QUANTIFICATION STANDARDS – THE ACHILLES HEEL OF CHLORINATED PARAFFIN DETERMINATION?

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Introduction

Chlorinated paraffins (CPs) are a very complex class of flame retardants, plasticizers and high-pressure lubricants that are commonly divided into groups by the length of their carbon chain or their overall chlorination degree. Main groups are short-chain chlorinated paraffins (SCCPs, C_{10}-C_{13}, 40-70% chlorine), medium-chain chlorinated paraffins (MCCPs, C_{14}-C_{17}, 30-70% chlorine) and long-chain chlorinated paraffins (LCCPs, $C_{>17}$, chlorination degree varies widely), with each group spanning several thousand compounds. Currently, the most commonly used commercially available reference standards are SCCP, MCCP or LCCP mixtures. These are only characterized by their overall chlorination degree and known to show impurities of other CP groups[1]. As recent interlaboratory studies have shown the choice of quantification standards to be among the major influencing factors of CP analysis[2], the present study compared the mixed standards most commonly used for quantification of different production batches to investigate differences and their possible influence on quantification results. Thus, need for better characterizable standards became apparent.[3]

Here we use electron capture negative ion high-resolution Orbitrap mass spectrometry (GC-ECNI-Orbitrap-HRMS)[6] in order to evaluate existing problems in CP analysis. For this purpose, 28 single chain length standards for SCCPs and MCCPs were investigated. Due to the wide variety of chlorination degrees available for every chain length it was possible to investigate the influence of overall chlorination degree, but also of different degrees of mixing two or more chain lengths together on response and linearity. Finally, the individual design of matrix-specific mixed standards is presented as an opportunity to improve low resolution mass spectrometry (LRMS) analysis.

Materials and methods

Standards and chemicals. Twenty-eight single chain CP standards (C_{10}-C_{17}, three or four degrees of chlorination per chain length) were synthesized at University of Hohenheim, Stuttgart, Germany using a method described elsewhere[3] and received as pure substance. Solutions of 100 ng/µL CP standard in cyclohexane (analysis grade, LGC standards, Wesel, Germany) were used to determine the chlorination degree independent response factors according to Yuan et al[4] and their homologue pattern. Experiments on linear range were conducted using solutions in cyclohexane with concentrations of 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5, 10, 15, 20, 30 and 40 ng/µL CP standard. A 0.05 ng/µL ε-HCH solution in cyclohexane derived from Dr. Ehrenstorfer, Augsburg, Germany, was used as additional injection standard.

Mixed SCCP, MCCP and LCCP standards originally purchased from Dr. Ehrenstorfer (Augsburg, Germany) with expiry dates between 2003 and 2021 were collected from laboratories across the European Union and analysed as 10 ng/µL solutions in cyclohexane.

Instrumental set-up and data processing. The instrumental set-up has been described in detail elsewhere[5,6]. In brief, a GC-ECNI-Orbitrap-HRMS instrument (Thermo Scientific, Waltham, MA, USA) was used at 120,000 mass resolution (FWHM, measured at *m/z* 200) in a full scan range spanning *m/z* 250-810. The processing method used accurate masses for the three most abundant isotopes of the [M-Cl]⁻ and [M-HCl]⁻ ions of each homologue to extract chromatograms. The GC-EI-MS/MS instrument (Agilent Technologies, Santa Clara, CA, USA) was used to monitor three different mass transitions, using the average concentration as result.

QAQC. All measurements were carried out in duplicate. Ions were considered positively identified when retention time, accurate mass and ion ratios of at least two isotopes matched the compound database. Fluctuations due to injection or different tuning were corrected by using ε-HCH as injection standard. If possible, all measurements were carried out using a freshly cleaned ion source and a new tune and mass calibration to ensure the system working at optimal conditions.

Results and discussion

Single chain length CP standards

Initial standard analyses via GC-ECNI-Orbitrap-HRMS revealed a difference between theoretical and calculated overall chlorination degree (Table 1). The highest deviations were always observed for the lowest chlorinated standards, hinting at a general difference caused by the ionisation technique chosen for this study. In this regard, comparisons with results from other instruments could be of interest.

Table 1: Characterisation of 28 single chain length standards by theoretical chlorine content (determined by elemental analysis) and calculated overall chlorination degree (based on GC-ECNI-HRMS measurements).

Chain length	Chlorine content SCCPs			Chain length	Chlorine content MCCPs		
	theoretical	calculated	difference		theoretical	calculated	difference
C_{10}	48.5%	31.9%	-16.6%	C_{14}	43.7%	53.5%	+9.80 %
C_{10}	50.2%	49.5%	-0.70%	C_{14}	51.4%	55.8 %	+4.36 %
C_{10}	56.7%	55.6%	-1.07%	C_{14}	59.4%	61.6 %	+2.20 %
C_{10}	61.0%	64.5%	+3.45%	C_{14}	63.0%	55.1 %	-7.82 %
C_{11}	47.7%	60.9%	+13.2%	C_{15}	39.5%	50.2 %	+10.7 %
C_{11}	52.2%	58.0%	+5.80%	C_{15}	51.0%	56.9 %	+5.91 %
C_{11}	59.0%	63.1%	+4.09%	C_{15}	55.0%	57.2 %	+2.14 %
C_{11}	69.0%	64.6%	-4.40%	C_{15}	66.8%	58.2 %	-8.65 %
C_{12}	43.5%	54.0%	+10.5%	C_{16}	39.6 %	49.9 %	+10.3 %
C_{12}	50.6%	58.5%	+7.97%	C_{16}	51.6 %	56.4 %	+4.79 %
C_{12}	60.0%	63.2%	+3.18%	C_{16}	53.0 %	57.5 %	+4.49 %
C_{13}	43.1%	59.2%	+16.2%	C_{17}	43.1 %	49.6 %	+6.57 %
C_{13}	52.4%	58.8%	+6.32%	C_{17}	50.0 %	52.2 %	+2.20 %
C_{13}	60.0%	61.6%	+1.62%	C_{17}	61.0 %	60.7 %	-0.33 %

Analysis of 28 single chain length SCCP and MCCP standards as twelve-point-calibration curves spanning 0.1 - 40 ng/µL total CP concentration revealed a slight deviation from linearity with decreasing relative chlorination degree for the present instrument. Whereas SCCP standards all showed very good linearity ($R^2 > 0.99$), MCCP standards resulted in calibration curves best described by second degree polynomials. As expected, the overall ECNI-MS response increased with increasing chlorination degree of the single chain CP standards. High-chlorinated CP standards showed a faster increase in response with increasing concentration, thus resulting in steeper calibration curves (e.g. Figure 1). This phenomenon was observed for all degrees of chlorination in the single chain CP standards. Therefore, further investigations were only conducted with the dominant homologue(s) of each single chain CP standard.

Exemplarily, C_{12}-CPs and C_{16}-CPs were chosen as representatives for SCCPs and MCCPs, respectively, and for the investigation of mixing effects with other chain lengths. For instance, isotope peaks of homologues with five carbon atoms and two chlorine atoms difference (e.g. $C_{12}H_{18}Cl_8$ and $C_{17}H_{30}Cl_6$) were shown to overlap and they cannot be resolved with low resolution MS.[7] Even though Orbitrap-HRMS is able to resolve these homologues by means of their exact masses, there was still a noticeable impact on the response. Typically, the response of the target

Figure 1: calibration curves for three different C_{17} chain length standards given as relative responses of the homologue $C_{17}H_{28}Cl_8$. For the highest chlorinated standard, regression coefficients are given for linear regression (red, dotted line) and second degree polynomial regression (grey).

homologue was increasing with increasing percentage of the interfering homologue (which, when measured alone did not give response to the target homologue). This was valid for both single chain standards of SCCPs (C_{12}-CPs, mixed with C_{17}-CPs) and MCCPs (C_{16}-CPs, mixed with C_{11}-CPs). Furthermore, the formerly very good linearity of the SCCP standard decreased gradually to the point where concentrations < 2 ng/µL were no longer sufficiently described by a linear equation. However, no increase in linearity was observed for the C_{16}-CP standard with increasing amounts of C_{11}-CP standard. Therefore, it seems to be easier to decrease linearity than to increase it by only combining two single chain length standards.
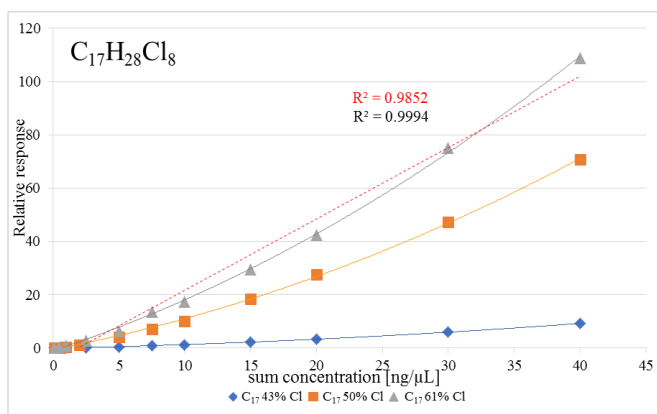
When adding single chain CP standards which differed only by one carbon in chain length, the C_{12}-CP standard showed the expected increase in response with increasing percentage of the second standard. Surprisingly, when increasing amounts of C_{17}-CPs were added to the C_{16}-CP standard, the response was decreasing despite the unchanged C_{16}-CP concentration. This was fully unexpected, as in general, C_{17}-CPs seemed to have higher responses than the C_{16}-CP standard when analysed separately. So, if an effect was expected then it was more likely that the total response of the combination would increase. The reasons for this phenomenon are as of yet still unknown and the consequences difficult to predict at the moment. Yet, this problem will most likely have consequences for all mixed CP standards: interdependency of different chain lengths subsequently means that the 'true' concentration even of the chain lengths in a mixed CP standard cannot be determined based on response alone. Therefore, chain length specific quantification using commercial mixed standards without information on chain specific concentrations, contamination by other CP groups or even variations in production is prone to errors due to over- or underestimation.

Commercial mixed standards

Between three and eight batches of commercial SCCP and MCCP standards were compared with each other using the same method (Table 2). The three mixed SCCP standards showed the lowest variations between homologues with a relative standard deviation (RSD) of 0.01-4.52% (Table 2). The agreement of MCCP standards was also very good (RSD 0.01-1.45%) except for the "MCCP 52% Cl" standard, where RSDs up to 12.5% were observed.

Table 2: Commercial SCCP and MCCP standards within a range of expiry dates, characterised by relative standard deviation, range of CP homologues, and percentages of total response by CP group

Standard	Expiry dates	n	RSD % of CP homologues	CP chain lengths present in standard	% of total response by CP groups		
					SCCPs	MCCPs	LCCPs
SCCP 51.5% Cl	2013-2018	4	0.01-0.45	C_{10}-C_{16}	98-99%	1.2-1.8%	n.a.
SCCP 55.5% Cl	2008-2022	8	0.01-4.52	C_{10}-C_{17}	95-97%	2.9-4.8%	n.a.
SCCP 63% Cl	2013-2022	3	0.01-2.87	C_{10}-C_{16}	99-100%	0.3-0.6%	n.a.
MCCP 42% Cl	2013-2020	3	0.01-1.98	C_{12}, C_{14}-C_{16}	0.2-0.4%	99-100%	n.a.
MCCP 52% Cl	2008-2021	7	0.01-12.8	C_{11}-C_{17}	0.9-10%	89-99%	n.a.
MCCP 57% Cl	2013-2019	3	0.01-1.38	C_{13}-C_{17}	0.5-0.6%	99-100%	n.a.
LCCP 36% Cl	2006-2018	4	0.11-34.5	C_{11}-C_{12}, C_{14}-C_{20}	6.6-7.5%	25-37%	55-75%
LCCP 49% Cl	2003-2019	5	0.01-3.13	C_{11}-C_{12}, C_{14}-C_{20}	2.6-2.8%	46-53%	47-52%
n.a. = not analysed n= no. of standards				All standards were originally obtained from Dr. Ehrenstorfer, Augsburg, Germany.			

LCCP standards could only be partly evaluated because longer chain lengths are unsuited for GC analysis. Within the limitations of the method, homologues in the "49% Cl LCCP" standard agreed well between different batches (RSD 0.01-3.31%), whereas the lower chlorinated "LCCP 36% Cl" standard showed a much higher variation of up to 34.5% RSD (Table 2). Since the same method was used, these variations must have originated from the standards themselves.

Interestingly, all commercial standards not only contained the correct chain lengths (C_{10}-C_{13}-CPs in the case of SCCPs and C_{14}-C_{17}-CPs in the case of MCCPs) but varying contributions of other chain lengths (Table 2). While contributions of MCCPs to SCCP standards and *vice versa* were usually small (1-5%), MCCPs caused up to 50% of the total standard response of LCCP standards. Especially the "LCCP 49% Cl" standard contained high
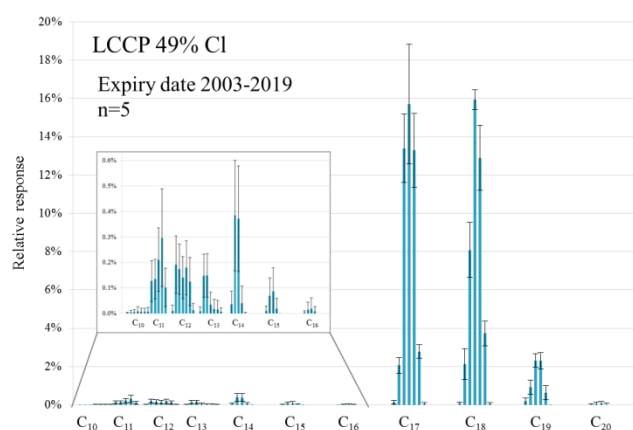

Figure 2: Mean relative CP homologue response pattern for the LCCP 49% Cl standard with standard deviation given as error bars.

amounts of C_{17}-CP homologues but also low amounts of SCCPs and other MCCPs (Figure 2). This problem has already been found for industrial CP products. Especially the high amounts of other CP groups in the LCCP standards should be kept in mind when using these standards for CP determination as they are not representative of one, but rather two CP groups. Overall, the impact of both variations throughout the production years and presence of other CP groups on low-resolution MS methods targeting the CP groups or total CP amount was considered to be small (1-5% contribution only). However, the high amount of MCCPs in the LCCP standards should be taken into account. Irrespectively, the lack of information provided by the certificate of analysis for these standards

and the possible influences of variations in CP groups on the overall response are a key-problem in CP analysis. Consequently, no conclusions as to real chain length or homologue concentrations in these mixtures can be derived. For this reason, CP determination on homologue level using these standard mixtures seems inadvisable. Therefore, there is a need that commercial CP mixtures should provide this information when sold.

Custom-made mixed standards

Using CP standard mixtures with known chain length composition, the quantification error can most likely be reduced. This can be achieved by creating customised mixtures from individual chain length CP standards. Moreover, in the interest of a comprehensive food control in case of maximum levels or restrictions going into effect on CPs, the focus should also be on less expensive LRMS instruments and corresponding methods. Therefore, a mixture made from individual single chain length standards customised to be similar to salmonids was created as described elsewhere[5] and used to quantify samples of known concentration from EURL's interlaboratory study on chlorinated paraffins in lard[2] by GC-EI-MS/MS. These samples were spiked with both SCCPs and MCCPs mimicking natural contamination, making them a good indicator for method performance in regular (food) samples. Compared to quantification results using either the "SCCP 55.5% Cl" or the "MCCP 42% Cl" standard mixtures, the custom made mixture resulted in much lower deviations from the theoretical values (Table 3).

Table 3: Theoretical total CP concentrations and resulting deviations for interlaboratory study samples analysed by GC-EI-MS/MS and three different calibration standards.

	Total CP concentration [ng/g lipid]			
Sample	Theoretical value	SCCP 55.5% Cl calibration	MCCP 42% Cl calibration	Custom-made mixed standard calibration
1805-LAB	125	82 (-34%)	110 (-10%)	110 (-9%)
1805-LAC	270	260 (-4%)	340 (+25%)	270 (+1%)
1805-LAD	125	82 (-35%)	110 (-14%)	110 (-9%)
1805-LAE	270	200 (-25%)	270 (+0.5%)	270 (+0.5%)

Based on these results, the creation of further custom made standard mixtures from single chain length CP standards seems to be prudent to improve method performance. Further investigations into the dependence on different food groups and their specific CP patterns could finally lead to a set of matrix-specific CP standards available for both LRMS and HRMS methods, which in turn might increase method comparability. Still, the true concentration of CPs in a sample is bound to be somewhat obscured by the influences on response described in this study, especially when differing standards or standard mixtures are used for quantification.

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