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Determination of toxaphene specific congeners in fish liver oil and feedingstuff using gas chromatography coupled to high resolution mass spectrometry

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ABSTRACT

A new method for the determination of nine toxaphene specific congeners in fish liver oil and feedingstuff has been developed. The samples were extracted using pressurized liquid extraction followed by a purification on silica and florisil columns. Identification and quantification were conducted using GC–(EI)-HRMS, and comparison with MS/MS detection was performed, using electron ionization and negative chemical ionization. Limits of detection were ranged from 0.01 to 0.22 $\mu g\,kg^{-1}$ (12% moisture) as required for feed samples. The calibration curves showed a good linearity for all congeners ($R^2 > 0.99$). Repeatability was below 9% for all the congeners and recoveries were in-between 73 and 86%. This analytical method was applied to the quantification of thirteen real samples collected within national monitoring plans for further risk assessment.

1. Introduction

Toxaphene is a complex mixture consisting in a group of several hundreds of congeners, mainly polychlorinated bornanes (Fig. 1), with an average chlorine content of 67–69% [1]. Systematic names of the individual compounds are simplified using shorthand nomenclature systems. The most commonly used is the numbering system according to Parlar et al. [2], where congeners bear the prefix "CHB" or "P#"). The list of monitored compounds in this study is described in Table 1.

Toxaphene is a nonsystemic insecticide with some acaricidial action and was used on crops and animals. It has been the most widely applied pesticide in many parts of the world and replaced DDT in the early 1970 s [3]. Toxaphene bioaccumulates in lipid-rich tissues. Transfer into the milk in animals and humans has been demonstrated [4]. It has been found to be highly toxic to fish [3] and rodents [1] and seems to be a human carcinogen (listed in Group 2B; IARC, 2001). Toxaphene has been banned in Europe for all uses since 1984 by the Council Directive 79/117/EEC [5] of 21 December 1978 prohibiting the introduction on the market and use of plant protection products containing certain substances, as amended by the Commission Directive 83/131/EEC [6] of 14 March 1983. In many other parts of the world the use of toxaphene is also currently banned. Due to its persistence, its bioaccumulation, its inherent toxicity and susceptibility to long-range transport, toxaphene is now internationally considered as one of the most worrying organochlorinated pollutants [7]. It is included in the list of persistent organic pollutants (POPs) of the Stockholm Convention. Nowadays, maximum tolerated limits are published for the sum of congeners P#26, P#50 and P#62 (Annex to Directive 2002/32/CE [8]). Human exposure is mainly due to food contamination and especially from fatty fishes [9], even if exhaustive occurrence data in food are not available. The determination of the total concentration of toxaphene is very difficult due to the presence of numerous congeners, each one with a specific response factor. Monitoring all the compounds is impossible due to the lack of standard solutions. Nevertheless, congeners are not all relevant: despite the large number of compounds that can be found in the technical mixture, only a reduced number of compounds are usually detected in biota because species with highly developed enzyme systems are able to metabolize most toxaphene components. As a result, only a few hepta, octa and nonachloro-homologues accumulate in fatty tissues of mammals and fish [10] while other congeners are rapidly metabolised. P#26, P#50 and P#62 were suggested as indicator compounds [10] because these congeners accumulate in the food chain and are predominant: the sum of the concentration of these three compounds normally amounts to approximately 8–50% of total toxaphene. There is some evidence that especially in fish, P#40, P#41, P#42a and P#44 are also present at measurable amounts [11–13]. Despite the fact that the toxicological properties of these congeners are still scarce, the European Food Safety Authority (EFSA) recommended to include these compounds in the monitoring plan [4]. Moreover,

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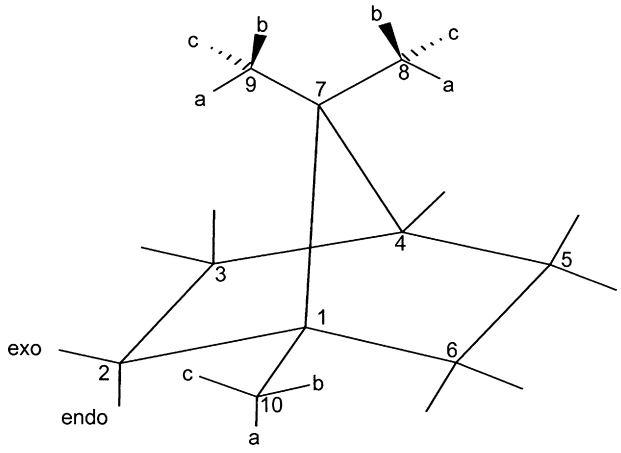

Fig. 1. Bornane skeleton structure with numbering of carbon atoms and endo and exo positions.

EFSA proposed to use P#32 as an indicator for recent contamination because this congener is a major constituent in technical toxaphene mixture and degrades easily in the environment. Toxaphene can be analysed using gas chromatography coupled to electron capture detection (GC/ECD) [14]. Currently, mass spectrometry is recognised as the target analytical tool to provide both selectivity and sensitivity. Electron ionisation (EI) and negative chemical ionization (NCI) techniques can be used, the latter offering the advantage of being both more selective and sensitive. Indeed, electron ionisation generates drastic fragmentation of toxaphene congeners due to the poor stability of the compounds. Negative ion chemical ionization allows a more gentle ionization, which produces mainly the molecular ion $[M]^-$. Nevertheless, the major drawback of NCI is the different response factor between the different congeners depending their ability to catch the negative charge [15]. For instance, P#62 has a very low response factor which limits the sensitivity of this signal. As for the nature of the mass spectrometers used for the analysis, either tandem mass spectrometry [16,17] or high resolution mass spectrometry [15,18,19] are mostly used, and allow an efficient measurement of toxaphene in biological matrices. In this paper, the development of an analytical method dedicated to toxaphene congeners is presented, and a comparison is made between tandem mass spectrometry and high resolution mass spectrometry. Application of the method to the occurrence determination of toxaphene congeners is performed in fish oil and fish feedingstuff.

Table 1
Relation between IUPAC systematic name and Parlar numbering for monitored compounds

IUPAC systematic name	Parlar numbering
2,2,5-endo,6-exo,8,9,10-heptachlorobornane	P#32
2-endo,3-exo,5-endo,6-exo,8,8,10,10-octachlorobornane	P#26
2-endo,3-exo,5-endo,6-exo,8,9,10,10-octachlorobornane	P#40
2-exo,3-endo,5-exo,8,9,9,10,10-octachlorobornane	P#41
2,2,5-endo,6-exo,8,8,9,10-octachlorobornane	P#42a
2-exo,5,5,8,9,9,10,10-octachlorobornane	P#44
2-endo,3-exo,5-endo,6-exo,8,8,9,10,10-nona-chlorobornane	P#50
2,2,5,5,8,9,9,10,10-nona-chlorobornane	P#62

2. Experimental

2.1. Chemicals and standards

All the organic solvents (Promochem, Wesel, Germany) were Picograde® quality. Silica (Fluka, Buchs, Switzerland), Florisil (Promochem, Wesel, Germany), sodium sulphate (Merck, Darmstadt, Germany) and sulphuric acid (Solvents Documentation Synthesis, Peypin, France) were of superior analytical quality. Toxaphene congeners and ^{13}C-labelled PCB were purchased from Cambridge Isotope Laboratories (Andover, USA) and Wellington Laboratory (Guelph, Canada). Standard solutions were prepared in toluene and stored in darkness at < +6 °C.

2.2. Sample preparation

Before extraction, each sample was dried in an oven at 80 °C for 48 h. Then, approximatively 1.5 g of each sample was weighed and spiked with 400 pg of ^{13}C-2,3,4,4′,5 PCB (#114) as internal standard. The extraction was performed using an automatic pressure liquid extractor (ASE 300, Dionex, Sunnyvale, USA). Feedingstuff were transferred into ASE cells (34 ml cells, which contain filter and approximatively one gram of diatomaceous earth). Pressure and temperature were set to 100 bar and 120 °C respectively. The extraction solvent was a mixture of toluene/acetone 70:30 (v/v), and three successive extraction cycles (5 min each) were performed with a 100% flush. The extracts were evaporated to dryness then dissolved in 25 ml of hexane for sample clean up. In the case of fish oil, samples were simply diluted in 25 ml of hexane after spiking.

2.3. Purification

Before purification, silica was washed with methanol and dichloromethane in order to remove interferences. Florisil was dried in an oven at 600 °C for 5 h. Then, 3% of water (w/w) was added and result phase was shaking vigorously. A two-step purification was performed, using successively silica and Florisil columns. After removal of fat on a multilayer silica gel column (packed with 2.5, 10 and 12.5 g of neutral, 22% and 44% of sulfuric acid activated G60 silica, respectively between two layers of anhydrous sodium sulphate) with 100 ml of hexane for elution, the extract was cleaned up on Florisil column (packed with 3 g of Florisil between two layers of anhydrous sodium sulphate) using 60 ml of hexane as eluting solvent. After addition of external standards (400 pg of $^{13}C_{12}$-PCB #111), the final sample extract was evaporated to dryness at 40 °C under a nitrogen stream and reconstituted by addition of 20 μL of toluene.

2.4. Instrumentation

The GC/HRMS detection was performed on a Hewlett-Packard 6890 (Palo-Alto, CA, USA) gas chromatograph, equipped with a DB-5MS column (30 m × 0.25 mm, 0.25 μm film thickness, produced by Agilent/J&W and purchased from Interchim, Montluçon, France) coupled to a Jeol JMS-700 high-resolution mass spectrometer (Jeol, Tokyo, Japan). The GC program was as follows: 80 °C (2 min), 20 °C/min until 200 °C (0 min), 5 °C/min until 250 °C (7 min) and then 10 °C/min until 300 °C (10 min). The injector was set to 230 °C while transfer line temperature was set to 280 °C. Injected volume was 2 μL in the splitless mode. Helium (N55) was used as carrier gas at the constant flow of 1 ml/min. The MS was used in the SIM mode with a resolution higher than 10000 (10% valley). The electron ionization energy was at 38–40 eV and the ion source temperature was set to 280 °C.

Table 2
Toxaphene congeners monitored transitions, using electronic ionization (70 eV)

Class	Congener	Transition 1 (T1)	Collision T1 (eV)	Transition 2 (T2)	Collision T2 (eV)
Hepta-CHB	P#32	233 > 197	5	195 > 159	10
Octa-CHB	P#26	231 > 195	5	195 > 159	10
	P#40	195 > 159	10	231 > 195	5
	P#41	195 > 159	10	231 > 195	5
	P#44	195 > 159	10	231 > 195	5
	P#42a	269 > 233	5	195 > 159	10
Nona-CHB	P#50	231 > 195	5	195 > 159	10
	P#62	195 > 159	10	231 > 195	5

Table 3
Toxaphene congeners monitored transitions, using negative ion chemical ionization (methane as reactant gas, pressure of 8.10^{-5} mbar)

Congener	Transition 1	Collision T1 (Ev)	Transition 2	Collision T2 (eV)
Hepta-CHB (P#32)	343 > 307	5	343 > 71	5
Octa-CHB (P#26; P#40; P#41; P#44; P#42a)	377 > 341	5	377 > 71	5
Nona-CHB P#50; P#62	413 > 377	5	413 > 71	5

For the GC–MS/MS analysis, a gas chromatograph (Agilent, 6890 Series, Palo-Alto, CA, USA) with a split/splitless injector was coupled to a Quattro-micro GC triple quadrupole analyzer (Waters, Micromass, Manchester, UK) operating in the electron ionization mode or the negative ion chemical ionization mode (using methane as ionization gas, pressure of 8.10^{-5} mbar). The injector was set to 230 °C while GC program and column were the same as previously described in this paragraph.

3. Results and discussion

3.1. Mass spectrometry

3.1.1. GC–MS/MS

Toxaphene analysis was studied using gas chromatography coupled to tandem mass spectrometry. Two ionization techniques were tested in order to allow for a comparison.

3.1.1.1. Electron ionization. The major problem in the analysis of toxaphene using electron ionization is the significant fragmentation of the analytes. A wide number of possibilities are available regarding precursor ion selection. In 2002, Gouteux et al. [16] showed that the choice of transitions depends on the chloration degree of the compound: the low mass-to-charge ratio should be used for octa-chlorobornane (P#26, P#40, P#41 and P#44) and the high mass-to-charge ratio should be used for nona-chlorobornane (P#50 and P#62). After scan mode analysis, several precursor ions were chosen and, for each one, few daughter ions were determined. Conditions of fragmentation were optimized in terms of collision energy and main results are shown in Table 2. Transition 125 > 89, which was reported as among the most sensitive signal in previous studies performed on ion trap was not confirmed as such on triple quadrupole in our study. Indeed, this transition is observed for all congeners, thus delivering an interesting generic signal, but not with the highest sensitivity. In terms of specific congener quantification, it was chosen not to use this transition, but to monitor specific transitions for each congener. Finally, 5 transitions were kept for efficient monitoring of target analytes.

3.1.1.2. Negative chemical ionization. Gas chromatography coupled to NCI–MS is the most popular method for the analysis of toxaphene. In order to evaluate this technique and to assess its performance with regard to electron ionization, an optimisation was conducted first. This ionisation mode allows to reduced fragmentation by comparison with electron ionization, and depending of the ionization mechanism, either proton abstraction or electron capture, quasi-molecular ion $[M–H]^-$ or molecular ion $M^{\bullet-}$ can be generated, respectively. Conditions of fragmentation were optimized in terms of collision energy, and final parameters are given in Table 3. A standard mixture containing every congener was injected in Multiple Reaction Monitoring (MRM) mode, in order to evaluate the performance of the technique. The sensitivity was found well-adapted to trace measurements of these analytes. The major drawback was the difference in term of relative response factor between each congener. As observed by Lau et al. [15], this difference of behaviour is prejudicial for toxaphene analysis, especially for congener P#62 for which the sensitivity is sometimes limited. As illustrated in Fig. 2, a mixture containing P#50 and P#62 at the same concentration (200 pg on-column) was injected in GC–(NCI)–MS/MS. The signals obtained for these nona-chlorobornane congeners were very different: while congener P#50 was detected with an excellent signal-to-noise ratio (230 and 340 respectively), P#62 was only detected (S/N > 3). The same congener mixture was ionised under EI conditions. As shown in Fig. 2, the EI mode generates equivalent signal whatever the congeners, in general with worse S/N. For the first reason, EI ionisation was chosen as the better strategy, since lower sensitivity could be compensated by more hyphenated mass analysers.

3.1.2. GC–EI-HRMS

For better sensitivity, high resolution mass spectrometry (double sector) was evaluated. Analysis of toxaphene using GC–EI-HRMS was already described [15,18,19] and as interestingly developed by Lau et al., the $[M–Cl]^+$ fragment ion was preferentially monitored for all the compounds, as described in Table 4. The major advantage in monitoring this ion was to generate and benefit from the same signal whatever the congeners belonging to the same congener group (identical chlorine atom number). Nevertheless, it is important to

Table 4
Accurate masses monitored, class of congeners and ion abundance ratios for toxaphene congeners, using EI-HRMS detection mode after electron ionization (38–40 eV)

Congener	Ion 1	Ion 2	Isotopic ratio
Hepta-CHB (P#32)	342.8963	344.8934	100/80
Octa-CHB (P#26; P#40; P#41; P#44; P#42a)	376.8573	378.8544	100/96
Nona-CHB P#50; P#62	410.8183	412.8154	89/100

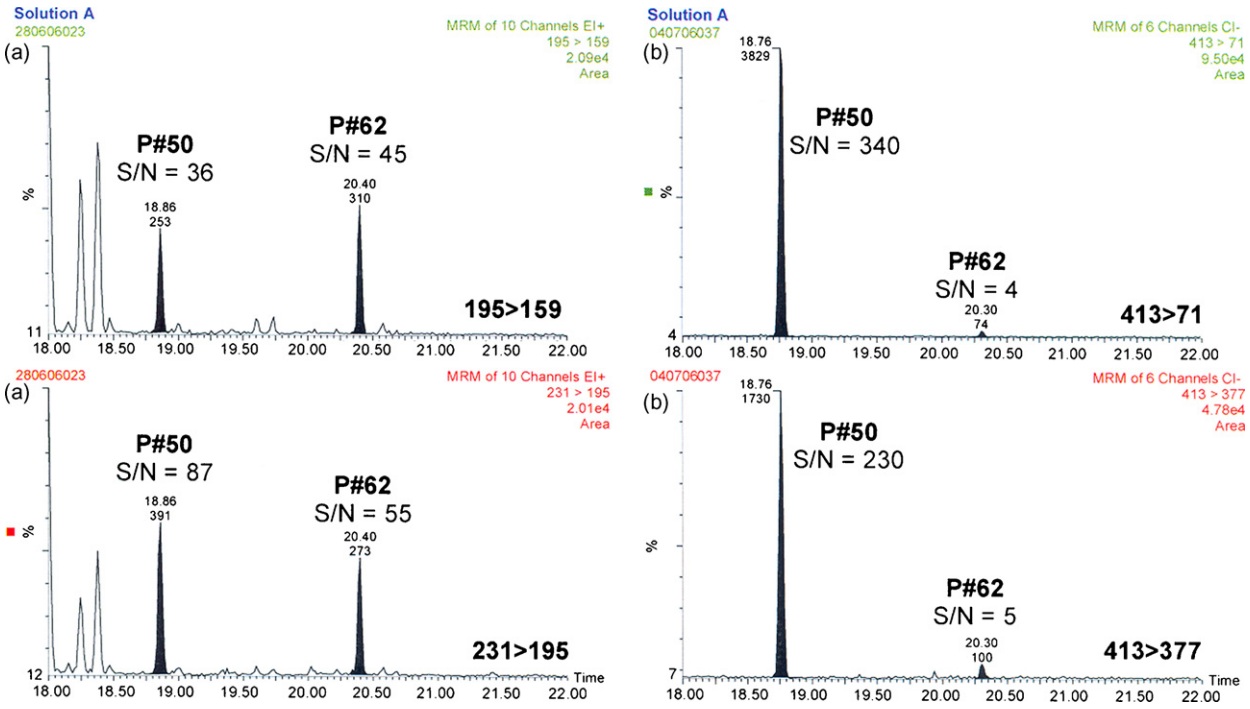

Fig. 2. Ion chromatograms (GC–MS/MS) characteristic of two toxaphene congeners (P#50 and P#62) observed on a triple quadrupole instrument after (a) electron ionization (transitions 195 > 159 and 231 > 195) and, (b) negative ion chemical ionization (transitions 413 > 71 and 413 > 377).

underline at this stage that different congeners are characterised by slightly different response factors, so that the quantification of a given congener obliges the availability of the corresponding standard.

3.1.3. Sensitivity comparison of MS/MS vs. HRMS

A fish oil sample was used to evaluate the respective performances of GC–MS/MS and GC–HRMS. As illustrated in Fig. 3, the results were significantly improved in HRMS com-

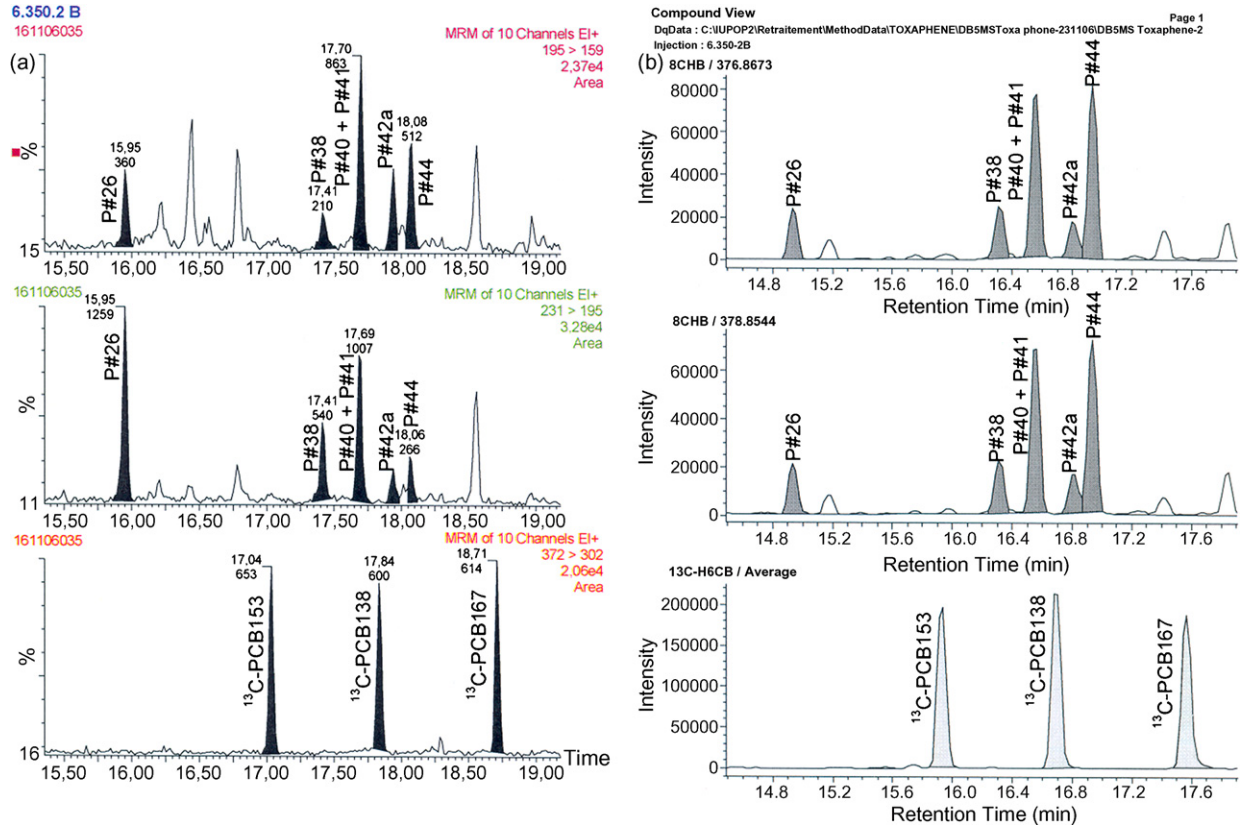

Fig. 3. Ion chromatograms corresponding to the analysis of a fish oil sample (concentrations between 0.9 μg kg^{-1} for P#44 and 5.6 μg kg^{-1} for P#42a) using: (a) EI–MS/MS detection (SRM mode) and (b) EI-HRMS detection (SIM mode).

Table 5
Performances of the purification method in term of repeatability, linearity, limits of detection and recoveries

Congeners	Repeatability (%)	Linearity (R^2)	LOD ($\mu g\,kg^{-1}$ [a])	Recovery (%)
P#32	7.62	0.9986	0.122	78.5
P#26	5.73	0.9999	0.076	82.1
P#40 + P#41	5.33	0.9998	0.012	81.0
P#42a	4.90	0.9992	0.221	73.9
P#44	3.19	0.9996	0.020	78.2
P#50	5.42	0.9992	0.019	85.6
P#62	7.19	0.9933	0.013	80.3

[a] $\mu g\,kg^{-1}$ relative to a feedingstuff with a moisture content of 12%.

pared to MS/MS measurements. The S/N ratio was found to be 3–10 times higher with high resolution mass detection, from P#26 to P#44. Same results have been observed for the other congeners, proving the better sensibility of HRMS detection mode.

3.2. Validation

As illustrated in Fig. 3, congeners P#40 and P#41 were co-eluted and no satisfying separation could be definitely achieved using a DB-5MS column. We thus decided to consider systematically the signal of these 2 compounds together. Repeatability was assessed on the basis of the extraction, purification and quantification of 6 sub-samples by 2 different operators. As described in Table 5, relative standard deviations were in the range [3.2%–7.6%]. These values were found clearly satisfying and adapted to the purpose of the analytical method. Linearity was assessed on the basis of seven calibration levels for each analyte, covering the concentration range of 0–80 $\mu g\,kg^{-1}$ of dry matter. Coefficients of determination (R^2) were better than 0.99 for all analytes, demonstrating the adequacy between the measured signal and the concentration. Absolute recoveries of each target congener have been evaluated by comparing the signal abundances obtained for one sample fortified before extraction and one sample fortified at the same concentration level just before the injection. Results were ranging 74–86%. Limits of detection (LOD) were estimated by extrapolation of the lowest point of the calibration curve, i.e. the concentration expected to induce a chromatographic peak with a signal-to-noise ratio higher than 3. Calculated LOD varied from 0.01 to 0.22 $\mu g\,kg^{-1}$ in feedingstuff characterised by a moisture content of 12%. These limits are fully compatible with a concentration range potentially met in fish oil and fish feedstuffing, and meet the European regulation fixing the maximum residue level at 100 $\mu g\,kg^{-1}$ (expressed by the sum of P#26, P#50 and P#62). Moreover, these performances are globally equal [17,20] or better [11] than those previously described in the literature.

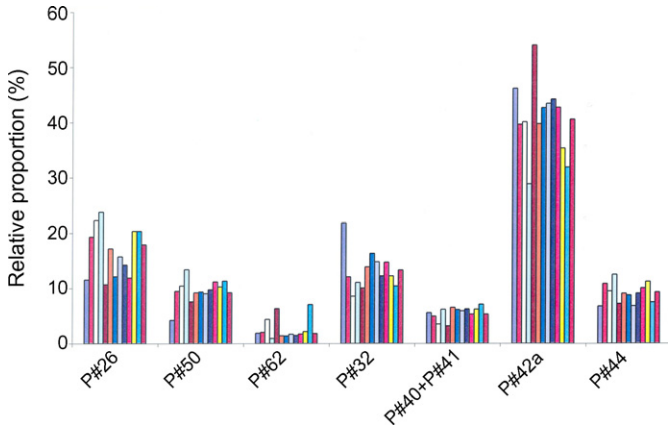

Fig. 4. Contamination profile for 13 analysed samples, expressed as individual relative proportion.

3.3. Application

The analytical procedure was applied to the determination of the 8 target toxaphene congeners in fish liver oils ($n = 5$) and fish feedingstuffs ($n = 8$) of different origin. Samples were analysed using the developed analytical method (GC–EI-HRMS). All samples were found compliant regarding the EU regulation: concentration for the sum of P#26, P#50 and P#62 was in-between 0.5 and 30 $\mu g\,kg^{-1}$ of dry matter at 12% moisture (maximum residue level is fixed at 100 $\mu g\,kg^{-1}$). The analysis of the contamination profile, as presented in Fig. 4, shows the relative proportion of each monitored congener as compared to the sum. As shown, the accumulation profiles follow the same pattern, in spite of differences in samples and origins. Moreover, we can observe that congener P#42a has the highest relative proportion (between 25% and 50% of the full toxaphene content), and it turns out to be one of the most toxic congeners [4]. This result shows the relative importance of monitoring this compound, even if nowadays this congener is not taken into account for regulation.

4. Conclusion

The present study related a new analytical method for toxaphene individual congener quantification, based on GC–EI-HRMS measurement. The advantages/drawbacks of using this technique as compared to MS/MS detection in EI or NCI mode have been described. Indeed, use of NCI allows ionisation without high fragmentation, but the different congeners do not have the same response factor and this becomes very prejudicial for the analysis of Parlar #62 (one of the most important compounds) which shows a very low sensitivity. Detection in MS/MS mode after EI ionisation allowed for a detection of all the compounds, but due to the high fragmentation of toxaphene congeners, the sensitivity was very low compared to HRMS. Indeed, a high number of transitions were observed using MS/MS detection, the spectrometric signal was also “diluted” decreasing significantly the detection level. The best alternative seems to be EI-HRMS, which enables to combine quasi-equal response factors between all the congeners, as well as the high sensitivity and specificity of an HRMS detection. Performance of this method has been evaluated and results are fit-for-purpose. In accordance with the opinion of the scientific panel on contaminants in the food chain [4], new congeners have been included in this method. Analysis of different samples have specially demonstrated the importance of congener P#42a in the toxaphene profile in food, and the repeatability of the profile from one sample to another.

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