Guidance Document on the Analysis of Chlorinated Paraffins

Sample preparation and quantification in food and feed

VERSION 1.9

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EURL POPs

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ABBREVIATIONS

GC – gas chromatograph(y)
LC – liquid chromatograph(y)
TIC – total ion current
FWHM – full width, at half maximum
TOF – time-of-flight (mass analyser)
CPs – chlorinated paraffins
SCCPs – short-chain chlorinated paraffins
MCCPs – medium-chain chlorinated paraffins
LCCPs – long-chain chlorinated paraffins
MOSH/MOAH – mineral oil saturated hydrocarbons, aliphatic hydrocarbons
LOD – limit of detection
LOQ – limit of quantification
RRFs – relative response factors
EFSA – European Food Safety Authority
CWG – Core Working Group

PREFACE AND SCOPE OF THIS DOCUMENT

Chlorinated paraffins (CPs) are high-production volume chemicals used in various industries as plasticizers, high-pressure lubricants or flame retardants. Annual CP production was predicted as superseding 1 million tons in 2016, with further increase expected especially on the Chinese market [1]. Depending on the desired properties, different alkane feedstocks are chlorinated to a specific degree. CPs are commonly categorized by alkyl chain lengths in three main groups: short-chain chlorinated paraffins (SCCPs, C_{10}- to C_{13}-alkanes, 40-70% chlorine), medium-chain chlorinated paraffins (MCCPs, C_{14}- to C_{17}-alkanes, 30-70% chlorine) and long-chain chlorinated paraffins (LCCPs, $C_{>17}$-alkanes, chlorination degree varies widely). All technical CP products are composed of several thousand congeners with varying degrees of chlorination. Analytically, these mixtures cannot be individually characterised at present, so there is little knowledge about individual chemical structures or whether any configurations predominate.

SCCPs have proven to be persistent [2], bioaccumulative [3–5] and toxic [6–8]. Some of these properties have also been reported for MCCPs [3–6,9–11] and LCCPs [4,5,12], and this data continues to grow, but currently only SCCPs have been regulated as POPs under the Stockholm Convention [13]. However, as SCCPs have declined in production and the manufacturing focus has moved to MCCPs/LCCPs [14], some of which have already shown evidence of PBT characteristics [11], it would be prudent to follow the precautionary principle and include these CPs as well, for surveillance.

The risk assessment of CPs published by the European Food Safety Authority (EFSA) early 2020 noted limitations in the toxicokinetic and toxicological database and limited data on the occurrence of SCCPs and MCCPs in food. A robust exposure assessment, a risk characterisation and consequently a complete risk assessment could not be performed [15]. A broad monitoring of CPs in food and the environment is needed to collect occurrence data for robust exposure estimates to identify any environmental concerns and in order to support regulatory efforts. However, the complexity of CP products engenders an analytical challenge and no general standardised analytical method could be established to date. So far there is only a standardised method for SCCPs drinking water available [16], which has been adapted for the analysis of sediments [17] and leather [18] and currently is in the process of adaptation for SCCPs and MCCPs in textiles [19].

For food and feed samples, a large variety of methods based on different principles and using many instruments has been reported in the literature [20–22]. Although interlaboratory studies showed marked improvement in comparability, still there are difficulties when different quantification methods or instrument types are being used [16,23,24]. These factors make laboratories often very hesitant to start establishing CP analysis.

To improve comparability and to support the exchange of information, the EURL for halogenated POPs (former EURL for Dioxins and PCBs) in Feed and Food has established a Core Working Group (CWG) on CPs in 2016. It quickly realised that one standardised analytical method for food and feed was not feasible given the diversity of methodologies and instrumentation, and focused instead on comparing method performance.

A current aim of the CWG is to compile guidance on a set of analytical parameters that would lead to satisfactory method performance, together with analytical standards and advice on the levels of quantification. This document compiles these parameters as well as an example of a method that provides the required level of performance. It is intended as general guidance for laboratories and particularly for those that do not have an existing method. In contrast to other guidance documents published by the EURL, the uncertainties in CP analysis (as elaborated further over the course of this document) do not support the establishment of validated analytical criteria that are commonly used for other contaminants. Thus, **the main goal of this document is to provide guidance to establish CP analysis in food and feed throughout the European Union as part of the EURL's official mandate and scope of work.**

1. Definition of analytes

Chlorinated paraffins are complex mixtures of several thousand compounds. As such, there is no binding definition or a definite CAS number available (currently, more than a dozen CAS numbers are active for chlorinated paraffins) to characterize the analytes or analyte groups. In fact, CPs currently produced often do not always adhere to the rough labels of SCCPs, MCCPs and LCCPs but are rather characterized by their overall average chlorination degree [25].

EFSA categorized CPs by carbon chain length (SCCPs: C_{10}-C_{13}, MCCPs: C_{14}-C_{17}, LCCPs: C_{18} and higher, [15]), while the Stockholm Convention listing for SCCPs is more specific, as C_{10}-C_{13}, min. 48% Cl [13]. In order to harmonize the analytical efforts and clarify reporting, the following analyte definitions are proposed.

1.1 General terms

Beside the actual analytes or analyte groups, there are also some other frequently used terms with differing definitions. This guidance document follows the IUPAC Gold Book as much as possible, with restrictions relevant to the scope. Stereochemistry is not taken into consideration in this document. Examples and interpretations are provided in **Annex VII**.

1.1.1 Isomer

One of several molecules with the same molecular formula but different line formulae, e.g. CH_3OCH_3 and CH_3CH_2OH, both with molecular formula C_2H_6O.

1.1.2 Congener

One of several molecules related to each other by origin, structure, or function.

1.1.3 Chlorinated paraffin (CP), or CP congener

A CP congener is a molecule of chemical formula $C_nH_{2n+2-x}Cl_x$, with a defined positioning of Cl atom substitutions on the carbon backbone, e.g. 2,3,5,6,9,10-hexachlorododecane.

1.1.4 CP homologue group

One complete group of CP isomers, e.g. the $C_{12}Cl_6$ homologue group contains all CP congeners with the molecular formula $C_{12}H_{20}Cl_6$. Sometimes shortened to “homologue” or called “congener group” in the literature.

1.1.5 CP isotopologues

One of the molecular entities of a CP congener that differ only in isotopic compositions, e.g. $^{12}C_{12}{}^{1}H_{20}{}^{35}Cl_6$, $^{12}C_{12}{}^{1}H_{20}{}^{35}Cl_5{}^{37}Cl$ and $^{12}C_{12}{}^{1}H_{20}{}^{35}Cl_4{}^{37}Cl_2$. They manifest as different peaks in the mass spectrum.

1.1.6 CP isotopomer

One of the molecular entities that, within a CP homologue group, exhibit the same isotopic composition, e.g. 2,3,5,6,9-$^{35}Cl_5$,10-^{37}Cl-dodecane and 2,3,5,9,10-$^{35}Cl_5$,8-^{37}Cl-dodecane.

1.1.7 CP isotopomer group

One entire set of CP isotopomers, e.g. the $^{12}C_{12}{}^{35}Cl_5{}^{37}Cl$ isotopomer group. The mass spectrum of each group corresponds to a single peak.

1.2 CP groups

The following parameters are based on mass spectrometric analysis. The criteria for positive identification of compounds (see **section 7.1**) should be applied to all data as far as possible. If sub groups (e.g. *ΣSCCPs, ΣMCCPs*) cannot be quantified with the method in use, then the corresponding sum parameter (e.g. *ΣCPs*) cannot be reported. Instead, the parameter "total CPs" should be applied.

1.2.1 Sum of SCCPs (ΣSCCPs)

Defined as the sum of SCCPs, calculated as C_{10}-C_{13} based on at least the sum of all corresponding Cl_5-Cl_9 congeners (five most abundant homologue groups for GC and LC instruments, more if possible).

1.2.2 Sum of MCCPs (ΣMCCPs)

Defined as the sum of MCCPs, calculated as C_{14}-C_{17} based on at least the sum of all corresponding Cl_6-Cl_{10} congeners (five most abundant homologue groups for GC and LC instruments, more if possible).

1.2.3 Sum of LCCPs (ΣLCCPs)

Defined as the sum of LCCPs, calculated as C_{18}-C_{36}. Due to the wide range of compounds included in this parameter, no further characterization is given at this time.

1.3 Sum of CPs (ΣCPs, $ΣCPs_{LC}$)

For GC-based methods, this is defined as sum of ΣSCCPs and ΣMCCPs. For LC-based methods, this definition is expanded to include ΣLCCPs. Due to this difference in definition, it is necessary to indicate if LCCPs are included in the sum parameter, e.g. by using an index ($ΣCPs_{LC}$ or alternatively $ΣCPs_{C10\text{-}Cx}$ if not the full range defined for ΣLCCPs has been analysed).

1.4 Chain length specific results ($ΣC_i$, *optional*)

Sum parameter for CPs of a certain carbon chain length. The same minimum homologue groups and criteria for positive compound identification as for the corresponding group parameters apply. Example: $ΣC_{14}$ – sum of all MCCPs with 14 carbon atoms, including at least congeners with six to ten chlorine atoms.

1.5 Total CPs (alternative parameter to sum of CPs)

Concentration of all CPs detected in a sample, without distinction of groups, chain lengths or homologue groups. Depending on the instrument used, this parameter may include LCCPs. Since this is meant as a parameter for semi-quantitative analysis only, no further distinction of the parameter by the instrument or method (e.g. through an index) is necessary. Total CPs can also be determined by non-mass spectrometric or low resolution-MS/MS methods, though appropriate sample preparation is necessary to remove co-eluting organohalogens (see also **section 2.2**).

In those cases, the full resulting peak hump should be integrated for quantification. Chromatographic peak humps caused by CPs are very distinct in their form independent of the GC instrument set-up used and therefore easily differentiated from mineral oil

(MOSH/MOAH) humps or similar signals (example in **Figure 1** and **2**). Typical mass transitions for GC-EI-MS/MS are m/z 102→67, m/z 102→65 and m/z 91→53 [26,27].

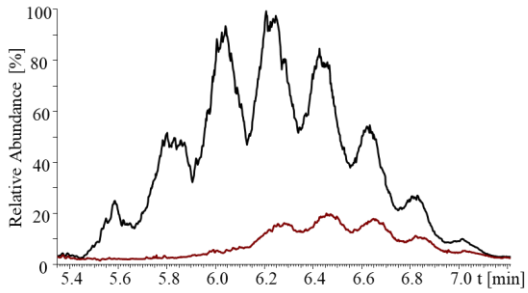


Figure 1. Total ion current chromatogram of a SCCP (black) and MCCP (red) standard obtained by GC-ECNI-HRMS.

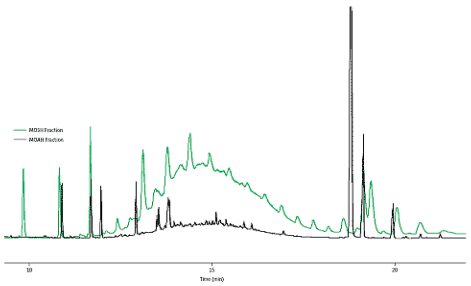


Figure 1. MOSH (green) and MOAH (black) compounds on Rxi-5Sil MS and Hydroguard-treated MXT with Allure Silica (Online LC/GC Coupling). Source: https://www.restek.com/chromatogram/view/GC_FS0500

Depending on choice of instrument and quantification method, the range of possible results includes a qualitative evaluation (CPs are found/not found), an estimation of total CP contamination levels (low/medium/high contamination) and a quantitative result for all CPs included in this analysis. For more information on the different types of results and how to report them, please refer to section 6 of this document (Reporting).

All of the definitions above should be applied as far as signals have been detected and could be quantified using the chosen quantification method. In general, results should only be reported alongside information on the method, instrument and possible restrictions used during quantification.

2. Sample preparation

2.1 Pre-cleaning and decontamination of laboratory glassware and equipment

Since CPs are currently in use in a variety of applications including as plasticisers in soft polymers, seals or computer components. It is vital to check the laboratory background and blank levels before sample preparation and analysis begins. These checks should be repeated during each sample batch even once the method is established, as blank levels might change over time (see also **section 7.3**). Solvents, reagents and particularly, automated systems should be checked during method development to ensure that the background is not significant. It is helpful to replace most plastic components in the laboratories with either Teflon or metal alternatives and pre-rinse all glassware.

Figure 2. Storage of glassware sealed with aluminium foil to keep out dust.

Especially freeze-drying has been shown to introduce large amounts of S/MCCPs (old instruments) or even LCCPs (especially new instruments) to the samples (Figure 4). Persisting contamination issues might also be resolved by heating and pre-extracting all chemicals and filters before use and checking the solvents directly for impurities. In general, pesticide-grade or analysis-grade solvents have proven to be mostly CP free.

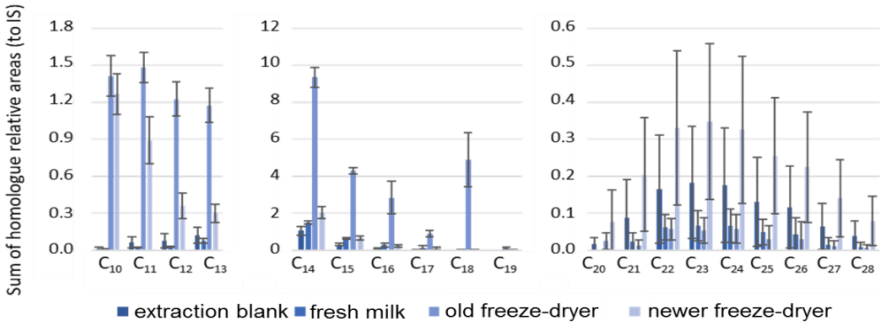


Figure 3: CP homologue groups summed by carbon chain length found in milk without drying, drying in an old freeze dryer and drying in a recently bought freeze dryer. Data and graphic courtesy of Marie Mézière, LABERCA-Oniris, France.

2.2 Possible extraction and sample clean-up methods

Reagents and equipment that are used for CP analysis are similar to those used for PCB and PCDD/F analysis and are commercially available. Methods of preparation for food or feed samples are described similarly in the literature. Basic schematics of the procedures used by participants of the first and second round of the EURL interlaboratory study on CPs [23] are given in Figure 5. Analysis of these schematics shows that the combination of sample preparation modules is not considered to be a major influencing factor for CP analysis.

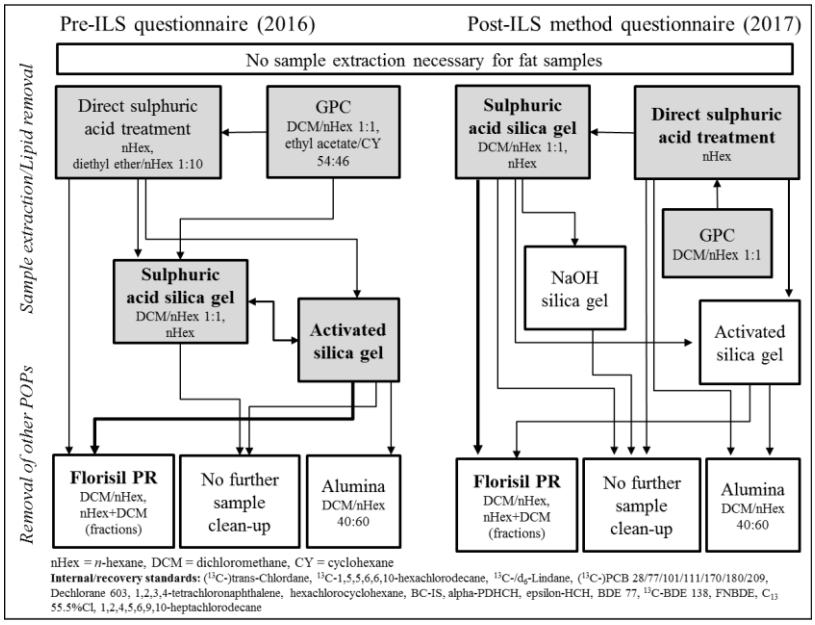


Figure 4. Sample preparation and clean-up methods reported by participants of EURL interlaboratory studies of CPs. Figure taken from Krätschmer and Schächtele, 2019.

Sample extraction can also be done in a similar way to PCDD/F and PCB analysis using heated solvents (e.g. Soxhlet or Twisselmann extraction), pressurized liquid extraction (PLE or ASE, several solvent combinations) or cold extraction (e.g. dichloromethane/n-hexane 1:1 v/v). An overview of reported sample extraction strategies is given in Annex I, with a full example method for (completely manual) sample preparation in Annex II.

3. General categorisation of instruments

3.1 Gas chromatography, liquid chromatography, direct introduction into the ion source

All analytical methods described in this document either separate analytes in a gaseous or liquid phase or offer no separation at all, as the analyte is directly introduced into the ion source. Depending on the choice of separation method, the resulting CP peaks will show very different characteristics:

Gas chromatography leads to a hump signal which is often further characterized by multiple noise-like shoulders, as is shown in Figure 6. Although the presence of other analytes or matrix signals might conceal the hump in a TIC, the extracted mass traces should reveal this or a similar shape. Even chain-length specific mixture standards or extracted homologue group mass traces will show at least two, more often three of these substructures. Depending on origin or sample matrix, the ratio between the sub-structures may vary; nevertheless, they will always be present and clearly defined (Figure 6). Each peak/shoulder does not represent a homologue or chain length group so separation by segmenting the TIC based on retention time only is not feasible.

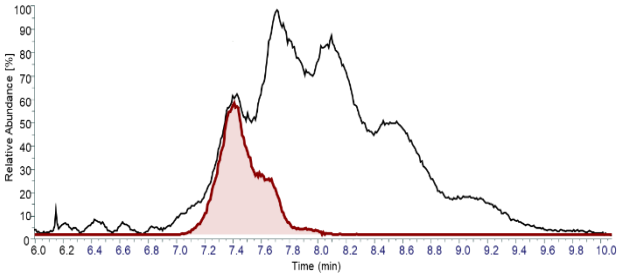


Figure 5: GC chromatogram of a mixture of SCCPs and MCCPs. Overlaid and filled in red is a corresponding extracted homologue peak (here: $C_{16}H_{27}Cl_7$).

Liquid chromatography does not yield hump shapes similar to those observed in GC, but rather comparatively smoother humps similar to those seen obtained from MOSH/MOAH analysis (Figure 2, Figure 7A). These types of shape do not always translate down into single chain length CP standards and extracted mass traces of homologue groups and appear, depending on signal smoothing, similar to GC chromatograms instead (Figure 7B).

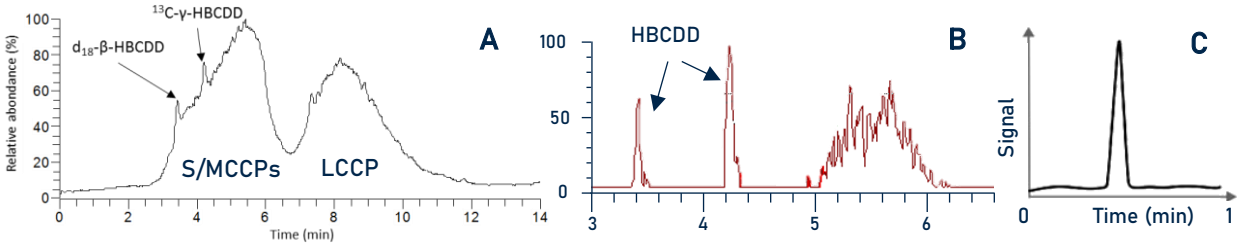


Figure 6: (A) LC chromatogram of a mixture of SCCPs, MCCPs and LCCPs. The latter appears as a second, separate hump with the gradient used in this method. No substructures are observed here. (B) The same shape is found in extracted homologue group chromatograms (here: $C_{11}H_{11}Cl_{13}$). Figures kindly provided by M. Mézière, LABERCA, France. (C) Direct injection APCI-QTOF-HRMS chronogram of CPs. This method does not allow for separation, so that all sample material enters the ion source at the same time. Taken from Bogdal, Alsberg et al. 2015 [37].

In contrast, **direct introduction** of sample material into the ion source of an LC-based system does not provide any chromatographic separation but rather a chronogram with a single peak indicating time of injection (Figure 7C). As there is no separation, analysis time is reduced to less than a minute. However, as there is no retention window, an important factor for correctly

identifying homologue groups in the observed data is not applicable. Still, all LC-based methods offer increased selectivity in the presence of several other halogenated POPs, which is helpful especially in samples with higher overall POPs contamination (e.g. fish samples). Additionally, only LC-based systems are able to detect and quantify LCCPs with chain lengths above C_{20}, which are less volatile and therefore not suitable for analysis in GC-based systems.

3.2 Semi-quantitative analysis

The goal of semi-quantitative analysis is determination of the general CP contamination level, i.e. total CP content of a sample. Mass spectrometric methods are not absolutely necessary to achieve this goal.

Electron capture detection (ECD), usually used with 1-30 m 100% Dimethylpolysiloxane (DB-1 or DM-1) columns, provides gas chromatograms that only show the CP hump. If used with a GC×GC set-up, ECD results can also give further information on CP groups and even homologue groups [28–30].

Another option would be **electron ionisation mass spectrometry ((GC-)EI-MS)**. Literature shows the use of (5%-Phenyl)-methylpolysiloxane (DB-5) columns of 15-60 m length and source electron energy of 70 eV. This comparatively hard ionisation method leads to strong fragmentation, which prevents easy access to further homologue group or pattern information. On the other hand, GC-EI-LRMS or MS/MS is often used in other routine analyses and therefore available in most laboratories. Electron ionisation coupled with high resolution MS on the other hand is able to quantify CP groups or even homologue groups [26,31,32]. An example instrument method can be found in **Annex III**.

3.3 Quantitative analysis of CP groups (chain length specific patterns)

In order to quantify SC-, MC- and LCCP groups separately, mass spectrometric detection with softer ionisation techniques is required. The most commonly used instrumentation for this analysis is GC (15-30 m DB-5) with **negative chemical ionisation/electron capture negative ion low resolution MS (NCI-LRMS, ECNI-LRMS)**. [25, 28–35]. In all cases, the most abundant [M-Cl]- signals are used for quantification of CP groups based on homologue group-specific masses. To cover all chosen mass signals, often several GC runs are necessary for the same sample. Methane is most commonly used as reaction/moderating gas but ammonia or argon can also be used. The temperature of the ion source can also be varied and LRMS can also be exchanged for high(er) resolution mass spectrometers. Recently, McGrath et al. [33] found evidence of strong matrix enhancement for CPs which also needs to be accounted for during quantification, i.e. through use of a suitable recovery standards (see **section 4.2**).

Atmospheric pressure chemical ionisation (APCI) with time-of-flight (TOF) MS detection is another soft ionisation technique. Direct injection without chromatographic separation [36–38] leads to one compressed signal with an analysis time of 1-2 min. Full scan mode allows post-run examination of all CPs by extracting specific data. Compared to ECNI-MS, fewer false positive results are expected. As for all LC-based methods, the addition of dichloromethane to the eluent before ionisation shifts the formation of fragment ions towards $[M+Cl]^-$, which is

supposed to show fewer interferences than $[M\text{-}Cl]^-$ CP ions and increases overall analyte responses through the higher chlorination degree of the ions. Special quantification methods and data treatment strategies are needed to process the raw data of the direct injection method.

More recently, **liquid chromatography with electrospray ionisation (LC-ESI-MS)**, sometimes with DCM enhancement, was also introduced for CP analysis [39,40]. Depending on available quantification standards and the resolution of the chosen mass spectrometer, quantification by chain lengths and possibly also determination of information on homologue group patterns is possible. Example instrument methods for LC-ESI-QTOF-MS and GC-ECNI-LRMS(/MS) can be found in **Annex III**.

3.4 Quantitative analysis of CP groups (homologue group patterns)

Attaining additional information on homologue group patterns needs high (R>20,000 FWHM) or even very high (R>100,000 FWHM) mass resolution, often in the form of Orbitrap, Iontrap, Fourier Transformation Ion Cyclotron Resonance (FT-ICR) or the newer TOF mass spectrometers. Due to the available configurations of these mass detectors, gas chromatography, liquid chromatography and direct injection of the sample into the ion source are possible.

Generally, the literature describes homologue group patterns derived from soft ionisation techniques **(ECNI/NCI, APCI, ESI)** as smaller fragment ions do not allow specific enough conclusions on the corresponding homologue group to be of use [34–38]. Example instrument methods for LC-HESI- HRMS and GC-ECNI-HRMS can be found in **Annex III**.

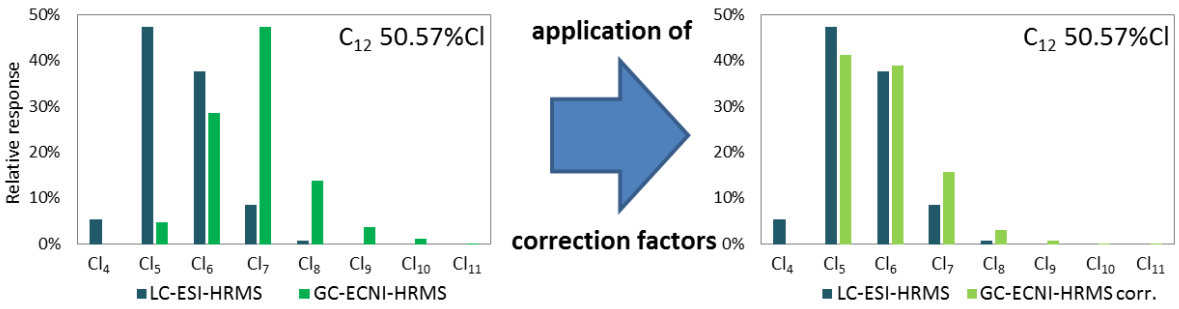


Figure 7: Effect of correction factors on GC-ECNI-HRMS response patterns compared to LC-ESI-HRMS response patterns.

Based on currently available methods, homologue group patterns have been found to have little comparability between instruments and quantification strategies [23,39]. Especially for ECNI-based results, the use of correction factors has been proposed to increase comparability [39]. Such corrections have the strongest effect on low chlorinated CP patterns, as ECNI-MS has a particularly low sensitivity to these and consequently overestimates higher chlorinated homologue groups in the pattern instead (**Figure 8**). A list of correction factors can be found in **Annex IV**.

3.5 Comparison of methods

Table 1:Summary of the methods presented in the previous sections with advantages and disadvantages.

Method	Quan level	Advantages	Disadvantages
GC-FID	total CP	• low chlorinated CPs detectable	• no information about congener pattern attainable • no specificity
GC-ECD	total CP	• low chlorinated CPs detectable • quick determination of total CP	• GCxGC needed for qualitative identification of CPs, otherwise only one “hump”
GC-EI-MS	MS/MS: total CP HRMS: ΣSCCPs, ΣMCCPs, ΣCPs; ΣC_i	• response is influenced neither by chlorination degree nor by the chain length • equipment available in most dioxin/ PCB laboratories	• high degree of fragmentation • no information about homologue group pattern attainable
GC-ECNI-MS	ΣSCCPs, ΣMCCPs, ΣCPs, ΣC_i HRMS: homologue group patterns	• equipment available in most dioxin/ PCB laboratories • most commonly used in publications → comparable results	• interferences with other organo halogenated compounds with same retention time (e.g. PCBs) • interferences within CP homologue groups (overlapping fragment ions and molecule ions; not with more expensive HRMS) • problems with low chlorinated CPs
direct injection APCI-MS	ΣSCCPs, ΣMCCPs, ΣLCCPs; ΣCP_{LC}, ΣC_i HRMS: homologue group patterns	• no column necessary (run time < 1min) • simultaneous quantification of MCCPs and LCCPs possible • less false positive results due to freak ions low interferences by DCM addition to eluent	• expensive equipment • complex quantification • authentic quantification standards necessary
LC-ESI-MS	ΣSCCPs, ΣMCCPs, ΣLCCPs; ΣCPs_{LC}, ΣC_i HRMS: homologue group patterns	• LCCPs detectable • low chlorinated CPs detectable • less false positive results due to freak ions low interferences by DCM addition to eluent	• expensive equipment • complex quantification • authentic quantification standards necessary • problems with high chlorinated CPs

As additional information to help choose an instrument, the next table shows the results of interlaboratory studies on CPs (in fortified fat samples) organised by the EURL sorted by instrument. Please keep in mind that although the instruments might have been similar, the rest of the methods might notably differ between laboratories!

Table 2: Interlaboratory study results given as z-scores, sorted by instrument set-up (x = no participation, n = number of z-scores, median z-score is given in brackets).

method	z-scores total CP	z-scores ΣCPs/ΣCP$_{LC}$	z-scores ΣSCCPs	z-scores ΣMCCPs
GC-FID	x	x	x	x
GC-ECD	-4.0 to -3.7 (-3.9) n=4	x	x	x
GC-EI-MS	MS/MS: -1.2 to 1.1 (-0.1) n=14	HRMS: -2.6 to -0.2 (-1.2) n=8	HRMS: -2.1 to -0.4 (-1.5) n=7	HRMS: -3.4 to -0.5 (-2.8) n=6
GC-ECNI-MS	x	LRMS: -2.5 to 5.0 (-1.3) n=31 HRMS: -1.5 to 2.5 (0.4) n=16	LRMS: -3.6 to 2.9 (-1.7) n=29 HRMS: -2.0 to 1.2 (-0.1) n=15	LRMS: -2.6 to 5.0 (-1.5) n=21 HRMS: -1.4 to 3.9 (1.3) n=16
direct injection APCI-MS	x	-4.0 to 2.5 (-0.4) n=17	-4.0. to 1.8 (-0.9) n=15	0.4 to 1.5 (1.1) n=3
LC-ESI-MS	x	-0.6 to 1.3 (0.2) n=8	-2.4 to 5.0 (0.6) n=12	-1.0 to 1.5 (-0.2) n=8

4. Choice of standards

4.1 Quantification standards

In terms of correctly quantifying SCCPs in the presence of MCCPs, no final conclusions concerning the choice of standards alone could be made during on the basis of results from the interlaboratory studies, although the very relatively good results for SCCPs achieved with single chain standards in combination with linear regression should be mentioned. In general, the need for more single chain standards (or any commercially available single chain standards in case of MCCPs) for more accurate quantification of MCCPs has become apparent. Due to the currently fast changing market on CP standards and the different requirements of each quantification method, no definitive recommendation for quantification standards can be given here. Instead, some general remarks are supposed to help choosing an appropriate set of standards:

- **A wide range of chlorination degrees and standards** appears to provide better results. Quantification based only on one single chain standard or one commercial technical mixture could not objectively reflect CP composition in the sample and is not recommended.

- **(Technical) mixture standards** can be used for ΣSCCPs, ΣMCCPs, ΣLCCPs or total CP analysis. Additional information on chain length specific concentrations would be helpful as some commercially available LCCP standards also contain MCCPs and in some cases even SCCPs, making ΣLCCPs results calculated based on their total response unreliable [40].

- **Multiple single-chain CP mixture standards** can be used for all quantification methods, allowing for a metrological retraceable quantification on chain length level (lowest comparable quantification level to date). SCCP single chain length standards as well as C_{14} and C_{16} MCCP single chain standards are commercially available from LGC/Dr. Ehrenstorfer, a full set of MCCP single chain length standards is available upon request from the EURL for method development purposes.

- A number of **configurationally defined individual CP congener standards** are available from Chiron (Trondheim, Norway) but the evidence suggests that at least the older ones are unsuitable without major adjustments of the methods. Apart from individual research requirements, these are not currently recommended. More suitable standards are currently in development as part of the Eurostars "Chloffin" project (www.chloffin.eu).

4.2 Recovery and syringe (injection) standards

***Recovery standard**: A standard that is added to the sample during the first step of preparation to account for losses during sample preparation, but not measurement. Often an isotope labelled compound for easy identification during analysis.*

***Syringe (injection) standard**: A standard that is added at the very end of sample preparation, immediately before instrumental analysis. Does not have to be structurally similar to the analyte, as it accounts for losses caused by problems with injection or other steps of instrumental analysis/measurement.*

The choice of recovery and syringe standards in publications also varied greatly, including isotope-labelled and unlabelled pesticides, PCBs, PBDEs and ^{13}C-1,5,5,6,6,10-hexachlorodecane (Dr. Ehrenstorfer, Augsburg, Germany). The latter is one of the very few available labelled single compound CP standards and therefore considered suitable for recovery control for GC methods. However, this standard is only barely or not at all detectable using LC-ESI-MS or LC-APCI-MS; here, a suitable CP alternative has yet to be produced. Notably, many participants in the EURL interlaboratory study scheme reported the use of no recovery or syringe standards.

No correlation between choice of recovery/syringe standard (or lack thereof) and z-scores achieved in interlaboratory exercises could be found. Schinkel *et al* [41] proposed $^{13}C_{15}H_{26}Cl_6$ as compromise for GC and LC users; as MCCP it should not only reflect behaviour of SCCPs but also cover MCCPs and shorter LCCPs. This compound is also assumed to be better detectable in LC-MS set-ups. Currently, this compound is not yet available as standard. Until such a standard is available, some minimum requirements for recovery standards are suggested below:

- the standard should be isotope labelled (e.g. ^{13}C, deuterated,…)
- as CPs are assumed to be mostly linear, the standard should also be based on a linear alkane

- alternatively, the standard should at least be of aliphatic nature in order to ensure properties similar to CPs during clean-up/sample preparation
- the standard should ideally be chlorinated to ensure similar behaviour to the analytes during sample preparation
- based on recent findings, the use of 6-MeO-BDE-157 as injection standard might account for matrix effects in GC systems; if that is also applicable for LC systems is currently unknown

Further information on currently available commercial standards, their advantages and disadvantages, and connected analytical conundrums when trying to quantify CPs can be found in a separate peer-reviewed publication written by members of this Core Working Group [42].

5. Quantification methods

In recent years, a general trend towards the use of high resolution mass spectrometers (HRMS), interfaced to gas chromatography (GC) [37,43,44] or high performance liquid chromatography (LC) [36,45,46] and increasingly complex data treatment regimens have been used.

The quantification methods presented here are suitable for use with HRMS instruments but can - with the exception of Gaussian and Barycentre method - also be adapted for use with other MS techniques. The methods shown in **Figure 9** were tested on three different instrument set-ups (GC-ECNI-HRMS, LC-ESI-HRMS and LC-ESI-TOF-MS) and were found to be comparable both to each other and to spiked amounts in fat samples. All methods presented have been applied to food samples and successfully participated in interlaboratory exercises.

Recently, a simplified quantification method for screening purposes has been presented in the literature [47]. However, such simplified methods carry a greater risk for under- or overestimation of results and are therefore currently still under investigation by the CWG CP.

5.1 Linear calibration corrected for chlorination degree

This method was primarily developed to account for GC-ECNI-MS and original linear calibration's dependency on the chlorination degree of the sample or standard analysed. The method first described by Reth *et al.,* (2005) [48] derives the total response factors and calculated chlorine contents for a set of CP mixture standards using the GC-ECNI-MS response of each analysed congener group divided by an internal standard.

A linear regression is typically then fit to the relationship between these parameters, although some laboratories have reported improved results using an exponential curve [33,49]. ∑SCCP or ∑MCCP concentrations in samples are determined by calculating the chlorine content in samples and deriving the corresponding total response factor from the calibration equation.

$$total\ response\ factor\ (sample) = ax + b \qquad \text{(Eq. 1)}$$

Where *a* is the slope of the linear regression, *x* the chlorine content calculated from ECNI analysis and *b* the axis intercept. The CP amount of the sample is then directly calculated as division of the summed relative total peak area from each of the analysed homologue groups by the calculated total response factor of that sample [31,50] (**Equation 2**).

$$CP\ amount\ (sample) = \frac{relative\ total\ area\ (sample)}{total\ response\ factor\ (calculated\ for\ the\ sample)} \quad \text{(Eq. 2)}$$

This method was also adapted in a modified version for the ISO method on CP analysis in drinking water, which uses multiple linear regression of the sum of SCCPs and peak areas of two distinct CP masses [51], although no applications of the ISO method to food or feed matrices have been reported.

Instrumental set-up needed: at least GC-ECNI-LRMS
Minimum requirements for standards: all standards applicable
Type of possible results: ΣSCCPs, ΣMCCPs, ΣCPs, ΣC_i

5.2 Gaussian curve method

The Gaussian curve method of Yuan *et al.* was published in 2017 [52] and has already been used for occurrence studies in food [34,53]. It was slightly modified for the purpose of integrating MCCPs [34]. In brief, the base assumption of the underlying mathematical model is a Gaussian distribution of the abundance of homologue groups within the same carbon chain length.

For a considered chain length, the homologue group-specific relative response factors (RRFs) are derived from observed responses of several single-chain CP standards of varying chlorination degrees after modulation in a way that matches as closely as possible the observed and theoretical Gaussian distributions of all standards **(Equations 3 and 4)**. An Excel sheet for calculating these RRFs is available from the EURL upon request.

$$f_i(C_nCl_m|x_m,\ \sigma_i) = \frac{1}{\sigma_i\sqrt{2\pi}} * e^{-\frac{(x_m - \%Cl_i)^2}{2{\sigma_i}^2}} \quad \text{(Eq. 3)}$$

$$RRF_i(C_nCl_m) = \frac{relative\ area_i(C_nCl_m)}{concentration_i(C_n) * \frac{f_i(C_nCl_m|x_m,\sigma_i)}{\sum_1^m f_i(C_nCl_m|x_m,\sigma_i)}} \quad \text{(Eq. 4)}$$

In the modified method, the calculated RRFs are not directly used for quantification but rather as a base to calculate theoretical concentrations of individual homologue groups for a set of calibration mixtures. Quantification itself is then done as a simple linear calibration with a mixed standard, which accounts for daily changes in analysis conditions by including calibration solutions in every measured batch. In general, the use of RRFs instead of RFs is recommended to correct for these daily changes, even if the calibration is not repeated with each batch.

Instrumental set-up needed: HRMS (detection of homologue groups)
Minimum requirements for standards: single chain length standards
Type of possible results: ΣSCCPs, ΣMCCPs, ΣCPs, ΣC_i, homologue group patterns

5.3 Barycentre method

The Barycentre method has only recently been reported for the first time [39]. It is derived from the method of Reth *et al.* [48] that calculates a standard mixture RRF depending on its total chlorination degree (%Cl). Here, instead of using a total chlorination degree of a standard, a "barycentre" is calculated for each single-chain standard, which is the mean number of chlorine per chain length (**Equation 5**):

$$n_{Cl} = \%Cl * \frac{n_C * m_C + (2 * n_C + 2) * m_H}{(1 - \%Cl) * m_{Cl} + \%Cl * m_H} \quad \text{(Eq. 5)}$$

with $\%Cl$ the provided chlorination degree of the standard, m_H, m_C and m_{Cl} the molar masses of the atoms, and n_C is the carbon chain length. A linear correlation between the single-chain standards' barycentres and RRFs is obtained for each chain length. Interpolation and extrapolation enable the attribution of RRFs for each SCCP and MCCP homologue. Linearity within the dynamic range is usually not controlled.

Instrumental set-up needed: HRMS (detection of homologue groups)
Minimum requirements for standards: single chain length standards
Type of possible results: ΣSCCPs, ΣMCCPs, ΣLCCPs, ΣCPs, ΣC_i, homologue group patterns

5.4 Average chain length concentration

A comparatively simple quantification strategy that focuses on the basic principles of validated analysis: generally, only values generated with standards that are metrological retraceable to pure substances are considered valid. Based on this principle, currently available CP standards allow in most cases only quantification on chain length level. Therefore, this method equalled all responses gained for homologue ions to the known sum concentration of the single-chain length standard in question.

$$c_{chain} = \left(\sum_{n_{Cl}=5}^{n} \frac{area_{homologue}}{RRF_{chain}}\right) / \mathrm{n} \quad \text{(Eq. 6)}$$

The average of all sum concentrations gained this way is then considered the quantification result for the corresponding chain length. As responses of the homologue groups with very high or very low chlorination are less robust because of instrumental lack of sensitivity, only homologue groups with Cl_5-Cl_8 are considered.

Instrumental set-up needed: at least GC-ECNI-LRMS
Minimum requirements for standards: technical mixture standards, single chain length standards
Type of possible results: ΣSCCPs, ΣMCCPs, ΣLCCPs, ΣCPs (estimation through total concentration: technical mixture standards), ΣC_i (needs single chain length standards)

5.5 Deconvolution method

The deconvolution method of Bogdal *et al*. [38] on technical CP mixtures is already well-integrated in the CPs analysis community [54,55]. It was recently adapted from technical mixture to single-chain length standards by Pērkons *et al.* [46]. Briefly, single-chain CP mixtures are injected within the same sequence as the sample extracts.

$$\begin{pmatrix} y_{1,1} & y_{2,1} \\ \vdots & \vdots \\ y_{1,n} & y_{2,n} \end{pmatrix} * \begin{pmatrix} x_1 \\ x_2 \end{pmatrix} = \begin{pmatrix} s_1 \\ \vdots \\ s_n \end{pmatrix} \quad \text{(Eq. 7)}$$

Each chain length profile of the samples *s* is then matched with a combination of two single-chain standards of different %Cl (y_1 and y_2), e.g. using the *R* programming environment. Then, the quantification of each chain length is performed using the corresponding combination of RRFs, assuming that the response factor is independent of the concentration.

Instrumental set-up needed: HRMS (detection of homologue groups)
Minimum requirements for standards: all standards applicable (wide range needed)
Type of possible results: ΣSCCPs, ΣMCCPs, ΣLCCPs, ΣCPs, ΣC_i, homologue group patterns

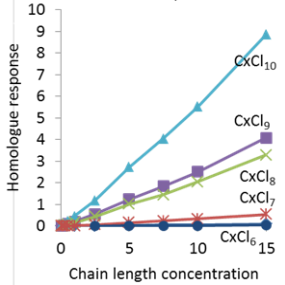
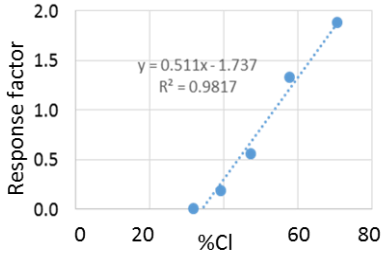
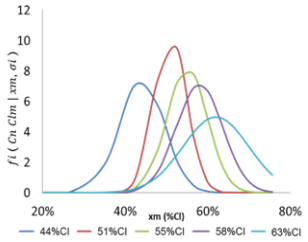
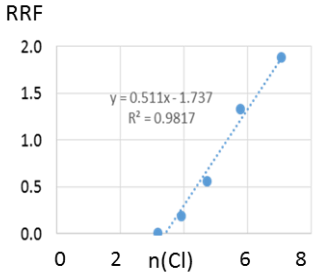
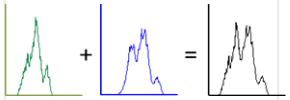
	Average mixture concentration	Linear calibration corrected for chlorination degree	Gaussian curve model	Barycentre	Deconvolution of spectra
Quan. level	ΣCP, ΣC_n	ΣCP, ΣC_n	ΣC_nCl_x	ΣC_nCl_x	Chain length
Principle	Average of the concentrations found for each of the homologue responses	RFs calculated from linear correlation between $\%Cl$ and RF of standards	RRFs calculated from Gaussian distribution assumption	RRFs calculated from linear correlation between $\%Cl$ and RRF of standards	Concentrations calculated from linear combination of standards
Model descrption	$c_{mix} = \left(\sum_i^n \frac{\text{area}_i(homologue)}{\text{RRF}_{mix}}\right) / n$ 	$c(spl) = \frac{\sum_i \frac{area_i(homo,\ spl)}{area_i(ISTD,\ spl)}}{a * \sum_i \frac{rel.\,area_i(homo,spl) * \%Cl_i(homo,spl)}{relative\ total\ CP\ area\ (spl)}} + b)$ 	$f_i(C_nCl_m \mid x_m, \sigma_i) = \frac{1}{\sigma_i\sqrt{2\pi}} * e^{-\frac{(x_m - \%Cl_i)^2}{2{\sigma_i}^2}}$ 	$n_{Cl} = \frac{\%Cl\ * (2m_H * n_C) + 2m_H + m_C * n_C}{m_{Cl} * (1 - \%Cl) + \%Cl * m_H}$ 	$\begin{pmatrix} y_{1,1} & y_{2,1} \\ \vdots & \vdots \\ y_{1,n} & y_{2,n} \end{pmatrix} * \begin{pmatrix} x_1 \\ x_2 \end{pmatrix} = \begin{pmatrix} spl_1 \\ \vdots \\ spl_n \end{pmatrix}$ 
Weakness	• Worked only for Cl_5-Cl_8 homologues (esp. GC) • Dependent on Cl-degree of quantification standard	• Linear regression does not always describe the correlation • Works only for sum concentrations, dependent on available standards	• Non-Gaussian data • Not enough chain length standards available • Atypical behaviour of fringe homologues	• High Cl homologue responses overestimated • Not enough chain length standards	• Dependence on fit of standards/technical mixtures • Not enough chain length standards available
Instrumental set-up	• At least GC-ECNI-LRMS • Can work for GC-EI-HRMS/MS	• At least GC-ECNI-LRMS • Can work for GC-EI-HRMS/MS	• HRMS (must be able to detect homologues)	• HRMS (must be able to detect homologues)	• HRMS (must be able to detect homologues)
standards	• Technical mixture standards • Single chain length standards	• All standards applicable	• Single chain length standards of different chlorination degrees	• Single chain length standards of different chlorination degrees	• All standards applicable • Technical products • Wide range needed

Figure 8: Summary of four possible quantification methods. Figure modified based on Meziere et al, 2020 [39].

6. Reporting

Results should be reported in ng/g wet weight and ng/g lipid, with additional report of the sample fat content and, if available, average CP chlorine content. In all cases, results should be given with two significant digits, rounded using the rules in section B.2 of ISO 80000-1:2009 (e.g. 150, 15, 1.5 or 0.15 ng/g lipid). Reports should also include:

1) A brief description of the analytical method used
2) Specification of the analytical instrument and data treatment strategy, including source parameters, mobile phase and modifier (LC only), type of ions included for quantification, mass resolution and tolerance (MS) and method-specific parameters like goodness of fit R^2 (deconvolution method)
3) (Method) limit of quantification and how it was determined
4) *Optionally, a comment regarding a check for matrix effects for the specific matrices reported*
5) *Optionally, expanded measurement uncertainty and how it was determined*

When homologue group patterns are reported, appropriate correction factors [39] shall be applied to improve comparability between instruments. Additionally, a brief description of how response patterns were obtained shall be given as part of the data treatment strategy specifications. Analytes should only be reported as specific as they have been validated for the quantification method in use (e.g. GC-ECD can only report total CP, GC-ECNI-MS can report ΣSCCPs and ΣMCCPs, but ΣC_i only after validation; LC-based methods report ΣCPs_{LC} instead of ΣCPs). For the purpose of comparing results it is absolutely important to discern between ΣCPs/ ΣCPs_{LC} and total CP amount.

7. Quality control and quality assurance

7.1 Identification and quantification criteria

Due to the large uncertainties and marked differences in experience between laboratories in the European Union at the moment, CWG CP has decided to add identification and quantification criteria at a later point in time in a separate annex to this document.

7.2 LOD/LOQ

For the different methods of establishing a method LOD or LOQ, please refer to the Joint Guidance Document on LOD and LOQ determination of the four Contaminant EURLs. The applicable criteria for the different methods are to be chosen from **section 7.1** or corresponding annexes of the present document as soon as they are available.

7.3 Measurement uncertainty

Measurement Uncertainty for CPs can currently only be estimated empirically.
The evidence from five interlaboratory studies suggests that a limit of ±50% from the assigned value may be satisfactory. Although improvements in comparability should always be the goal, a general measurement uncertainty of ±50% seems more prudent at this time. However, this

value will be reviewed based on analytical improvements and cumulative data from further interlaboratory exercises.

7.4 Continuous QC measures

To ensure a stable level of analytical results, certain continuous quality control or quality assurance measure should be followed:

- regular participation in interlaboratory study or proficiency test schemes (EURL, Quasimeme,…)
- regular check of blank levels (ideally with each sample batch)
- use of PT/ILS material as QC samples, ideally with each batch
- once available, use of certified reference material (CRM)
- especially in the case of LC-based methods, new matrices should be checked for ion suppression or enhancement effects, e.g. by using standard addition.

As long as the certification campaign to gain a CP CRM has not concluded, the use of ILS material or other internal QC material is recommended to check all stages of sample preparation and analysis (one QC sample in each sample batch).

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