w.] or [pg/g fat]
% Lipid content (food)					
% Moisture content (feed)					
Extraction method used					
Purification method used					
Quantitation method used					
Other relevant information					

Note: The concentrations may also be reported as toxic equivalents (TEQs) by multiplying the individual congener concentrations with the relative potency (REP) value for the respective congener. For co-eluting congeners, the higher of the two REP values should be used in accordance with the precautionary principle. Both, concentrations as well as TEQ values may also be summarised giving a summed PCN concentration (ΣPCN and ΣPCN-TEQ). As a set of TEF values for PCNs has not yet been established, the relative potency values that are used should be correctly referenced – this will allow identification of the REP values used and also allow recalculation if/when TEF values are established.

4. Literature

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