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Final Dissertation

Development and validation of analytical methods based on GC-MS/MS Triple Quadrupole instrument for the analysis of POPs in food and feed matrices

Chiara Calaprice

Supervisors

Prof. Pietro Mastrorilli (Poliba)
Prof. Carlo Zambonin (Uniba)
Dott.ssa Cosima Damiana Calvano (Uniba)
Prof. Jean François Focant (ULg)

Co-ordinator of the Research Doctorate Course

Prof. Michele Mossa (Poliba)

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POLITECNICO DI BARI

SCUOLA INTERPOLITECNICA DI DOTTORATO

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Tesi Finale

Sviluppo e validazione di metodi analitici basati sulla spettrometria di massa a Triplo Quadrupolo per l'analisi di composti organici persistenti (POPs) in prodotti per l'alimentazione umana e animale

Chiara Calaprice

Supervisori

Prof. Pietro Mastrorilli (Poliba)
Prof. Carlo Zambonin (Uniba)
Dott.ssa Cosima Damiana Calvano (Uniba)
Prof. Jean François Focant (ULg)

Co-ordinatore della Scuola di Dottorato

Prof. Michele Mossa (Poliba)

4 Aprile 2016

To my beloved grandparents

EXTENDED ABSTRACT *(eng)*

This thesis focused on the development and validation of analytical methods for the detection and quantification of Persistent Organic Pollutants (POPs) in biological matrices, namely food and feed samples. POPs are a group of chemical compounds listed after the Stockholm Convention in 2001, with demonstrated toxicity and dangerousness for environment, animals and humans.

In this work of thesis, special attention was reserved to some selected POPs: polychlorodibenzo-*p*-dioxins (PCDDs), polychlorodibenzofurans (PCDFs), usually referred to as "dioxins", and polychlorinated biphenyls (PCBs), as there is a big concern about these contaminants in Taranto, a city in the Southern Italy very close to Bari, my home town. Taranto, indeed, is characterized by a large industrial area with a steel mill, several incinerators and a refinery in few kilometre radius (Di Leo et al., 2014). This work has been done in collaboration with the University of Liège (Belgium) where a consolidated expertise in the field of POP measurements was available, especially in terms of dioxin analyses.

In Chapter 1 the main steps, from sample preparation to data elaboration, of a validated confirmatory method for dioxin and PCB detection in food and feed using gas chromatography coupled to tandem mass spectrometry Triple Quad instrument (GC-MS/MS Triple Quad) have been described. This method was developed at the University of Liège in the framework of the last updates of the EU Regulation that in 2014 allowed confirmatory quantitative analysis of dioxins with Triple Quad. This method was the starting point of this work of thesis, because it was used for all dioxin and PCB quantifications. In Chapter 2 and chapter 3 alternative clean-up approaches for dioxin analysis in fatty food matrices have been developed using different automated systems. These works have been done in the framework of solvent and time saving for high throughput analytical methods in dioxin analysis. In chapter 2, an already existing automated system, DEXTech™ from LCTech GmbH (LCTech GmbH, Bahnweg 41, 84405 Dorfen, Germany) was used for sample clean-up, but a completely new clean-up approach was developed with this. GC-MS/MS Triple Quad instrument, as well as Magnetic Sector High Resolution Mass Spectrometry (HRMS) instrument were employed for final quantification, to demonstrate the

suitability of our newly developed clean-up approaches whatever the instrumental detection. In Chapter 3 PowerPrep™ automated system from Fluid Management System (FMS Inc., 580 Pleasant Street, Watertown, MA 02472, USA) was used and our routine sample clean-up approach was modified in order to enhance the efficiency and to reduce cost and solvent consumption of the analysis. In Chapter 4 the main method for dioxin analysis was adapted for the integration of Dechloranes in the list of the analytes targeted in the regular control for dioxins in food and feed. Dechloranes are a family of 6 organochlorinated compounds with structure similar to Mirex, also called Dechlorane, a POP listed in the Stockholm convention. Dechloranes have been found in human blood of people from Europe (Brasseur et al., 2014) and in this work an analytical method for Dechlorane detection was developed and validated to investigate food as a possible route of exposure for humans in Europe, where no production plant has been identified so far. The analytical method was used to analyse 88 food and feed real sample and to give an idea of Dechlorane daily dietary intake.

Dioxins, PCBs, GC-MS/MS Triple Quadrupole, method validation, Dechloranes

EXTENDED ABSTRACT *(ita)*

Questo lavoro di tesi è stato incentrato sullo sviluppo e la validazione di metodi analitici per la rivelazione e la quantificazione di composti organici persistenti (Persistent Organic Pollutants, POPs) in campioni biologici, in particolare campioni di cibo per l'alimentazione umana e animale. Il termine "POPs" comprende un gruppo di composti chimici, elencati durante la Convenzione di Stoccolma nel 2001, la cui tossictà e pericolosità per l'uomo, gli animali e l'ambiente sono dimostrate. In questo lavoro di tesi è stata dedicata particolare attenzione ad alcuni POPs, come diossine (polychlorodibenzo-*p*-dioxins, PCDDs), furani (polychlorodibenzofurans, PCDFs) e bifenili policlorurati (polychlorinated biphenyls, PCBs), poichè questi composti suscitano molta preoccupazione per la salute umana nella città di Taranto, una città a Sud dell'Italia, situata vicino a Bari, la mia città natale. Taranto, infatti, è caratterizzata dalla presenza di una acciaieria, vari inceneritori e una raffineria, concentrate nell'area industriale della città e non molto distanti fra loro. Questo lavoro di tesi è stato svolto in collaborazione con l'Università di Liegi (Belgio), che possiede esperienza decennale ed internazione nell'ambito dell'analisi di diossine.

Nel Capitolo 1 di questo lavoro di tesi sono descritti i passaggi principali dell'analisi delle diossine, dalla preparazione del campione all'analisi strumentale utilizzando la tecnica di gas cromatografia accoppiata alla spettrometria di massa con analizztore a Triplo Quadrupolo (GC-MS/MS Triple Quad). Questo metodo analitico, che è stata la base di questo lavoro di tesi, è stato sviluppato all'Università di Liegi nel quadro degli ultimi aggiornamenti della Regolamentazione Europea in materia di diossine, che nel 2014 ha accettato l'utilizzo del Triplo Quadrupolo per l'analisi quantitativa di questi composti. La maggior parte delle analisi descritte in questo lavoro sono state effettuate con il Triplo Quadrupolo sulla base del suddetto metodo analitico. Nei Capitoli 2 e 3 sono descritti degli approcci alternativi per il clean-up del campione, utilizzando sistemi automatici. In particolare nel Capitolo 2, per il clean-up del campione, è stato utilizzato il DEXTech™ della compagnia tedesca LCTech (LCTech GmbH, Bahnweg 41, 84405 Dorfen, Germany), per il quale è stato sviluppato un approccio completamente nuovo per il suo utilizzo. Le successive analisi quantitative sono state fatte con il Triplo Quadrupolo e con il Settore Magnetico, per dimostrare la validità dell'approccio proposto a prescindere dalla tecnica impiegata

per l'analisi strumentale finale. Nel Capitolo 3, per il clean-up del campione, è stato utilizzato il PowerPrep™, prodotto dalla compagnia americana FMS (FMS Inc., 580 Pleasant Street, Watertown, MA 02472, USA); il metodo di clean-up utilizzato routinariamente nel nostro laboratorio a Liegi, è stato opportunamente midificato con l'obiettivo di ridurre i tempi e i costi della preparativa del campione. Infine, l'analisi strumentale è stata effettuata con il Settore Magnetico. Infine, nel Capitolo 4, il metodo analitico per l'analisi di diossine, è stato modificato per consentire l'analisi simultanea di diossine e di Declorani, che sono una famiglia di composti potenzialmente pericolosi. Il primo di questi composti è stato il mirex, noto anche come Declorano, che è inserito nella lista dei POPs della Convenzione di Stoccolma. I Declorani sono stati trovati in campioni di sangue umano in soggetti provenienti dalla Francia. In questo lavoro di tesi, è stato sviluppato e validato un metodo analitico per la rivelazione di questi composti in campioni destinati all'alimentazione umana ed animale, per capire se la catena alimentare rappresenta una possibile via di esposizione a questi composti in Europa, dove non è stato identificato alcun impianto di produzione di Declorani. Il metodo analitico sviluppato è stato applicato a 88 campioni reali di varie matrici alimentari e un apporto giornaliero di Declorani è stato stimato sulla base delle abitudini alimentari della popolazione belga.

Diossine, PCBs, GC-MS/MS Triple Quadrupole, validazione di metodi analitici, Declorani

LIST OF GENERAL ABBREVIATIONS

ABN	Acid Basic Neutral silica column
ARRF	Average Relative Response Factor
AhR	Aryl hydrocarbon Receptor
ASE	Accelerated Solvent Extraction
ATSDR	Agency for Toxic Substances and Diseases Registry
CART	Centre for Analytical Research and Technology
CONTAM	Panel of EFSA on Contaminants in the Food Chain
co-PCBs	Coplanar PCBs (#77, 81, 126, 169)
DCM	Dichloromethane
DL	Detection limit
DL-PCBs	Dioxin-like PCBs
DoE	Design of Experiment
DP	Dechlorane Plus
EC	European Commission
EFSA	European Food Safety Authority
EI	Electron Ionization
EPA	Environmental Protection Agency
EURL	European Reference Laboratories
f_{anti}	Ratio between the concentration of DP_{anti} and DP_{syn} isomers
FCD	Face Centered Design
FFD	Full Factorial Design
GC-MS/MS	Gas chromatography – tandem mass spectrometry
HC	High Capacity
HCCPD	Hexachlorocyclopentadiene
HRMS	High Resolution Mass Spectrometry

ID	Isotope Dilution
iLOQ	Instrumental Limit of Quantitation
I-PCBs	Indicator PCBs (# 28, 52, 101, 138, 153, 180)
ISTD	Internal Standard, often labelled
lb	Lower bound (approach of reporting final result in dioxin analysis)
LOQ	Limit of Quantitation
LVI	Large Volume Injection
LRMS	Low Resolution Mass Spectrometry
ML	Maximum residual Level
MO-PCBs	Mono-*ortho* PCBs (#105, 114, 118, 123, 156, 157, 167, 189)
MRM	Multiple Reaction Monitoring
NDL-PCBs	Non-dioxin like PCBs
NRL	National Reference Laboratory
PBMS	Performance-based Measurement System
PCBs	Polychlorinated biphenyl
PCDDs	Polychlorinated dibenzo-*p*-dioxins
PCDFs	polychlorinated dibenzofurans
PCP	Pentachloro phenol
PLE	Pressurised Liquid Extraction
ppb	Part-per-billion (10^{-9})
ppm	Part-per-million (10^{-6})
ppq	Part-per-quadrillion (10^{-15})
ppt	Part-per-trillion (10^{-12})
PTV	Programmable Temperature Vaporization
QC	Quality Control
QCE	Quality Control egg
QCG	Quality Control fat

QCL	Quality Control milk
QIST	Quadrupole Ion Storage tandem-in-time mass spectrometer
QQQ	Triple Quadrupole
RRF	Relative Response Factor
S/N	Signal to Noise ratio
SIM	Single Ion Monitoring
ST ROU	Standard routine
TCDD	Tetrachloro dibenzo-*p*-dioxin
TEF	Toxic Equivalent Factor
TEQ	Toxic equivalents
ub	Upper bound (approach of reporting final result in dioxin analysis)
VF	Vent Flow
VP	Vent Pressure
WHO	World Health Organization

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CHAPTER IV

INTRODUCTION

Persistent Organic Pollutants, often indicated with the acronym “POPs”, are carbon-based chemicals addressed in the Stockholm Convention signed by 151 Countries in 2001. The Stockholm Convention has as its objective the environmental, as well as human and biota health protection from POPs.

Initially the list of POPs included only 12 compounds, 10 intentionally and 2 non-intentionally produced. With time the list has been updated and new compounds showing POP characteristics have been included. In fact POPs are considered all the chemicals that exhibit common characteristics such as chemical and physical stability under environmental conditions, semi-volatility, low solubility in water, and inherent toxicity. The combination of these chemical and physical properties results in long-range transport and in bioaccumulation. Indeed POPs are found in regions far from where they have been used or released. Due to their lipophilicity and their environmental and metabolic persistence they accumulate in the food-chain and high concentrations have been detected in animals and humans.

In the Stockholm convention list, POPs are grouped in Annexes A, B and C (Table I.1) based on the measure to be taken on them by parties: compounds in Annex A must be eliminated (neither produced or used); chemical in Annex B must be restricted in the production and use; compounds in Annex C are unintentionally produced and result as by-products from other industrial processes, so their unintentional release must be restricted until the complete elimination.

Table I.1: List of POPs decided by the Stockholm convention (Stockholm Convention official(www.pops.int/)

Annex A	**Annex B**	**Annex C**
Aldrin (P)	DDT (P)	Hexachlorobenzene (HCB) (UP)
Chlordane (P)	Perfluorooctane sulfonic acid, its salts and perfluorooctane sulfonyl fluoride (IC)	Pentachlorobenzene (UP)
Chlordecone (P)		Polychlorinated biphenyls (PCB) (IC)
Dieldrin (P)		Polychlorinated dibenzo-*p*-dioxins (PCDD) (UP)
Endrin (P)		Polychlorinated dibenzofurans (PCDF) (UP)
Heptachlor (P)		
Hexabromobiphenyl (IC)		
Hexabromocyclododecane (HBCD) (IC)		
Hexabromodiphenyl ether and heptabromodiphenyl ether (IC)		
Hexachlorobenzene (HCB) (P and IC)		
Alpha hexachlorocyclohexane (P)		
Beta hexachlorocyclohexane (P)		
Lindane (P)		
Mirex (P)		
Pentachlorobenzene (P and IC)		
Polychlorinated biphenyls (PCB) (IC)		
Technical endosulfan and its related isomers (P)		
Tetrabromodiphenyl ether and pentabromodiphenyl ether (IC)		
Toxaphene (P)		

P = pesticide; IC = industrial chemical; UP = unintentional product

Aim of this work

This thesis focused on the development and validation of analytical methods for POP detection and quantification in biological matrices, namely food and feed. Special attention was reserved to some selected POPs: toxic PCDDs, PCDFs and PCBs, as there is a big concern about these contaminants in Taranto (Italy), a city in the Southern Italy characterized by a large industrial area with a steel mill, several incinerators and a refinery (Di Leo et al., 2014). The collaboration with the University of Liège started because of the consolidated expertise owned in POPs field, especially in dioxin analysis. Experimental activities described in the chapters have been carried out mainly at the University of Liège, in collaboration with the CART (Centre for Analytical Research and Technology), which is referred to as "our laboratory" during the following dissertation.

In Chapter 1 the main steps, from sample preparation to data elaboration, of our validated confirmatory method for PCDD/Fs and dioxin-like (DL-) PCBs detection in food and feed have been described. Our method is based on gas chromatography coupled to tandem mass spectrometry Triple Quad instrument (GC-MS/MS Triple Quad) in the framework of the last updates of the EU Regulation in this field (L'Homme et al., 2015).

In Chapter 2 alternative clean-up approaches for dioxin analysis in fatty food matrices have been shown. These approaches were developed using an already existing automated system, DEXTech™ from LCTech GmbH (LCTech GmbH, Bahnweg 41, 84405 Dorfen, Germany), but they were based on a completely new column set in the framework of faster, cheaper and more environmental sustainable processes. In this project instrumental quantification has been done mainly with GC-MS/MS Triple Quad instrument, but also with Magnetic Sector high resolution mass spectrometry (HRMS) instrument to demonstrate the suitability of the clean-up approaches whatever the instrumental detection.

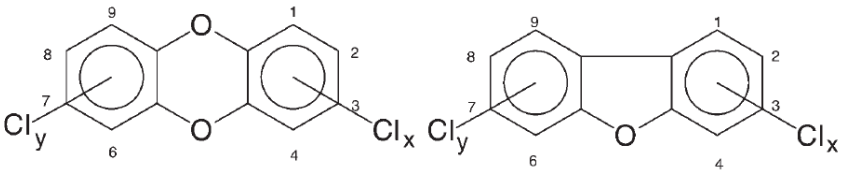
In Chapter 3 alternative approaches based on our routine clean-up method have been reported. New procedures were based on PowerPrep™ automated system from Fluid Management System (FMS Inc., 580 Pleasant Street, Watertown, MA 02472, USA) and new programs were implemented in the framework of solvent and time saving for high throughput analytical methods.

In Chapter 4 an analytical method for Dechlorane detection in food and feed, as well as a first estimation of Dechlorane dietary intake for people from Europe has been described.

Dechloranes are organo-chlorinated compounds with structure similar to Mirex, also called Dechlorane, which is the only Dechlorane compound listed in the Stockholm convention at the present. Dechloranes have been found in human blood of people from Europe (Brasseur et al., 2014) where no production plant has been identified. In this work, food was investigated as a possible route of exposure, as probably Dechlorane compounds might show toxicological effects similar to Mirex. For Dechlorane analysis, the main method for dioxins was modified in order to integrate Dechlorane detection in the regular control for dioxins and have a multi-analyte method. The work reported in Chapter 4 has been published in peer-reviewed journal.

Scientific context of the research about Dioxins and PCBs

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and their analogues polychlorinated dibenzofurans (PCDFs) are well known persistent organic pollutants (POPs) detectable in trace amount but in almost all compartments of the global ecosystem, environment and biota. General structures are based on planar, tricyclic aromatic ethers, which can have up to 8 chlorine atoms attached to carbon atoms 1 to 4 on one ring, and 6 to 9 on the other ring (Figure I.1).



Polychloro dibenzo-p-dioxins
PCDDs

Polychloro dibenzofurans
PCDFs

Figure I.1: General structures of PCDDs and PCDFs.

Depending on the number and the position of the chlorine atoms on the rings, one can have many chemically different PCDD/Fs, each of which is called a “congener”. Compounds, or congeners, with the same number of chlorine atoms are called “homologues” and homologues with different substitution pattern are called “isomers”. The term “dioxins” - note the plural - is frequently used to refer to 75 congeners of PCDDs and 135 congeners of PCDFs (Table I.2), for a total of 210 compounds.

Among these 210 compounds, 17 congeners, 7 PCDDs and 10 PCDFs (listed later in Table I.3) can have chlorine atoms in the positions 2, 3, 7, and 8 (but not only) of the parent molecule, assuming a planar structure, showing toxicity to many laboratory animals, resistant towards chemical, biological, and physical attack. Thus these compounds accumulate in the environment and in organisms as animals and humans (Fiedler, 2002).

Table I.2: Possible number of isomers within homologue groups for PCDD and PCDF.

Homologue	PCDD isomers	PCDF isomers
Monochloro-	2	4
Dichloro-	10	16
Trichloro-	14	28
Tetrachloro-	22	38
Pentachloro-	14	28
Hexachloro-	10	16
Heptachloro-	2	4
Octachloro-	1	1
Total	75	135

Polychlorinated biphenyls (PCBs) are a class of nonpolar, chlorinated hydrocarbons with a biphenyl nucleus on which 1 to 10 of the hydrogens have been replaced by chlorine, generating 209 discrete compounds, called “congeners” as for dioxins (Figure I.2).

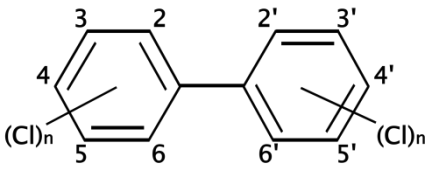


Figure I.2: General structure of PCBs.

PCBs having the same chlorination degree are called “homologues”, and homologues with different substitution patterns are referred to as “isomers”. For example homologues trichlorobiphenyls have 24 isomers. Due to the high number of congeners, PCBs have been arranged in ascending numeric order and assigned a IUPAC number from 1 to 209. The numbering system of carbon atoms in PCBs is shown in Figure I.2. As commonly for aromatic rings, positions 2, 2', 6, and 6' are called *ortho* positions; 3, 3', 5, and 5' are called *meta* positions, and positions 4 and 4' are called *para* positions. PCB benzene rings can be coplanar or not, depending on the steric and electronic effects of substitu-

ents, especially in the *otho* position, where the replacement of hydrogen atoms with larger chlorine atoms forces the benzene rings to rotate out of the planar configuration.
The benzene rings of non-*ortho* substituted PCBs assume a planar configuration and so these compounds can be called “coplanar congeners” (co-PCBs). Also some mono-*ortho* (MO-) substituted PCB congeners are planar. The benzene rings of those PCBs having more than two chlorine atoms in *ortho* position cannot assume a planar configuration and are indicated as “non-planar congeners”. The chlorination pattern of the PCBs determines the final PCB spatial configuration and also the toxicity of the substance as explained in the following paragraph.

1. Dioxin and PCB structure related toxicity

Among dioxins, 2,3,7,8-tetrachloro dibenzo-*p*-dioxin (2,3,7,8-TCDD) is considered the most toxic compound, as it has one of the lowest known LD_{50} (lethal dose to 50% of the population) values. It takes only 0.6 µg/kg of body weight to kill male guinea pigs (Schwetz B.A., 1973, Fiedler, 2002). The polychlorinated dibenzofurans are only slightly less toxic; for example, the LD_{50} of 2,3,7,8-TCDF is about 6 µg/kg for male guinea pigs (Van den Berg et al., 2006). It is important to underline that the toxicity of dioxins varies dramatically from species to species; for example, 2,3,7,8-TCDD is about 500 times less toxic to rabbits than it is to guinea pigs. Other 2,3,7,8-substituted dioxin and furan congeners are also toxic, and many of these compounds have both acute and chronic effects.
PCDD and PCDF toxicity is related mainly to their capacity to interact with the cytosolic specific protein called the aryl hydrocarbon receptor (AhR). The binding to the Ah receptor constitutes a first and necessary step to initiate the toxic and biochemical effects. Dioxin 2,3,7,8-TCDD (Figure I.3) is the congener with the highest affinity to AhR and so it is the most toxic PCDD congener.

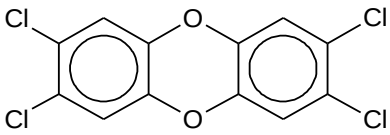


Figure I.3: 2,3,7,8-TCDD structure.

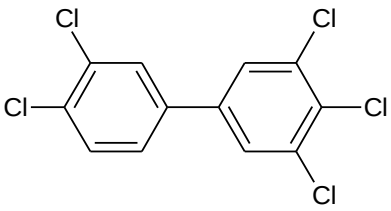
2,3,7,8-TCDD is planar and highly symmetric. The AhR binding affinities of 2,3,7,8-TCDF, 1,2,3,7,8- and 2,3,4,7,8-PeCDF are in the same order of magnitude as that observed for 2,3,7,8-TCDD. With increasing chlorination, receptor-binding affinity decreases, but still it was recognized that all PCDD/PCDF substituted at least in position 2, 3, 7, or 8 (Table I.3) are highly toxic and thus major contributors to the overall toxicity of the dioxin mixture. Moreover they persist in the environment and accumulate in food chain (Fiedler, 2002).

Table I.3: Toxic PCDD and PCDF congeners.

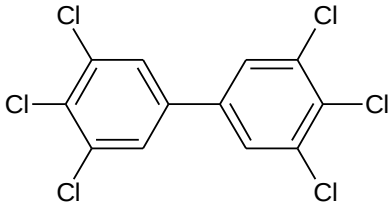
	PCDD congener	**Acronym**
1	2,3,7,8-tetrachlorodibenzo-*p*-dioxin-	2,3,7,8-TCDD
2	1,2,3,7,8-pentachlorodibenzo-*p*-dioxin	1,2,3,7,8-PeCDD
3	1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin	1,2,3,4,7,8-HxCDD
4	1,2,3,6,7,8-hexachlorodibenzo-*p*-dioxin	1,2,3,6,7,8- HxCDD
5	1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin	1,2,3,7,8,9- HxCDD
6	1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin	1,2,3,4,6,7,8-HpCDD
7	octachlorodibenzo-*p*-dioxin	OCDD
	PCDF congener	**Acronym**
1	2,3,7,8-tetrachlorodibenzofuran	2,3,7,8-TCDF
2	1,2,3,7,8-pentachlorodibenzofuran	1,2,3,7,8-PeCDF
3	2,3,4,7,8-pentachlorodibenzofuran	2,3,4,7,8-PeCDF
4	1,2,3,4,7,8-hexachlorodibenzofuran	1,2,3,4,7,8-HxCDF
5	1,2,3,6,7,8-hexachlorodibenzofuran	1,2,3,6,7,8-HxCDF
6	1,2,3,7,8,9-hexachlorodibenzofuran	1,2,3,7,8,9-HxCDF
7	2,3,4,6,7,8-hexachlorodibenzofuran	2,3,4,6,7,8-HxCDF
	1,2,3,4,6,7,8-heptachlorodibenzofuran	1,2,3,4,6,7,8-HpCDF
9	1,2,3,4,7,8,9-heptachlorodibenzofuran	1,2,3,4,7,8,9-HpCDF
10	octachlorodibenzofuran	OCDF
	Total	17

In this manuscript, the terms “dioxin“ and “dioxins” should be interpreted as including these selected polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans showing high toxicity and for this reason regulated from the EU.

Based on structural characteristics and toxicological effects, PCBs are divided into dioxin-like PCBs (DL-PCBs) showing toxicological properties similar to dioxins and non dioxin-like PCBs (NDL-PCBs) which do not share the dioxin’s toxic mechanism of AhR binding (European Food Safety Authority, 2010), but their toxicological effects are related to different action mechanisms. PCB congeners showing dioxin-like toxicity have planar and symmetric spatial configuration due to their chlorination pattern, and in fact they are non-*ortho*-PCBs, 4 congeners indicated also as coplanar PCBs (co-PCBs), and 8 mono-*ortho* (MO-) PCBs with only one chlorine atom in *ortho* position. It is the planar structure that leads to the same toxicity as the dioxins, and in fact the most toxic PCB congeners, namely, 3,3',4,4',5-pentachlorobiphenyl (PCB 126) and 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169) are approximate isostereomers of 2,3,7,8-TCDD, whit similar structural and spatial configuration (Safe S. et al., 1985). Toxic DL-PCBs and are listed in Table I.4.



PCB 126



PCB 169

Figure I.4: Examples of planar structure of the two most toxic dioxin-like PCBs.

Table I.4: Toxic dioxin-like PCBs.

	co-PCBs	Acronym
1	3,3',4,4'-tetrachlorobiphenyl	PCB 77
2	3,4,4',5- tetrachlorobiphenyl	PCB 81
3	3,3',4,4',5-pentachlorobiphenyl	PCB 126
4	3,3',4,4',5,5'-hexachlorobiphenyl	PCB 169
	MO-PCBs	**Acronym**
1	2,3,3',4,4'-pentachlorobiphenyl	PCB 105
2	2,3,4,4',5- pentachlorobiphenyl	PCB 114
3	2,3',4,4',5- pentachlorobiphenyl	PCB 118
4	2',3,4,4',5- pentachlorobiphenyl	PCB 123
5	2,3,3',4,4',5- hexachlorobiphenyl	PCB 156
6	2,3,3',4,4',5'- hexachlorobiphenyl	PCB 157
7	2,3',4,4',5,5'- hexachlorobiphenyl	PCB 167
8	2,3,3',4,4',5,5'- heptachlorobiphenyl	PCB 189
	Total	12

NDL-PCBs are compounds expressing non-dioxin-like toxicity, and structurally speaking in these molecules the two phenyl rings are not in the same plane because of more than one chlorine atom in the *ortho* position (the planar structure is not the favourite because of the steric and electronic effect of more than one chlorine atom in the *ortho* position). NDL-PCBs have been shown to elicit neurological, endocrine, immunological and carcinogenic effects; in particular, several international agencies classify PCBs as probably carcinogenic to humans, even if in general, the non-*ortho* and mono-*ortho* substituted congeners are more potent than the di-*ortho* substituted congeners (ATSDR, 2000).
As explained later in this Introduction, PCBs were industrially produced as mixtures and marketed with the commercial name of Aroclor. After stopping PCB production, in order to evaluate possible contamination from Aroclor, six congeners (# 28, 52, 101, 138, 153, and 180 in Table I.5) were chosen as indicators for contamination from Aroclor on the basis of their relative ratio. The Scientific Panel on Contaminants in the Food Chain of the European Food Safety Authority (CONTAM Panel of EFSA), in its Scientific Opinion related to the presence of NDL-PCBs in feed and food, noticed that the sum of the six indi-

cator PCBs represented about 50% of the total NDL-PCB in food (European Food Safety Authority, 2010). For this reason NDL-PCBs are also referred to as “indicator PCBs” (I-PCBs).

Table I.5: List of I-PCBs.

	I-PCBs	Acronym
1	2,4,4’-trichorobiphenyl	PCB 28
2	2,2’,5,5’-tetrachorobiphenyl	PCB 52
3	2,2’,4,5,5’-pentachorobiphenyl	PCB 101
4	2,2’,3,4,4’,5’-hexachorobiphenyl	PCB 138
5	2,2’,4,4’,5,5’- hexachorobiphenyl	PCB 153
6	2,2',3,4,4',5,5'-heptachlorobiphenyl	PCB 180
	Total	6

2. Dioxin sources and environmental contamination

PCDDs and PCDFs were never produced intentionally as marketable products but they were unwanted by-products of industrial and combustion processes (Hites, 2011), that are considered “primary sources” (Fiedler, 2002). Due to their chemical, physical and biological stability PCDD/PCDF from “primary sources” are transferred to other matrices and enter the environment. Such “secondary sources” are sewage sludge/biosludge, compost, or contaminated soils and sediments.

2.1. Primary sources

Primary sources of environmental contamination with PCDD/PCDF in the past were due to production and use of chloro-organic chemicals. The propensity to generate PCDD/PCDF during synthesis of chemical compounds decreases in the following order: chlorophenols>chlorobenzenes>aliphatic chlorinated compounds>inorganic chlorinated compounds. Factors favourable for the formation of PCDD/PCDF are high temperatures, alkaline media, presence of UV-light, and presence of radicals in the reaction mixture/chemical process (Fiedler, 2002).

One of the classic example is the formation of 2,3,7,8-TCDD as by-product in the production of 2,4,5-trichlorophenol (also known as Dowicide 2, one product in the Dowicide antimicrobial series from Dow chemical company), which was synthesized by the reaction of 1,2,4,5-tetrachlorobenzene with sodium hydroxide (NaOH). Dimerization of the resulting 2,4,5-trichlorophenol produced small amounts of 2,3,7,8-TCDD, which contaminated the final commercial product (Figure I.5)

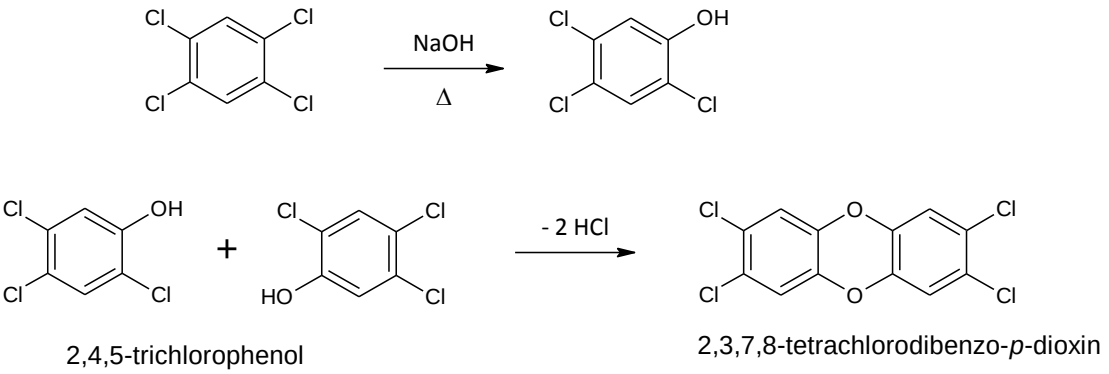


Figure I.5: 2,4,5-trichlorophenol dimerization in acidic media forming 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Although dioxins were present at very low levels in some commercial products, they entered the environment with uncontrolled release, causing very big environmental and health issues for animals and human, as described in some examples in the following paragraph about secondary sources of contamination.

At the present, changes in industrial processes have resulted in the reduction of PCDD/PCDF contamination in other products, but still a little amount of PCDD/PCDF into the environment via water and to soils derives also from kraft pulp (process for the production of pure cellulose fibres from lignin in alkali conditions) and paper mills, because of the wood treated with pentachlorophenol (PCP) or other chlorinated preservatives. Other PCP treated materials include textiles, leather goods, and cork products. However, today's dioxin input is mainly due to thermal processes. One (but not the only) of several examples) are waste incinerators, that are burning together a great variety of entry materials, at high temperature, in excess of oxygen and in presence of a catalyst. In these

conditions carbon and chlorine containing materials (PVC, chloroparaffines, organic dyes or inorganic chlorine are just little examples) lead easily to dioxin production. Most recent technology is aimed to reduce emissions of PCDD/Fs in the environment but it does occur in some extent.
PCB based electric fluids are another important source of contamination from dioxins in landfills.

2.2. Secondary sources

Secondary sources of PCDD/PCDFs are environmental and biota matrices contaminated by the primary emission sources and after the accidental contamination happened in the past, where toxicological effects of dioxins were not yet known.

Likely the first example of dioxin accidents was the "Chick Edema Disease" occurred in 1957 in the US, when millions of checks died mysteriously (Hites, 2011). After several researches, cause was traced to the fatty acids added to the chicken's feed and coming from hides for tanning industry. Fatty acids in fact were produced from the saponification of the fat removed from animal hides. In the end of the '50s, 2,3,4,6-tetrachlorophenol (sold as Dowicide 6) was added as a preservative to hides for tanning industry. Removed fat saponification caused tetrachlorophenol dimerization and 1,2,3,7,8,9- and 1,2,3,6,7,8-hexachlorodibenzo-*p*-dioxin production (similar reaction as reported in Figure I.5), that contaminated fatty acids for feed.
Another historical example happened in Vietnam where in the '70s, 2,4,5-trichlorophenol and hence 2,3,7,8-TCDD (see Figure I.5) were contaminants of the herbicide Agent Orange used by the U.S in large amount during the war in Vietnam (from 1955 to 1975). This caused proven environmental and health problems to veterans exposed to Agent Orange, whose tumour incidence was higher than the average.
In the '60s in a city called Times Beach in Missouri, chemical waste oil containing kilograms of 2,3,7,8-TCDD was first withdrawn for disposal, but then mixed with other waste oils and re-used for several purposes, as for example spray for indoor dust control. This "ingenuousness" caused animal death and environmental issues.

A very famous accident occurred in Europe, in Italy, in a northern town called Seveso, where in 1976 chemicals from a production plant using 2,4,5-trichlorophenol, and hence producing dioxins as by-products, were released into the environment as a safety measure for vessel overpressure. Within few days in that area chicken and rabbits died and children had first skin diseases. The area was immediately evacuated for decontamination, with social and economic consequences. With the time, several chloracne cases were recorded among exposed people, as well as higher female children natality from exposed fathers.

Unfortunately the list of accident of dioxin contamination is longer and some other examples are reported later because they involve also PCBs.

2.3. Fate and transport

Due to their high lipophilicity and low water solubility, after their introduction in the environment, PCDD/PCDFs are primarily bound to particulate and organic matter in soil and sediments. Their resistance to degradation and semi-volatility caused their transport over long distances and their past released into the environment still contributes to contemporary exposure. Despite their low solubility, dioxins are slowly released from sediments into water and they can be adsorbed by biota, assimilated by small fishes and so enter the food chain. Due to their lipophilicity and metabolic resistance, they are concentrated and accumulated in fatty tissues and tend to bioaccumulate in higher animals, including humans (Buckley-Golder, 1999).

3. PCB sources and fate

3.1. Industrial production

Contrary to PCDDs and PCDFs, PCBs were industrially produced as complex mixtures of several congeners for a variety of uses, including dielectric fluids, capacitor and transformer components because of their good electrical insulating properties, as well as additives in plasticizers or lubricants. PCBs were used in minor extent also in pesticides, inks, paints, flame retardants and many other applications. (Erickson, 1997). The list of PCB uses is very long and it involves common domestic goods; their chemical and physical stability led to their commercial utility on one side and to environmental contamination problem on the other side.

PCBs were produced via direct chlorination of biphenyl with gaseous chlorine at high temperature and in presence of $FeCl_2$ as catalyst. The extent of chlorination was controlled with reaction time (Figure I.6).

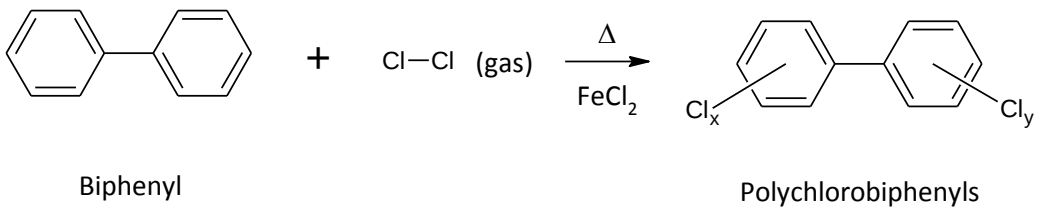


Figure I.6: PCB general production reaction.

The major producer was the USA with Monsanto Corporation (St. Louis, MO) that marketed PCBs under the name Aroclor®. But, due to their commercial importance, PCBs were produced all over the world as shown in Table I.6 (Breivik et al., 2002)

Table I.6: PCB total production in tons as reported in literature (Breivik et al., 2002). Total amount in tons is the global production over the period 1930-1993.

Producer	Country	Start	Stop	Amount (tons)
Monsanto	USA	1930	1977	641 246
Geneva Ind.	USA	1971	1973	454
Kanegafuchi	Japan	1954	1972	56 326
Mitsubishi	Japan	1969	1972	2 461
Bayer AG	West Germany	1930	1983	159 062
Prodelec	France	1930	1984	134 654
S.A. Cros	Spain	1955	1984	29 012
Monsanto	UK	1954	1977	66 542
Caffaro	Italy	1958	1983	31 092
Chemko	Czechoslovakia	1959	1984	21 482
Orgsteklo	USSR (Russia)	1939	1990	141 800
Orgsintez	USSR (Russia)	1972	1993	32 000
Xi'an	China	1960	1979	8 000
Total		1930	1993	1 325 131

Most likely the true cumulative global production has been higher, but at least these numbers give and idea of the production scale.
In 1977 the USA banned PCB production because of their potential carcinogenicity (ATSDR, 2014), but globally PCB production stopped in 1993 with Russia, despite the documented toxic effects of these compounds on biota.

The Aroclor mixtures are identified by a four-digit numbering code in which the first two digits indicate the type of mixture and the last two digits indicate the approximate chlorine content by weight percent. For example, Aroclor 1242 is a chlorinated biphenyl mixture of varying amounts of mono- through heptachlorinated homologues with an average chlorine content of 42% (Hutzinger et al. 1974).

Table I.7: Average molecular composition (wt%) of some Aroclor.

Homologues	Aroclor						
	1221	1232	1016	1242	1248	1254	1260
0	10						
1	50	26	2	1			
2	35	29	19	13	1		
3	4	24	57	45	22	1	
4	1	15	22	31	49	15	
5				10	27	53	12
6					2	26	42
7						4	38
8							7
9							1

As reported in Table I.7 main constituents of commercial mixtures are homologues with 3 to 7 chlorine atoms, which include very toxic DL-PCBs and NDL-PCBs, that therefore are used as indicators of contamination from Aroclor mixtures.

Among other impurities, commercial PCB mixtures contained tetra- and penta-CDF congeners as reaction by-products. The presence of PCDFs in PCB mixtures has been documented at µg/g level (part per million, ppm) and may account for some toxicological properties of PCB mixtures; PCDDs have not been found in marketed products (Erickson, 1997).

3.2. Secondary sources

Secondary PCB sources, after the stop of their production, are industrial processes where PCBs are produced as by-products, and items already containing PCBs, that release them with time. Outdated or illegal landfills are new sources of PCBs, as well as the burning of PCB-containing products, that has introduced large volumes of PCBs into the environment.

PCBs can incidentally be produced as by-products of other industrial processes using hydrocarbons and chlorine. These by-products are referred to as “incidental PCBs”. Industrial processes include manufacture of chlorinated solvents, chlorinated alkanes or some

pigments, as well as the thermal degradation of waste in incinerators. In all these cases, the composition and the amount of by-products is not exactly known and traceable (Erickson, 1997).

Due to the long service life of many PCB-containing items and the use of PCBs in some durable, relatively inert products, PCB-containing materials are still currently disposed and processed in waste and recycling operations, that can be a new source of PCBs if operations are not carried out properly (EPA, 2003).

PCBs are present in building demolition material, not always properly disposed of; they are in dumps, landfills and wastewater treatment plant sludge, and sometimes they enter the environment because of the releases into sewers and streams, improper disposal of PCB-containing equipment in non-secured landfill sites and municipal disposal facilities, and by other routes (such as sea dumping).

In the past, PCB food contamination episodes have occurred and they introduced big quantities of toxic persistent compounds (PCBs and their contaminants) in the food chain. Just an example was the “Yusho” accident, in Japan in 1968 (Tanabe et al., 1987) and the analogue “Yu-Cheng” accident in Taiwan in 1979 (Soongl D. K., 1997) where rice oil for food was accidentally mixed with PCB containing oil used as heat exchange fluid in the production factory.

In Europe, namely in France in the ‘ 70s, French cheese was accidentally contaminated with technical oil from farm engines and some poultry was poisoned with PCB-contaminated plastic wire netting; accident happened also in The Netherlands and in Germany in the ‘80s and ‘90s, contaminating environment, food and feed with dioxins and PCBs (Covaci et al., 2008). But it was in 1999 that the most mediatised crisis occurred and it was the “Belgian PCB/dioxin crisis”, when PCB containing transformer waste oil was inadvertently mixed with recycled fats used in the production of animal feeds and further distributed to chicken and pig farms. PCB oil was contaminated with dioxins and this resulted in the slaughter of thousands of animals, with resulting political and economic crisis.

3.3. Fate and transport

Some of the PCB commercial mixtures applications were “open ended” (component of dust control formulations, paints, and inks, carbonless copy paper, flame retardants, pesticide additives) and resulted in widespread low level releases to environmental compartments. Closed and controlled uses, such as dielectrics within electric equipment have still resulted in environmental release because of spills, improper handling and improper disposal. This caused local but very high concentration contamination. Once in the environment, PCBs had the same fate as dioxins because of similar chemical and physical properties, such as high lipophilicity and stability. PCBs were adsorbed on the organic matter of sediments and soil. With time they have been transported also to remote areas and widespread in different sites, so that, at the present, PCBs are ubiquitous environmental pollutants.

PCBs volatilize from both soil and water, and once in gas phase, they can be transported long distances in air, and then redeposited by settling or scavenging by precipitation. This cycling process continues indefinitely and is referred to as the grasshopper effect (EPA, 2001). Clear evidence of the atmospheric deposition of PCBs is the presence of PCBs in remote areas of the planet and their accumulation in polar bears. From the environment, they were taken up by small organisms and fishes and entered the food chain, where they tend to bio-accumulate as dioxins: PCBs have been found in animal adipose samples, milk, sediments and numerous other matrices.

4. Risk assessment, Toxic Equivalent Factor (TEF) and European Regulation

Media and public opinion spotlighted the Belgian dioxin crisis and the lack of food safety, so early in 2000 the European Union (EU) was pushed to start an efficient monitoring program to ensure the proper quality of European food and feed (Focant, 2012). As a consequence, starting from 2000, the European Commission (EC) began to propose legislation to regulate Maximum Residual Levels (MLs) for PCDDs, PCDFs and DL-PCBs in foodstuffs and feed products, as well as guidelines for analytical methods to support and implement continuous monitoring of food and feed. PCDDs, PCDFs and PCBs exist as

mixtures of congeners and this complicates the risk evaluation. As all these chemicals have similar actions on the AhR but different potencies, a toxic equivalent factor (TEF) was developed to assess the impact of all these compounds on human and environmental health, as well as for regulatory control of exposure to these mixtures.
TEF values are based on the relative toxicity of a chemical in comparison with 2,3,7,8-TCDD, which is the most toxic congener with a TEF value of 1. Other 2,3,7,8-substituted PCDD, PCDF and PCB congeners, with similar planar structure, and hence similar interactions with AhR receptor, have been assigned a TEF value. In 1997 an expert meeting was organized in Stockholm by the World Health Organization (WHO) to determine TEF values for specific congeners based on existing literature toxicological data available at that moment (Van.den.Berg et al., 1998). Included congeners had

- Structural similarity to 2,3,7,8-TCDD
- Capacity to bind to the aryl hydrocarbon receptor (AhR);
- Capacity to elicit AhR-mediated biochemical and toxic responses;
- Persistence and accumulation in the food chain

In 2005, a further WHO expert panel updated TEF values for dioxin-like compounds (Van den Berg et al., 2006). They reaffirmed the characteristics necessary for the inclusion of a compound in the WHO's TEF list, but they changed TEF value for some congener based on new and updated toxicological data. As said, 2,3,7,8-TCDD is the most toxic congener with a TEF value of 1 and all other congeners have lower TEFs ranging from 0.00001 to 0.5 (Table I.8).

Table I.8: WHO 1198 and 2005 TEF values for dioxin-like compounds. Numbers in bold indicate a change in TEF values from 1998 to 2005

	Compound	WHO 1998 TEF	WHO 2005 TEF
	PCDDs		
1	2,3,7,8-TCDD	1	1
2	1,2,3,7,8-PeCDD	1	1
3	1,2,3,4,7,8-HxCDD	0.1	0.1
4	1,2,3,6,7,8-HxCDD	0.1	0.1

5	1,2,3,7,8,9-HxCDD	0.1	0.1
6	1,2,3,4,6,7,8-HpCDD	0.01	0.01
7	OCDD	0.001	**0.0003**
	PCDFs		
1	2,3,7,8-TCDF	0.1	0.1
2	1,2,3,7,8-PeCDF	0.05	**0.03**
3	2,3,4,7,8-PeCDF	0.5	**0.3**
4	1,2,3,4,7,8-HxCDF	0.1	0.1
5	1,2,3,6,7,8-HxCDF	0.1	0.1
6	1,2,3,7,8,9-HxCDF	0.1	0.1
7	2,3,4,6,7,8-HxCDF	0.1	0.1
8	1,2,3,4,6,7,8-HpCDF	0.01	0.01
9	1,2,3,4,7,8,9-HpCDF	0.01	0.01
10	OCDF	0.0001	**0.0003**
	co-PCBs		
1	PCB 77	0.0001	0.0001
2	PCB 81	0.0001	**0.0003**
3	PCB 126	0.1	0.1
4	PCB 169	0.01	**0.03**
	MO-PCBs		
1	PCB 105	0.0001	**0.00003**
2	PCB 114	0.0005	**0.00003**
3	PCB 118	0.0001	**0.00003**
4	PCB 123	0.0001	**0.00003**
5	PCB 156	0.0005	**0.00003**
6	PCB 157	0.0005	**0.00003**
7	PCB 167	0.00001	**0.00003**
8	PCB 180	0.0001	**0.00003**

Risk assessment based on the TEF approach starts from the important assumption that the combined effects of the different congeners are dose or concentration additive. Therefore, TEF values of each congener, multiplied by its concentration, can be used to calculate the toxic equivalent (TEQ) concentration of an environmental or biological sample. TEQ calculation is based on the following formula, and allows the estimation of sample global toxicity.

$$TEQ = \sum_{n=1}^{7} [PCDD]_n * TEF_n + \sum_{m=1}^{10} [PCDF]_m * TEF_m + \sum_{q=1}^{12} [PCB]_q * TEF_q$$

TEFs and TEQs are used for risk characterization and regulation purposes because they allow converting quantitative analytical data for individual PCDD/PCDF and PCB congeners into a single parameter representing the global toxicity of the sample, the TEQ. At the present the reference regulation for maximum residual levels (MLs) of dioxins in food and feed stuff in Europe are based on TEQ levels and the most recent are the EU Regulation 1881/2006 with all its amendments for food, and the Directive 2002/32/CE with all its amendments for MLs in feed.

In order to support a continuous monitoring program for food and feed safety in Europe and ensure rapid action in case of non-compliant samples, dedicate legislation was done by the EC for analytical methods of sampling and analysis for the control of PCDD/Fs and DL-PCBs in food and feed. The most recent versions are the Commission Regulation 589/2014 for analytical methods for quantitative analysis in food, and the Commission Regulation 709/2014 for analytical methods for quantitative analysis in feed, both released as amendments of previous Regulation arisen after the Belgian dioxin crisis. These Regulations describe the criteria to meet when doing dioxin analysis for confirmatory or screening purposes without laying down just one analytical method to follow, so actually there is a great variety of an analytical procedures to perform dioxin and PCB analysis in food and feed, from sample preparation to instrumental detection. In particular a major recent update is the recognition of gas chromatography (GC) triple quadrupole mass spectrometry (GC-MS/MS Triple Quad) as a confirmatory tool for checking compliance with MLs. Triple Quad instruments are cheaper and more widespread instruments than Magnetic Sector high resolution mass spectrometers (HRMS), and so, thanks to this modification in the EU Regulation, the number of laboratories participating to food safety monitoring program can increase.

In this thesis dioxin analysis has been carried out on the basis of the routine procedure developed in our laboratory (CART at the University of Liège). This procedure has been

modified in the sample preparation part related to the automated clean-up, with the aim of reducing solvent and time consumption, in order to have more environmental sustainable, cheaper and faster procedures. From the instrumental point of view, a validated method based on GC-MS/MS Triple Quad instrument (L'Homme et al., 2015b) has been used to assess the recoveries of the alternative clean-up methods, as described in Chapters 1, 2 and 3. Chapter 1 reports the description of the instrumental method and all the steps followed for its validation. The work load of the author of this thesis has been related to method optimization and continue usage, rather than to the initial development.

Scientific context of the research about Dechloranes

Dechloranes are organo-chlorinated compounds sharing a bicycle [2,2,1] heptane structure. The first of these compounds was called Mirex, or Dechlorane, and it was extensively used as pesticide and as additive for flame retardants in the USA during the '60s and the '70s until 1978 when it was banned because of its toxicity, persistence and bioaccumulation (Kaiser, 1978). In fact Mirex is in the POPs list of the Stockholm convention (Table I.1), but other Dechloranes, namely Dechlorane Plus, syn- and anti-isomers, (DP), Dechlorane 602 (Dec 602), Dechlorane 603 (Dec 603,), Dechlorane 604 (Dec 604), Chlordene Plus (CP) are unregulated and they are currently used as replacement of Mirex or decabromodiphenyl ether (deca-BDE, BDE-209) for their pesticide and flame retardant properties. They are extensively used as additives in various synthetic products such as nylon or plastic like polypropylene, as well as in electronic devices (Sverko et al., 2011). They have recently been reported at low levels in environmental samples, or in dust collected from various environments (Dodson et al., 2012, Cao et al., 2014). Biota and humans are exposed to these chemicals and in fact very recent human biomonitoring studies have reported levels at the ng/g lipid level in breast milk from Canada (Zhou et al., 2014), as well as in human serum from Norway (Cequier et al., 2015) and France (Brasseur et al., 2014), even though no production sources have been found in Europe.
In this thesis, with the goal of understanding the extent and the origin of human exposure to Dechloranes, food consumption was investigated as a possible route of exposure for people from Belgium, as, due to their similarity to Mirex, other Dechloranes can be subject to bioaccumulation and long range transport. Because of the emerging character of these analytes, the first part of the study has been dedicated to the development of a specific method for the analysis of 6 Dechloranes (Dec 604 was not detectable at the level of interest). The sample preparation procedure currently applied for dioxin analysis demonstrated to be suitable for Dechlorane analysis; final extracts were injected and quantitated by means of GC-MS/MS Triple Quad, while usually HRMS instruments have been used for such compounds; the method was validated following the applicable guidelines of the stringent EU Regulation for dioxin analysis (589/2014 and 709/2014), and finally Dechlorane levels have been assessed in 88 selected food and feed samples to produce a first estimate of Dechlorane dietary intake for the Belgian population. Details

are reported in Chapter 4. The results of this work have been published in peer-reviewed journal.

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CHAPTER I

Description of a validated gas chromatography-triple quadrupole mass spectrometry method for confirmatory analysis of dioxins and dioxin-like polychlorobiphenyls in feed.

1. Introduction

After the "Dioxin/PCB crisis" of Belgium in 1999, the European Union began intense controls of the food and feed web. Dedicate legislation was done by the European Commission (EC) for setting maximum residual levels in food and feed, as well as for methods of sampling and analysis for the control of PCDD/Fs and DL-PCBs in environmental and biological matrices. TEFs and TEQs, calculated from quantitative and toxicological data for individual PCDD/PCDF and PCB congeners, were used for regulation purposes to have a single parameter representing the global toxicity of the sample. As already reported in the Introduction, at the present in Europe the reference regulation for maximum levels (MLs) of dioxins and PCBs in food and feed stuff are the EU Regulation 1881/2006 (with all its amendments) for food, and the Directive 2002/32/CE (with all its amendments) for MLs in feed.

When dioxin controls started, each laboratory had its own method of analysis; no standardized or harmonized method was available. The European Commission then, rather than a single standardized method, adopted a performance-based measurement system (PBMS) approach to allow laboratories to use their own methods as far as they were able to meet the requirements in terms of sensitivity, selectivity and accuracy. So, harmonized quality criteria for biological and gas chromatography coupled to mass spectrometry (GC-MS) measurements were defined and proposed as guidelines (Malisch R. et al., 2001, Behnisch P.A. et al., 2001). The general concept of harmonized quality criteria gave the flexibility of maintaining already existing methods with maybe the need to modify or to improve the analytical procedure. Doing so, the possibility of adding new knowledge and technology with time was let open (Focant and Eppe, 2013).

One of the major harmonized quality criteria for the confirmatory method was the use of ^{13}C-labelled isotope dilution (ID) technique for exact congener identification and quantitation, and the use of gas chromatography (GC) coupled to Magnetic Sector High-Resolution mass spectrometers (GC-HRMS) for trace level analysis, as it was the most sensitive and selective instrument at that time. Other available mass analyzers, high or low resolution, such as time-of-flight (TOF), single quadrupoles (Q), and quadrupole ion storage tandem-in-time (QIST), were not yet sensitive enough for the very low detection levels required for dioxin analysis (Focant et al., 2005).

Anyway, technical progresses of the last decade in the area of GC coupled to tandem-in-space MS (GC-MS/MS) using triple quadrupole analyzers (QQQ), led this modern instrumentation to exhibit performances similar to GC-HRMS (García-Bermejo et al., 2015, Kotz A et al., 2011, Sandy C et al., 2011, Ingelido et al., 2012). Based on these reports, a working group formed within the network of European Reference Laboratory (EU-RL) and National Reference Laboratories (NRLs) of EU Member States has successfully investigated the capability of GC-MS/MS Triple Quadrupole instruments for potential use as an alternative method to HRMS for quantitative confirmatory analysis of dioxins. Basic principles of these two instruments are so different that some specific criteria were proposed for method validation when using GC-MS/MS Triple Quad (Kotz et al., 2012), for example for the calculation of the quantitation limit (limit of quantitation, LOQ) that is a crucial parameter in dioxin analysis as described later in this chapter. These criteria were accepted at the EU level and new legislation was issued: the EU Regulation 589/2014 (EC, 2014a) and the EU Regulation 709/2014 (EC, 2014b), referring to the use of GC-MS/MS Triple Quad instruments as an appropriate confirmatory technique for checking compliance with the MLs in food and feed control, respectively.

In addition to regular control at maximum levels, the investigation of low background levels is also of prime interest for risk assessment. In this case it is important to establish congener patterns at very low levels, typically below one fifth of the level of interest in food-feed, to identify the source of a possible contamination. At the moment, for such measurements the use of GC-HRMS is still recommended to attain sufficient sensitivity, as studies about the use of GC-MS/MS Triple Quad are still in progress.

In this chapter, a fully validated method for the control of PCDDs, PCDFs, and DL-PCBs in vegetable oil using GC-MS/MS Triple Quad is described, from sample preparation to

instrumental quantification. The method, developed and validated on vegetable oil, has been used for the instrumental quantification of dioxins in the experimentation of this overall work of thesis, with modification and optimization for matrices other than vegetable oil. In fact, most of the determinations described in the following chapters have been carried out using GC-MS/MS Triple Quad instrument, except for determinations in Chapter 3 where a GC-HRMS method has been used. The full description of the method validation process is out of the scope of this chapter and it has been already reported elsewhere (L'Homme et al., 2015b), so only aspects relevant for the successive discussion are reported, such as acquisition parameters (multiple reaction monitoring (MRM) transitions), retention time, selectivity, accuracy (trueness and precision), measurement uncertainty. Special focus is dedicated to the proper establishment of blank levels and LOQ because no homogeneous criteria for the calculation have been reported in the Regulation up to date. Method description is based on vegetable oil for feed as sample matrix even if the method has been applied to several matrices, because vegetable oil was used in the validation process and validation parameters have been calculated referring to vegetable oil maximum levels, as required by the EU Regulation. The reference EU Regulation is the number 709/2014 (EC, 2014b) for the analytical method and the Directive 2002/32/EC (EC, 2002) for the MLs of dioxins and PCBs in feed.
Nevertheless the global method is applicable to other food and feed matrices, using the appropriate sample amount and sample preparation (described for egg and milk in Chapter 2) and checking/adapting instrumental performances.

2. General main steps of analytical methods for dioxin and PCB analysis

The characteristics of modern methods for PCDD, PCDF and DL-PCB determination are the consequence of several factors related to their chemical, physical and toxicological properties, as well as to the presence of strict Regulation due to the legal, political, economic and social importance of this analysis (Reiner et al., 2006). The exceptionally high toxicity of 2,3,7,8-TCDD and related compounds, their lipophilicity and their resistance to metabolic degradation necessitate reaching very low detection limits (DLs), in the order of part-per-quadrillion (ppq, 10^{-15} g of 2,3,7,8-TCDD per g of sample), that are required es-

pecially for bioaccumulation and chronic effects evaluation rather than acute toxicological effects. So, for dioxin analysis, analytical methods are required to be sensitive, selective and to reduce analysis time, as well as to generate robust data that could withstand scrutiny in a court of law.

The first and important barrier to successful analysis at trace level analysis is the separation of interesting compounds from the bulk sample matrix and from all other organic chemicals that could interfere in the selective quantification of toxic PCDD, PCDF and PCB congeners. For this reason analytical methods include extensive sample preparation with several and delicate separation steps.

From the instrumental point of view, it is also necessary to ensure selectivity and sensitivity and, since the past, this brought to the necessity of using methods based on isotope dilution–High Resolution Mass Spectrometry (ID-HRMS) technique. Characteristics of analytical methods are also influenced by the need for regulators to evaluate the combined toxicity of mixture of compounds with similar toxicological mechanism to 2,3,7,8-TCDD, but widely varying potencies, and this aspect was taken into account with the introduction of TEF and TEQ.

In this context, the general analytic approach for dioxin analysis at trace levels includes 4 main steps aimed to isolate each toxic PCDDs, PCDFs and PCBs from the sample matrix and to identify each congener with high accuracy:

1. Efficient extraction of lipophilic compounds and transfer to an appropriate organic solvent. Isotope labelled standards of each congener are added before or after fat extraction depending on the sample matrix as stated in the reference Regulation;
2. Separation of toxic congeners from all other organic co-extracted compounds via sample clean-up, ensuring very high dioxin recoveries and the highest rate of elimination of all other compounds and the matrix effect;
3. Separation of toxic and regulated PCDD/Fs and PCBs from other less toxic congeners with very efficient and selective gas chromatography;
4. Recording of characteristic molecular fragments of toxic congeners using a very sensitive and selective mass spectrometer (HR Magnetic Sector or LR Triple Quadrupoles instruments).

These steps are quite general and applicable to environmental or biological matrices. The detailed operations effectively change from matrix to matrix, according to its chemical composition, and from lab to lab as well, based on the technologies employed.
Moreover, with time, thanks to the EU policy to reduce POP's emission and production, as well as the intense monitoring program to check dioxin levels in environment and biota, average dioxin levels have decreased and so analytical methods, from sample preparation to instrumental detection, are required to be more sensitive, more selective, but also faster, cheaper and more environmental sustainable. Hence, each step of the main dioxin analysis scheme is still evolving and has been optimized with time. Thanks to the introduction of more efficient technologies, also the Regulation is evolving, as described in this chapter.

3. Chemicals and consumables

As reported in the EU Regulation 709/2014 (paragraph 3 about Quality assurance requirements), solvent and consumables have to be checked for possible influence on final results. In all the analysis, solvents (hexane, dichloromethane, toluene) were Picograde® reagents (LGC Promochem, Wesel, Germany) and solvent batches were tested to investigate analyte contamination before use. Nonane puriss analytical-reagent grade standard for GC were purchased from Fluka (Steinheim, Germany). Silica gel was purchased from Macherey-Nagel (Macherey-Nagel GmbH & Co KG, Düren, Germany) Disposable PTFE columns for PowerPrep™ automated clean-up system were obtained from Fluid Management Systems (FMS Inc., Waltham, MA, USA). Chromatographic pure grade helium gas, 99.9999% alphagaz 2 was purchased from Air Liquide (Paris, France). Technical N27 grade liquid CO_2 was used for PTV cooling (Air Liquide, Paris, France).
In our accredited ISO 17025 routine laboratory, two fractions are normally collected out of the clean-up system and injected for instrumental quantification: fraction one (F1) containing indicator (I-) and mono-*ortho* (MO-)PCBs, and fraction two (F2) containing coplanar (co-)PCBs, PCDFs and PCDDs. All the congeners were quantitated with Isotope (ID) dilution technique against their corresponding ^{13}C-labbeled internal standard. Recoveries were assessed adding recovery standard solutions to each fraction to check they were in

the range 60 – 120 % as stated in the EU Regulation 709/2014 (paragraph 6.2). However, recoveries did not affect final quantification of native compounds, because corresponding isotope labelled internal standard was added for each congener (Table 1.1), and the final quantification of the native compound was based on the ratio native *vs* labelled, that was not influenced by any analyte loss.

Table 1.1: Labelled and recovery standard of each analyte.

	PCDD congener	**Labelled ISTD**	**Recovery standard**
1	2,3,7,8-TCDD	2,3,7,8-TCDD ($^{13}C_{12}$, 99%)	1,2,3,4-TCDD ($^{13}C_{12}$, 99%)
2	1,2,3,7,8-PeCDD	1,2,3,7,8-PeCDD ($^{13}C_{12}$, 99%)	1,2,3,4-TCDD ($^{13}C_{12}$, 99%)
3	1,2,3,4,7,8-HxCDD	1,2,3,4,7,8-HxCDD ($^{13}C_{12}$, 99%)	1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)
4	1,2,3,6,7,8- HxCDD	1,2,3,6,7,8- HxCDD ($^{13}C_{12}$, 99%)	1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)
5	1,2,3,7,8,9- HxCDD	1,2,3,7,8,9- HxCDD ($^{13}C_{12}$, 99%)	1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)
6	1,2,3,4,6,7,8-HpCDD	1,2,3,4,6,7,8-HpCDD ($^{13}C_{12}$, 99%)	1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)
7	OCDD	OCDD ($^{13}C_{12}$, 99%)	1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)
	PCDF congener	**Labelled ISTD**	**Recovery standard**
1	2,3,7,8-TCDF	2,3,7,8-TCDF ($^{13}C_{12}$, 99%)	1,2,3,4-TCDD ($^{13}C_{12}$, 99%)
2	1,2,3,7,8-PeCDF	1,2,3,7,8-PeCDF ($^{13}C_{12}$, 99%)	1,2,3,4-TCDD ($^{13}C_{12}$, 99%)
3	2,3,4,7,8-PeCDF	2,3,4,7,8-PeCDF ($^{13}C_{12}$, 99%)	1,2,3,4-TCDD ($^{13}C_{12}$, 99%)
4	1,2,3,4,7,8-HxCDF	1,2,3,4,7,8-HxCDF ($^{13}C_{12}$, 99%)	1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)
5	1,2,3,6,7,8-HxCDF	1,2,3,6,7,8-HxCDF ($^{13}C_{12}$, 99%)	1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)
6	1,2,3,7,8,9-HxCDF	1,2,3,7,8,9-HxCDF ($^{13}C_{12}$, 99%)	1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)
7	2,3,4,6,7,8-HxCDF	2,3,4,6,7,8-HxCDF ($^{13}C_{12}$, 99%)	1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)
8	1,2,3,4,6,7,8-HpCDF	1,2,3,4,6,7,8-HpCDF ($^{13}C_{12}$, 99%)	1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)
9	1,2,3,4,7,8,9-HpCDF	1,2,3,4,6,7,8-HpCDF ($^{13}C_{12}$, 99%)*	1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)
10	OCDF	OCDF ($^{13}C_{12}$, 99%)	1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)
	Co-PCBs	**Labelled ISTD**	**Recovery standard**
1	PCB 77	PCB 77 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)
2	PCB 81	PCB 81 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)
3	PCB 126	PCB 126 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)
4	PCB 169	PCB 169 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)
	MO-PCBs	**Labelled ISTD**	**Recovery standard**
1	PCB 105	PCB 105 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**
2	PCB 114	PCB 114 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**
3	PCB 118	PCB 118 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**
4	PCB 123	PCB 123 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**

5	PCB 156	PCB 156 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**
6	PCB 157	PCB 157 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**
7	PCB 167	PCB 167 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**
8	PCB 189	PCB 189 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**
	I-PCBs	**Labelled ISTD**	**Recovery standard**
1	PCB 28	PCB 28 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**
2	PCB 52	PCB 52 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**
3	PCB 101	PCB 101 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**
4	PCB 138	PCB 138 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**
5	PCB 153	PCB 153 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**
6	PCB 180	PCB 180 ($^{13}C_{12}$, 99%)	PCB 80 ($^{13}C_{12}$, 99%)**
	* 1,2,3,4,7,8,9-HpCDF is quantitated using 1,2,3,4,6,7,8-HpCDF ($^{13}C_{12}$, 99%) as internal standard because the corresponding labelled compound is used as recovery standard for hexa-, hepta- and octa-congeners. ** PCB 80 ($^{13}C_{12}$, 99%) recovery standard for co-PCBs, collected in fraction 2 with dioxins and furans, has different concentration than PCB 80 ($^{13}C_{12}$, 99%) recovery standard for MO- and I-PCBs that are collected in fraction 1 after sample clean-up		

Regulated MO-PCBs (including PCBs #105, 114, 118, 123, 156, 157, 167, 189) and I-PCBs (PCBs #28, 52, 101, 138, 153, 180) were quantitated with isotope dilution measuring the ratio of native *vs* labelled internal standard. Internal standard spiking solution for MO-PCBs (MBP-MKX) was purchased from Wellington Laboratories (Guelph, Canada), internal standard for I-PCB was from Cambridge Isotope Laboratories (CIL, Andover, MS, USA). Recovery standard EC-1414 from CIL, containing 3,3',5,5'-TetraCB (PCB 80 $^{13}C_{12}$, 99%) was used to assess possible losses during sample preparation with internal standard *vs* recovery standard ratio. The calibration curve for MO-PCBs and I-PCBs was prepared using EC-5179 (native I-PCBs), EC-4987 (native MO-PCBs) solutions from CIL, as well as labelled internal standard and recovery standard solutions listed above.

Regulated PCDD/Fs 2,3,7,8-substituted congeners and coplanar co-PCBs (PCBs #77, 81, 126, 169) were quantitated using ^{13}C-labelled internal standards EDF-4144 from CIL. Recoveries were measured with recovery standards (EDF-4145 solution from CIL): 1,2,3,4-TCDD ($^{13}C_6$, 99%) was used for recoveries of tetra- and penta- chlorinated compounds, 1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%) for hexa-, hepta-, and octa- chlorinated species, and 3,3',5,5'-TCB (PCB 80 $^{13}C_{12}$, 99%) for PCBs. Calibration curve standards for this fraction were purchased from CIL (EDF-4143).

To facilitate sample spiking in our routine lab, an internal standard mixture containing ^{13}C-labelled PCDDs, PCDFs, co-PCBs, and MO-PCB was prepared (ST-ROU); I-PCBs internal standard was added separately. For each sample 20 µL of ST-ROU and 10 µL of I-PCBs were added, resulting in a final amount in pg reported in Table 1.2.

Table 1.2: Labelled Internal Standard spike levels and Recovery Standard spike level

	Labelled ISTD	**pg**
1	2,3,7,8-TCDD ($^{13}C_{12}$, 99%)	25
2	1,2,3,7,8-PeCDD ($^{13}C_{12}$, 99%)	25
3	1,2,3,4,7,8-HxCDD ($^{13}C_{12}$, 99%)	60
4	1,2,3,6,7,8- HxCDD ($^{13}C_{12}$, 99%)	60
5	1,2,3,7,8,9- HxCDD ($^{13}C_{12}$, 99%)	60
6	1,2,3,4,6,7,8-HpCDD ($^{13}C_{12}$, 99%)	60
7	OCDD ($^{13}C_{12}$, 99%)	125
	Labelled ISTD	**pg**
1	2,3,7,8-TCDF ($^{13}C_{12}$, 99%)	25
2	1,2,3,7,8-PeCDF ($^{13}C_{12}$, 99%)	25
3	2,3,4,7,8-PeCDF ($^{13}C_{12}$, 99%)	25
4	1,2,3,4,7,8-HxCDF ($^{13}C_{12}$, 99%)	62.5
5	1,2,3,6,7,8-HxCDF ($^{13}C_{12}$, 99%)	62.5
6	1,2,3,7,8,9-HxCDF ($^{13}C_{12}$, 99%)	62.5
7	2,3,4,6,7,8-HxCDF ($^{13}C_{12}$, 99%)	62.5
8	1,2,3,4,6,7,8-HpCDF ($^{13}C_{12}$, 99%)	62.5
9	OCDF ($^{13}C_{12}$, 99%)	125
	Labelled ISTD	**pg**
1	PCB 77 ($^{13}C_{12}$, 99%)	24
2	PCB 81 ($^{13}C_{12}$, 99%)	24
3	PCB 126 ($^{13}C_{12}$, 99%)	36
4	PCB 169 ($^{13}C_{12}$, 99%)	48
	Labelled ISTD	**pg**
1	PCB 105 ($^{13}C_{12}$, 99%)	2000
2	PCB 114 ($^{13}C_{12}$, 99%)	2000
3	PCB 118 ($^{13}C_{12}$, 99%)	2000
4	PCB 123 ($^{13}C_{12}$, 99%)	2000
5	PCB 156 ($^{13}C_{12}$, 99%)	2000
6	PCB 157 ($^{13}C_{12}$, 99%)	2000

7	PCB 167 ($^{13}C_{12}$, 99%)	2000
8	PCB 189 ($^{13}C_{12}$, 99%)	2000
	Labelled ISTD	**pg**
1	PCB 28 ($^{13}C_{12}$, 99%)	2000
2	PCB 52 ($^{13}C_{12}$, 99%)	2000
3	PCB 101 ($^{13}C_{12}$, 99%)	2000
4	PCB 138 ($^{13}C_{12}$, 99%)	2000
5	PCB 153 ($^{13}C_{12}$, 99%)	2000
6	PCB 180 ($^{13}C_{12}$, 99%)	2000

	Recovery standard	**pg**
F1	PCB 80 ($^{13}C_{12}$, 99%)	2000
F2	1,2,3,4-TCDD ($^{13}C_{12}$, 99%)	12.5
	1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)	31.25
	PCB 80 ($^{13}C_{12}$, 99%) for co-PCBs	24

4. Sample preparation for vegetable oil in our laboratory

As reported in the Introduction of this thesis, the experimental activity was carried out at the University of Liège, at CART laboratory, that is a ISO 17025 accredited national reference laboratory for dioxin and PCB analysis. Sample preparation in this work of method validation was done according to the validated method used for routine analysis.

For vegetable oil, sample preparation doesn't include fat extraction as the matrix is already pure fat. Anyways sample clean-up is still indispensable in order to remove matrix interferences, isolate and fractionate trace level dioxins from interfering chlorinated compounds, PCB included. The procedure used is described in detail in Chapter 3, that reports the development of alternative clean-up approaches based on our routine method. Briefly, an aliquot of 4 grams of vegetable oil was weighted, diluted in 10 mL of hexane and spiked with IS solution (20 µL of internal standard ST-ROU and 10 µL of I-PCBs solution). Then the sample was directly cleaned-up according to our routine procedure that includes a preliminary fat digestion with a manually packed acidic silica column, followed by clean-up and fractionation with PowerPrep™ automated system from FMS (Fluid Management System Inc.).

Preliminary lipid digestion was done with disposable glass column manually packed as follows: (from the bottom) glass wool, 5 g Na_2SO_4, 5 g neutral silica, 20 g 44% acidic silica, 20 g 22% acidic silica. Hexane was used for column conditioning and elution. Collected eluate was evaporated to approximately 10 mL before the introduction in PowerPrep™ automated system for further clean-up and for fractionation of I- and MO-PCBs (usually found at part-per-billion (ppb) level in food and feed) from PCDDs, PCDFs and co-PCBs (usually found at part-per-trillion (ppt) level). PowerPrep™ system was equipped with 3 column setup: standard multilayer silica column (4 g acidic, 2 g basic, 1.5 neutral), basic alumina (11 g), carbon AX-21 column (0.34 g). Two fractions were collected out of the system: fraction 1 (F1) eluted by forward elution of alumina/carbon column with a mixture of hexane/dichloromethane 50/50 and containing I- and MO-PCBs; fraction 2 (F2) collected by backflush elution of the carbon column with toluene and containing co-PCBs, PCDFs and PCDDs. Solvent volumes were reduced to approximately 500 µL in dedicated tubes using a sensor-equipped TurboVap II Workstation (Caliper Life Science, Teraflene, Belgium), to be then transferred in GC vials containing nonane (90 µL for F1 and 4 µL F2) as keeper. Final evaporation for solvent exchange to nonane was done with RapidVap (Labconco, Kansas City, MO, USA). Recovery standard was added to each fraction before instrumental analysis to assess recoveries.

5. GC-MS/MS Triple Quad instrumental setup

For quantitative analysis, a 7000C GC-MS/MS Triple Quad system from Agilent (Palo Alto, CA, USA) was used, according to a validated method developed in our laboratory (L'Homme et al., 2015b). Briefly, the triple Quad instrument was equipped with a 7890B GC oven, a programmable temperature vaporization (PTV) inlet, and a 7693A automated liquid sampler (ALS). PTV inlet was operated in solvent vent mode and cooled by liquid CO_2; its temperature program was the same for both fractions F1 and F2 and was chosen after an Experimental Design (DoE)(L'Homme et al., 2015a): start at 45°C (3 min) and ramp at 720°C/min until 320°C; vent flow of 100 mL/min at pressure of 10 psi for 2.8 min. Purge flow was set to 1200 mL/min after 5 min to avoid memory effect in the inlet. GC column was the classic DB-5ms 60 m × 250 µm × 0.25 µm (Agilent). Injection volume

was 5 µL for F2 and 2 µL for F1, and GC oven temperature program was different for the two fractions as well: for F2, oven program started at 120°C (5 min), ramped up at 25°C/min until 250°C (5 min), then 3°C/min until 285°C (15 min) for a total runtime of 41.6 min; for F1 the program started the same, but temperature was set at 285°C for 0 min for a total run time of 26.6 min. The transfer line temperature was held at 280°C in both cases. On the MS side, 7000C electron ionization (EI) ion source was heated at 280°C and operated at 70 eV. Quadrupoles were held at 150°C, nitrogen collision gas was flowing at 1.5 mL/min, and helium quench gas at 2.25 mL/min. Quads resolution was set to unit mass, which by default corresponds to peak width of 0.7 Da at half height.

6. Main features of the validated instrumental method with GC-MS/MS Triple Quad instrument

The method was validated using vegetable oil as a reference because it is a challenging matrix with the lowest ML (1.5 pg TEQ/g (ppt)). The method is still valid for matrices with higher ML, after double checking possible interferences or co-elution problems due to the matrix effect.

Maximum levels for vegetable oil, as well as other feed matrices are reported in the Directive 2002/32/EC and its amendments. Table 1.3 summarizes the MLs for vegetable oil, expressed in product basis normalized at 12% moisture content, which is the criterion used for all feed matrices. Average sample amount for vegetable oil in our laboratory was 4 g.

Table 1.3: MLs for vegetable oil, expressed in product basis normalized at 12% moisture.

Parameter	ML
pg WHO_{2005}-PCDD/F TEQ/g	0.75
pg WHO_{2005}-PCDD/F-DL PCB TEQ/g	1.50
Sum of NDL PCBs ng/g	10

6.1. Validation criteria in the EU Regulation

The validation of the GC-MS/MS Triple Quad method was done according to all the validation criteria listed in the recent EU Regulation No 709/2014 about analytical methods for dioxins and PCBs in feed, summarised in the first part of Table 1.4 for dioxins and DL-PCBs, and in the second part of Table 1.4 for NDL-PCBs.

Table 1.4: Criteria to be met when using GC-MS/MS for confirmatory PCDD, PCDF and DL-PCBs analysis in feed, according to the EU Regulation 709/2014.

Dioxins and dioxin-like PCBs

Criteria	Ref.*	PCDD/Fs and DL-PCBs
Specific criteria for GC-MS/MS: Multiple reaction monitoring (MRM) transitions	6.5	Monitoring 2 specific precursors with each specific product ion transition for all labelled and unlabelled analytes Maximum deviation from refrence Qual/Quant ion intensities ±15% Quadrupole resolution set at unit
Detectable quantity	5.1	PCDD/Fs = upper femtogram (10^{-15} g) co-PCBs = low pictogram (10^{-12} g) MO-PCB = nanogram (10^{-9} g)
Calibration range	6.4	Cover the relevant range (from 1/5x to 2x ML)
LOQ minimum value	5.5.2	1/5 of the maximum level for the selected matrix
LOQ calculation	2(b) and Kotz et al., 2012	LOQ calculated from average blank levels If no detection in blanks, LOQ = iLOQ, iLOQ = 10σ of the average 8 replicate injections of the lowest calibration point, giving a RRF with acceptable (≤30%) and

		consistent (RSD ≤ 15%) deviation to the ARRF
Acceptable difference between upper-bound and lower-bound at maximum level	6.1	Difference < 20%
Validation in the range of the maximum level	5.4.1	Demonstrate performances at 0,5x, 1x, 2x with acceptable RSD_r (x = ML)
High selectivity (specificity)	5.2 and 6.3	Sample preparation has to separate PCDD/Fs from interfering chlorinated compounds as NDL-PCBs and polychlorinated diphenyl ethers; Gas-chromatographic separation of 1,2,3,4,7,8-HxCDF and 1,2,3,6,7,8-HxCDF, 25% peak to peak
High Accuracy (trueness and precision)	5.3 and 5.6	Trueness: difference ±20% of certified value Precision (RSD_R) <15%
Blanks and QCs	5.4.2	Record control charts
Recovery check	6.2	Feed with fat content <10% ISTD added prior to extraction; Recoveries in the range 60 to 120 % for each congener
Measurement Uncertainty	2.2	Expanded measurement uncertainty with cover factor 2 (95% level of confidence)
Reporting of results	8	x ± U (expanded measurement uncertainty) results in the same units and the same significant figure as MLs set in the Regulation
* Ref.: number of the pertinent paragraph in the EU 709/2014		

NDL-PCBs

Criteria	**Ref.**	**NDL-PCB**
Specific criteria for GC-MS/MS: Multiple reaction monitoring (MRM) transitions	3.3	Monitor 1 precursor and 2 product ions Tolerance ratio ±20% if rel. intens. >50% Tolerance ratio ±25% if rel. intens. 20-50% Quadrupole resolution set at unit
Detectable quantity Calibration range		nanogram (10^{-9} g)
Calibration range	4	It has to include 0.5x and 2x the ML
LOQ minimum value		1/5 of the maximum level for the selected matrix
LOQ calculation		

Acceptable difference between upper-bound and lower-bound at maximum level		Difference ≤ 20%
Validation in the range of the maximum level	4	Demonstrate performances in the range 0.5 - 2x (x = ML)
High selectivity (specificity)	3.1 and 3.2	Deviation from ISTD retention time ±0.25%
High Accuracy (trueness and precision)		Trueness: difference ±30% of certified value Precision (RSD_R) ≤ 20%
Blanks and QCs	5 and 6	Blank < 30% of ML Quality control samples
Recovery check	7.3	Recoveries in the range 50 – 120 %
Measurement Uncertainty	10.4	Expanded measurement uncertainty with cover factor 2 (95% level of confidence)
Reporting of results	10	x ± U (expanded measurement uncertainty) results in the same units and the same significant figure as MLs set in the Regulation

Some of these criteria, such as LOQ calculation and MRM transitions, are specifically defined in the Regulation for GC-MS/MS Triple Quad or Magnetic Sector instrument, as for some aspect the two allowed techniques work in substantial different way. Briefly, GC-HRMS works in Selected Ion monitoring (SIM) and it does have noise, so signal to noise (S/N) ratio is used for LOQ calculation; GC-MS/MS Triple Quad is a tandem technique and works in Multiple Reaction Monitoring (MRM), so the quadrupoles are filtrating also the noise and S/N ratio does have no sense. For this reason LOQs are calculated from blank levels or form instrumental performances (Kotz et al., 2012) as better explained in paragraph 6.4. The other requirements, for example selectivity, upper-bound and lower-bound differences, calibration range, etc., are the same for both instrumental approaches. As shown in the second part of Table 1.4, criteria for NDL-PCB measurement in food and feed are generally less stringent than for PCDD/Fs and DL- PCBs and MLs are at the ng/g (ppb) levels, which makes their proper measurement easier to attain. However in our laboratory, the strict criteria of PCDD/Fs and DL-PCBs measurement were applied to NDL-PCBs for the validation.

6.2. MRM transitions

Measurements with GC-MS/MS Triple Quad were carried out in Multiple Reaction Monitoring (MRM) mode in tandem mass spectrometry. This is an important difference in comparison with the HR Magnetic Sector instrument that cannot work in MRM, but in Selected Ion Monitoring (SIM). The EU Regulation reports specific criteria for GC-MS/MS and demands to monitor, for each congener, two specific precursor ions with one specific product ion (point 6.5 in the Regulation 709/2014). In our method, for the most toxic PCDD/Fs and co-PCBs, three MRM transitions were followed, two for quantitation ('Quant transitions') and one for qualification ('Qual transition') purposes. The two Quant transitions corresponded to the same precursor ion, for a total of two specific precursor ions and three specific product ions for each congener, to prevent integration of interferences. The most intense Quant transition was used for congener quantification as reported later. For MO- and I-PCBs one Quant and one Qual transition were followed, for a total of two MRM transitions as the minimum requirement of the EU Regulation. Table 1.5 shows Quant and Qual transitions of all the congeners.

Tale 1.5: MRM Qual and Quant transitions recorded for all the congeners

PCDD congener	**Native Quant**	**Native Qual**	**Labelled Quant**	**Labelled Qual**
2,3,7,8-TCDD	319.9 -> 256.9, 258.9	321.9 -> 258.9	331.9 -> 267.9, 269.9	333.9 -> 269.9
1,2,3,7,8-PeCDD	355.9 -> 292.9, 290.9	353.9 -> 290.9	365.9 -> 301.9, 303.9	367.9 -> 303.9
1,2,3,4,7,8-HxCDD	389.8 -> 326.9, 328.8	391.8 -> 328.8	403.8 -> 339.8, 337.9	401.8 -> 337.9
1,2,3,6,7,8- HxCDD	389.8 -> 326.9, 328.8	391.8 -> 328.8	403.8 -> 339.8, 337.9	401.8 -> 337.9
1,2,3,7,8,9- HxCDD	389.8 -> 326.9, 328.8	391.8 -> 328.8	403.8 -> 339.8, 337.9	401.8 -> 337.9
1,2,3,4,6,7,8-HpCDD	423.8 -> 360.8, 362.8	425.8 -> 362.8	437.8 -> 373.8, 371.8	435.8 -> 371.8
OCDD	457.7 -> 394.8, 396.8	459.7 -> 396.8	469.7 -> 405.8, 407.8	471.7 -> 407.8
PCDF congener				
2,3,7,8-TCDF	303.9 -> 240.9, 242.9	305.9 -> 242.9	315.9 -> 251.9, 253.9	317.9 -> 253.9
1,2,3,7,8-PeCDF	339.9 -> 276.9, 274.9	337.9 -> 274.9	351.9 -> 287.9, 285.9	349.9 -> 285.9
2,3,4,7,8-PeCDF	339.9 -> 276.9, 274.9	337.9 -> 274.9	351.9 -> 287.9, 285.9	349.9 -> 285.9
1,2,3,4,7,8-HxCDF	373.8 -> 310.9, 312.9	375.8 -> 312.9	385.8 -> 321.9, 323.9	387.8 -> 323.9
1,2,3,6,7,8-HxCDF	373.8 -> 310.9, 312.9	375.8 -> 312.9	385.8 -> 321.9, 323.9	387.8 -> 323.9
1,2,3,7,8,9-HxCDF	373.8 -> 310.9, 312.9	375.8 -> 312.9	385.8 -> 321.9, 323.9	387.8 -> 323.9
2,3,4,6,7,8-HxCDF	373.8 -> 310.9, 312.9	375.8 -> 312.9	385.8 -> 321.9, 323.9	387.8 -> 323.9

1,2,3,4,6,7,8-HpCDF	407.8 -> 344.8, 346.8	409.8 -> 346.8	419.8 -> 355.8, 357.88	421.8 -> 357.8
1,2,3,4,7,8,9-HpCDF	407.8 -> 344.8, 346.8	409.8 -> 346.8		
OCDF	441.7 -> 378.8, 380.8	443.7 -> 380.8	453.7 -> 389.8, 391.8	455.7 -> 391.8
Co-PCBs				
PCB 77	289.9 -> 219.9, 221.9	291.9 -> 221.9	301.9 -> 231.9, 233.9	303.9 -> 233.9
PCB 81	289.9 -> 219.9, 221.9	291.9 -> 221.9	301.9 -> 231.9, 233.9	303.9 -> 233.9
PCB 126	323.9 -> 253.9, 255.9	325.9 -> 255.9	335.9 -> 265.9, 267.9	337.9 -> 267.9
PCB 169	359.9 -> 289.9, 287.9	357.8 -> 287.9	371.9 -> 301.9, 299.9	369.9 -> 299.9
MO-PCBs				
PCB 105	325.9 -> 255.9	327.9 -> 257.9	337.9 -> 267.9	339.9 -> 269.9
PCB 114	325.9 -> 255.9	327.9 -> 257.9	337.9 -> 267.9	339.9 -> 269.9
PCB 118	325.9 -> 255.9	327.9 -> 257.9	337.9 -> 267.9	339.9 -> 269.9
PCB 123	325.9 -> 255.9	327.9 -> 257.9	337.9 -> 267.9	339.9 -> 269.9
PCB 156	359.9 -> 289.9	361.9 -> 291.8	371.9 -> 301.9	373.9 -> 303.8
PCB 157	359.9 -> 289.9	361.9 -> 291.8	371.9 -> 301.9	373.9 -> 303.8
PCB 167	359.9 -> 289.9	361.9 -> 291.8	371.9 -> 301.9	373.9 -> 303.8
PCB 189	393.8 -> 323.8	395.8 -> 325.8	405.8 -> 335.8	407.8 -> 337.8
I-PCBs				
PCB 28	256.0 -> 186.0	258.0 -> 188.0	268.0 -> 198.0	270.0 -> 200.0
PCB 52	289.9 -> 219.9	291.9 -> 221.9	301.9 -> 231.9	303.9 -> 233.9
PCB 101	325.9 -> 255.9	327.9 -> 257.9	337.9 -> 267.9	339.9 -> 269.9
PCB 138	359.9 -> 289.9	361.9 -> 291.8	371.9 -> 301.9	373.9 -> 303.8
CB 153	359.9 -> 289.9	361.9 -> 291.8	371.9 -> 301.9	373.9 -> 303.8
PCB 180	393.8 -> 323.8	395.8 -> 325.8	405.8 -> 335.8	407.8 -> 337.8
Recovery standard				
1,2,3,4-TCDD ($^{13}C_6$, 99%)			325.9 -> 262.9, 264.9	327.9 -> 264.9
1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%)			419.8 -> 355.8, 357.8	421.8 -> 357.8
PCB 80 ($^{13}C_{12}$, 99%)			301.9 -> 231.9, 233.9	303.9 -> 233.9

Peak area of the most intense Quant transition was used for congener quantitation, calculated through the ratio with the area of the corresponding labelled ISTD according to *Equation 1* of the Isotope Dilution (ID) technique. For all the calculations, chromatograms without smoothing were used to stay as close as possible to raw data and to avoid any artificial effect on the signal and then congener quantification. The experimental ratio between Quant and Qual transition was monitored as well, to double check for interfer-

ences, and it could deviate maximum ±15% from the value assessed during the calibration as required by the Regulation. In case of higher deviation, peak integration was double checked to verify the signal of the Quant transition was not coming from an interfering compound. Actually, the whole procedure for MRM transitions limited the risk of integrating signals from interfering compounds.

$$RRF_i = \frac{area_{native}}{area_{ISTD}} * \frac{concentration_{ISTD}}{concentration_{native}} = \frac{area_{native}}{area_{ISTD}} * \frac{amount_{ISTD}}{amount_{native}} \qquad (Eq.1)$$

Relative response factors (RRF) for each congener (*i*) were assessed from the calibration curve using the same acquisition parameters of consecutive analyses. Congener Average Relative Response Factor (ARRF) reference value was calculated as the congener average RRF of all the calibration points, replicates included.
Dwell times and acquisition windows were set so to have an acquisition frequency of ten data points per peak.

6.3. Calibration range and retention time

The calibration range was chosen according to the guidelines of the Regulation: the lowest calibration point had to include the LOQ, which had to be 1/5 of the ML, and twice the ML because the method had to be validated at 0.5x, 1x and 2x ML (paragraph 5.4.1 of the Regulation). Calibration ranges are reported in Table 1.6, together with retention times, which were locked to PCB 105 to maintain the original setup in the acquisition and quantitation method even after cutting the chromatographic column. Deviation from retention time of individual labelled internal standards was also checked for the absence of interferences. The labelled internal standard eluted always few seconds before the unlabelled compound and a tolerance of 3 seconds was accepted for the retention time of the target compound.

Table 1.6: Calibration ranges for all the congeners

PCDDs	Retention time min	Lowest cali point pg/µL	Highest cali point pg/µL
2,3,7,8-TCDD	20.72	0.016	0.800
1,2,3,7,8-PeCDD	24.31	0.016	0.800
1,2,3,4,7,8-HxCDD	27.97	0.016	0.800
1,2,3,6,7,8- HxCDD	28.10	0.040	2.000
1,2,3,7,8,9- HxCDD	28.46	0.080	4.000
1,2,3,4,6,7,8-HpCDD	32.93	0.400	10.000
OCDD	39.35	4.000	120.000
PCDFs			
2,3,7,8-TCDF	20.30	0.016	0.800
1,2,3,7,8-PeCDF	23.26	0.016	0.800
2,3,4,7,8-PeCDF	24.06	0.016	0.800
1,2,3,4,7,8-HxCDF	27.02	0.016	0.800
1,2,3,6,7,8-HxCDF	27.15	0.016	0.800
1,2,3,7,8,9-HxCDF	27.79	0.016	0.800
2,3,4,6,7,8-HxCDF	28.94	0.016	0.800
1,2,3,4,6,7,8-HpCDF	31.09	0.080	4.000
1,2,3,4,7,8,9-HpCDF	33.92	0.016	0.800
OCDF	39.76	0.016	0.800
Co-PCBs			
PCB 77	17.71	0.320	8.000
PCB 81	18.02	0.320	8.000
PCB 126	20.92	0.320	8.000
PCB 169	24.17	0.320	8.000
MO-PCBs			
PCB 105	19.66	1.000	80.000
PCB 114	19.12	1.000	80.000
PCB 118	18.74	1.000	80.000
PCB 123	18.62	1.000	80.000
PCB 156	22.51	1.000	80.000
PCB 157	22.71	1.000	80.000
PCB 167	21.56	1.000	80.000
PCB 189	25.76	1.000	80.000
I-PCBs			
PCB 28	14.19	4.000	1000.000

PCB 52	14.79	4.000	1000.000
PCB 101	16.81	4.000	1000.000
PCB 138	20.46	4.000	1000.000
PCB 153	19.43	4.000	1000.000
PCB 180	23.14	4.000	80.000

6.4. *Limit of Quantitation (LOQ) and detectable quantity*

The proper establishment of the limit of quantitation (LOQ) is one of the major differences between the GC-HRMS method and the GC-MS/MS Triple Quad method. In GC-HRMS, LOQs are calculated from S/N (signal/noise) ratio, but this cannot be calculated on the GC-MS/MS Triple Quad because the two quadrupoles working in tandem are filtering ions, with the effect of deeply reducing also the noise and making S/N calculation meaningless. The EU Regulation 709/2014, at point 2, reports a definition of the LOQ for GC-MS/MS instruments, but it does not explain precisely how to calculate it. Such a definition of the LOQ can lead to several interpretation and way of calculation. This is undesirable as improper establishment of LOQs of individual congeners might lead to inaccurate final result calculations and inaccurate method performances estimation.

In our laboratory LOQ was calculated first from the levels of each congener in procedural blanks. As reported in the Regulation, procedural blank analysis shall be performed by carrying out the entire analytical procedure, omitting only the sample. Hence, for vegetable oil the blank consisted of 10 mL of hexane spiked with IS solutions and processed as the matrix.

For LOQ assessment, 12 procedural blanks were prepared and analysed. For each congener, the average level (in absolute amount, pg) and the standard deviation (*s*) were calculated. Specific congener LOQ was calculated as the average value plus 6 times *s (Equation 2)*; doing so, whenever an experimental signal is higher than LOQ, it is statistically coming from the sample and not from the background.

$$LOQ_i = average + 6s \qquad \textit{(Eq. 2)}$$

When a congener was not detected in the blank, the LOQ was set equal to the "instrumental LOQ" (iLOQ) for this congener, after checking its value was included in the calibration range. iLOQs for each congener were calculated according to the guidelines of a report from a EU core working group composed of members from EU national reference laboratories (NRLs) and expert laboratories in Europe (Kotz et al., 2012). Eight replicate injections of the lowest calibration point were done. This point could be "acceptable" if, for each congener, the difference between "punctual" average RRF and ARRF (calculated on all the calibration points) was lower than 30% and it was "consistent". For our laboratory, point consistency meant that the standard deviation of each punctual congener RRF was lower than 15%, even if this criterion is not clarified in the Regulation. After checking lowest calibration point acceptability, average value and standard deviation (s) of the eight replicate injections were calculated for each congener (j), and eventually the iLOQ (in pg) was determined according to *Equation 3*, as ten times the standard deviation of the eight replicates.

$$iLOQ_j = 10s \qquad \textit{(Eq. 3)}$$

LOQs from the blanks and iLOQs were eventually compared to estimate method LOQ in pg for each congener, even when blank level was zero. At this point, LOQ in pg for each congener was divided by average sample amount (in our laboratory it is 4g for vegetable oil) to obtain "matrix LOQ" in pg/g and then multiplied by the corresponding TEF value to calculate the LOQ in pg WHO_{2005} TEQ/g. Table 1.7 reports data and final LOQ values of our method for vegetable oil.

Table 1.7: Average blank levels and corresponding standard deviation for 12 blanks, derived LOQ calculation, iLOQ, and final vegetable oil LOQs in pg/g fat and in pg TEQ/g fat. Vegetable oil sample amount was 4 g.

PCDDs	**Average blk pg**	**SD blk pg**	**LOQ pg**	**iLOQ pg**	**Matrix LOQ pg/g**	**Matrix LOQ pg WHO_{2005}TEQ/g**
2,3,7,8-TCDD	0.000	0.000	0.000	0.017	0.004	0.004
1,2,3,7,8-PeCDD	0.000	0.000	0.000	0.033	0.008	0.008
1,2,3,4,7,8-HxCDD	0.000	0.000	0.000	0.027	0.007	0.001
1,2,3,6,7,8- HxCDD	0.043	0.083	0.543	0.033	0.136	0.014
1,2,3,7,8,9- HxCDD	0.007	0.021	0.130	0.071	0.033	0.003
1,2,3,4,6,7,8-HpCDD	0.403	0.259	1.959	0.056	0.490	0.005
OCDD	1.910	1.094	8.477	0.464	2.119	0.001
				Sum LOQs	**PCDD**	**0.036**
PCDFs						
2,3,7,8-TCDF	0.043	0.067	0.442	0.052	0.111	0.011
1,2,3,7,8-PeCDF	0.566	0.309	2.420	0.021	0.605	0.018
2,3,4,7,8-PeCDF	0.015	0.052	0.327	0.021	0.082	0.025
1,2,3,4,7,8-HxCDF	0.018	0.045	0.288	0.016	0.072	0.007
1,2,3,6,7,8-HxCDF	0.018	0.037	0.240	0.021	0.060	0.006
1,2,3,7,8,9-HxCDF	0.050	0.112	0.722	0.024	0.181	0.018
2,3,4,6,7,8-HxCDF	0.027	0.047	0.311	0.015	0.078	0.008
1,2,3,4,6,7,8-HpCDF	0.504	0.266	2.101	0.053	0.525	0.005
1,2,3,4,7,8,9-HpCDF	0.005	0.011	0.069	0.048	0.017	0.000
OCDF	0.275	0.355	2.407	0.070	0.602	0.000
				Sum LOQs	**PCDF**	**0.098**
				TOTAL LOQ (pg WHO_{2005} TEQ/g) dioxins		**0.134**
Co-PCBs						
PCB 77	59.038	26.790	219.776	0.037	54.944	0.005
PCB 81	2.328	2.095	14.898	0.030	3.725	0.001
PCB 126	1.387	0.519	5.829	0.077	1.457	0.112
PCB 169	0.000	0.000	0.000	0.071	0.018	0.001
				Sum LOQs	**Co-PCBs**	**0.119**
MO-PCBs						
PCB 105	282.561	166.864	1283.747	2.109	320.937	0.010
PCB 114	25.376	13.189	104.508	1.504	26.127	0.001
PCB 118	947.212	526.532	4106.404	1.930	826.601	0.027

PCB 123	16.829	7.603	62.449	1.537	15.612	0.000
PCB 156	24.772	11.983	96.669	1.897	24.167	0.001
PCB 157	4.580	3.032	22.774	1.287	5.693	0.000
PCB 167	64.943	35.941	280.591	2.067	70.148	0.002
PCB 189	2.514	1.011	8.581	1.626	2.145	0.000
				Sum LOQs	**MO-PCBs**	**0.045**
				TOTAL LOQ (pg WHO_{2005} TEQ/g) dioxins and DL-PCBs		**0.298**
I-PCBs						
PCB 28	2322.316	837.194	7345.480	3.928	1836.370	
PCB 52	4642.084	1685.366	14754.278	6.530	3688.570	
PCB 101	3029.900	1216.934	10331.506	2.733	2582.877	
PCB 138	305.718	144.509	1172.771	1.587	293.193	
PCB 153	361.613	150.079	1262.084	1.469	315.521	
PCB 180	88.992	27.838	256.021	0.904	64.005	
				Sum LOQs	**8780.535**	

As reported before (Table 1.3), MLs for vegetable oil are 0.75 pg WHO_{2005}-PCDD/F-TEQ/g, 1.50 pg WHO_{2005}-PCDD/F-PCB-TEQ/g (including co-PCBs and MO-PCBs), and 10 ng/g (10000 pg/g) for the sum of the 6 NDL-PCBs. As shown in Table 1.7, for our method the calculated LOQ in pg WHO_{2005}-PCDD/F-TEQ/g is 0.134, so ~18% of the ML; the LOQ in WHO_{2005}-PCDD/F-PCB-TEQ/g is 0.298, that is ~20% of the ML. Only the sum of the NDL PCBs is not compliant with the Regulation because it is higher than 20%. This is however due to a fairly high contamination of the laboratory environment during the period of this study and method development, and it not related to the Triple Quad instrument or the method itself. The same, in fact, was observed with GC-HRMS instrument. Nevertheless, even under these unfavourable laboratory conditions, every single of the 29 congeners contributing to the TEQ calculation fulfilled the criteria of the Regulation. Calculated LOQs were also compliant with the minimum detectable quantity criterion fixed in the Regulation, in the order of upper femtogram (10^{-15} g) for dioxins, low pictogram (10^{-12}g) for co-PCBs and of nanogram (10^{-9}g) for MO-PCBs.

As already reported, the LOQ is a crucial parameter because of the reporting system of the final results, as LOQ is the threshold values used to determine upper-bound, medium-bound, and lower-bound values. The lower, medium and upper bound approaches consist in reporting respectively zero, LOQ/2 and LOQ whenever the measured level is below the LOQ for a target compound. The value calculated with the upper bound approach is used to check for sample compliance to MLs, and this is the reason why LOQ calculation is crucial in dioxin analysis.

6.5. Selectivity

Criteria for selectivity in the Regulation are the same if working with HRMS or Triple Quad instruments. Peaks relative to 1,2,3,4,7,8- and 1,2,3,6,7,8-hexachlorinated furans congeners (HxCDFs) have to show maximum 25% overlapping. Chromatographic separation of other peaks is ensured when this criterion is fulfilled. In the method developed, these two congeners were baseline separated (Figure 1.1)

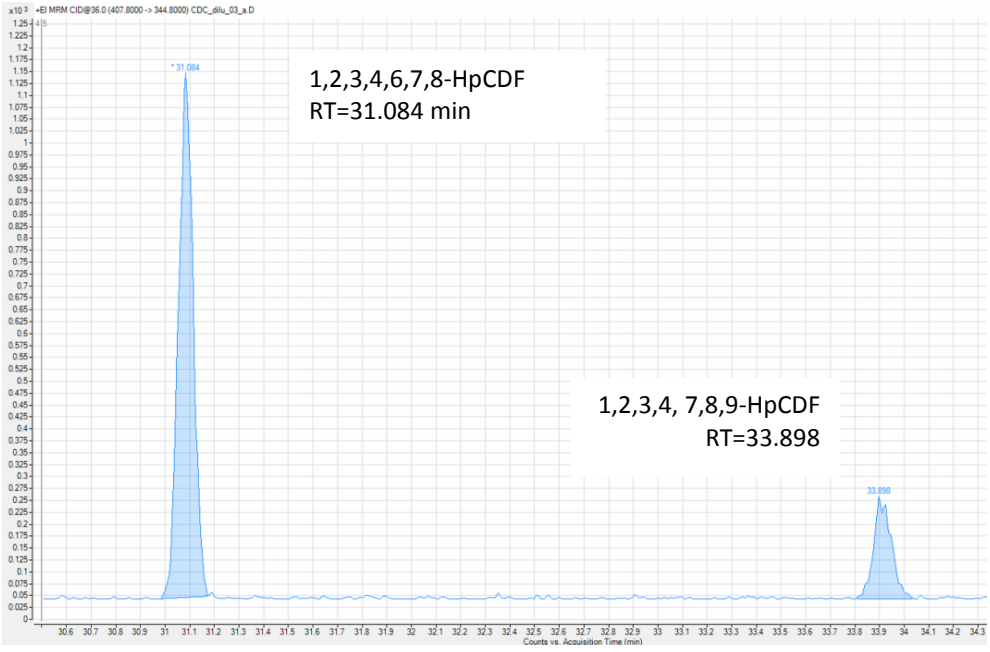

Figure 1.1: Chromatogram of the two heptafurans, showing their clear separation (non-smoothed signals).

Also hexachlorodibenxo dioxins (Figure 1.2), whose separate elution is challenging,, were properly separated using 60 m chromatographic column.

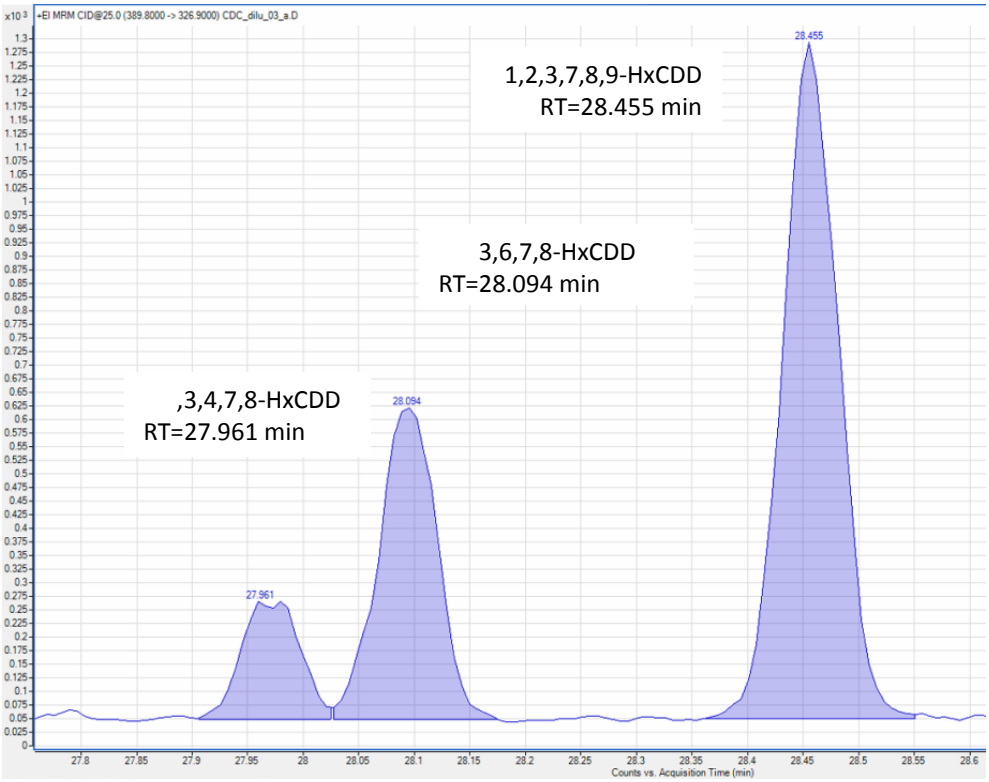

Figure 1.2: Chromatogram of hexadioxins (non-smoothed signal).

6.6. Accuracy: trueness and precision

The results of the accuracy test carried out during method validation are reported to show method performances and the reliability of the data shown in the next chapters.

Fortified vegetable oil (sunflower oil) was used to assess method accuracy for PCDD/Fs, co-PCBs, and MO-PCBs at half ML (ML/2), ML, and twice ML (2ML). Matrix blank was checked and no congener was found in the unfortified vegetable oil. Six samples for each level were processed over three days (two samples for each level per day) providing also within lab reproducibility data; ten procedural blanks were also processed. Results were

obtained and reported by independent operators who operated without knowing target levels. Table 1.8 shows the results of the accuracy experiments.

Table 1.8: Results of the accuracy test.

	Experimental value pg WHO_{2005} TEQ/g	SD pg WHO_{2005} TEQ/g	RDS %	Target value pg WHO_{2005} TEQ/g	Bias %
PCDD/Fs					
ML/2	0.41	0.03	7.1	0.40	2.36
ML	0.78	0.04	5.7	0.79	-1.54
2ML	1.60	0.03	2.2	1.58	1.30
DL-PCBs					
	0.31	0.03	9.0	0.33	-7.00
	0.59	0.02	3.4	0.65	-8.53
	1.26	0.02	1.6	1.30	-3.42

These results met the EU Regulation criteria, that set the bias <20% and within lab reproducibility <15%. The method, then, showed to be accurate and reliable.

7. Conclusions

This chapter described the GC-MS/MS Triple Quad acquisition method for dioxin and PCB analysis used in this work of thesis. When this work of thesis was done, the method was already developed and validated for vegetable oil, but in this work, the GC-MS/MS method was optimized in some parameters, because it was applied to matrices other than vegetable oil. In fact, it was used for the assessment of levels and recoveries in the next chapters, which report the development of alternative clean-up approaches using automated systems, as well as the development of an analytical method for Dechlorane detection in food and feed samples. Quantitative analysis was performed mainly with GC-MS/MS Triple Quad to determine recovery rates, as well as levels in QC samples (pork, milk and egg), with the aim of evaluating the suitability of the new clean-up strategy proposed.

Moreover, this GC-MS/MS acquisition method was used for the first time in our laboratory, for Dechlorane detection in food and feed, which in general is a novelty because usually Dechloranes are usually detected with GC-HRMS instruments.

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CHAPTER II

Development of a new dioxin/PCB clean-up and fractionation procedure for an existing automated system

1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs), have been recognised as highly toxic and ubiquitous environmental contaminants. The most toxic congeners of these three organochlorine families are PCDDs and PCDFs with chlorine in the 2,3,7,8-positions, the planar CBs (non-*ortho* substituted), and semiplanar (mono-*ortho* and di-*ortho* substituted) (Safe, 1990). The determination of these individual congeners in food and feed samples involves sophisticated and delicate multistage sample preparation because of the extremely low concentrations of PCDD/PCDFs and co-PCBs (part per trillion, ppt) and the presence of many other co-extracted and more concentrate organic compounds, such as lipids. For this reason, after fat extraction, a clean-up step is of prime importance and is aimed to the elimination of these co-extracted analytes and to the separation of some PCBs and dioxins to avoid successive interferences during instrumental quantification. In addition to the clean-up procedure, high-resolution gas chromatography (HRGC) coupled with high-resolution mass spectrometry (HRMS) is essential for the accurate and specific determination of these compounds, characterised by structure-related toxicity and a great number of congeners. Recently Triple Quadrupole low resolution (LR)MS instruments have shown performances that demonstrate their suitability for confirmatory analysis, recognized by EU Regulation (2014a, 2014b).

Most of the clean-up methods described in the literature are based on more than one column-chromatographic step and are therefore very time and solvent consuming. Mainly, clean-up methods are based on lipid oxidation via sulfuric acid coated silica gel column, PCBs and PCDD/Fs fractionation by means of Florisil® or alumina column, and eventually activated charcoal to complete fractionation and isolate planar compounds because of geometric interaction with the planar structure of the carbon. Adopted sorbents affect final

fractionation profile, also related to instrumental acquisition method: usually 2 fractions per sample are collected in the end of the sample preparation, but some laboratories fractionate up to 4 fractions; then, based on the fractionation profile, 2 or more chromatographic runs are carried out for quantification. Many routine laboratories collect and quantify 2 fractions containing I- and MO-PCBs together and co-PCBs, PCDFs and PCDDs separately, because their average levels in real food and feed samples are in the part per billion (ppb) range, 1000 times higher than average levels of co-PCBs, PCDDs, and PCDFs that are found at ppt level and quantified in a separate GC-MS run. For some laboratories, separate quantification of mono- and di-*ortho* PCBs from co-PCBs is important also because PCB 126 and 169, with the highest TEFs among PCBs, can be subject to instrumental interferences from other PCBs with possible overestimation (Bernsmann T. et al., 2014). Other laboratories regularly collect and analyse 2 fractions with different composition: all the PCBs (I-, MO- and co-PCBs) are collected in the same fraction and PCDD/Fs are separate, with no instrumental quantification issue (Aries et al., 2004, Fürst, 2006).Some other laboratories are further splitting co-PCBs from PCDD/Fs, collecting 4 fractions per sample, namely I-PCBS, MO-PCBs, co-PCBS and PCDD/Fs, and doing 3 injections, one for I- and MO-PCBs, one for co-PCBs and one for PCDD/Fs. This is because PCB 126 and 169 have retention time very close to 2,3,7,8 TCDD and 1,2,3,7,8 PeCDD respectively (difference around 0.2 min depending on chromatographic setup) with quantification issues if the level of co-PCBs is higher than dioxins one for the same samples

The goal of the present work was to develop new and high throughput clean-up approaches for an existing automated system, the DEXTech™ from LCTech GmbH. The new strategies were based on silica and carbon columns commercially available for DEXTech™ system, and on manually packed alumina column prepared in our laboratory. The two methods lead to different final fractionation profiles due to the use of different carbon sorbents after the fractionation with alumina. New methods showed a great improvement in terms of solvent and time saving in comparison with the classic clean-up method routinely used for the same system.

2. Materials and methods

2.1. Chemicals and consumables

Solvents (hexane, dichloromethane, toluene) were Picograde® reagents (LGC Promochem, Wesel, Germany). All solvent batches were tested for investigated analyte contamination before use. Nonane and dodecane puriss analytical-reagent grade standard for GC were purchased from Fluka (Steinheim, Germany). Water was obtained from a Milli-Q Ultrapure water purification system (Millipore, Brussels, Belgium). Ethanol, ammonium hydroxide solution 28-30% and ethyl ether were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium sulphate and diatomaceous earth were purchased from VWR International (Radnor, PA, USA). Neutral and basic aluminium oxides were purchased from Sigma-Aldrich. Disposable multilayer silica and carbon columns for the automated clean-up DEXTech™ system were obtained from LCTech (LCTech GmbH, Dorfen, Germany).

Ten mL glass syringes from Microsyringes (ILS Innovative Labor Systeme GmbH, Stützerbach, Germany) and/or sterile disposable plastic syringes from B. Braun (Melsungen, Germany) were used for injection. Chromatographic pure grade helium gas, 99.9999% alphagaz 2 was purchased from Air Liquide (Paris, France). Technical N27 grade liquid CO_2 was used for PTV cooling (Air Liquide, Paris, France).

In our accredited ISO 17025 routine laboratory, in dioxin analysis after all the analytical procedure, two fractions are collected and injected for instrumental quantification: one containing I- and MO-PCBs, and one containing co-PCBs, PCDFs and PCDDs. Regulated MO-PCBs (including PCBs #105, 114, 118, 123, 156, 157, 167, 189) and I-PCBs (PCBs #28, 52, 101, 138, 153, 180) were quantitated with isotope dilution measuring the ratio of native *vs* labelled internal standard. Internal standard spiking solution for MO-PCBs (MBP-MKX) was purchased from Wellington Laboratories (Guelph, Canada), internal standard for I-PCB was from Cambridge Isotope Laboratories (CIL, Andover, MS, USA). Recovery standard EC-1414 from CIL, containing 3,3',5,5'-TetraCB (PCB 80) ($^{13}C_{12}$, 99%) was used to assess possible losses during sample preparation with internal standard vs recovery standard ratio. The calibration curve for MO-PCBs and I-PCBs was

prepared using EC-5179 (native I-PCBs), EC-4987 (native MO-PCBs) solutions from CIL, listed above internal standard solutions and recovery standard solutions.
Regulated PCDD/Fs 2,3,7,8-substituted congeners and coplanar co-PCBs (PCBs #77, 81, 126, 169) were quantitated using ^{13}C-labelled internal standards EDF-4144 from CIL. Recoveries were measured with recovery standards (EDF-4145 solution from CIL): 1,2,3,4-TCDD ($^{13}C_6$, 99%) was used for recoveries of tetra and penta chlorinated compounds, 1,2,3,4,7,8,9-HpCDF ($^{13}C_{12}$, 99%) for hexa, hepta, and octa chlorinated species, and 3,3',5,5'-TCB ($^{13}C_{12}$ PCB 80, 99%) for PCBs. Calibration curve standards for this fraction were purchased from CIL (EDF-4143).
To facilitate samples spiking in our routine lab, an internal standard mixture containing ^{13}C-labelled PCDDs, PCDFs, co-PCBs, and MO-PCB was prepared (ST-ROU); I-PCBs internal standard was added separately. 20 µL of ST-ROU and 10 µL of I-PCBs solution are called labelled IS solution trough this work.

2.2. Sample preparation

During the first part of this work, model samples, consisting of 2 mL of hexane spiked with IS solution (20 µL of internal standard ST-ROU and 10 µL of I-PCBs solution), were used for method development. Model samples were directly injected in the clean-up system with no preliminary sample preparation step, to assess recovery rates related to this part of the analysis only. After method development, one of the two methods was applied to quality control (QC) samples, consisting of pork fat (QCG), freeze-dried egg yolk (QCE), and milk (QCL) to evaluate method performances on real fatty food matrices. Procedural blanks were analysed in the same series of corresponding QCs and were respectively pure hexane for pork fat, diatomaceous heart for egg yolks and ultrapure water for milk.
Normally if sample is not already pure fat, the first step of a dioxin analysis is fat extraction because dioxins are lipophilic compounds. In considered QCs, fat extraction was not necessary only for pork fat, accelerated solvent extraction (ASE™ 350, Dionex, Thermo Fisher Scientific) was used for egg yolks, and liquid-liquid extraction was used for milk. For pork fat, 5 g were directly diluted in hexane and spiked with labelled IS before sample clean-up.

For egg yolk, 5 g of freeze-dried sample were first mixed with diatomaceous heart and then extracted with ASE system and solvent toluene/ethanol 90/10 at 150°C for 5 minutes for 2 cycles. Extracts were filtrated over Na_2SO_4 and solvent was evaporated until dryness for fat determination. The extracted fat, approximately 2.5 g, was spiked with labelled IS solutions prior introduction in the clean-up system. Diatomaceous earth was used as blank and followed the same procedure.
For QC milk samples, liquid-liquid extraction was carried out: 80 mL were poured in a separation funnel, 20 mL of NH_3 and 80 mL of ethanol were added, the funnel was shaken and 100 mL of ethyl ether was added and the solution was left for 30 minutes to allow separation of the two phases; eventually hexane was added and left 30 minutes until the clear separation of aqueous and organic phase. This latter was transferred in a round flask for gravimetric determination of fat content, which was spiked with labelled IS solutions after solvent evaporation. The blank was 80 mL MQ ultrapure water following the same procedure as the sample.
Samples were injected in the DEXTech™ automated system for clean-up using the procedure with basic alumina described in the following sections.

2.2.1. DEXTech™ system for sample clean-up

The aim of this work was to develop an alternative approach for sample clean-up using an already existing automated system, DEXTech™ from LCTech GmbH (Figure 2.1).

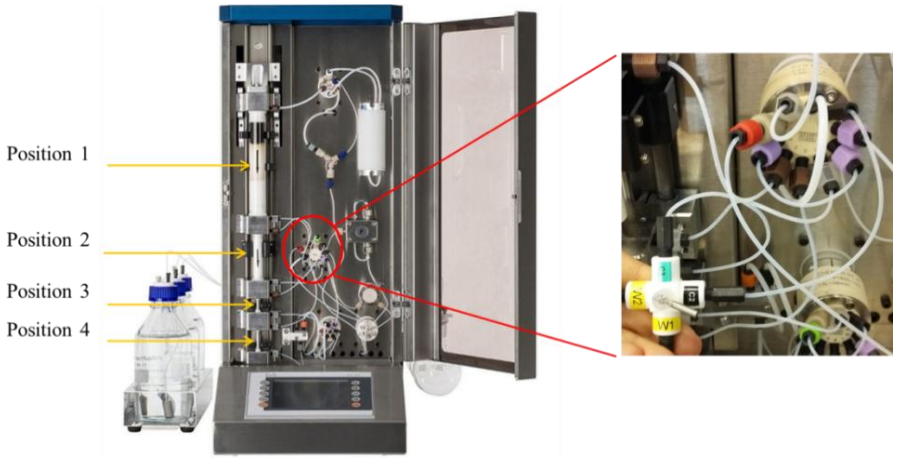

Figure 2.1: (left) DEXTech™ system in its classic configuration; (right) in this work a T valve was added to central valve to fulfil some steps.

DEXTech™ system was equipped with 4 main valves (Figure 2.2), from the top: injection valve, solvent valve, hexane/DCM valve and toluene valve. Injection valve was connected to the injection port and the sample loop, whose size was 15 mL in the standard configuration; solvent valve was connected to solvent bottles placed on the left of the instrument; hexane/DCM valve is connected in port 3 with solvent and only hexane/DCM mixture can enter this valve; toluene valve is connected in port 2 with solvent valve and only toluene can enter it. Such valve design allowed only definite paths, which gave more robustness but less flexibility to the system.

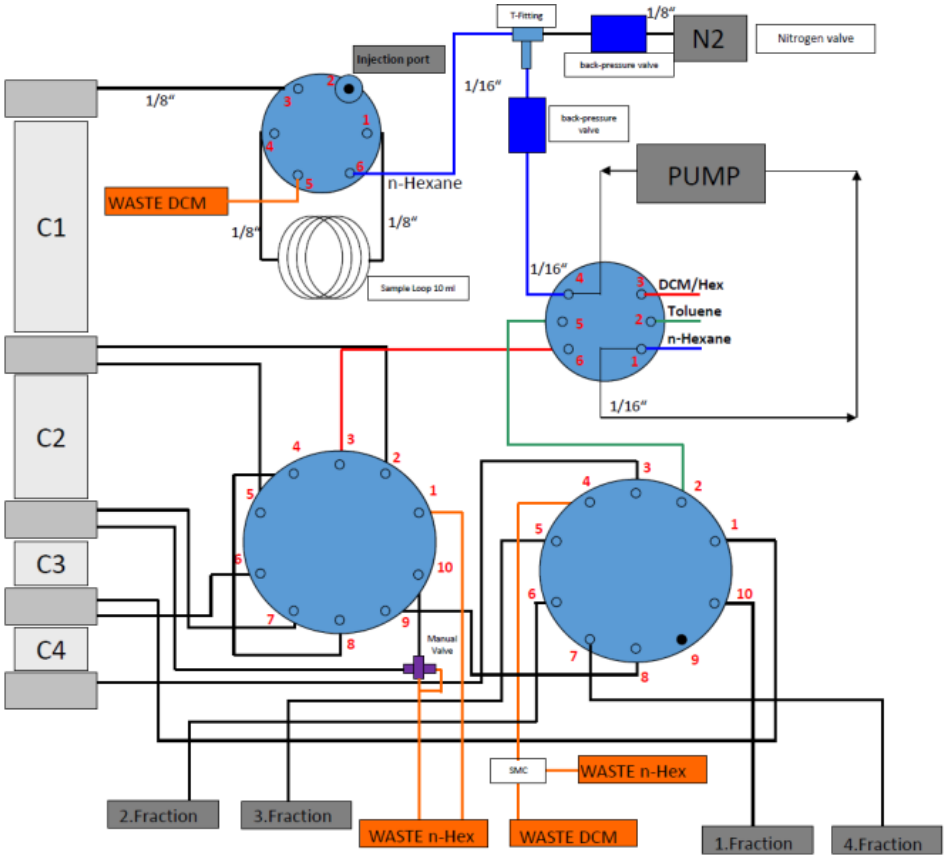

Figure 2.2: Schematic view of DEXTech™ system: column slot (left), system valves (in blue, from top to bottom): injection valve, solvent valve, hexane/DCM valve, toluene valve.

Columns were placed on the left side of the instrument and they had to be in a precise order due to electronic locking of the system. Classic configuration was based on 4 column set: multilayer silica column (acid-basic-neutral, fat capacity up to 5 g), Florisil® column in position 2 (C2) and two carbon columns, one in position 3 (C3) containing Norit:celite for PCBs fractionation (C_{small}), and one in position 4 (C4) with Carbopack C:celite for planar compounds separation (C_{large}). The silica column was used for lipid degradation, Florisil® and two carbon columns were used for fractionation: Florisil® separated first PCBs from PCDDs and PCDFs, then C_{small} divided co-PCBs from other PCBs

with geometrical interactions and charge transfer mechanisms, and C_{large} was used for further purification of PCDD/Fs fraction.
For the alternative approach developed in this work the system was modified by adding a T valve to the hexane/DCM valve, and sample loop size was increased from 15 mL to 25 mL. The present configuration of the system didn't allow fully automated sequence, as manual tubing connection was needed before carrying out some steps requiring solvent paths different than standard ones. Column set included 3 columns: commercial multi-layer silica column, manually packed alumina column and one of the two commercial carbon columns.

2.3. Instrumental quantification

Two fractions were collected out of the system: the first by forward elution of alumina/carbon column with a mixture of hexane/dichloromethane 50:50 (fraction 1, F1) containing mainly PCBs, and the second by carbon column backflush with toluene (fraction 2, F2), containing mainly dioxins, furans and, in case, co-PCBs depending on the carbon column connected. Solvent volumes were reduced to approximately 500 µL in dedicated tubes using a sensor-equipped TurboVap II Workstation (Caliper Life Science, Teraflene, Belgium), to be then transferred in GC vials containing nonane (90 µL for F1 and 4 µL F2) as keeper. Final evaporation for solvent exchange to nonane was done with RapidVap (Labconco, Kansas City, MO, USA). Recovery standard was added to each fraction prior the instrumental analysis.
For quantitative analysis, a triple Quad instrument 7000C GC-QQQMS/MS system from Agilent (Palo Alto, CA, USA) was used with a validated method developed in our laboratory (L'Homme et al., 2015b). Briefly, the triple Quad instrument was equipped with a 7890B GC oven, a programmable temperature vaporization (PTV) inlet, and a 7693A automated liquid sampler (ALS). PTV inlet was operated on solvent vent mode and cooled by liquid CO_2; its temperature program was the same for both fractions F1 and F2 and was chosen after an experimental design (L'Homme et al., 2015a): start at 45°C (3 min) and ramp at 720°C/min until 320°C; vent flow of 100 mL/min at pressure of 10 psi for 2.8 min. Purge flow was set to 1200 mL/min after 5 min to avoid memory effect in the

inlet. GC column was the classic DB-5ms 60 m x 250 µm x 0.25 µm (Agilent). Injection volume was 2 µL for F1 and 5 µL for F2, and GC oven temperature program was different for the two fractions as well. For F2 oven program started at 120°C (5 min), ramped up at 25°C/min until 250°C (5 min), then 3°C/min until 285°C (15 min) for a total runtime of 41.6 min. For F1 the program started the same, but temperature was set at 285°C for 0 min. The transfer line temperature was held at 280°C in both cases. On the MS side, 7000C electron ionization (EI) ion source was heated at 280°C and operated at 70 eV. Quadrupoles were held at 150°C, nitrogen collision gas was flowing at 1.5 mL/min, and helium quench gas at 2.25 mL/min. Quads resolution was set to unit mass, which by default corresponds to peak width of 0.7 Da at half height.

3. Results and discussion

3.1. Classic clean-up scheme

Classic clean-up method for DEXTexh system was based on 4 column set: multilayer silica- Florisil®-two carbon columns. Classic procedure followed the same well-proven principle as the manual CVUA-MEL clean-up (Bernsmann T. et al., 2014).

Organic solvent from Soxhlet extraction or pure fat was dissolved in 2 mL toluene and 3 mL n-hexane, and manually injected with a syringe into the sample loop. The process (Table 2.1) starts with a column conditioning step (from 1 to 3). Then sample is automatically collected with hexane from the sample loop and loaded with pure hexane on C1 silica column for lipid oxidation (steps 4 and 5). After 4 minutes C1/C2/C3 are connected in line and fraction 1 (F1) containing I-PCBs is collected after carbon column C3, while MO- and co-PCBs are loaded on C3 (step 6). In step 7, Florisil® column C2 is backflushed with hexane/DCM 50/50 mixture to transfer dioxins and furans from the top of the Florisil® to the carbon column C4. Afterwards C3 is eluted with hexane/DCM (50/50) mixture to collect fraction 2 (F2) of MO-PCBs (step 8). C3 is backflushed with toluene in step 9 to collect co-PCBs (F3). Finally in step 10 carbon column C4 is backflushed with toluene to collect fraction 4 (F4) containing planar PCDD/Fs. Solvent flow rate was always set at 7 mL/min, except in step 9 where it was 1 mL/min.

Table 2.1: Detailed description of classic method steps with the automated system. C1 = silica column, C2 = Florisil® column, C3 = carbon column Norit:celite (C_{large}), C4 = carbon column Carbopack C:celite (C_{small}), w = waste, F1 = fraction 1 of I-PCBs, F2 = fraction 2 of MO-PCBs, F3 = fraction 3 of co-PCBs, F4 = fraction 4 of PCDDs and PCDFs.

	Column	Solvent	Process	Time (min)	Flow (mL/min)
1	C3 - w	Hex/DCM	Conditioning carbon C3	1	7
2	C4 - w	Hex/DCM	Conditioning carbon C4	1	7
3	C1 - w	Hex	Conditioning silica	6	7
4	Sample loop/C1 - w	Hexane	Load silica	4	7
5	C1/C2/C3 – w	Hex	Load Florisil® and carbon C3	1	7
6	C1/C2/C3 - F1	Hex	Collect F1 – I-PCBs	20	7
7	C2/C4 - w	Hex/DCM	Load carbon C4 back-flushing Florisil®	7	7
8	C3 – F2	Hex/DCM	Flush carbon C3 forward for F2 – MO-PCBs	8	7
9	C3 – F3	Toluene	Backflush carbon C3 for F3 – co-PCBs	8	1
10	C4 – F4	Toluene	Backflush carbon C4 for F4 – PCDD/Fs	8	7
	Run time (min)			64	
	Vol Tot (mL)			400	

Hexane (mL)	DCM (mL)	Toluene (mL)
276.5	59.5	64

This clean-up procedure takes 64 minutes and needs 400 mL of solvent. Fractions F1 and F2 are put together before instrumental analysis and quantified in the same chromatographic run; F3 and F4 are quantified separately for a total of 3 chromatographic runs per sample.

Figure 2.3 gives a visual summary of steps from 6 to 10 related to fraction collection.

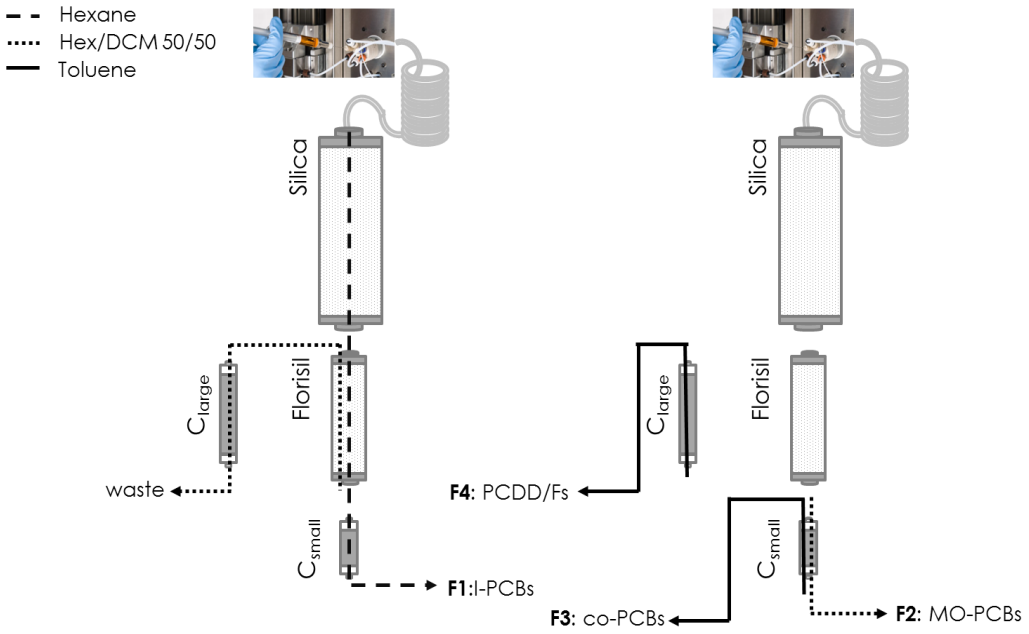

Figure 2.3: Visual scheme of classic clean-up method with 4 column set: (left) steps from 6 to 7; (right) steps from 8 to 10.

3.2. Alternative approach scheme

In the alternative approach proposed, 3 column set was used with the aim of solvent and time reduction for high throughput protocols. In the classic method, Florisil® and two carbon columns are used for fractionation as Florisil® is not able to quantitatively separate *ortho*- and non-*ortho* PCBs, while complete separation is possible with alumina (Loos R., 1997). In fact, in the developed method, alumina started the fractionation of planar and non-planar compounds (including *ortho*- and non-*ortho* PCBs) which distribute at different depth into the column. Then only one carbon column was needed to complete the fractionation, based on geometrical and charge transfer interactions. Final fractionation depends on the properties of the carbon sorbent (Concejero et al., 2001).
Main steps of the alternative method are described here (Table 2.2) and method development is detailed in the following discussion. The method started with a short column

conditioning involving silica, alumina and carbon column in line, mainly to check for possible leaks or backpressure caused by manual column packing, before sample injection into the system (step 1). Sample was injected in 25 mL sample loop and loaded with hexane on C1 multilayer silica connected to manually packed basic alumina column (C2) in position 2 (step 2). In this step C1 and C2 were connected to the waste turning the additional T valve and outlying a flow path not allowed in the classic system layout. The main advantages of this configuration were that lipid degradation products formed on the silica were purged out of the system, contrary to the classic method, and meanwhile analytes were trapped on the basic alumina column. Manual connection of tubing was necessary in step 3 to elute alumina column fraction towards carbon column in position 3 (C_{large} or C_{small}) with hexane/DCM 50/50 mixture and collect fraction 1 (F1) containing PCBs. In the meantime, planar compounds eluting from alumina were trapped on the top of carbon column because of electronic and geometrical interactions, to be collected in fraction 2 (F2) by backflushing with toluene in step 4.

Table 2.2: Main steps of the alternative clean-up approaches using 3 column set: silica (C1) – alumina (C2) - carbon (C3) column.

	Column	Solvent	Process
1	C1/C2/C3 - w	Hexane	Conditioning system
2	Sample loop/C1/C2 - w	Hexane	Load silica and alumina, purge
3	C2/C3 – F1	Hexane/DCM	Elute alumina, load carbon, collect F1
4	C3 – F4	Toluene	Backflush carbon C3 for F2

The alternative method used only 3 columns instead of 4, and only 4 steps were needed for complete sample clean-up. Final method performances and fractionation profile were different based on carbon column used to complete fractionation, as described in the following discussion. Figure 2.4 gives a visual summary of the new method from step 2 to 4.

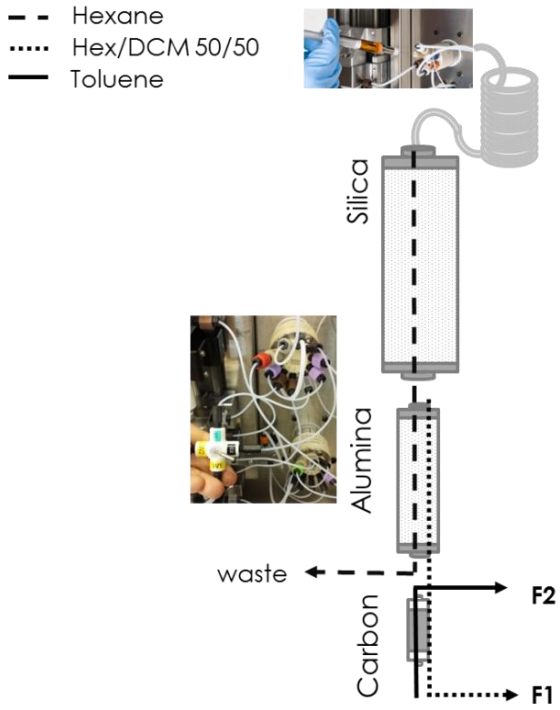

Figure 2.4: Alternative clean-up scheme with 3 column set and basic alumina. Two fractions were collected and their composition varied with the carbon column used. A T valve was added to the central valve of the system to allow purging after alumina column, which was not possible in the classic configuration of the system.

This method was developed step by step optimizing solvent volume and time. Flow rate was always hold at 7 mL/min in all the steps as in the classic method.

3.2.1. Conditioning step

The conditioning step was considerably shortened in comparison to the classic method, because no difference in background levels was found when skipping this step. Just a short preliminary silica-alumina and carbon column flushing in a row with hexane was maintained to check for possible leaks or backpressure in the automated system. In some cases in fact, the system recorder high backpressure and stopped automatically. Likely, as the alumina column was manually packed, little experimental variation in sorbent amount or filters placement into the column, led to solvent flow hindrance and resulting backpressure. Conditioning step took 8 minutes, which corresponded to the sum of the dead volumes of each column: 6 minutes for standard multilayer silica, 1 minute for alumina and 1 minute for the carbon.

3.2.2. Silica column elution profile

Multilayer silica column elution profile was studied to assess the volume of hexane required to bring all the analytes out of the silica and to load them onto the alumina. Commercial multilayer silica column with fat capacity up to 5 g were used. Its exact composition was unknown, but basically it contained layers of Na_2SO_4, 44% acidic, neutral and basic silica.

For method development, samples consisted of 2 mL of hexane spiked with ^{13}C labelled IS of all the congeners and injected in the automated clean-up system with a glass syringe. Elution profiles were outlined collecting fractions every 5 minutes directly after the silica column with hexane flowing at 7 mL/min. Each fraction was evaporated and spiked with RS to assess the percentage of compounds eluting during time. Figure 2.5 showed the elution profiles of I- and MO-PCBs, indicated as PCB fraction, and co-PCBs, PCCDs and PCDFs, called dioxin fraction even if no fractionation occurred on the silica column. This was due to our normal instrumental setup, detecting PCBs and dioxins separately.

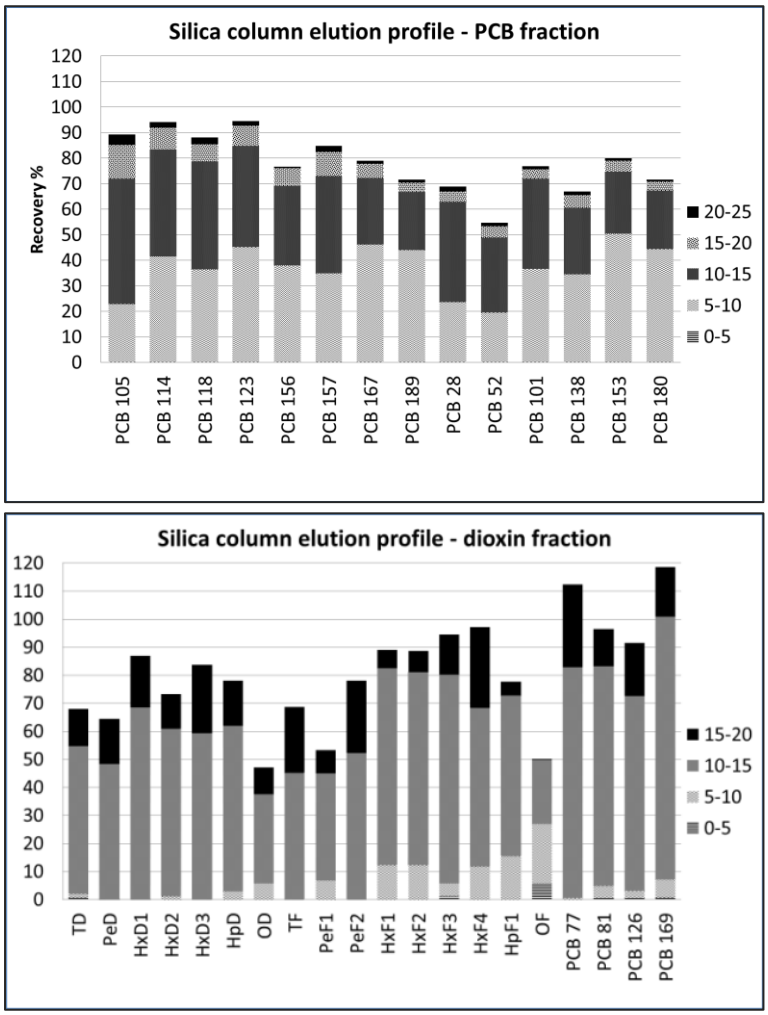

Figure 2.5: (top) I- and MO-PCBs silica column elution profile from 0 to 25 minutes of n-hexane flowing at 7 mL/min; (bottom) co-PCBs, PCDD/Fs silica column elution profile from 0 to 20 minutes in the same conditions.

These plots showed that PCBs elution was faster than PCDD and PCDF one, and that complete elution out of the silica took place within 20 minutes. Nevertheless, in order to compensate possible pump fluctuation, in the following tests 25 minutes was set as time value.

3.2.3. Alumina column

The alumina column was manually packed with commercial alumina. Both commercial neutral and basic alumina were already activated at Brockmann grade I, with ~150 mesh particle size and 58Å pore size. Normally, basic alumina has a pH of 9-10 and it is used to separate basic and neutral compounds stable to alkali; neutral alumina has pH 6 – 8 and is used for less polar compounds (1994). Basic and neutral alumina can be prepared in various activity grades (I to V) based on the Brockmann scale:

Activity grade	I	II	III	IV	V
Water added (wt %)	0	3	6	10	15

Grade I is prepared by heating alumina until complete water removal, typically overnight at 400-450°C; the other grades are prepared by adding water to Grade I to deactivate it. In this work, the alumina column needed for PCB and dioxin fractionation was manually packed using the same scheme and the same components of the commercial Florisil® column for the DEXTech™ system (Figure 2.6).

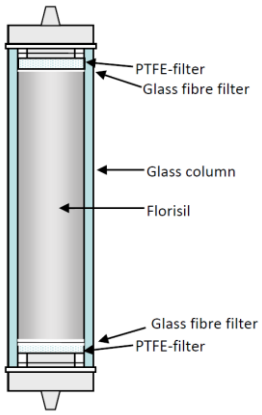

Figure 2.6: Packing scheme and components used in the commercial Florisil® column.

Alumina column size had to be the same as commercial Florisil® because of the electronic locking of the automated system based on fixed dimension. Such a column was

able to host 10-11 g of sorbent, and this represented a constraint for method development, as well as the silica column size. Alumina sorbent, in fact, had to be active enough to trap all the analytes, preventing their loss into the waste despite using an imposed limited amount of maximum 11 g, and despite 175 mL of hexane flowing at 7 mL/min. Figure 2.7 showed the experiment designed to assess the suitability of alumina material: 2 mL of hexane spiked with labelled IS of all the congeners were injected in the system and loaded/eluted with 175 mL of hexane on the silica column connected to manually packed alumina column and to the waste. Afterwards alumina was eluted with hexane/DCM 50/50 mixture to calculate recovery rates, which had to be in the range 60-120 % as required by EU Regulation. Two fractions were collected for each experiment: the waste and alumina eluate, to check if interesting compounds were lost in the waste or were trapped on the alumina column and then eluted with hexane/DCM 50/50 mixture. Only PCBs elution was assessed, being the first eluting compounds, followed by dioxins and furans.

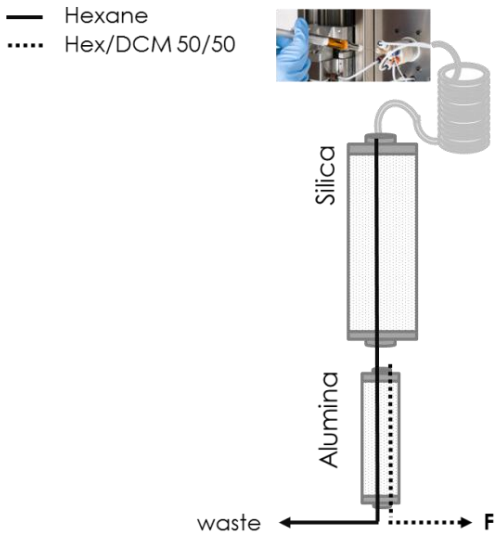

Figure 2.7: Flow scheme of the experiment designed to assess the suitability of alumina: the model sample was injected and loaded on the silica column connected in line with the alumina column end ending into the waste. Two fractions were collected and analysed to check the compound distribution: the waste and the fraction eluting from alumina column

Preliminary tests using commercial Brockmann I neutral and basic alumina with no additional treatment showed immediately that for our purposes basic alumina was more effective than neutral, revealing that sorbent basicity played an important role in interaction with compounds. For this reason successive testing was focused only on basic alumina.
Super-basic materials were prepared according to reported procedures (Ramos et al., 1997, Xie et al., 2006) but these materials lead recovery rates lower than 60% established by EU Regulation. Then thermal activation of alumina was assessed as a possible way to enhance material basicity by water and CO_2 removal from the active sites. Thermal treatment suggested in EPA 1613 method was tried first: basic alumina was baked for 30 hours at 600°C and cooled down to 130°C in desiccators before use. Material so prepared showed higher recovery rates than before, but some compounds were still lost in the waste in our experimental conditions (Figure 2.8).

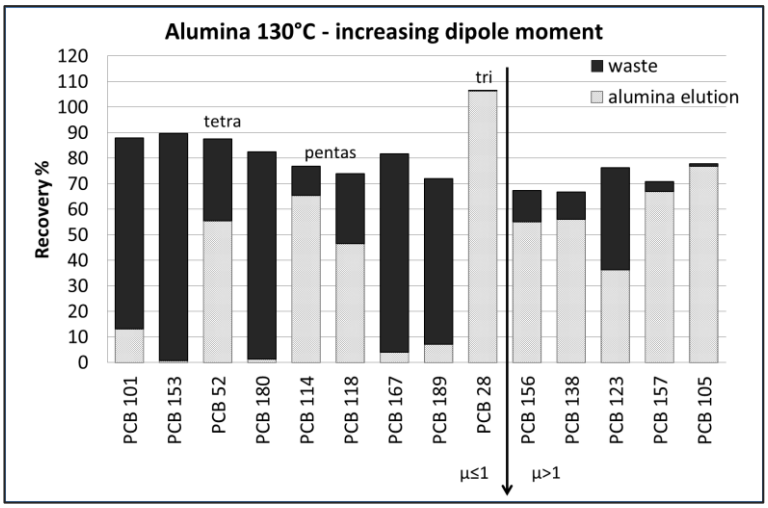

Figure 2.8: Distribution of PCBs between waste and alumina column when using thermal treatment in EPA 1613, cooling down alumina at 130°C after 30 h backing at 600°C.

Even if not usable in the method development, this experiment was very important to reveal interaction mechanism between compounds and alumina sorbent. Results displayed that, taking into account PCBs dipole moment (μ) calculated in a previous work with molecular modelling (Pirard et al., 2002), polar PCBs (μ > 1) were more retained on the sor-

bent (light grey bars in Figure 2.8) than less polar PCBs ($\mu < 1$), which were released in the waste (dark bars in Figure 2.8). PCB 28, 52, 114 and 118, the less chlorinated PCBs, were an exception. This behaviour was attributable to two kinds of interactions between basic alumina and the analytes: dipolar interactions, responsible for higher retention of polar PCBs, and hydrogen bonding between alumina basic sites and acidic hydrogens of less chlorinated PCBs.

In view of this, EPA 1613 thermal treatment was modified and alumina, after backing, was cooled down in desiccator until 200-250°C after baking, and not until 130°C as suggested in EPA method. The idea was to assure that moisture and CO_2 were not re-adsorbed on active sites during material cooling. For each experiment column was packed when the material was still hot and it was immediately inserted into the automated system for conditioning with hexane (step 1 Table 2.2). So prepared material was more active and no compound was lost in the waste as shown in Figure 2.9.

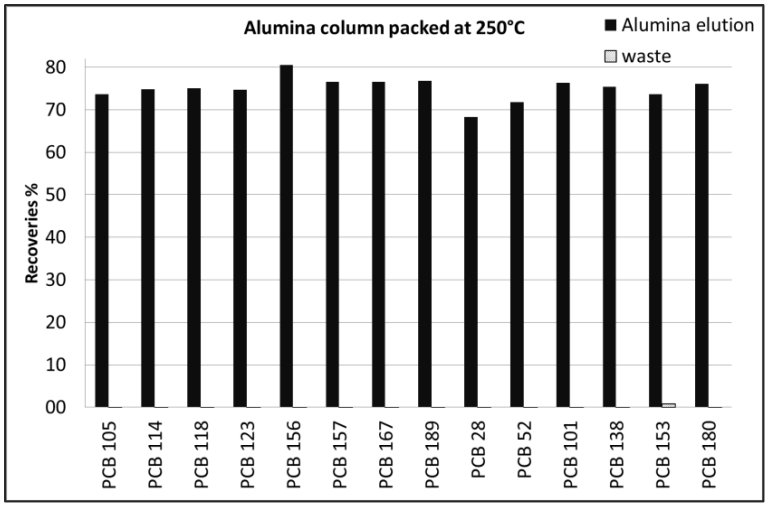

Figure 2.9: PCB distribution between the waste and alumina eluate when using basic alumina backed at 600°C for 30h and cooled down in desiccator until 200-250°C.

After this experiment, tests were made using basic alumina columns dry-packed after backing the sorbent at 600°C for 30h and using the sorbent when it was still hot at ap-

proximately ~250°C. It is important to underline that even if alumina column was packed with hot material, it was operating at room temperature during all sample clean-up steps.

3.2.4. Carbon column

The last step of method development was the connection of the carbon column in line with the alumina for PCB and dioxin complete fractionation. Two carbon columns were commercially available C_{large} (Carbopack C:celite) and C_{small} (Norit:celite) exhibiting different fractionation properties (Concejero et al., 2001). In this set of experiments, 2 mL of hexane spiked with labelled IS of all the congeners were injected in the system and loaded on the alumina column with 175 mL of hexane. Alumina fraction was eluted with hexane/DCM 50/50 mixture towards the carbon column and fractions were collected after the carbon to assess its elution profile and recoveries.

3.2.4.1. C_{large} - Carbopack C:celite elution profile

C_{large} column, containing Carbopack C:celite mixture, whose position is the number 4 in the classic system configuration, was placed in position 3 after the alumina column. A dummy column was placed in position 4 to allow system locking. Fractionation profile of C_{large} flushing with hexane/DCM mixture was calculated to assess the volume needed to elute all the compounds. The first fraction was collected after 5 minutes and then every minute until 8 min in order to avoid oversplitting and to allow a better assessment of required volume (Figure 2.10).

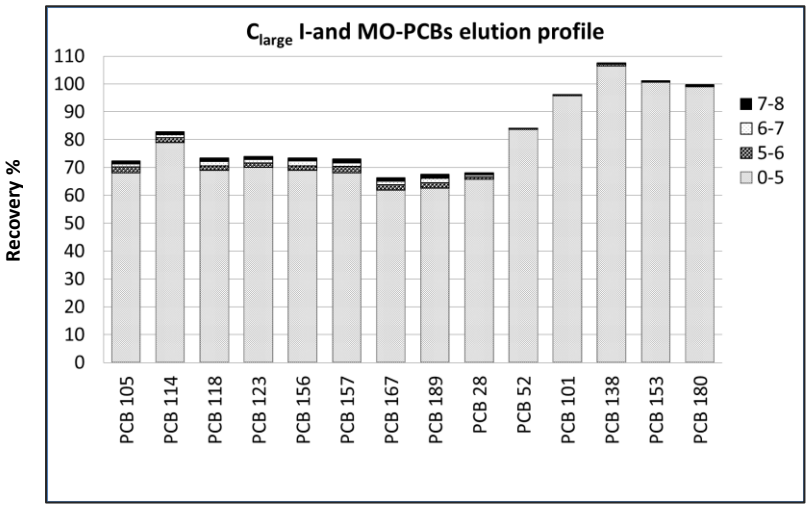

Figure 2.10: C_{large} column PCB elution profile. Carbon was eluted with hexane/DCM 50/50 mixture at 7 mL/min in forward direction.

I- and MO-PCBs elution time using C_{large} was 6 minutes with hexane/DCM 50/50 mixture flowing at 7 mL/min. With this column co-PCBs were collected together with other PCBs in forward direction flush, despite their different planar structure. Whereas PCDDs and PCDFs were still trapped on the top of the carbon column and were collected in backflush with toluene for 8 minutes as in the classic method (Table 2.1). Recoveries were in the range 60-120% for all congener in both fractions.

So, the clean-up method using silica-alumina-Carbopack C:celite columns lead to this fractionation profile: all the PCBs, I-, MO- and co-PCBs were collected in fraction 1, PCDDs and PCDFs were found in fraction 2. Method steps and performances are reported in Table 2.3.

Table 2.3: “Alumina – Carbopack C” method steps and performances in terms of run time and total sample volume.

	Column	Solvent	Process	Time (min)	Flow (mL/min)
1	C1/C2/C3 - w	Hex	Conditioning system	8	7
2	Sample loop/C1/C2 - w	Hex	Load silica and alumina, purge	25	7
3	C2/C3 – F1	Hex/DCM	Elute alumina, load carbon, collect F1	6	7
4	C3 – F2	Toluene	Backflush carbon C3 for F2	8	7
	Run time (min)			47	-26.6%
	Vol Tot (mL)			329	-17.8%

If compared with classic method, “alumina-Carbopack C method” allowed about 18% of solvent saving and 27% of time saving (See Table 2.5 for comparison)

3.2.4.2. C_{small} – Norit:celite elution profile

C_{small} carbon column, containing Norit:celite mixture is usually found in position 3 in classic system configuration. In this work, C_{small} was still in position 3, but a dummy column was placed in position 4, just for electronic system locking. 2 mL of hexane spiked with labelled IS of all the congeners were injected in the system, loaded on the silica and alumina column and finally eluted from the alumina with hexane/DCM 50/50 mixture towards the carbon column. Fractions were collected every 2 minutes for 8 minutes after the carbon and injected on the Triple Quad for recovery and fractionation profile assessment (Figure 2.11)

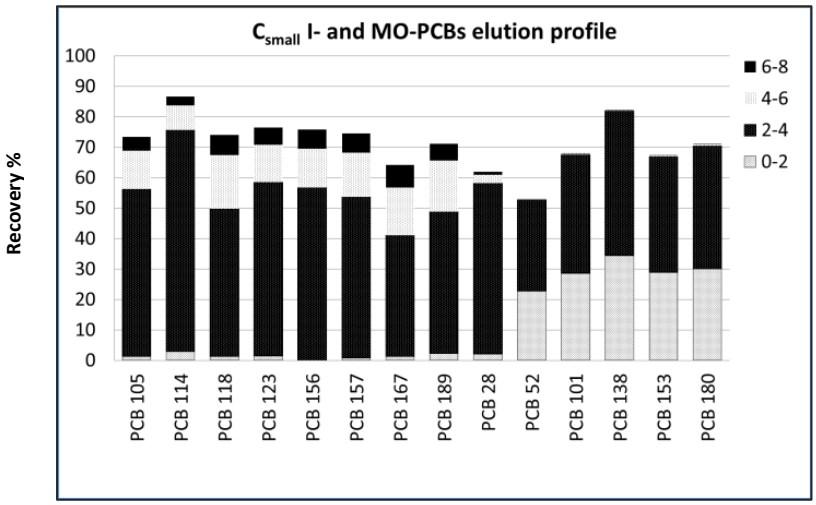

Figure 2.11: C_{small} column PCB elution profile. Carbon was eluted with hexane/DCM 50/50 mixture at 7 mL/min in forward direction.

C_{small} demonstrated higher interaction with PCBs than C_{large}; in fact 8 minutes instead of 6 were necessary to collect fraction 1, indicating that PCBs elution was slowed down by interactions with the sorbent. Actually C_{small} was already used in the classic method for PCBs fractionation, as it showed more effective interactions and discrimination capacity for PCBs. In fact, with this column co-PCBs were collected together with dioxins in fraction 2 by backflushing the carbon column with toluene at 7 mL/min. In this set of experiments, 3 fractions were collected in backflush to assess the minimum volume required to elute the carbon, the first after 4 minutes and then every 2 minutes until 8 minutes (Figure 2.12).

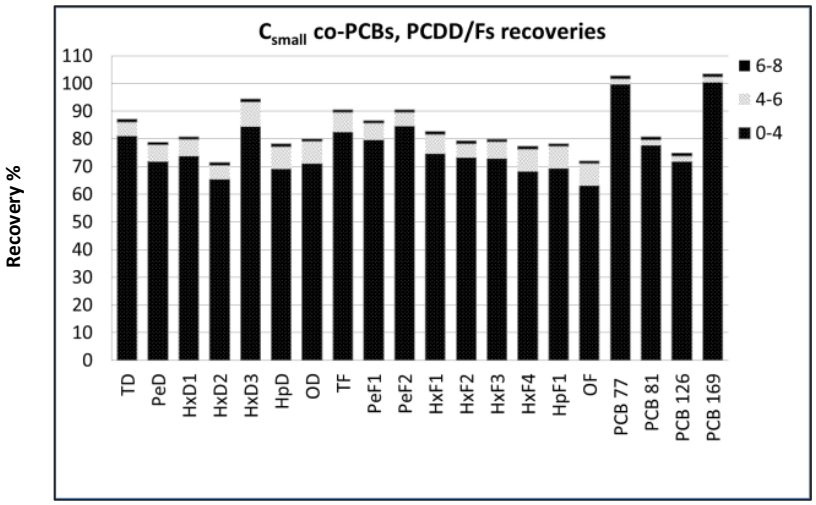

Figure 2.12: C_{small} column co-PCB, PCDD/F elution profile. Carbon was eluted with toluene at 7mL/min in backflush and several aliquots were collected.

Thus, final "alumina-Norit method" showed similar performances to the previous one with a time saving of 27% and volume saving of 18% (Table 2.4), but with a different fractionation profile due to the different carbon type used.

Table 2.4: "Alumina – Norit" method steps and performances, as time - solvent consumption.

	Column	**Solvent**	**Process**	**Time (min)**	**Flow (mL/min)**
1	C1/C2/C3 - w	Hex	Conditioning system	8	7
2	Sample loop/C1/C2 - w	Hex	Load silica and alumina, purge	25	7
3	C2/C3 – F1	Hex/DCM	Elute alumina, load carbon, collect F1	8	7
4	C3 – F2	Toluene	Backflush carbon C3 for F2	6	7
	Run time (min)			47	-26.6%
	Vol Tot (mL)			329	-17.8%

Both developed methods, the one with Carbopack C:celite and the one with Norit:celite column, brought to column, solvent and time saving as well as global analysis cost reduction as shown in Table 2.5, maintaining recovery rates in the range 60-120% as required from EU Regulation.

Table 2.5: Comparison of time and solvent consumption between classic and alternative methods.

	Method	Time (min)	Vol tot (mL)	Hexane (mL)	DCM (mL)	Toluene (mL)
Features	Classic	64	400	276.5	59.5	64
	Alumina-Carbopack C	47	329	252	21	56
	Alumina-Norit	47	329	259	28	42
Saving	Alumina-Carbopack C	27%	18%	9%	65%	13%
	Alumina-Norit	27%	18%	6%	53%	34%

One can choose between the two developed methods according to the following instrumental acquisition method.

3.3. QC samples

The alumina-Norit method was applied to fatty food matrices to test its applicability to real samples. The choice of this method rather than the alumina-Carbopack C method was related to the final fractionation profile, which matched with our instrumental acquisition method. Quality control samples prepared in our ISO 17025 routine lab were used namely pork fat (for feed), egg yolk and milk. QCs were spiked with native compounds at a level close to the maximum allowed level for each specific matrix (pork fat: Directive 2006/13/CE, egg and milk: EU Regulation 1259/2011 emending EU Regulation 1881/2006). A minimum of 3 samples were processed for each matrix together with one procedural blank, prepared following the same sample preparation, and run with each series. Blank levels were subtracted from the final result.

Table 2.6 showed reference and average experimental levels measured for each QCs.

Table 2.6: Maximum allowed values and experimental values for analysed QCs.

Matrix	acronym	Maximum allowed value pg TEQ/g fat	Average experimental level QC pg TEQ/g fat
Pork fat (for feed)	QCG	3	2.6
Egg	QCE	5	5.5
Milk	QCL	5.5	5.5

All QC control charts were outlined in our ISO 17025 laboratory according to a validated procedure, using substantially different clean-up procedure and HRMS for final quantification as described in Chapter 3.

3.3.1. QCG

Approximately 5 g of 3 QC pork fat samples were weighted and cleaned-up with the alumina-Norit method. Two fractions were collected and quantitated, experimental levels, after blank subtraction, were divided by sample amount and corrected with TEF values to calculate total pg TEQ/g fat. Figure 2.13 showed control charts used in our routine ISO 17025 laboratory and experimental levels found with alumina-Norit clean-up method. Average value, lower and upper limit were calculated in our routine laboratory using a different clean-up approach.

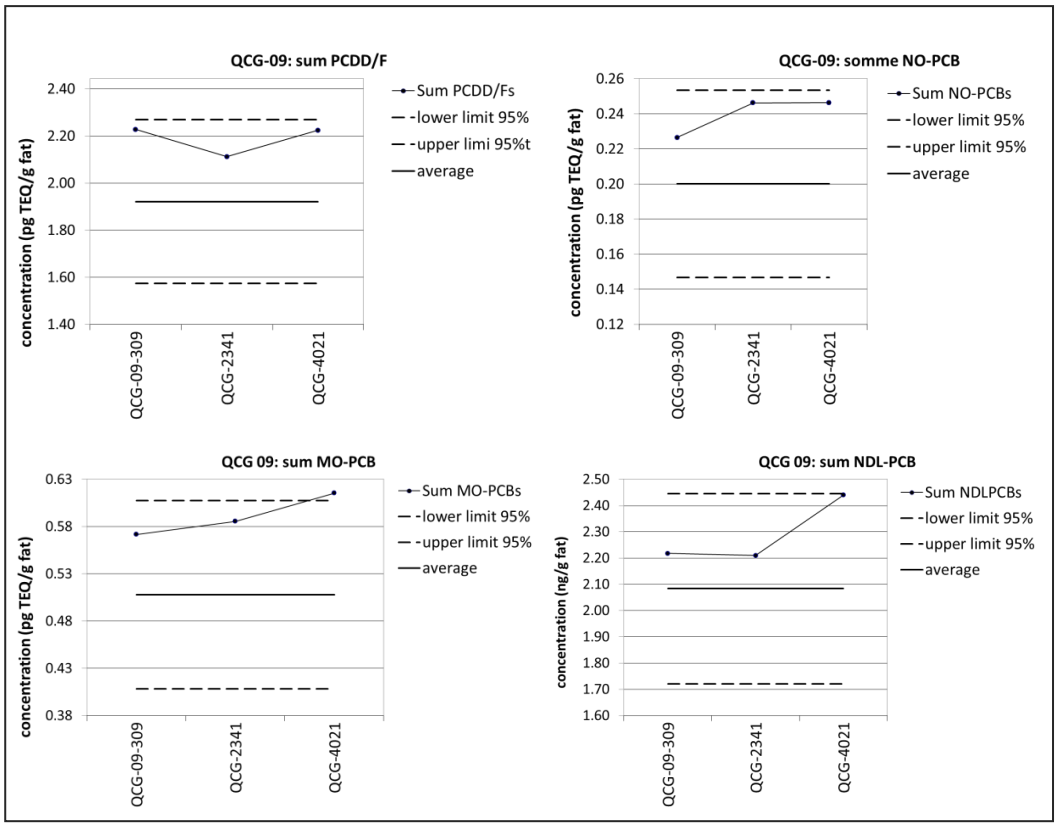

Figure 2.13: Control charts for QC pork fat.

Experimental levels using alumina-Norit method were not statistically different from expected values obtained with our validated procedure. Only one QC sample had MO-PCB and I-PCBs (NDL-PCBs) sum slightly higher than expected value. This result was attributed to a small memory effect deducible from the control chart trend in both graphs. Likely memory effect occurred because QC pork fat amount corresponded to maximum silica column fat capacity (up to 5 g), but it could be removed adding a further washing step between samples. In spite of this small inconvenient, these results showed the suitability of "alumina-Norit" method to clean-up pork fat.

3.3.2. QCE and QCL

Freeze dried QC egg yolks were processed as described in the experimental section. Extracted fat was spiked with IS solutions and diluted with 5 mL of hexane to be injected in the automated system and cleaned-up accordingly to “alumina-Norit” method. Figure 2.14 (top) showed the comparison between reference values and average experimental values of three QC samples, which were not statistically different and demonstrated the applicability of “alumina-Norit” method to egg yolks. The same approach was applied to milk QCs and similar results were obtained (Figure 2.14, bottom).

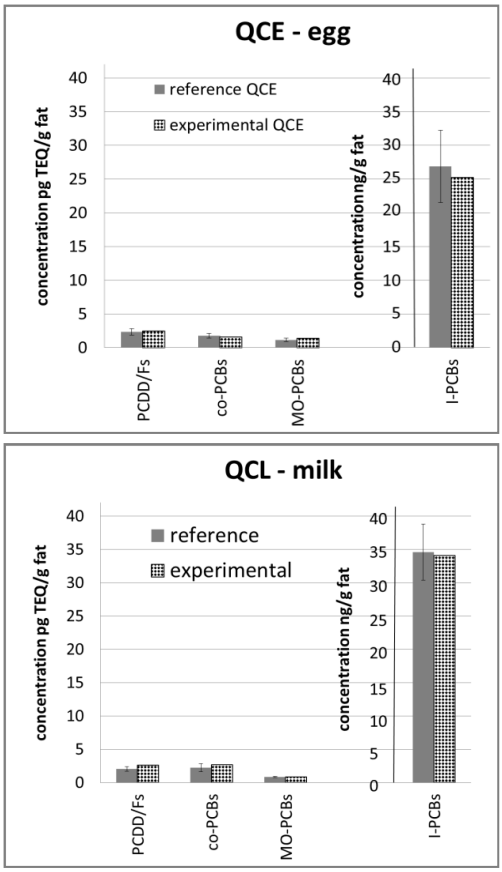

Figure 2.14: Reference and experimental values for egg yolk (top) and milk (bottom) quality control samples. PCDD/Fs, MO- and co-PCBs are expressed in pg TEQ/g fat; I-PCBs are in ng/g as they don’t have a TEF assigned.

In both cases, some egg yolk and milk samples were not valid due to interferences in instrumental quantification of hexa- and hepta-dioxins and furans, which heavily contribute to final TEQ calculation. For these samples, fraction 2, the most critic one, looked yellowish even after clean-up, indicating the presence of undesirable compounds, which was probably related to manual packing of the alumina column because it occurred randomlµy. Maybe high temperature packing had a negative effect on column components not designed for this scope. The clean-up method seemed to be not robust but nevertheless reliable because it led to accurate TEQ calculation when no interferences were released.

4. Conclusions

Alternative clean-up approaches were developed and implemented in an already automated clean-up system. This work was aimed to reduce global cost of the analysis in comparison with the currently used method. New approaches were based on 3 column set instead of 4, namely multilayer silica, basic alumina and one carbon column. Multilayer silica and two carbon columns were commercially available, while alumina column was manually packed after proper thermal treatment based on a modified EPA 1613 procedure. The two approaches arose from the availability of two different carbon columns leading to different final fractionation profiles: with Carbopack C:celite column, I-, MO- and co-PCBs were collected in fraction 1, and PCDD/Fs were found infraction 2, with Norit:celite column, co-PCBs were found in fraction 2 together with dioxins. It is thus possible to choose the appropriate protocol based on the consecutive instrumental analysis. The new developed approaches allowed time and solvent saving, respectively 27% and 18%, as well as global cost reduction, giving recoveries in the range 60-120% as required by EU Regulation.

The “Alumina-Norit” method was applied to QC samples of pork fat, egg yolk and milk to assess its performances on real fatty matrices and results showed that the clean-up method was accurate, despite some issues related to manual packing. A full validation was not carried out because of the high variability of the manual packing and because it was out of the scope to validate a non-fully automated method using manually packed column. These preliminary results, however, could be a starting point for full validation of a clean-up method using DEXTech™ system (equipped with a T valve that switches

automatically) and alumina columns produced industrially. Implementing a fully automated alumina column method would be interesting due to global cost reduction of this method.

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CHAPTER III

Revisiting an existing dioxin/PCB clean-up and fractionation procedure to reduce solvent and time consumption

1. Introduction

Alternative automated clean-up approaches using PowerPrep™ system from Fluid Management Systems (FMS Inc., Waltham, MA, USA) have been implemented and tested on food and feed matrices for the low level analysis of polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, dioxin like and non-dioxin like regulated PCBs. This work was carried out in the perspective of a cheaper and more sustainable procedure, as well as faster a high throughput method for food safety (Abad et al., 2000, Fujita et al., 2009).
In the first part of the work our validated procedure employing PowerPrep™ was optimized for low fat matrices; in the second part, a fully automated approach was tested and compared to our routine procedure for fat content up to 4 g.

2. Materials and method

2.1. Chemicals and consumables

Solvents (hexane, dichloromethane, toluene, methanol) were Picograde® reagents (LGC Promochem, Wesel, Germany). All solvent batches were tested for the investigated analytes contamination before use. Nonane puriss analytical-reagent grade standard for GC was purchased from Fluka (Steinheim, Germany). Disposable PTFE columns for the automated clean-up were obtained from Fluid Management Systems (FMS Inc., Waltham, MA, USA).
Chromatographic pure grade helium gas, 99.9999% alphagaz 2 was purchased from Air Liquide (Paris, France). Technical N27 grade liquid CO_2 was used for PTV cooling (Air Liquide, Paris, France). Disposable PTFE columns for the automated clean-up were ob-

tained from Fluid Management Systems (FMS Inc., Waltham, MA, USA). Reagent standards for native and ^{13}C labelled PCDD/Fs and regulated PCBs, as well as calibration curve solutions and recovery standards were the same described in Chapter II. Again, analytes were quantitated by adding isotope dilution and ^{13}C labelled IS solution prior clean-up procedure (20 µL of internal standard mixture ST-ROU (^{13}C-labelled PCDDs, PCDFs, co-PCBs and MO-PCB) and 10 µL ^{13}C I-PCBs solution). The recovery standard was added to injection vial preceding the instrumental analysis for each fraction.

2.2. Sample preparation

For method development, only standard solutions were tested to assess recovery rates. 10 mL of hexane were spiked with IS solution and cleaned-up to check final recovery rates. Thereafter, QC pork fat and sunflower oil were processed. None of these two matrices required fat extraction as they were already pure fat. The QC was prepared in our routine laboratory and spiked at the maximum allowed level (EU Directive 2006/13/CE), sunflower oil was a food sample sent to our routine laboratory for regular analysis and its levels were unknown. An aliquot of 4 g of each sample was weighted, spiked with labelled IS solution and diluted in 60 mL of hexane to decrease viscosity and to disperse the fat. The procedural blank, composed of 10 mL of hexane for both the fatty matrices, was processed following exactly the same procedure as the samples. Samples were automatically aspired and loaded on the PowerPrep™ system equipped with disposable PTFE columns. In this work, different column sets and instrumental setups on PowerPrep™ system were tested.

First setup was the standard one based on multilayer ABN silica (4 g acid, 2 g basic, 1.5 neutral), basic alumina (11 g), and carbon (0.34 g AX21) column, suitable for low fat content matrices (<1 g fat). A second setup using 4 column set, with higher fat capacity, was proposed as follows: high capacity (HC) acidic silica column (45 g acidic silica 44%), multilayer mini silica column (2 g acid, 1 g basic, 0.5 g neutral), mini alumina column (6 g basic) and carbon column (0.34 g AX21) were used for fat amount up to 4 g. Mini silica and alumina together with standard carbon column are usually referred to as FMS Mini Dioxin prep kit. QC pork fat for feed and commercial sunflower oil were cleaned up with this instrumental setup to test its performances

As the carbon column didn't change, the fractionation profile was the same in all the cases and the two fractions were collected out of the automated system: fraction 1 (F1) containing I- and MO-PCBs, and fraction 2 (F2) containing co-PCBs and PCDD/Fs. Fraction solvents were evaporated to approximately 500 µL in dedicated tubes using a sensor-equipped TurboVap II Workstation (Caliper Life Science, Teraflene, Belgium) and then transferred in GC vials containing nonane (90 µL for F1 and 4 µL F2) as keeper. The final evaporation for complete solvent exchange to nonane was done with RapidVap (Labconco, Kansas City, MO, USA). Recovery standard was added to each fraction prior the instrumental analysis.

2.2.1. PowerPrep™ automated system for sample clean-up

PowerPrep™ accurate description is out of the scope of this dissertation and is reported elsewhere (Focant et al., 2001, Focant et al., 2006) but some aspects are worth to be highlighted for the following discussion. The sample can be automatically loaded into the system thanks to an aspiration valve (Figure 3.1, left). PowerPrep™ is designed to host disposable PTFE columns after adding Teflon screw fittings, giving the possibility of accommodating columns with variable size. Column set can be constituted by 3 or 4 columns as shown later in the discussion. Column connection (Figure 3.1, right) is very flexible and customizable because of several 2 ways valves connected to each column; carbon column in particular is connected to double 2 way valves to allow backflush; 6 way valves are used for solvent introduction and final collection. Such valve system allows several flow schemes, column connection and disconnection via simple instrument software. This design gives very flexible and customizable machine for several uses.

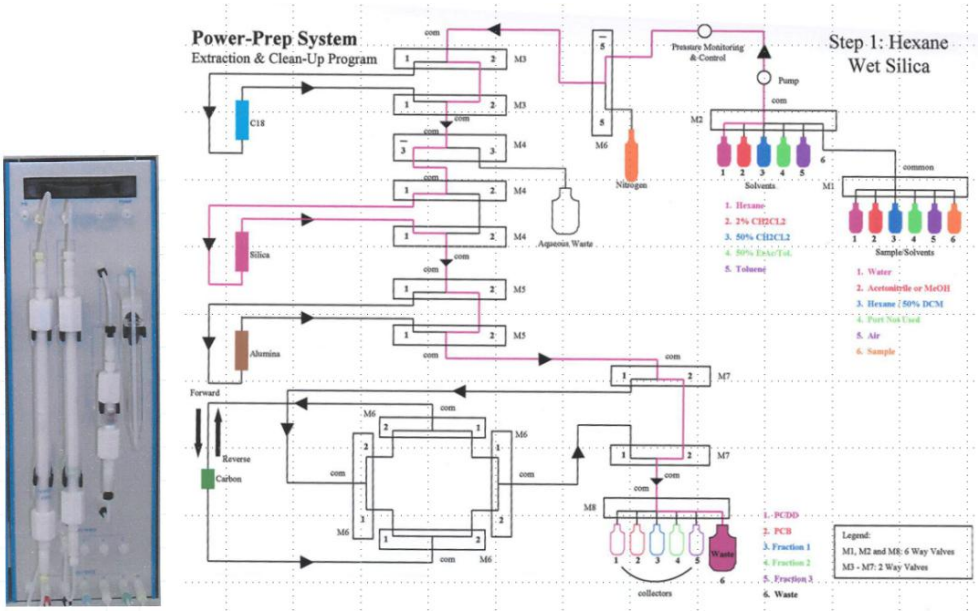

Figure 3.1: (left) PowerPrep™ column slot and automated sample introduction system; (right) valves and flow scheme.

2.3. Instrumental quantification

GC-HRMS (sector instruments) was used for the detection and quantification according to a fully validated method routinely used for the food and feed official controls in our laboratory.

Measurements of fraction 2 with 7 PCDDs, 10 PCDFs and 4 non-ortho DL-PCBs were carried out on an Autospec Premier (Micromass, Manchester, United Kingdom) connected by a heated transfer line (275 °C) to an Agilent 7890 Series (Palo Alto, CA, USA) gas chromatographer equipped with a GC Pal (CTC Analytics AG, Zwingen, Zwitzerland). The column was a VF-5MS (50 m × 0.2 mm internal diameter × 0.33 µm film thickness) from Agilent. Helium was used as the carrier gas at constant flow rate of 1.0 mL/min. A volume of 5 µl out of the final extract in nonane (9 µl) was injected into programmable temperature vaporization (PTV) injector from Gerstel working in solvent vent mode with vent flow at 50 mL/min and vent pressure 40 kPa. Technical N27 grade liquid CO_2 was used for PTV cooling (Air Lquide, Paris, France). The injector initial temperature was set at 40°C and hold for 3 minutes; then the temperature was increased to 320°C with a

720°C/min ramp. This temperature was hold for 4 minutes before a second increase to 330°C, and kept until the end of the GC analysis. The oven temperature was maintained at 60 °C for 5 min, ramped at 70 °C/min to 200 °C, ramped at 3.2 °C/min to 235 °C for 1.5 min, ramped at 3.2 °C/min to 270 °C for 10 min, and finally ramped at 15 °C/min to 310 °C for 13 min. The total separation time was 55 min. The HRMS instrument was operated in selected ion monitoring (SIM) mode with mass resolution of at least 10,000 at a 10 % valley. The specificity was insured by monitoring the signal of two ions and comparing their ion ratio to the theoretical chlorine isotope ratio.

Measurements of the 8 WHO mono-*ortho* PCBs and 6 I-PCBs were carried out on a MAT95 (Thermo Fisher Scientific, Bremen, Germany) connected by a heated transfer line (275 °C) to an Agilent 6890 Series gas chromatographer equipped with a A200S autosampler. The column was an HT-8 (25 m × 0.22 mm ID × 0.25 µm film thickness) (SGE, Villebon, France). Helium was used as the carrier gas at constant flow rate of 0.8 mL/min. A volume of 2 µl from the final extract in nonane (100 µl) was injected into a split/splitless injector held at 275 °C in splitless mode. A focus liner with glass wool form Agilent was used for injection. The oven temperature was maintained at 140 °C for 2 min, ramped at 15.0 °C/min to 220 °C held for 7.5 min, ramped at 6.0 °C/min to 250 °C, ramped at 2.0 °C/min to 265 °C, and finally ramped at 28 °C/min to 320 °C. The total separation time was 30 min. The HRMS instrument was operated in selected ion monitoring (SIM) mode with a static resolving power of 10,000. Two ions were monitored for both native and labelled compounds for isotope ratio check.

More details on instrumental quantification are reported in a previous work (Focant et al., 2001).

3. Results and discussion

3.1. Classic sample clean-up in our laboratory

In our laboratory, sample clean-up is based on two main steps: manually packed acidic silica column followed by automated clean-up with PowerPrep™ using a 3 column set, namely standard multilayer acidic-basic-neutral (ABN) silica column, basic alumina and

carbon column (AX-21) (Focant et al., 2004, Pirard et al., 2002, Focant and De Pauw, 2002). This approach has a fat capacity of up to 7 g and is outlined to have preliminary lipid digestion which intensely reduces fat content up to maximum 1 g for all the matrices, followed by automated clean-up which is the same for any matrix. This procedure is validated, robust, and represents a standardized approach for a great variety of food and feed matrices.

3.1.1. Manual step

In our laboratory, preliminary lipid digestion is done with disposable glass column manually packed with acidic silica as follows (Figure 3.2): from the bottom, glass wool, 5 g Na_2SO_4, 5 g neutral silica, 20 g 44% acidic silica, 20 g 22% acidic silica. Column is first conditioned with 150 ml of hexane, and then sample is loaded and eluted with 150 mL of hexane. Eluted solvent is evaporated to approximately 10 mL before being introduced in the automated system. This manual step consumes 300 mL of hexane and takes from 30 to 45 minutes depending on fat nature.

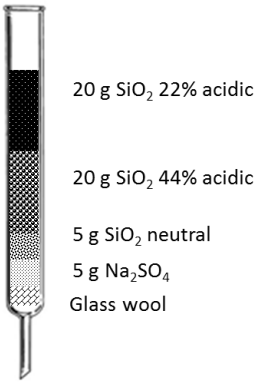

Figure 3.2: Manually packed acidic silica column for lipid digestion

3.1.2. Automated step

In our laboratory, automated clean-up is based on PowerPrep™ 3 column setup: standard multilayer silica column (4 g acidic, 2 g basic, 1.5 neutral), basic alumina (11 g), carbon AX-21 column (0.34 g). The program follows the steps describe in Table 3.1.

Table 3.1: Detailed description of the automated and validated method used in our laboratory for routine analysis, C1 = standard silica column, C2 = standard alumina column, C3 = carbon column (AX-21), w = waste, F1 = fraction 1 of I-PCBs and MO-PCBs, F2 = fraction 2 of co-PCBs, PCDDs and PCDFs. Total time and solvent consumption related only to the automated clean-up, and the manual + automated clean-up, that included time and solvent required for the preliminary lipid digestion with manually packed silica column.

	Column	Solvent	Process	Volume (mL)	Flow (mL/min)
1	Tubing – w	Hexane	Fill tubing	15	10
2	C2 – w	Hexane	Wet alumina column	12	10
3	C3 – w	Hexane	Wet carbon column	20	10
4	C1 – w	Hexane	Condition silica	100	10
5	Tubing – w	Toluene	Change to toluene	12	10
6	C3	Toluene	Pre-elute with toluene	40	10
7	Tubing – w	Hexane/DCM	Change to 50/50	12	10
8	C3 – w	Hexane/DCM	Pre-elute with 50/50	20	10
9	Tubing – w	Hexane	Change to hexane	12	10
10	C3 - w	Hexane	Pre-elute with hexane	30	10
11			Add sample	100	5
12	C1/C2 – w	Hexane	Elute silica with hexane	90	10
13	Tubing – w	Hexane/DCM	Change to 50/50	12	10
14	C2/C3 – F1	Hexane/DCM	Elute alumina with 50/50, load carbon - collect F1	100	10
15		Hexane	Change to hexane	12	10
16		Hexane	Flush with hexane	10	10
17	C3 – F2	Toluene	Elute carbon backflush with toluene - collect F2	90	5

	Time (min)	Volume (mL)	Hexane (mL)	DCM (mL)	Toluene (mL)
Automated system	67.7	587	373	72	142
Manual + automated clean-up	97.7	887	673	72	142

The program starts filling PowerPrep™ tubing with hexane. Then hexane flows through alumina column, which is the most sensitive to air moisture, for conditioning. Carbon column is first wet with hexane and then cleaned/conditioned with all the three solvents in order: toluene, hexane/DCM (50/50 mixture) and hexane. Last solvent is hexane to avoid uncontrolled fractionation during the following process. Silica column is conditioned with 100 mL of hexane before sample loading. Automatic aspiration introduces the sample into the system on the silica for fat degradation. In this step, silica column is connected in line with alumina and the waste of the system, so that degradation products are purged out, while analytes are trapped on the alumina. In the following step, alumina is connected to carbon column and eluted with hexane/DCM 50/50 mixture to collect fraction 1 (F1) after the carbon, and to load planar compounds on the carbon, which are collected in the last step in fraction 2 (F2) with toluene in backflush. The whole method involves washing column/conditioning steps (from 1 to 10) and additional steps indicated as “change” solvent (5, 7, 9, 13, 15 and 16), where desired solvent is pumped only into the tubing before going to columns to avoid uncontrolled processes due to solvent mixtures.
Collected fractions are evaporated, solvents are exchanged to ultrapure nonane and RS are spiked to each fraction before instrumental analysis.

3.2. Alternative approaches

3.2.1. Samples with fat content lower than 1 g

Two alternative clean-up approaches based on routine clean-up method in Table 3.1 were studied to reduce solvent and time consumption. The difference between the two methods, both indicated as “standard” because based on standard 3 column set, was related to silica column conditioning: “short standard method” included silica column condi-

tioning, which was shortened in “fast standard method”. Both approaches were suitable for low fat content matrices up to 1 g fat.

3.2.1.1. Short standard method

For method development, model samples consisting of 10 mL of hexane mimicking our extracts were spiked with IS solution. Clean-up solvent volumes were optimized step by step, measuring column and system dead volumes; silica, alumina and carbon column elution profiles were outlined to insure the use of the minimum solvent amount. Detailed description of method steps was reported in Table 3.2.

Table 3.2: “Short” and “fast” standard methods based on standard method in Table 3.1. Short method still had silica column conditioning, which was removed in the fast method (see step 4).

				SHORT	FAST	
	Column	**Solvent**	**Process**	**Volume (mL)**	**Volume (mL)**	**Flow (mL/min)**
1	Tubing - w	Hexane	Fill tubing	15	15	10
2	C2 – w	Hexane	Wet alumina	12	12	10
3	C3 – w	Hexane	Wet carbon	6	6	10
4	C1 - w	Hexane	Condition/wet silica	100	12	10
5			Add sample	100	100	5
6	C1/C2 - w	Hexane	Elute silica with hexane	70	70	10
7	C2/C3 – F1	Hexane/DCM	Elute alumina with 50/50, collect F1 – load carbon	100	100	10
8	C3 – F2	Toluene	Elute carbon backflush with toluene - collect F2	90	90	5

New approaches started with filling the tubing to eliminate air from the system. 15 mL were required as in the reference method, and this amount give and idea of the dead volume of the system. Thereafter, short wetting/conditioning step was carried out mainly to check for possible leaks before sample introduction into the system. A minimum solvent amount was used after dead volumes assessment.

“Short standard method”, as well as “fast standard method”, was based on classic method, skipping less relevant steps. “Wash carbon column” steps (from 5 to 10 in the reference method) were eliminated because commercial columns are produced in clean rooms and sealed under vacuum to prevent any adsorption. Silica column elution volume was set at 70 mL instead of 100 mL as a consequence of elution profile studies. Alumina and carbon column elution volumes stayed the same as the reference after confirmation of elution profiles. “Change solvent” steps in the reference method (5, 7, 9, 13, 15) were removed because solvent exchange can take place directly onto the column with no effect on fractionation. With the same logic, step 16 in the reference method was deleted because clean tubing from hexane/DCM 50/50 mixture before flushing toluene into the carbon column was not necessary. All the following discussion implies that preliminary steps from 1 to 4 of short method have been carried out at the beginning of the run. Fast method was developed later shortening only step 4 from 100 to 12 mL and calculating recoveries.

From step 1 to 4, silica and alumina column dead volumes were measured as the time required for hexane at 10 mL/min passing through the column and it was found equal to 12 mL for both. Dead volume including tubing, namely the volume required for hexane at 10 mL/min to reach the waste after passing through the column, was 20 mL for both. Carbon column dead volume was 6 mL as this column was considerably smaller in size. Silica column conditioning in the “short standard method” was maintained the same as the reference method (Table 3.1, step 4). This step was shortened in the “fast” method and final performances were compared to assess if column conditioning could be skipped or not.

After column wetting and conditioning, the sample was automatically aspired and loaded on the silica (step 5). Silica column elution profile (Figure 3.3) was calculated to assess the minimum solvent amount required for the complete elution of the column. Aliquots were collected directly after the silica column every minute for 7 minutes for PCB fraction (I- and MO-PCBs, F1), and every 2 minutes for 8 minutes for dioxin fraction (co-PCBs, PCDD/Fs, F2), as dioxins are the last eluting compounds. Fraction profiles are shown separately even if no fractionation occurred after the silica because of our instrumental setup.

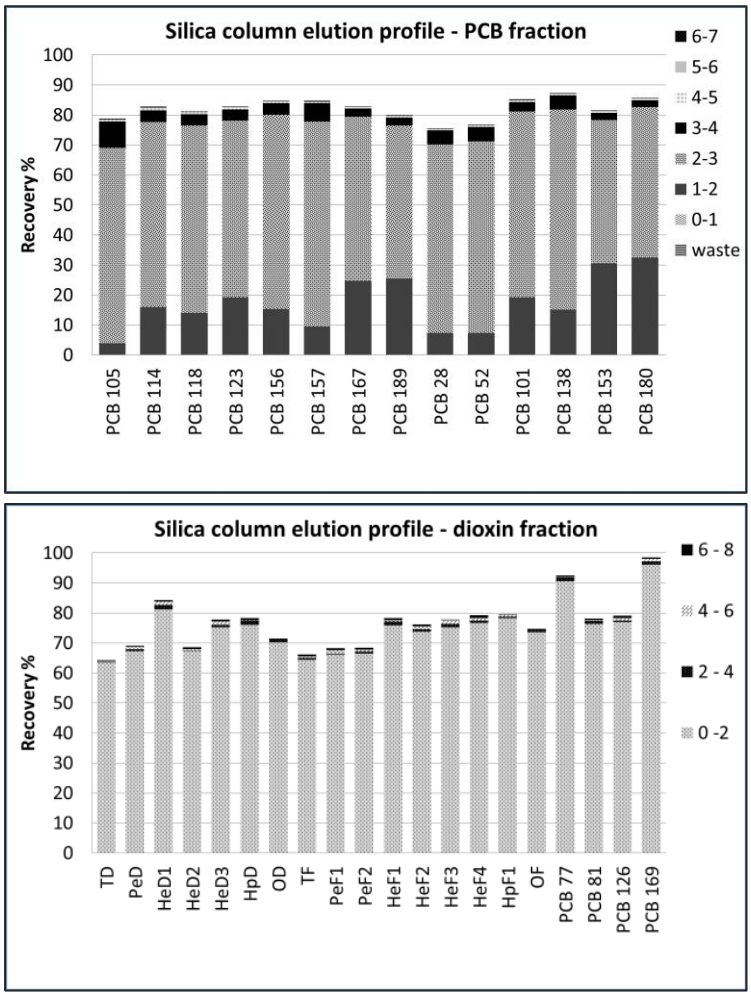

Figure 3.3: Silica column elution profiles of (top) PCB fraction (I- and MO-PCBs, F1) and (bottom) dioxin fraction (co-PCBs, PCDDs and PCDFs, F2).

This experiment showed that PCB fraction was faster and eluted in only 4 minute, while dioxin fraction required 6 minutes, namely 60 mL of hexane at 10 mL/min. In further experiments, 70 mL of hexane were used to compensate possible pump fluctuation and the effect of lipid degradation in real samples. It can happen, in fact, that by-products from lipid degradation generate backpressure and solvent flow fluctuation, slowing down the elution out of the silica.

To assess the volume needed for the complete elution from alumina in step 7 of the method, the alumina elution profile of dioxin fraction was drawn collecting fractions directly after the alumina column every 2 minutes for 10 minutes. Results indicated that 80 mL of hexane/DCM 50/50 mixture at 10 mL/min was required (Figure 3.4). Co-PCBs were eluted faster than dioxins and took only 4 minutes and 40 mL of solvent, and this result was considered an estimation of the volume needed by PCBs of fraction 1, for which the elution profile was not studied. Dioxins and furans required 8 minutes (80 mL of solvent). In further experiments, after connecting also carbon column in line, 100 mL of mixture were used in step 7 to take into account pump fluctuation and the slowing down effect of the interactions with carbon when collecting PCBs of fraction 1 (F1). This result fitted with the value set in our classic reference method, where 100 mL of mixture are employed in the classic method.

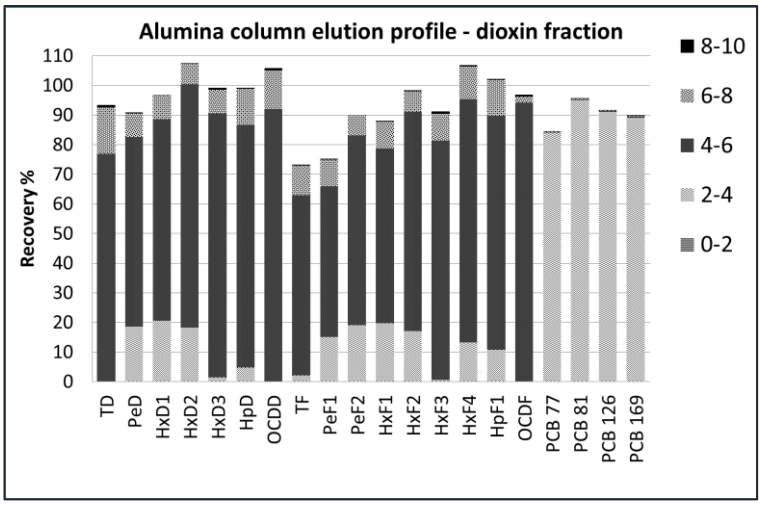

Figure 3.4: Alumina column elution profile with hexane/DCM (50/50) mixture at 10mL/min for dioxin fraction containing co-PCBs, PCDDs, and PCDFs. Likely other PCBs eluted as fast as co-PCBs within 4 min and 40 mL of solvent.

Carbon column elution profile (not shown) demonstrated good fitting with the reference method as 90 mL of toluene in backflush were needed to collect fraction 2.

Final method indicated as “short standard method” has been shown in Table 3.2 and it can represent an alternative to the validated standard automated method of our laboratory (Table 3.1).
“Short standard method” demonstrated comparable performances in terms of recovery rates to the reference clean-up method for low fat content matrices and it allowed time and solvent saving (Table 3.3).

Table 3.3: “Short standard method” performances compared to reference automated method in our routine laboratory.

	Method	Time (min)	Volume (mL)	Hexane (mL)	DCM (mL)	Toluene (mL)
Features	Reference	67.7	587	373	72	142
	Short standard method	48.3	393	253	50	90
Saving	Short standard method	29%	33%	32%	31%	37%

It is important to underline that the comparison in Table 3.3 with the reference method does not include the manual step but only the automated clean-up, which has fat capacity lower than 1 g. “Short standard method” could be applied also to process higher fat amount only after a preliminary fat degradation with manually packed acidic silica column.

3.2.1.2. Fast standard method

Further investigation was carried out with the aim of assessing if silica column conditioning (step 4) was relevant on recovery rates or it could be reduced to column dead volume. For this purpose “short standard method” scheme was modified into “fast standard method” reducing hexane volume in step 4 from 100 mL to 12 (Table 3.2). 10 mL of hexane spiked with IS solutions were processed and recovery rates for fraction 1 and 2 demonstrated that conditioning was not crucial and could be reduced to column dead volume, as recovery rates were still in the range 60 – 120% as required by the EU Regulation (Figure 3.5)

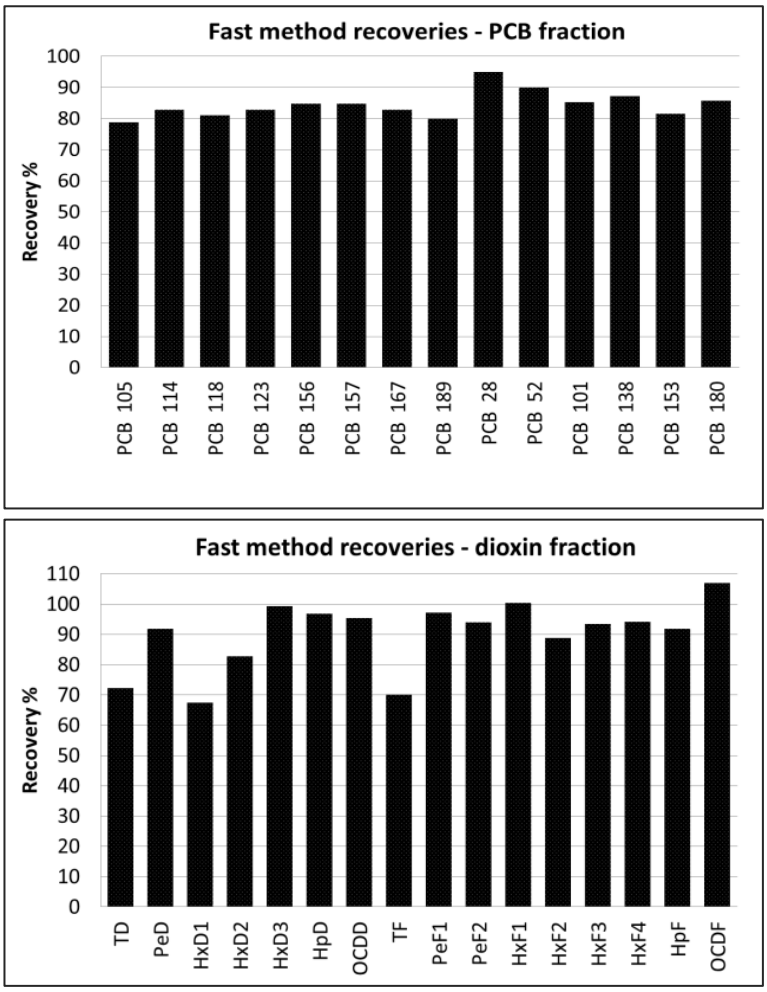

Figure 3.5: Recovery rates for PCB fraction (left) and dioxin fraction (right) when running "fast standard method" on the PowwerPrep.

Fast method performances allowed considerable saving as shown in Table 3.4

Table 3.4: “Fast standard method” performances compared to reference automated method in our routine laboratory.

	Method	Time (min)	Volume (mL)	Hexane (mL)	DCM (mL)	Toluene (mL)
Features	Reference	67.7	587	373	72	142
	Fast standard method	39.5	305	165	50	90
Saving	Fast standard method	42%	48%	56%	31%	37%

Alternative clean-up methods developed by eliminating less relevant steps of our classic automated clean-up method, led to considerable time and solvent saving, maintaining recovery rates in the range 60 -120 % as required by EU Regulation. Short standard method, including silica column conditioning, allowed around 30% time and solvent saving, fast standard method saving was 40% time and around 50% solvent, as column conditioning was skipped. Both methods were applicable to low fat content matrices (<1 g, as this was fat capacity of standard silica column), or to process up to 7 g of fat after preliminary fat degradation with manually packed acidic silica column.

3.2.2. Samples with fat content higher than 1 g

Alternatively, an additional clean-up method was studied to process sample with higher fat amount using only PowerPrep™ automated system without manually packed acidic silica clean-up. In this case a 4 column set was needed: high capacity (HC) acidic silica column, mini silica column, mini basic alumina column and carbon column (Figure 3.6, left). Mini columns had the same diameter but around half of the size of the standard columns (Figure 3.6, right); carbon column was the standard one.

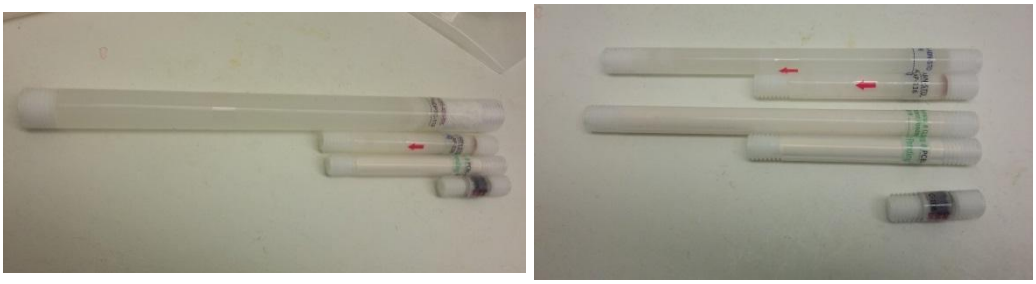

Figure 3.6: (left) 4 column setup used in this part of the work. (Right) mini column size compared to standard columns.

HC acidic silica column from FMS contains 45 g of 44% acidic silica so its fat capacity is up to 4 grams. This column has Teflon chips on the top (Figure 3.7) to disperse the sample avoiding rapid exothermic fat oxidation on silica surface. Indeed, in case of rapid oxidation fact degradation by-products could form agglomerates, being lipophilic compound in a polar environment, and generate backpressure.

Figure 3.7: Top of the high capacity acidic silica column, equipped with Teflon chips

The method, using 4 column set, was called “Jumbo method” because of the dimension of HC silica column. Such column set was chosen for a double purpose: first, to replace our manual acidic silica column with similar column integrated in a whole automated system; second, to test if standard column size can be reduced to mini column size, especially alumina column. This design in fact promoted acidic lipid degradation in the jumbo column, pH regulation in the multilayer mini silica, and final fractionation in alumina and carbon column.
The clean-up program was based on previous studies in our laboratory (Focant and De Pauw, 2002) and did not include conditioning step in view of the previous results.

Table 3.5: Main steps of “Jumbo method”.

JUMBO METHOD					
	Column	**Solvent**	**Process**	**Volume (mL)**	**Flow (mL/min)**
1	Tubing - w	Hexane	Fill tubing	15	10
2	C3 - w	Hexane	Wet alumina	6	10
3	C4 - w	Hexane	Wet carbon	6	10
4	C1/C2 - w	Hexane	Condition HC-mini silica	60	10
5			Add sample	100	5
6	C1/C2/C3 - w	Hexane	Elute silica with hexane	150	10
7	C3 – F1	Hexane/DCM	Elute alumina with 50/50, collect F1 – load carbon	70	10
8	C4 – F2	Toluene	Elute carbon backflush with toluene - collect F2	90	5

The method started filling tubing to remove air from the system. Then 6 mL of hexane, half of the volume used for standard columns, were used to wet mini alumina column since its size was about the half of the standard one; as the carbon column was the same as previous method, 6 mL of hexane were used for its soaking; 60 mL of hexane were needed to wet HC and mini silica columns connected in line to each other. After sample aspiration and loading, 150 mL (2.5 dead volumes) of hexane were used to elute HC and mini silica columns for fat degradation and alumina column loading. Mini alumina column required 70 mL of hexane/DCM 50/50 mixture at 10 mL/min for complete elution of compounds, partly collected in fraction 1 after carbon column (I- and MO-PCBs), and partly loaded on the top of the carbon and eluted in fraction 2 by backflushing the column with toluene (co-PCBs and PCDD/Fs). “Jumbo” method took 48.7 minutes and 397 mL of solvent in total to clean-up 4 g of fat.

“Jumbo method” performances were compared with the complete manual+automated clean-up approach used in our routine laboratory and it brought to more than 50% time and solvent saving (Table 3.6), though it is important to notice that the Jumbo method was suitable to process up to 4 g of fat instead of 7 g normally processed by the reference method.

Table 3.6: Jumbo method performances compared to manual+automated clean-up methods in our laboratory.

	Method	Time (min)	Volume (mL)	Hexane (mL)	DCM (mL)	Toluene (mL)
Features	Manual+automated standard	97.7	887	673	72	142
	Jumbo	48.7	397	272	35	90
Saving	Jumbo	50%	55%	60%	51%	37%

“

Jumbo method” led to about 50% time and solvent saving, still giving good performances after tests on real fatty matrices, as shown in the following tests on real food and feed matrices.

3.2.2.1. Test on feed and food samples

4 g of two fatty matrices, QC pork fat for feed and sunflower oil, as well as one procedural blank (10 mL of hexane spiked with IS solutions) were processed with “Jumbo method” and 4 column set to check recovery rates and the quality of the final extracts (Figure 3.8).

Figure 3.8: Test on food and feed samples with Jumbo method and 4 column set, from the left: blank, QC pork fat and commercial sunflower oil.

Samples were diluted in 60 mL of hexane in order to decrease their viscosity and to disperse the fat to reduce exothermic oxidation on the acidic silica column. After wetting the column and checking for leaks (steps from 1 to 4 in Table 3.5), samples entered the system by automated aspiration and were loaded on the high capacity and mini silica columns with hexane at 10 mL/min (step 6). As the sample interacted with the sorbent, lipid oxidation occurred and the sorbent turned darker. As shown in Figure 3.8, pork fat lipid degradation (central sample) seemed to occur faster than sunflower oil oxidation (sample on the right), as the sorbent was very dark until the middle of the column, whereas for sunflower oil fat degradation seemed to be slower as sorbent looked brownish for all column length. A possible explanation to this experimental evidence can be found in the different composition of these matrices, in particular in triglyceride composition, reported in Table 3.7 (O'Brien, 2009).

Table 3.7: Triglyceride composition of pork fat and sunflower oil.

Matrix	Triglyceride composition, %	Typical	Range
Pork fat (lard)	SSS Trisaturatedl	Lard composition is variable	2 to 5
	SSU Disaturatedl	and it depends on animal diet.	25 to 35
	UUS Monosaturated	Only ranges can be defined	50 to 60
	UUU Unsaturated		10 to 30
Sunflower oil	SSS Trisaturatedl	0.3	
	SUS Disaturatedl	2.9	
	SSU Disaturated	0.2	
	SUU Monosaturated	26.6	
	UUU Unsaturated	70.2	

Sunflower oil is mainly constituted by unsaturated triglyceride, unlike pork fat which contains more saturated triglycerides. One hypothesis to explain why sunflower oil oxidation is slower than lard oxidation, is the presence of competitive reactions acid catalyzed occurring instead of fat hydrolysis. In acid media, in fact, water and alcohol can add to double bond of unsaturated chains of triglycerides to form alcohols and ethers (electrophilic addition), which are further oxidized but in a second step. According to this, lard oxidation is faster because of the presence of less unsaturated chains for competitive reaction.

The Jumbo method demonstrated its suitability for application to real samples as no interferences preventing instrumental analysis were found in final extracts. Hexadioxin peaks have been chosen as an example of the chromatographic quality because these compounds are the most difficult to separate and are very sensitive to interferences. Figure 3.9 showed hexadioxin peaks for QC pork fat. Peak shape and separation were very good indicating lack of interferences. Chromatographic quality was comparable to the blank (Figure 3.9, bottom).

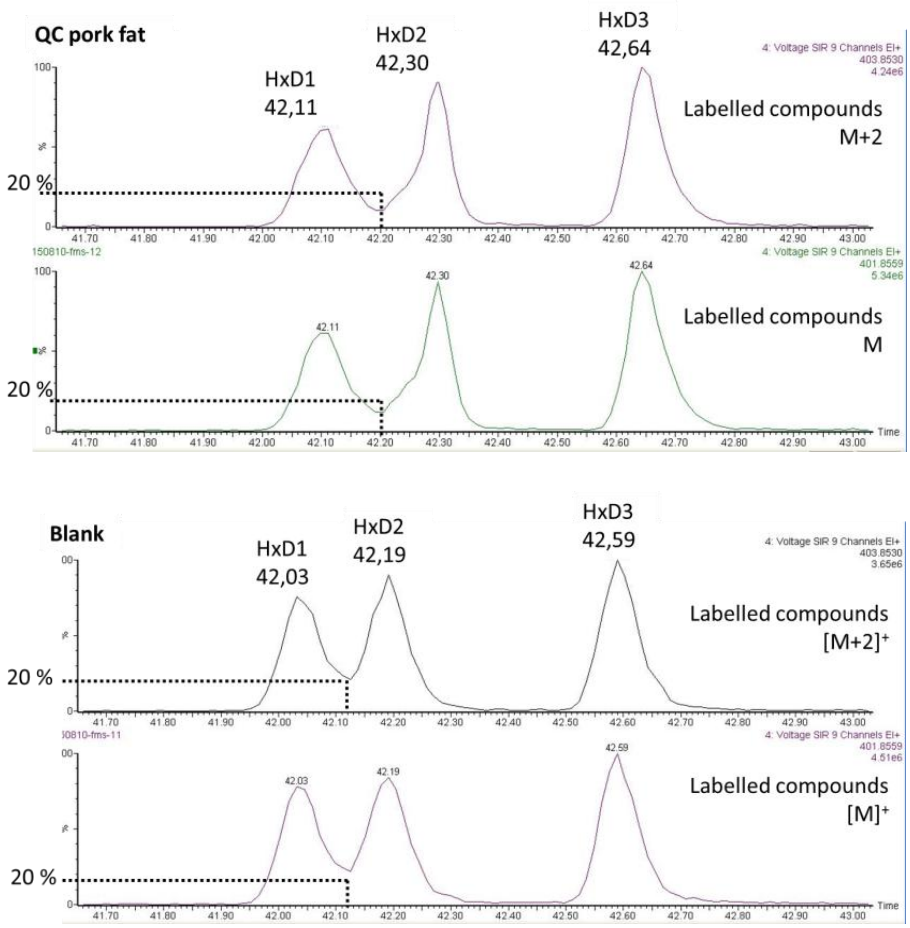

Figure 3.9: (top) hexadioxin chromatogram (labelled compound transitions) of QC pork fat processed with Jumbo method. Hexadioxin 1 and 2 (HxD1 and HxD2) have separation higher than 20%. (Bottom) procedural blank chromatogram.

For vegetable oil PCB peaks (#123, 114, 118, 105) are shown to give an example of chromatographic quality of PCB fraction cleaned-up with the “Jumbo method” (Figure 3.10).

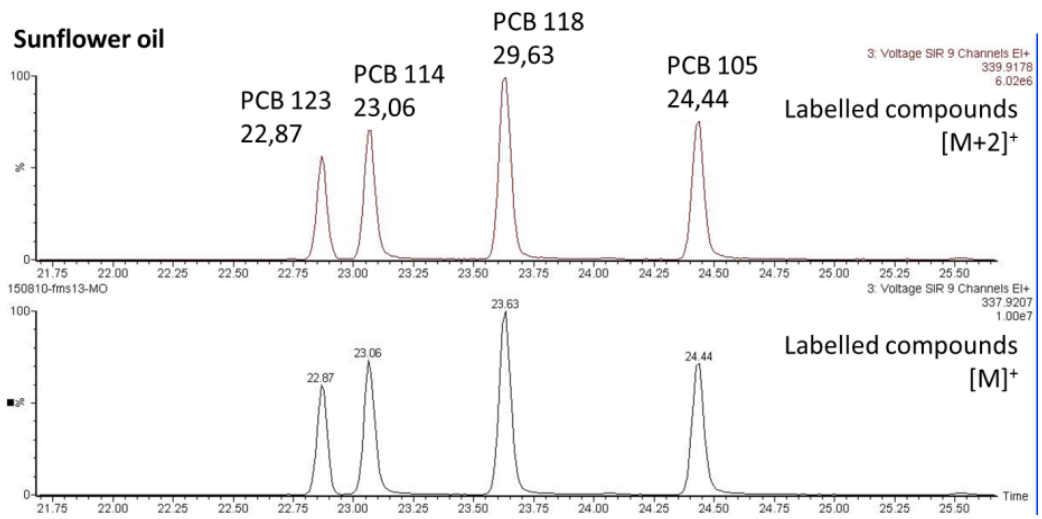

Figure 3.10: (top) PCB (#123, 114, 118, 105) chromatogram (labelled compound transitions) of vegetable oil processed with Jumbo method.

3.3. Alumina sorbent features

It is worth to compare the performances of alumina columns in different methods explored up to know, supposing that alumina used for manually packed columns (Chapter II) and alumina in commercial FMS columns underwent the same intense thermal treatment for moisture removal (600°C for 30h), and that both the materials had the same average particle size.

Alumina mini column showed the best performances if compared with standard FMS commercial columns and the alumina used for manually packed columns.

Table 3.8: Comparison between the performances of basic alumina used in the method with manually packed columns, and in the method with commercial mini columns.

Parameter	Alumina in the manually packed column	Alumina in the standard commercial column	Alumina in the mini commercial column
Amount	10 g	11 g	6 g
Column	7 cm x 1.7 mm ID	16 cm x 0.9 mm ID	8 cm x 0.9 mm ID
Hexane amount	175 mL	90 mL	150 mL
Solvent flow	7 mL/min	10 mL/min	10 mL/min
Ratio sorbent/solvent	0.058	0.122	0.04

For manually packed columns in Chapter II, 7 cm x 1.7 mm ID glass tube was filled with 10 g of basic alumina, which were able to hold compounds after 175 mL of hexane flowing at 7 mL/min. In the present case, commercial mini columns contained 6 g of basic alumina in a PTFE 8 cm x 0.9 mm ID tube and were able to hold compounds despite 150 mL flowing of hexane at 10 mL/min. On the other hand, standard alumina column contained 11 g of sorbent in a PTFE 16 cm x 0.9 mm ID column and it was flushed with 90 mL of hexane at 10 mL/min (Table 3.8).

Alumina mini columns showed the best performances among the alumina columns studied, as the ratio between the amount of sorbent in the column and the amount of solvent flushing the column with hexane (during fat degradation on the silica column) was the smallest (0.04). From these results, one could think that sorbent amount in the standard FMS column is exceeding the necessary quantity, as mini columns, despite the apparently low amount, showed performances suitable for the scope, even in hard conditions. Moreover, mini commercial column seemed to be very active also in comparison with alumina in the manually packed columns, despite the smaller sorbent amount and the higher solvent flow.

4. Conclusions

Alternative clean-up approaches were developed for the PowerPrep™ system from FMS with the aim of having high throughput method for dioxin analysis with time, solvent and global cost reduction. Approaches were based on our validated clean-up method consisting of a preliminary lipid digestion with a manually packed acidic silica column, followed by automated clean-up with PowerPrep™ equipped with a 3 column set: standard ABN silica, alumina and carbon column. "Short" and "fast standard methods" used the same column set and were suitable for low fat content samples (< 1 g) or they had to come after a preliminary acidic digestion with manually packed acidic silica column to have fat capacity of up to 7 g. New methods were designed from our routine automated method removing less crucial steps, namely column washing/conditionings and tubing washing. Silica, alumina and carbon column elution profiles were studied to assess minimum solvent amount for column elution. "Fast standard method" showed also that initial silica column conditioning could be skipped without affecting final recoveries and extract quality. Alternative automated approaches allowed from 30 to 50% saving in terms of solvent and time.

"Jumbo method" was developed as a fully automated alternative to our entire clean-up procedure including the manual and the automated steps. Jumbo method used 4 column set on the PowerPrep™: high capacity acidic silica column, mini ABN silica, mini alumina and carbon column, for a total fat capacity of up to 4 g and about 50% solvent and time saving in comparison to our whole clean-up procedure. After development, the method was tested on pork fat QC and commercial vegetable oil to check the quality of the final extracts, and it demonstrated to be suitable for trace level measurement of dioxins and PCBs. Standar and mini FMs alumina column performances were compared and mini column demonstrated to be suitable for the fractionation of dioxins, furans and PCBs processing up to 4 g of fat. Likely, standard FMS alumina column is oversized and it could be replaced by mini column with solvent and time saving.

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CHAPTER IV

Based on the following publication

L'Homme B., Calaprice C*., Calvano C.D., Zambonin C., Leardi R., Focant J.-F., (2015)*
"Ultra-trace measurement of Dechloranes to investigate food as a route of human exposure"
Chemosphere. 2015 Nov; 139:525-33. Doi: 10.1016/j.chemosphere.2015.07.043.

** These authors contributed equally to this work*

1. Introduction

In this project, the main method for dioxin analysis was modified in order to integrate a new class of compounds, Dechloranes, in the regular controls for dioxins.
This study started after the detection of Dechloranes in human blood of people from France (Brasseur et al., 2014) even if no production site has been identified in Europe. The study was aimed to determine the main route of human exposure to these compounds and the investigation was started from food, as it is the main route of exposure to dioxins.

In this part of the work of thesis, an analytical method from sample preparation to instrumental detection with GC-MS/MS Triple Quad was developed to allow the assessment of Dechlorane levels in food and feed, with the aim of investigating the contribute of food consumption to human exposure to these chemicals. The analytical approach was based on the fully validated method using isotope dilution (ID) and GC-MS/MS Triple quad instrument for the measurement of dioxins in food and feed at the low pg level under the European Legislation (L'Homme et al., 2015) with the aim of allowing simultaneous analysis of dioxins and Dehcloranes during regular food safety controls. Instrumental parameters were optimized to reach very high sensitivity at ppb levels. For this purpose, programmed temperature vaporizing (PTV) injector in solvent vent mode for large volume injection (LVI) was used; experimental design, in particular, full factorial design (FFD) and

face-centered design (FCD) were used to select optimum values for the three most relevant factors for us, such as vent flow, vent pressure, and vent temperature. On the MS side, specific multiple reaction monitoring (MRM) transitions were selected from Dechlorane fragmentation pattern, that followed a retro Diels-Alder reaction of the norbornene moiety of the molecule (Brasseur et al., 2012; Shen et al., 2012). The method was validated following the applicable criteria of the EU Regulation for dioxins, as dedicated regulation for Dechloranes is not yet available and MLs have not been assessed.

2. Dechlorane structures

Dechloranes are a family of organo-chlorinated compounds sharing a bicyclo [2,2,1] heptene structure, resulting from a Diels–Alder reaction between one or two molecules of hexachlorocyclopentadiene (HCCPD) with various cyclic dienophiles. The first of these compounds was Mirex, also called Dechlorane, which is included in the Annex A of the list of POPs drawn up during the Stockholm Convention (www.pops.int/), including chemicals whose production and use have to be eliminated because of their intrinsic toxicity. In fact, since 1978, Mirex is no longer made or used in the United States, but it was extensively used in the 1960s and 1970s as a pesticide to control fire ants, and also as a flame retardant additive under the trade name Dechlorane® in plastics, rubber, paint, paper, and electrical goods from 1959 to 1972, because it does burn easily (ATSDR, 2000). Since that time several studies were carried out on Mirex, highlighting its toxicity and negative effects on biota and humans (Keisr, 1978).
After Mirex was banned, structural similar compounds were synthetized to replace Mirex since no restriction was put on them. Dechlorane compounds are Dechlorane Plus, *syn*- and *anti*-isomer (DP, $C_{18}H_{12}Cl_{12}$), Dechlorane 602 (Dec 602, $C_{14}H_4Cl_{12}O$), Dechlorane 603 (Dec 603, $C_{17}H_8Cl_{12}$), Dechlorane 604 (Dec 604, $C_{13}H_4Br_4Cl_6$) and Chlordene Plus (CP, $C_{15}H_6Cl_{12}$) (Figure 4.1) (Inchem, 1984).

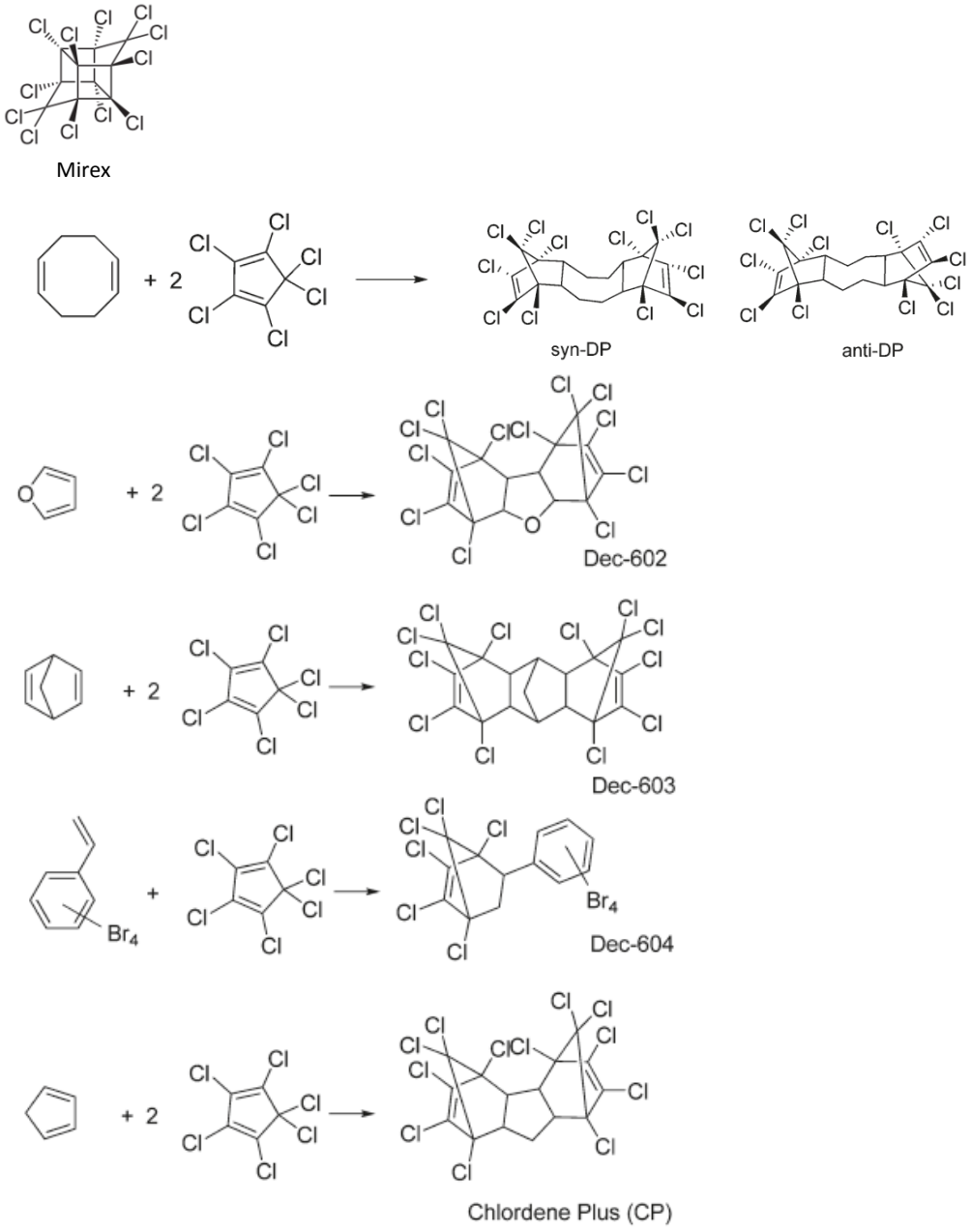

Figure 4.1: Dechlorane structures

3. Dechlorane sources, environmental and biological contamination

DP *syn*- and *anti*-isomers are currently produced by OxyChem in Niagara Falls, New York, and by Anpon in Huai'an in China (Sverko et al., 2011). The isomers in the technical mixture are in a ratio 1:3 for the *anti* isomer, that is thermodynamically more stable. DPs are currently used as flame retardants in electrical hard plastic connectors in televisions and computer monitors, wire coating, and furniture (Betts, 2006). Several studies showed the presence of DP isomers and other Dechloranes in the environmental (Hoh et al., 2006, Shen et al., 2010, Jia et al., 2011, Peng et al., 2014) and in biota samples (Guerra et al., 2011, Kim et al., 2014, Sühring et al., 2015), highlighting POP features of low solubility in water, chemical and physical stability, biopersistence and hence long range transport, bioaccumulation and biomagnification in the food chain. Very recent human biomonitoring studies have reported levels at the ng/g lipid in human serum from Norway (Cequier et al., 2013) and France (Brasseur et al., 2014), as well as in breast milk from Canada (Zhou et al., 2014).

4. Materials and methods

4.1. Chemicals and consumables

Solvents (hexane, dichloromethane, toluene, methanol) were Picograde® reagents (LGC Promochem, Wesel, Germany). All solvent batches were tested for investigated analyte contamination before use. Nonane and dodecane puriss analytical-reagent grade standard for GC were purchased from Fluka (Steinheim, Germany). Water was obtained from a Milli-Q Ultrapure water purification system (Millipore, Brussels, Belgium). Ethanol, ammonium hydroxide solution 28-30% and ethyl ether were purchased from Sigma Aldrich (St. Louis, MO, USA). Silica gel was purchased from Macherey-Nagel (Macherey-Nagel GmbH & Co KG, Düren, Germany). Disposable PTFE columns for the automated clean-up were obtained from Fluid Management Systems (FMS Inc., Waltham, MA, USA). Chromatographic pure grade helium gas, 99.9999% alphagaz 2 was purchased from Air Liquide (Paris, France). Technical N27 grade liquid CO_2 was used for PTV cooling (Air

Liquide, Paris, France). Sodium sulphate and diatomaceous earth were purchased from VWR International (Radnor, PA, USA).

Dechlorane quantitation was performed with Isotope Dilution (ID) technique, using the labelled internal standards available at the moment of the work. DP syn $^{13}C_{10}$-labelled internal standard and Dec 602 $^{13}C_{10}$-labelled internal standard, as well as DP syn and DPanti standards were supplied by Cambridge Isotope Laboratories (CIL, Andover, MS, USA). Mirex standard was purchased from Cluzeau Info Labo (France); Dec 602, Dec 603 and Dec 604 standards were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada); CP standard was bought from Wellington Laboratories (Guelph, ON, Canada). DP syn $^{13}C_{10}$-labelled internal standard was used for the quantitation of DP isomers, while Dec 602 $^{13}C_{10}$-labelled internal standard was used for Mirex, Dec 602, Dec 603, Dec 604 and CP based on retention time proximity. The EC-1414 solution of $^{13}C_{12}$-labelled PCB 80 was purchased from CIL and used as recovery standard for all the compounds. Calibration curves were prepared from stock standard solutions at 100 µg/mL and consisted in 5 to 7 calibration points.

4.2. Samples

After method development with standard solutions, a total of 88 samples were analysed to assess average levels in food and feed. Sample matrices consisted in chicken, pork, pure animal fat, salmon, milk, eggs, vegetable oil for feed, feed additives, and corn. 77 samples out 88 were randomly selected from samples entering our ISO17025 accredited laboratory for monitoring controls on PCDD/Fs and PCBs; the remaining 11 samples (5 salmons and 6 chickens) were collected from regular shops and supermarkets in the area of Liege, Belgium, in January 2015, to have a complete overview of several food matrices. During the study, 16 procedural blanks were also analysed.

4.3. Sample preparation: extraction and clean up

Sample preparation procedure, extraction and clean-up included, was the same used for polychlorodibenzo-*p*-dioxin (PCDD), polychloro-dibenzofuran (PCDF), and polychlorobiphenyl (PCB) analysis in our laboratory. Detailed description of the procedures for all ma-

trix typologies is out of the scope of this report and it has been already published (Focant et al., 2001, Focant et al., 2006, Pirard et al., 2002). Briefly, for all the matrices (except already pure fat) fat extraction was required because of Dechlorane lipophilicity (as dioxins), and it was performed by Accelerated Solvent Extraction (ASE™ 350, Dionex, Thermo Fisher Scientific). Only for milk, liquid-liquid extraction was required and the procedure is reported in Chapter 2 of this thesis. Samples were spiked with internal standards before fat extraction for salmon and feed, and directly on the extracted fat for the other matrices, according to the guidelines of the EU Regulation for dioxin analysis, as no guidelines are available for Dechloranes. Afterwards, samples underwent a double step clean-up in the same framework as dioxins and PCBs: first step was a manually packed acidic silica column for intense lipid digestion, and second step was automated clean-up and fractionation of dioxins and PCBs with PowerPrep™ automated system (FMS Inc, Waltham, USA), equipped with standard ABN silica, alumina and carbon column (Focant et al., 2004). As for PCDD/F and PCB classical clean-up, two fractions were collected and sent to instrumental analysis. Dechloranes were collected in fraction 1 (F1) with non-planar species collected by forward elution of the alumina-carbon column with hexane/DCM 50/50 mixture. This fraction was injected for Dechlorane analysis after evaporation of solvents in a dedicated tube using a sensor-equipped TurboVap II Workstation (Caliper Life Science, Teraflene, Belgium) and after subsequent evaporation in GC vials containing nonane (90 µL) as a keeper using a RapidVap (Labconco, Kansas City, MO, USA). The $^{13}C_{12}$-labelled PCB 80 recovery standard was added before instrumental analysis. Blanks were processed following the same procedure of their corresponding matrix according to the Table 4.1. Some matrices had the same procedural blank because followed the same extraction procedure.

Table 4.1: Procedural blanks for several food and feed matrices in our laboratory

Corresponding matrix	Blank	number
Chicken	10 mL of hexane	9
Pork		
Egg		
Pure fat		
Salmon		
Vegetable oil (for feed)		
Milk	80 mL Ultrapure water	3
Feed additives	Na_2SO_4 and diatomaceous earth mixture	4
Corn		
	Total	16

4.4. GC-MS/MS conditions

Instrumental analysis was carried out with GC-MS/MS Triple Quad instrument from Agilent (Palo Alto, CA, USA), namely a 7890G gas chromatographer coupled with a 7000C Triple Quadrupole mass spectrometer. The GC was equipped with a programmable temperature vaporization (PTV) inlet, and a 7693A automated liquid sampler (ALS). 5 µL out of 100 µL of the final purified extract were injected into the PTV inlet operating in solvent vent mode and cooled by liquid CO_2. The inlet temperature program and solvent vent parameters were optimized by means of an experimental design and were: inlet initial temperature of 45°C for 1.3 min, ramped up at 720°C/min to 320°C and hold until the end of the run; vent flow was set at 120 mL/min at vent pressure of 10.5 psi. Purge flow was set to 1200 mL/min after 5 min to insure complete evacuation of the inlet and prevent from possible memory effects. All the computations and graphs were performed using multiple linear regression routines written in Matlab (Mathworks Inc., Natrick, USA). The GC column was the same used for dioxin analysis, namely an Agilent DB-5ms ultra inert (60 m x 0.25 mm x 0.25 µm), in order to allow Dechlorane analysis when doing routine controls for dioxins and PCBs without changing instrumental layout. The GC oven temperature program was characterised by a sharp ramp because of Dechlorane high molecular weight: start at 140°C for 2.6 min, ramp at 100°C/min to 320°C and hold for 21.1 min (total run time of 25.5 min). The transfer line temperature was held at 320°C. On the

MS side, electron ionization (EI) ion source at 70eV energy and temperature of 280°C was used. Temperature of the quadrupoles was set at 150°C and multiple reaction monitoring (MRM) transitions were recorded at 'wide' mass resolution, which means 1.2 Da on the Agilent software. Ultrapure Nitrogen at 1.5 mL/min and Helium at 2.25 mL/min were used as collision gas and quench gas in the collision cell, respectively. Dwell times were selected during method optimization to increase the sensitivity as much as possible and to adjust the acquisition frequency to get ten data points per peak. Calibration and autotune were performed in the EI high sensitivity mode. Retention time locking was performed with PCB 105 allowing change and reinstallation of the column while keeping reproducible retention times. Mass Hunter version B.07.00 was used for acquisition and quantitative analysis.

5. Results and discussion

5.1. Sample clean-up

The study was aimed to the development of a multi-analyte procedure that included Dechloranes during regular controls for dioxins and PCBs, as from previous experience in our laboratory, it was known that typical dioxin sample preparation procedures were usable for Dechlorane measurements in serum and that Dechloranes could be collected in fraction 1 together with non-planar compounds (Brasseur et al., 2014).
Due to the lipophilic nature of Dechloranes, fat extraction procedures for each matrix were maintained the same as dioxin analysis. For sample clean-up, matrices with fat amount higher than blood were processed and stronger lipid acidic degradation was required. So, our routine sample clean-up approach, manual and automated stages, was checked step-by-step with recovery experiments, to investigate possible degradation of Dechlorane compounds under such acidic treatment. Figure 4.2 reports the detailed fractionation study where Dechlorane recovery rates were measured after each clean-up step. 10 mL of hexane spiked with 100 pg/µL mixture of all the compounds were cleaned up following the procedure one step at a time; eluate was collected and evaporated to approximately

500 µL and then transferred to an injection vial already containing 90 µL of nonane for solvent exchange before $^{13}C_{12}$-labelled PCB 80 recovery standard was added.

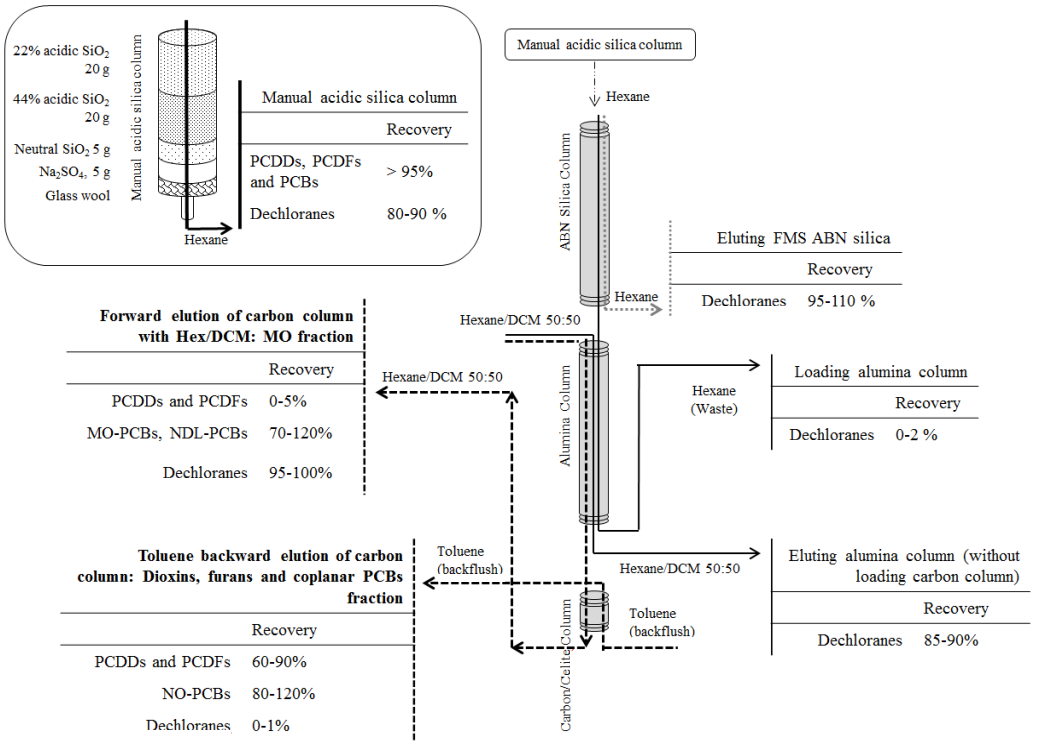

Figure 4.2: Recovery rates for Dechloranes using our Dioxin clean-up and fractionation procedure. Arrows and charts referring to the same step have the same layout. From top to bottom: manual acidic silica column eluted with hexane (detail on the top left corner); standard commercial ABN silica column eluted with hexane (grey dotted line); basic alumina loaded with hexane (to the waste) and eluted with hexane/DCM 50/50 mixture (black continuous lines); and carbon columns loaded and eluted with hexane/DCM 50/50 mixture for the collection of fraction 1, containing I- and MO-PCBs, as well as Dechloranes (black dotted line); carbon column backflush elution with toluene for fraction 2 collection containing PCDDs and PCDFs (black dotted line).

These experiments confirmed that all Dechlorane compounds were present in fraction 1 of non-planar PCBs, and revealed that no acidic degradation occurred during the clan-up, as recovery rates for all the analytes were close to 100%. This demonstrated that

Dechlorane analysis can be integrated in a multi-analyte method including already dioxins and PCBs, without modifying sample preparation layout.

5.2. Experimental design for optimization of PTV parameters

5.2.1. Structure of the experimental design

Since no preliminary data about Dechlorane background levels in food and feed matrices in Europe, and no Regulation about MLs was available, a wide dynamic range including ultra-trace levels was considered in the study. To insure method sensitivity a Design of Experiment (DoE) was performed for the programmed temperature vaporizing (PTV) inlet, as the first interface affecting method sensitivity. PTV inlet was working in solvent vent mode, so initial inlet temperature (T), vent flow (VF), and vent pressure (VP) were chosen as the most relevant factors for the experimental design, because they effected sample transfer into the column and hence method sensitivity.
A preliminary 2^3 full factorial design (FFD) was performed to explore the experimental domain and to assess a suitable model to postulate for a further more rigorous experimental design investigation. According to the screening results, central point and boundaries of the experimental domain were chosen. Thereafter, in this smaller domain (Table 4.2), experiments of a face-centred design (FCD) on three factors were done to calculate the coefficients of the postulated model in *Equation 1*, where quadratic effects and interactions between variables are taken into account.

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_{12} + b_{13}x_{13} + b_{23}x_{23} + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad \textit{(Eq.1)}$$

where:

- x_i is the experimental factor representing initial inlet temperature (T) for *i*=1, solvent vent flow (VF) for *i*=2 and solvent vent pressure (VP) for *i*=3;
- x_{ij} is the factor representing the interaction between factor *i* and *j*, or the quadratic factor if *i=j*;

- b_i is the effect of factor *i;*
- b_{ij} is the effect of the interaction between factor *i* and factor *j*, or the quadratic effect for *i=j*;
- b_0 is the response in the centre of the domain, where all the factor are set equals to 0;
- y is the response under studying.

In this work, two responses were monitored for each compound: peak area (y_1), to be maximised and peak symmetry (y_2), to be hold in the range 0.9 and 1.1 (perfectly symmetric peak at 1), as explained later. Both responses were calculated in the quantitative analysis software of the instrument.
To calculate such a model it was necessary to set, for each variable, three levels in the experimental domain, a low (-1), a middle (0) and a high (+1) one.

Table 4.2: Experimental domain and factor levels of the FCD.

Levels of the factors in the experimental design	**-1**	**0**	**+1**
x_1 = T, initial inlet temperature (°C)	30	45	60
x_2 = VF, vent flow (mL/min)	20	60	100
x_3 = VP, vent pressure (psi)	1	10.5	20

A total of 17 experiments (Experimental Matrix in Table 4.3), including three replicates in the centre, were conducted in random order using a 10 pg/µL mixture of all the Dechloranes in nonane. The three replicates in the centre were done to improve the mathematical characteristics of the model, and in this case it didn't add further experimental work, as replicates were easily run in high-throughput by using the auto-sampler.

Table 4.3: Experimental Matrix of the Face Centered Design with 3 replicates in the center.

	x_1 = T	x_2 = VF	x_3 = VP
1	30	20	1
2	60	20	1
3	30	100	1
4	60	100	1
5	30	20	20
6	60	20	20
7	30	100	20
8	60	100	20
9	30	60	10.5
10	60	60	10.5
11	45	20	10.5
12	45	100	10.5
13	45	60	1
14	45	60	20
15	45	60	10.5
16	45	60	10.5
17	45	60	10.5

5.2.2. Peak area

Figure 4.3 shows the significant effects (the coefficients of the quadratic model) calculated for peak area (y_1) and for each compound. At the explored concentration, 10 pg/µL, Dec 604 was not detectable and was therefore not represented. Significant coefficients were almost the same for all compounds, and were mostly related to temperature (b_1 and b_{11}), which had a negative quadratic effect on peak area, and to vent flow (b_2), which had a positive linear effect. Vent pressure (b_3) was not statistically significant, but close to have a significant negative linear effect for Mirex and DP syn.

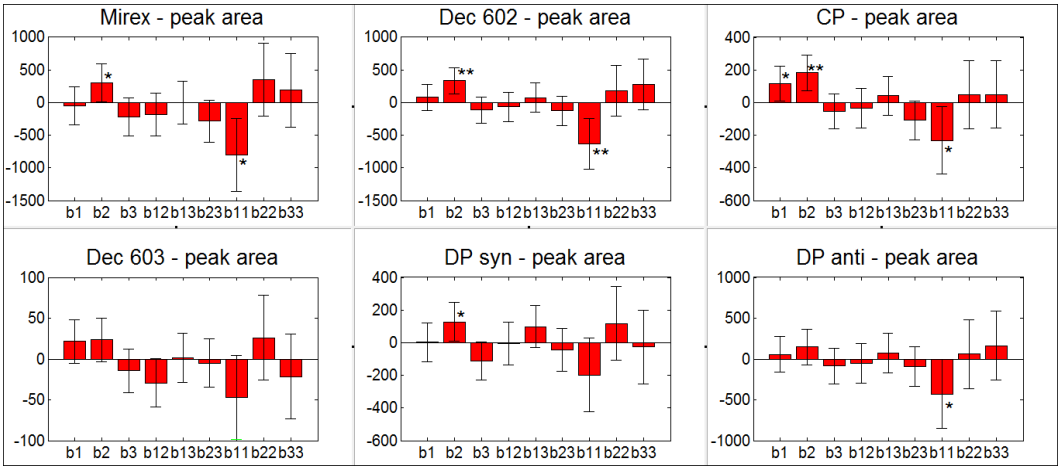

Figure 4.3: Bar plot of the coefficients of the postulated model for peak area. Brackets correspond to the confidence intervals at p=0.05; the stars indicate the significance of the coefficients (*=p<0.05, **=p<0.01, ***=p<0.001)

Peak area dramatically decreased with temperature above and below 45°C because of its strong negative quadratic effect, and mostly increased with vent flow. Vent pressure, even if not statistically significant, showed an overall negative effect on peak area. Table 4.4 reports a summary of the significant effects for peak area and the action to take to maximise it.

Table 4.4: Significant effects for peak area and values to set for each factor.

Goal	**Significant effect**	**Levels to set to maximize peak area**	**Values to set to maximize peak area**
To be maximized	$b_{11} < 0$	T = 0	T = 45°C
	$b_2 > 0$	VF = +1	VF = 100 mL/min
	$b_3 < 0$	VP = -1	VP = 1 psi

For all the models no lack of fit was observed, since variance of residuals was comparable to the variance of replicates.

5.2.3. Peak symmetry

Peak symmetry was calculated at 10% height of the peak as the ratio between the distance from the tail part of the peak to the centre of the apex, and from the front part of the peak to the centre of the apex (Figure 4.4).

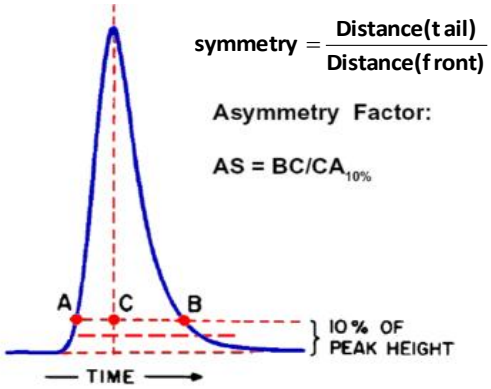

Figure 4.4: Calculation of peak symmetry done by the software of the instrument used in this work.

A symmetry value of 1.0 meant that the peak was balanced, whereas values lower and higher than 1 indicated respectively peak fronting and tailing. In this work, peak symmetry values between 0.9 and 1.1 were considered as acceptable, although the best symmetry was reached at 1.0. This was because peak symmetry was calculated on raw experimental data without smoothing and experimental variation of the peak apex from the centre of the peak was expected.

Figure 4.5 shows the effects calculated for peak symmetry (y_2). In this case, temperature, vent flow and their interaction were significant for some analytes $(b_1, b_2, b_{12} \neq 0)$, while vent pressure (b_3) was almost significant (positive effect) at 95% confidence for Mirex only.

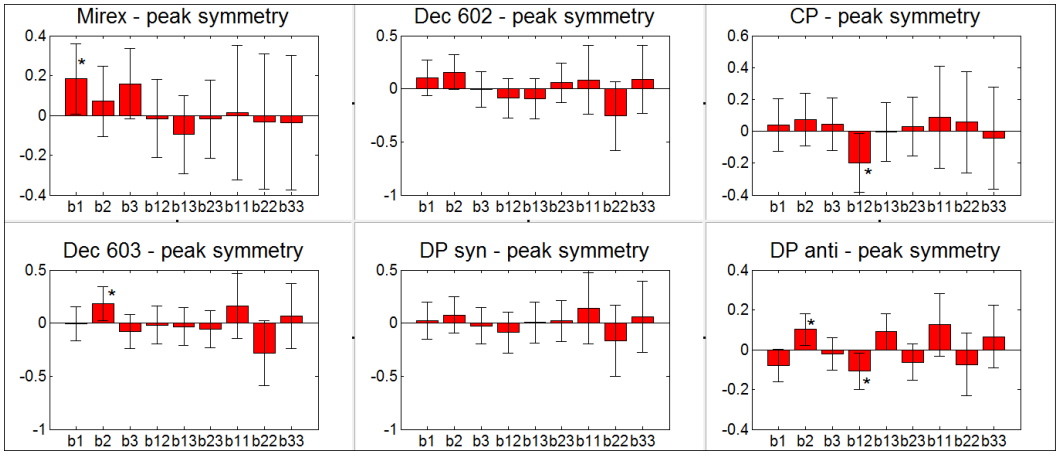

Figure 4.5: Bar plot of the coefficients of the model for peak symmetry for all the detectable Dechloranes. The brackets correspond to the confidence intervals at p=0.05; the stars indicate the significance of the coefficients (*=p<0.05)

Vent flow had a positive significant effect on peak symmetry (and also on peak area). Vent pressure, even if not statistically significant for both responses, showed a rather positive effect on peak symmetry, and an overall negative effect on peak area. Table 4.5 reports a summary of the significant effects for peak symmetry and the action to take to hold it in the range 0.9 – 1.1.

Table 4.5: Significant effects for peak symmetry and values to set for each factor.

Goal	**Significant effect**	**Levels to set to have symmetric peak**	**Values to set to have symmetric peak maximize peak area**
To be maintained in the range 0.9 – 1.1	$b_1 > 0$	T = +1	T = 60°C
	$b_2 > 0$	VF = +1	VF = 100 mL/min
	$b_3 > 0$	VP = +1	VP = 20 psi

5.2.4. Optimum values for inlet parameters.

The goal of the experimental design was to assure method sensitivity, namely to have maximum peak area with symmetric peak shape (peak symmetry between 0.9 and 1.1),

not only to maximise one of the two responses. For this reason, even if the optimum vent pressure to maximize peak area appeared to be 1 psi, this value led to peak fronting (peak symmetry <1) as shown by response surfaces and contour plots reported in Figure 4.6, referring to Mirex as an example.

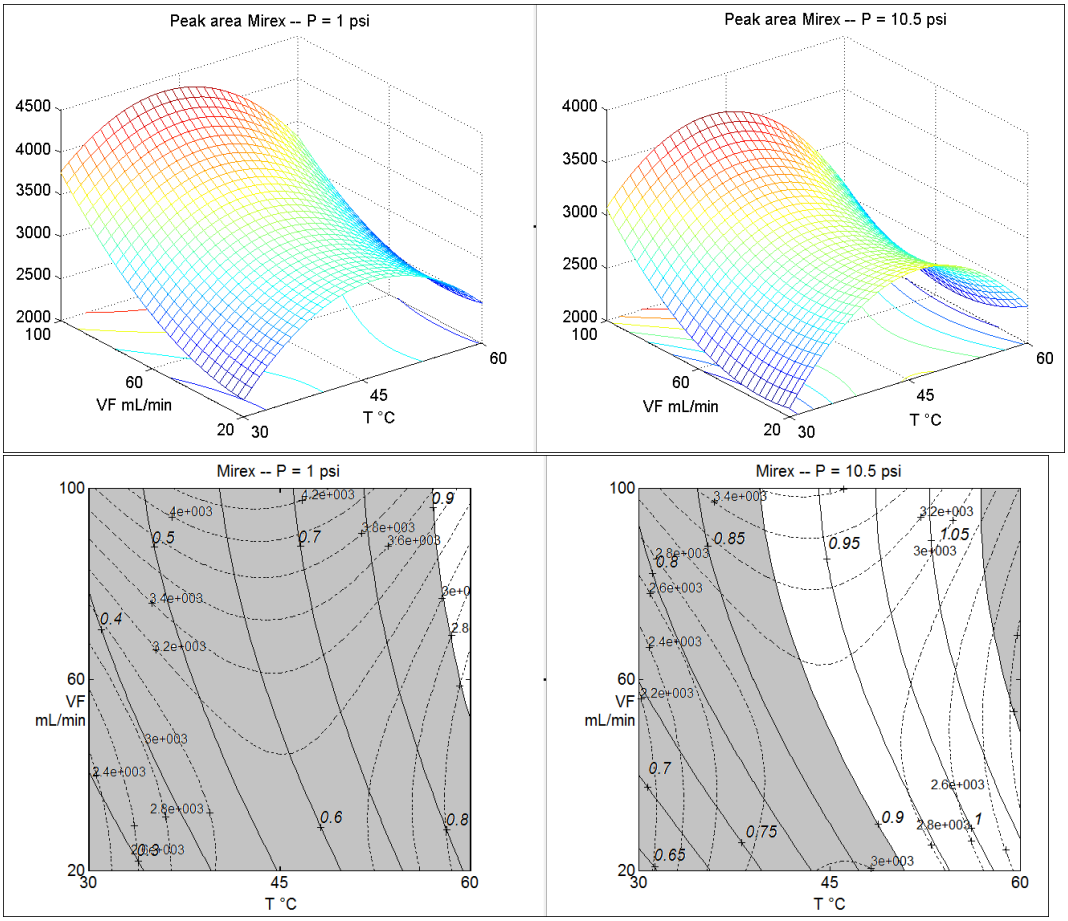

Figure 4.6: Top: peak area response surfaces for Mirex in temperature-vent flow planes at different vent pressure levels: 1 psi (left) and 10.5 psi (right). Bottom: overlapped contour plot of peak area (dashed line) and peak symmetry (continuous line) for Mirex at vent pressure 1 psi and 10 psi. The grey area shows un-acceptable values (in italic) for peak symmetry in the experimental domain (outside the 0.9-1.1 range).

From response surfaces and contour plots in Figure 4.6 it was possible to visualize that the most profitable combination between the two responses was to set vent pressure at

10.5 psi (intermediate level in the domain), vent flow at 100 mL/min (highest level in the domain) and temperature at 45°C (intermediate level in the domain) (Table 4.6)

Table 4.6: Optimum values of the factors to maximise peak area of symmetric peaks.

Peak area	Peak symmetry	Optimum values
T = 45°C	T = 60°C	T = 45°C
VF = 100 mL/min	VF = 100 mL/min	VF = 100 mL/min
VP = 1 psi	VP = 20 psi	VP = 10.5 psi

To go further, from the peak area response curves of Figure 4.6, it was evident that peak area might increase even more outside the experimental domain when increasing vent flow, while still keeping vent pressure at 10.5 psi for peak symmetry. So, three replicates at a vent flow of 120 mL/min (outside the experimental domain), temperature at 45°C and vent pressure at 10.5 psi were recorded to check whether or not the response increased significantly in the expected direction. Indeed, the peak area in these conditions was statistically higher (proved by a t-test) than at vent flow at 100 mL/min. At this point, even without a full optimization but still with enough improvements using the experimental design for Dechloranes analysis, the best injection conditions were settled as vent flow (VF) at 120 mL/min, temperature (T) at 45°C, and vent pressure (VP) at 10.5 psi.
The experimental design was useful not only to set optimum parameters for PTV inlet, but also to highlight a memory effect in the inlet. In fact, when running replicate injections for the experimental design, a possible memory effect emerged. To confirm this, 20 replicate injections were done in a row and actually peak area increased for consecutive runs, highlighting a real peak area carry over. To avoid this, inlet purge flow was increased from 100 mL/min set as default value, to 1200 mL/min (the maximum instrumental level) to insure complete inlet evacuation after each run.

5.3. Instrumental method development and validation

Analytical method for Dechlorane detection was developed and validated accordingly to the applicable criteria (Table 4.7) of the stringent EU legislation for dioxin measurement in

feed (and in food), and schematised in Table 1.3 of Chapter 1, as no dedicated regulation has been issued on Dehcloranes so far.

Table 4.7: Applicable criteria of the EU Regulation 709/2014 for the validation of an analytical method for Dechlorane detection in food and feed metrices.

Criteria	
MRM transitions	2 transitions were recorded, 1 Quant and 1 Qual
Detectable quantity	No requirement, no ML stated
Calibration range	Based on instrumental performances
LOQ minimum value	No requirement, no ML stated
LOQ calculation	The same approach used for dioxin analysis
Acceptable difference between upper-bound and lower-bound at maximum level	No requirement
Validation in the range of the maximum level	No requirement, no ML stated
High selectivity (specificity)	Verified, but with no specific requirements
High Accuracy (trueness and precision)	Not assessed, no proficiency test available
Blanks and QCs	Not needed for this study
Recovery check	Acceptable range 60 – 120%
Measurement Uncertainty	Not assessed, no proficiency test available; accurate assessment was to needed for the scope of this study
Reporting of results	The same as for dioxin analysis

Some of the EU Regulation criterion was not applicable for method validation in this case, because no MLs have been stated for Dechloranes, or because at the present there is no proficiency test available. Moreover, fortified sample were not prepared and measurement uncertainty was not precisely calculated because this work was aimed to assess average Dechlorane levels in food and feed, in order to estimate an average dietary intake for people from Europe and evaluate food as a possible route of exposure for humans.

Measurements of all targeted Dechloranes were performed by isotopic dilution (ID) using the commercially available labelled standards ($^{13}C_{10}$-labelled Dec 602 and $^{13}C_{10}$-labelled DP syn). Because of similar structures and retention times, $^{13}C_{10}$-labelled Dec 602 was

used as internal standard for Mirex, Dec 602, Dec 603 and CP, while $^{13}C_{10}$-labelled DP syn was the internal standard for DP isomers. Dec 604 was not detectable at concentration lower than 100 pg/µL and so at the level of interest in this study. For this reason further dissertation does not include this compound.

5.3.1. MRM transitions

For each compound two MRM transitions were monitored, one for quantitation and one for confirmation (usually the quantitation transition with a +2 Da offset) allowing identification of interferences or wrong integration. MRM transitions were determined by a set of experiments using a 100 pg/µL mix solution: first a full mass spectrum of every compound was recorded by full scan in the first quadrupole (MS1 scan). In the next experiment, the base peak was selected as precursor ion, fragmented in the collision cell at different collision energies (from 5 to 40 eV) and all the fragments were detected in full scan of the last quadrupole. Eventually, the most intense peak was selected as the product ion for the final MRM transition. Collision energy and dwell time for ach compound were further optimised to obtain the highest signal with at least 10 acquisition points. MRM transitions are listed in Table 4.8.

Table 4.8: MRM transitions of Dechlorane compounds

Compound	Retention time min	Native Quant	Native Qual	Labelled ISTD
Mirex	10.36	272.0 -> 237.0	273.8 -> 236.8	Dec 602 ($^{13}C_{10}$, 99%)
Dec 602	11.228	272.0 -> 237.0	274.0 -> 239.0	Dec 602 ($^{13}C_{10}$, 99%)
CP	12.327	272.0 -> 237.0	274.0 -> 239.0	Dec 602 ($^{13}C_{10}$, 99%)
Dec 603	15.883	262.8 -> 227.9	264.8 -> 229.9	Dec 602 ($^{13}C_{10}$, 99%)
Dec 604	17.092	419.7 -> 340.7	422.0 -> 343.0	Dec 602 ($^{13}C_{10}$, 99%)
DP syn	22.083	272.0 -> 237.0	274.0 -> 239.0	DP syn ($^{13}C_{10}$, 99%)
DP anti	24.085	272.0 -> 237.0	274.0 -> 239.0	DP syn ($^{13}C_{10}$, 99%)
Dec 602 ($^{13}C_{10}$, 99%)	11.227	277.0 -> 242.0		
DP syn ($^{13}C_{10}$, 99%)	22.079	277.0 -> 242.0		
PCB 80 ($^{13}C_{12}$, 99%)	8.02	303.9 -> 233.9		

MRM transitions for all the Dechloranes were quite similar because of the similarity of the chemical structures, but good chromatographic separation allowed unequivocal identification of each compound. Possible fragmentation paths are illustrated in Figure 4.7. For Dec 602, CP and DP isomers, the parent ion was the hexachlorocyclopentadiene (HCCPD) ion, formed in the ion source via a retro Diels-Alder reaction. In fact, this reaction was thermodynamically favoured as its products were stable conjugated compounds, and in the case of Dec 602, even aromatic (furan). After fragmentation in the ion source, the HCCPD ion was further fragmented in the collision chamber losing one chlorine atom (Cl, -35 m/z), and hence the final MRM transition for those compounds was m/z 272 > m/z 237 (25 eV collision energy (CE)). Mirex also fragmented according to this transition, not because of a retro Diels-Alder reaction, but rather because two HCCPD ions were formed, which are thermodynamically stable because of the electronic effects of the chlorine atom. For Dec 603, the HCCPD ion coming from retro Diels-Alder reaction was still observed, but it was subject to interferences preventing from proper quantitation in real samples. So for the MRM transition, a parent ion at m/z 262.8 was chosen. This ion was likely coming from the fragmentation in the middle ring with the concomitant loss of one chlorine atom (Cl, -35 m/z). The product ion subsequently consisted in the loss of one chlorine atom, hence the transition for Dec 603 was m/z 262.8 > m/z 227.8 (25 eV CE).

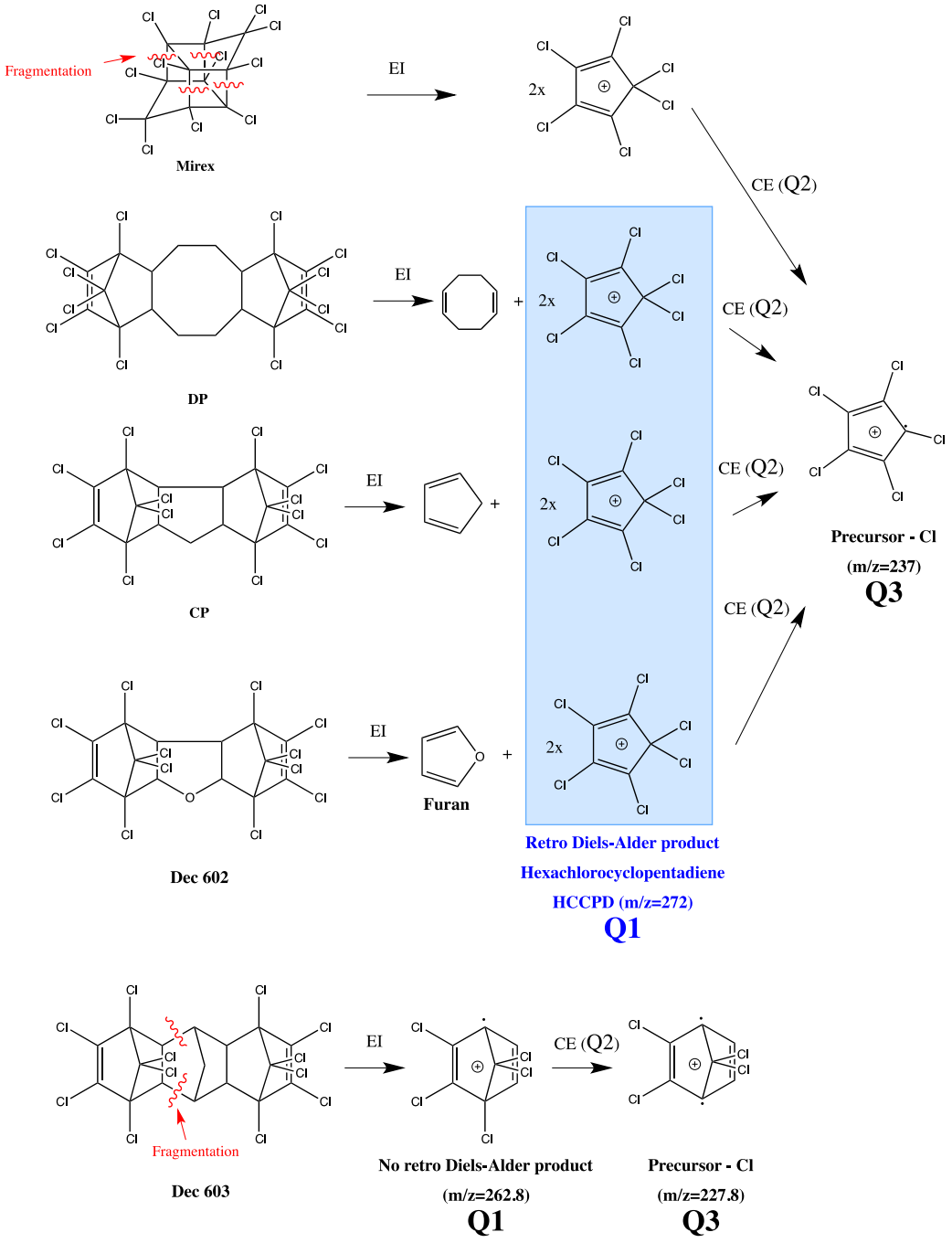

Figure 4.7: Possible fragmentation paths for Dechloranes when ionizing in EI at 70 eV, with subsequent fragmentation at collision energy (CE) 25 eV.

5.3.2. Calibration range

The calibration range (Table 4.9) varied for each compound, based on its own dynamic range, reproducibility at low and high level, and average response factor deviation.

Table 4.9: Calibration curve data for target Dechlorane compounds.

Compound	Calibration curve range pg/µL	Calibration points	R^2	Average Response factor (ARRF)*
Mirex	0.5 - 250	7	0.9997	1.4995
Dec 602	3 - 100	5	0.9993	1.2809
CP	0.5 - 100	7	0.9983	0.7754
Dec 603	0.5 - 100	7	0.9961	0.1788
DP syn	50 - 200	5	0.9995	1.0857
DP anti	3 - 200	5	0.9867	8.4338

*ARRF was the average response factor calculated on all the calibration points in the calibration range

In particular for DP syn isomer lowest calibration point was higher than for the other compounds. Likely DP syn labelled standard contained native compound impurities at trace levels, giving inaccurate concentration at very low levels.

5.3.3. Limit of Quantitation (LOQ)

Limits of quantitation were assessed with the same approach used for dioxins and PCBs in our laboratory with Triple Quad instrument and reported in Chapter 1. So, based on the EU Regulation, these LOQs were assessed either based on blank levels or from the instrumental limits of quantitation (iLOQs) when no signal was recorded in blanks. In any case, LOQ values were kept inside the calibration range for each compound (Table 4.10).

Table 4.10: LOQ values for Dechloranes in food and feed matrices analysed in this work.

Compound	Milk	Chicken	Pork	Egg	Fat	Vege-table oil	Salmon	Feed additive	Corn (feed)
	pg/g fat						pg/g wet weight		
Mirex	0.13	0.07	0.07	0.20	0.13	0.13	0.04	0.04	0.02
Dec 602	0.85	0.43	0.43	1.20	0.75	0.75	0.25	0.26	0.09
CP	0.13	0.07	0.07	0.20	0.13	0.13	0.04	0.04	0.02
Dec 603	0.28	0.07	0.07	0.20	0.125	0.13	0.04	0.04	0.02
DP syn	12.50	7.14	7.14	20.00	12.50	12.50	4.24	4.40	1.49
DP anti	5.11	1.94	1.93	5.42	3.39	3.39	1.15	1.54	0.52

In this work, 16 procedural blanks were analysed and in general Dechlorane levels were very small, often below the lowest calibration point. iLOQs, by definition, were related to instrumental repeatability and stability, and it allowed to set the lowest calibration point as the one with acceptable and consistent deviation from the ARRF. Anyway, iLOQ values were below the lowest calibration point for all the compounds and hence in almost all the cases LOQs coincided with the lowest calibration point. LOQs varied with sample matrix because of the different sample intake and values were reported in pg/g fat for most matrices, except for salmon, feed additives, and corn, for the sake of harmonization with the EU legislation for dioxins.

5.3.4. Selectivity

In this work, the same instrumental setup as for dioxin analysis was used, so chromatographic column length was 60 meters. This column was more than enough to allow complete Dechlorane compounds separation as illustrated in Figure 4.8, that shows signals recorded for 10 pg/µL solution: (from the top) $^{13}C_{10}$-labelled PCB 80 recovery standard, Mirex in the first acquisition segment, Dec 602 and CP in the second acquisition segment, Dec 603 in the third segment, DP isomers (syn and anti) in the last segment. The forth acquisition segment was dedicated to Dec 604 acquisition but this compound was not detectable at the considered level.

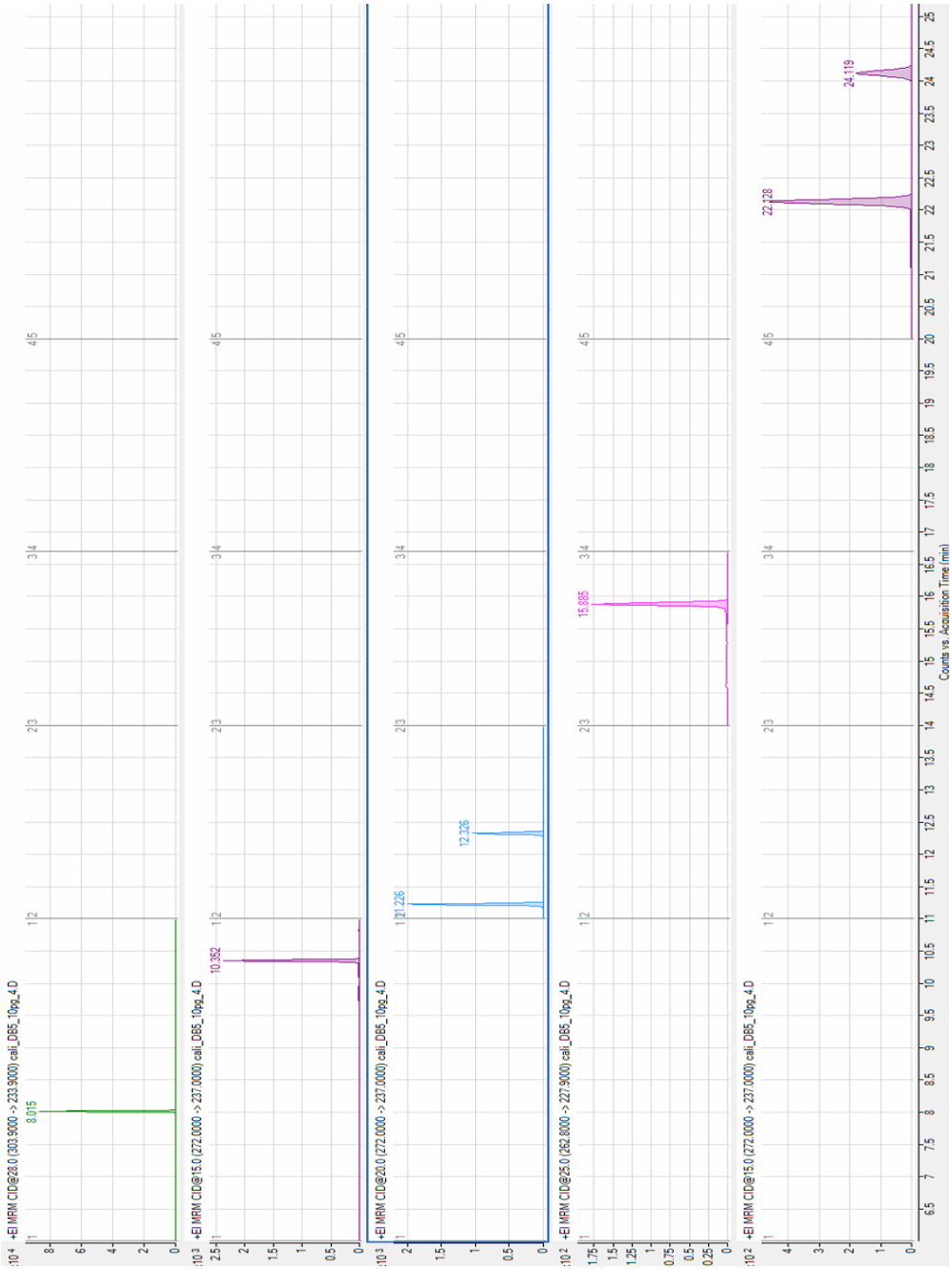

Figure 4.8: Dechlorane typical chromatogram showing very good chromatographic separation.

5.3.5. Result reporting

Also for result reporting, guidelines from the EU Regulation for dioxins were followed. In fact, as described in the next paragraph, Dechlorane levels were reported in terms of lower-bound (lb) and upper-bound (ub). The first approach consisted in reporting zero whenever the experimental level measured was below the LOQ for the target compound, hence an underestimated the measured level. On the other hand, the upper-bound approach consisted in reporting the LOQ value whenever the signal measured was below the LOQ, even when no signal at all was recorded; this approach led to overestimation of final measured levels. As for dioxins, also in this case, the LOQ assessment and value was a very important parameter affecting final results.

5.4. Dechlorane levels in food and feed matrices

The analytical method was developed with the aim of assessing Dechlorane levels in food and feed samples and average daily intake for human based on food habits of the Belgian population. A total of 88 samples from 9 different food and feed matrices, as well as 16 blank samples were prepared following specific sample procedures. Six Dechlorane concentrations were measured and Dec 604 was not quantitated due to the very low response at the level of interest. Results were calculated according to the method of reporting used for dioxin analysis under the EU Legislation in terms of lower-bound (lb) and upper-bound (ub). Table 4.11 reports Dechlorane levels calculated with the upper bound approach on the 88 food and feed samples analysed. For the same samples, also TEQ levels from dioxin and PCB analysis were reported, in order to demonstrate that the selected samples were not particularly contaminated from dioxins and could be considered as representative of what is actually available on the Belgian food market.
For some matrices, measured values were below LOQ, so LOQ was reported as a value and resulting standard deviation (SD) was not computable (indicated with a dash in Table 4.11).

Table 4.11: Levels of selected Dechloranes measured by GC-MS/MS Triple Quad in various food and feed matrices

	Milk		Chicken		Pork		Egg		Fat	
	pg/g fat									
Compound	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Mirex	0.50	0.24	0.11	0.07	0.21	0.25	0.21	0.02	0.43	0.68
Dec 602	0.89	0.13	0.43	-	0.43	-	1.28	0.23	0.75	-
CP	0.26	0.24	0.07	-	0.08	0.02	0.94	2.08	0.16	0.09
Dec 603	1.06	1.21	0.13	0.07	0.26	0.39	2.76	6.29	0.57	1.10
DP syn	12.50	-	7.14	-	7.14	-	20.00	-	12.50	-
DP anti	5.11	-	1.93	-	3.84	3.86	6.27	2.04	6.60	9.21
Sum DPs	17.61	-	9.08	-	10.98	3.86	26.27	2.04	19.10	9.21
Sum 6 Dechloranes	20.31	1.37	9.82	0.10	11.96	3.80	31.47	8.61	21.01	9.35
PCDD/Fs and DL-PCBs TEQ	1.12	0.20	0.40	0.01	0.48	0.23	0.91	0.04	0.93	0.14

	Vegetable oil		Salmon		Feed additive		Corn (feed)	
	pg/g fat		pg/g wet weight					
Compound	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Mirex	0.13	-	1.98	1.43	0.07	0.10	0.01	0.46
Dec 602	0.75	-	0.31	0.10	0.26	-	0.09	-
CP	0.20	0.10	0.30	0.46	0.04	-	0.01	-
Dec 603	0.50	0.53	0.58	1.11	0.04	-	0.01	-
DP syn	12.50	-	4.24	-	4.43	0.13	1.49	-
DP anti	6.61	3.17	1.15	-	6.86	15.05	0.52	-
Sum DPs	19.11	3.17	5.39	-	11.29	15.14	2.01	-
Sum 6 Dechloranes	20.68	3.60	8.56	2.03	11.71	15.13	2.14	0.46
PCDD/Fs and DL-PCBs TEQ	0.20	0.00	0.62	0.61	0.39	0.33	0.20	0.00

Figure 4.9 represents upper bound and lower bound values for selected matrices, as respectively the top and bottom of the box. Error bars represent ub+2*standard deviation.

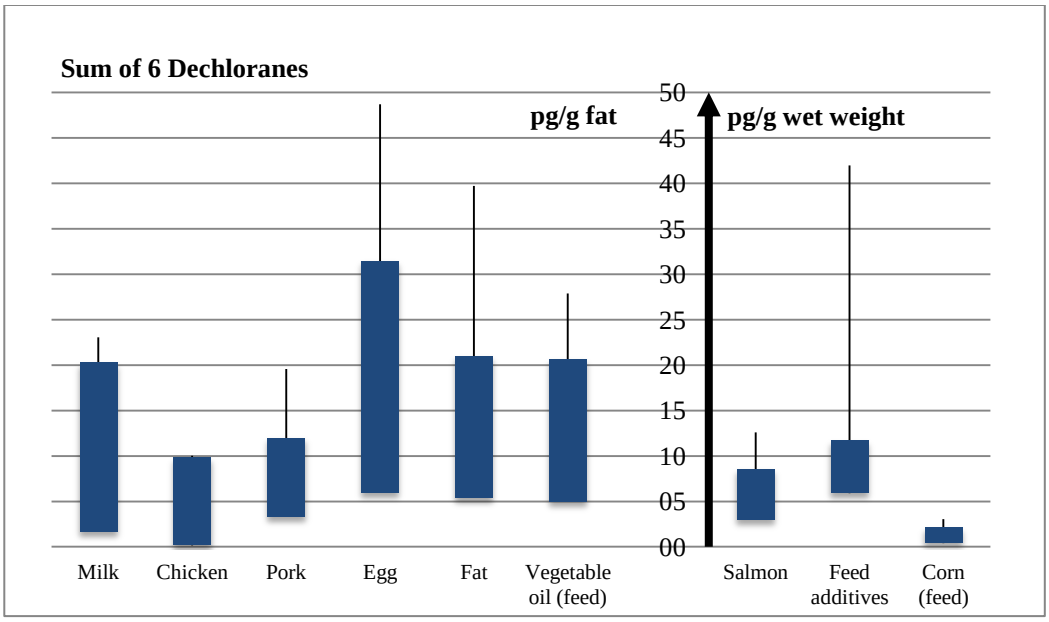

Figure 4.9: Average levels of the sum of the 6 Dechloranes in different food and feed matrices. Boxes represent lower-bound (bottom), and upper-bound (top) results and the error bars are the upper-bound + 2*standard deviation. Data are expressed on lipid weight basis on the left of the y axis and on product weight basis on the right. Salmon values are given in wet weight basis following the European Regulation for dioxins (Commission Regulation EU 1881/2006).

The "true" average levels were likely inside the interval defined by the boxes. The scope of this work was the assessment of Dechlorane average levels and, from these results, it was possible to say that they were in the order of low pg/g fat or wet weight (ppb) in the selected food and feed matrices.

Lower bound results, reported at the bottom of the boxes for each matrix, gave a rough estimation of background contamination levels for each matrix: for each compound, whenever above LOQ, the experimental level was reported, otherwise zero. Matrices with lower bound level close to zero had the lower contamination background, like for example chicken and corn; egg and feed additives had the highest background levels.

Even though the reporting approach was different from other works, it was possible to assess that DP levels recorded in this study were lower than already reported in Korea (Kim et al., 2014), and they were in the same order than the ones reported in foodstuffs from Japan (Kakimoto et al., 2014), as shown in Figure 4.10 and 4.11, where the contribution of the 6 Dechloranes for all matrices is reported in upper-bound and lower bound approach respectively.

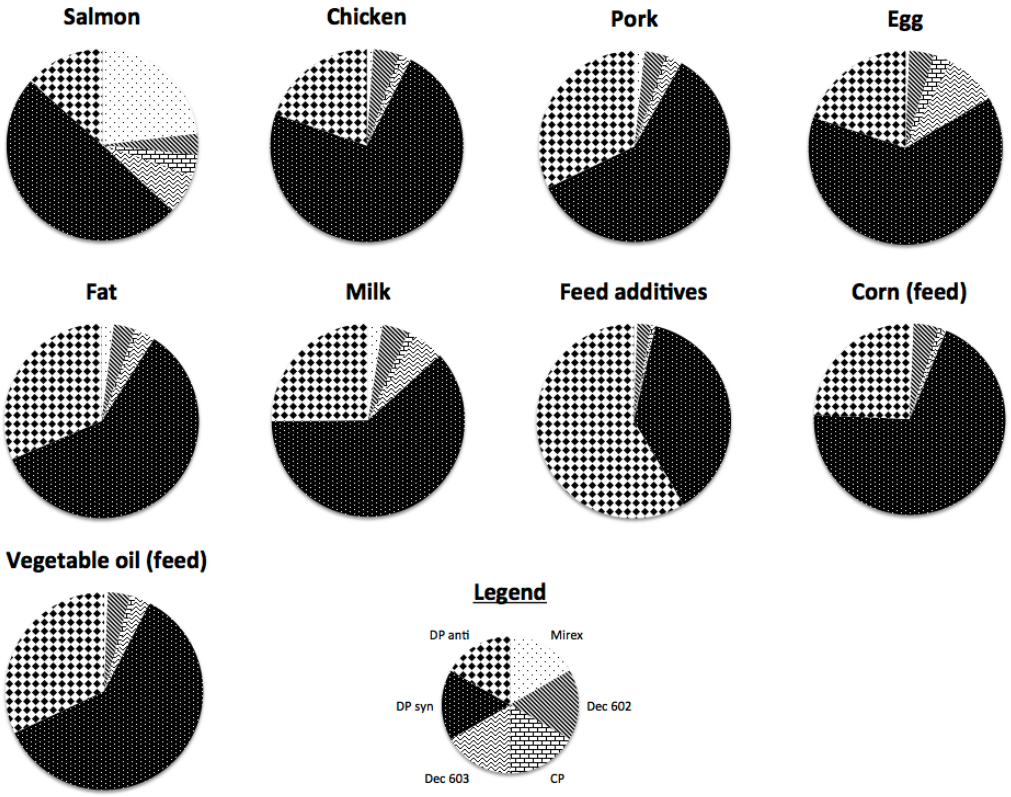

Figure 4.10: Relative contribution of the 6 Dechloranes to the sum for each investigated matrix, based on upper-bound results.

In particular, Figure 4.10 was very informative, as only levels actually recorded on the real samples were displayed.

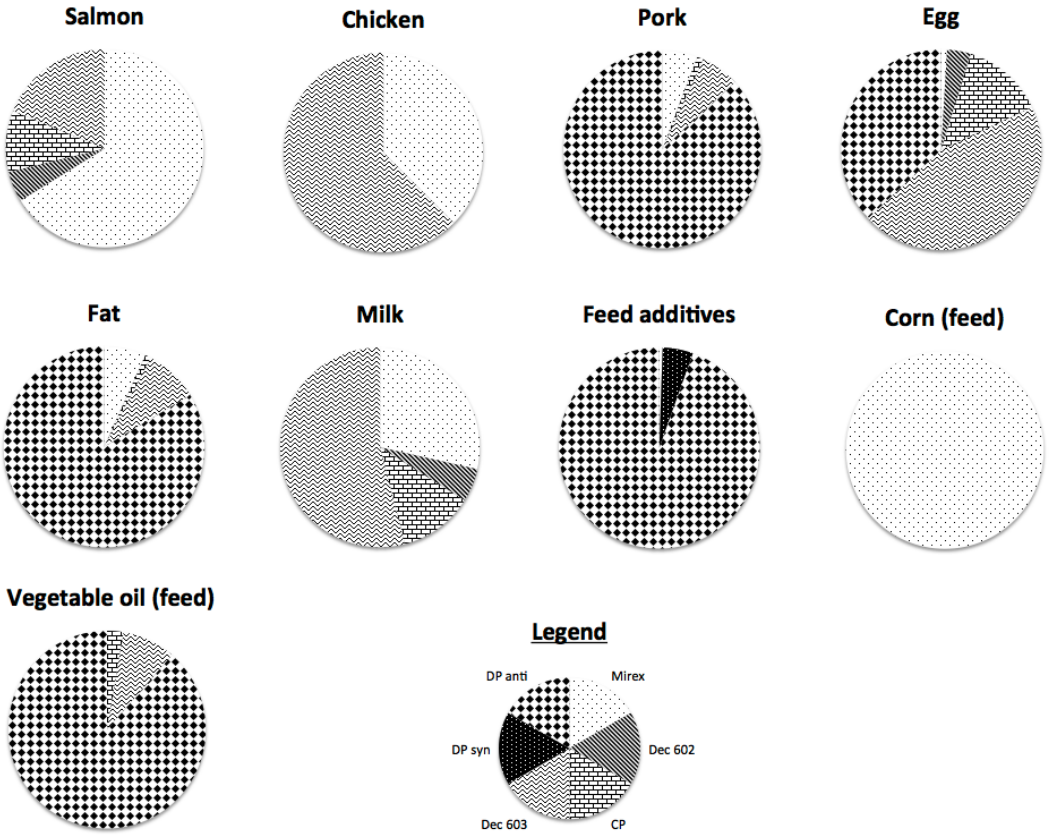

Figure 4.11: Relative contribution of the 6 Dechloranes to the sum for each investigated matrix, based on lower-bound results.

DP isomers were found in almost all the matrices. In particular, two out of the seventeen feed additive samples exhibited high levels of DP anti (43.9 pg/g and 49.6 pg/g), and lower levels of DP syn. The fractional abundance of the DP anti isomer (f_{anti}, calculated by dividing the concentration of DP anti by the sum of the concentrations of both DP isomers) was different from 0.3, that is the composition of the industrially produced technical mixture. A fractional abundance assessment was not possible because of the high DP syn isomer LOQ, which prevented from low level quantification of this compound.

Also Mirex was detected in many matrices. In salmon, relatively high levels of Mirex were measured, above LOQ for all the samples taken into account (n=8), even if its production is banned and even if salmon samples bought from regular Belgian food market. More-

over, Mirex was found very frequently in fatty samples like in various pure animal fat (50% of samples), in pork (75% of samples), and in milk (87% of samples). Mirex was the only chemical measured above the LOQ in corn, resulting in a single component pie chart, but levels were very low, as displayed in Figure 4.11.

5.5. Estimation of Dechlorane dietary intake

Dechlorane levels measured in food were used to estimate an average daily intake based on food habits of the Belgian population following the last food survey in Belgium (De Vriese et al., 2005) and Belgian food composition tables (NUBEL, 2010). The selected matrices were not accounting for all food consumption, but commonly consumed goods were selected, with the aim of giving preliminary data and to understand if food consumption could be a significant route of human exposure to Dechloranes for people from Europe. The aim was to produce a first time point for further, more detailed studies. Daily dietary intake was based on the overestimated result (obtained with the upper-bound approach) to produce a worst-case scenario. Results of these calculations are presented in Table 4.12

Table 4.12: Estimated average dietary intake for the sum of 6 Dechloranes (Mirex, Dec 602, Dec 603, CP, DP syn, DP anti) measured in selected food matrices in Belgium.

	Estimated dietary consumption			**Sum 6 Dechloranes (see Table 4.11)**	**Dechloranes sum intake**
	g/day	g fat/100g	g fat/day	pg/g fat or pg/g ww	pg/day
Salmon	2.7	16.5	0.4	8.6	23.1
Chicken	18.4	9.3	1.7	9.8	16.8
Pork	30.2	9.2	2.8	12.0	33.2
Egg	9.6	11.3	1.1	31.5	34.1
Milk	89.1	1.6	1.4	20.3	29.0
Estimated Dechloranes dietary intake (pg/day)					**136.2**

Reported levels suggested that Dechloranes enter the food chain and bioaccumulate, hence humans are exposed via consumption of food, even if it is not possible to estimate if food is the main route of exposure, as no data are available at the present for environmental samples and environmental human exposure, considering the relevant levels previously reported in human blood. From what was seen at this stage, no class of foodstuff can be highlighted as strongly contributing to human exposure to Dechloranes. More extensive and larger studies should be carried out to better understand fates of Dechloranes and estimate other route of exposure, such as indoor dust.

6. Conclusions

A dedicated analytical method has been developed for the measurement of six members of the Dechlorane family (Mirex, Dec 602, Dec 603, CP, DP syn, DP anti) in food and feed. The use of ID and optimized PTV inlet parameters, as well as the use of GC-MS/MS Triple Quad instrument, allowed trace level detection (ppb level) for all target compounds, except Dec 604. The optimization steps focused on the improvement of the sensitivity, from sample clean-up to sample injection and detection. 88 samples from 9 different matrices, representing commonly consumed goods, were analyzed in order to preliminary assess a daily intake of Dechloranes from food products. Levels in food and feed samples were found to be close to the method limits of quantitation, except for some samples, whose contamination appeared to be significantly higher. An estimation of the dietary intake showed that food was probably not the single route of exposure. Further studies are necessary to understand contamination sources by Dechloranes and whether or not detected levels could be harmful to human. Reported levels can be integrated in a global study considering dust and other possible routes of exposure to investigate the contribute of each source to human exposure. The analytical method developed for Dechlorane detection at trace levels in food can be adapted, optimized and validated for Dechlorane measurements in different matrices.

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The degree of responsibility and work load of the author for the paper

"Ultra-trace measurement of Dechloranes to investigate food as a route of human exposure".

Chemosphere. 2015 Nov; 139:525-33. Doi: 10.1016/j.chemosphere.2015.07.043.

Step		**Degree of responsibility and work load of the author**
Planning and preparation		Major
Execution:	Sample preparation	Medium
	Instrumental analysis	Medium
	Data elaboration	Major
Preparation of the paper		Medium
Presentation at conferences		Major

CONCLUSIONS

In this thesis analytical methods for POP detection in food and feed were developed and validated. For instrumental quantification GC-MS/MS Triple Quad instrument was mainly used, in addition to GC-HRMS Magnetic Sector. Part of the work of this thesis focused on dioxins and PCBs, the other part was aimed to the quantification of emerging contaminants, Dechloranes.

In the dioxin and PCB framework, new high throughput clean-up approaches were developed and validated for dioxin and PCB detection in food and feed using automated systems. They demonstrated to be faster and more environmental and economically convenient in comparison to existing procedures. Almost all the analyses were carried out with GC-MS/MS Triple Quad instrument, as recently allowed from the EU Regulation in 2014.
For the DEXTech™ system, new approaches were based on 3 column set, namely multilayer silica, basic alumina, and a single carbon column, instead of classical 4 column set using Florisil® and two carbon columns. Multilayer silica and two carbon columns were commercially available, while alumina column was manually packed after proper thermal treatment based on a modified EPA 1613 procedure. Two approaches arose from the availability of two different carbon columns and led to different final fractionation profiles: with Carbopack C:celite column, I-, MO- and co-PCBs were collected in fraction 1, and PCDD/Fs were found infraction 2, with Norit:celite column, co-PCBs were found in fraction 2 together with dioxins. Laboratories willing to use alumina columns can choose the appropriate protocol based on the consecutive instrumental analysis. The new developed approaches allowed time and solvent saving, respectively 27% and 18%, as well as global cost reduction, giving recoveries in the range 60-120% as required by EU Regulation.
“Alumina-Norit” method was applied to QC samples of pork fat, egg yolk and milk to assess its performances on real fatty matrices and results showed that the clean-up method was accurate, despite some issues related to manual packing. A full validation was not carried out because of the high variability of the manual packing of alumina column. However, preliminary results of this work show that alumina columns can be used in the DEX-

Tech™ system after slight modification of the system, because allow good quality sample clean-up in accordance with EU Regulation. Alumina method can represent a valid alternative to the classic method based on Florisil®, allowing faster and cheaper sample clean-up.

In Chapter 3 alternative clean-up approaches for PowerPrep™ system from FMS were proposed, with the aim of having high throughput method for dioxin analysis with time, solvent and global cost reduction. Alternative approaches were based on the validated clean-up method used at the University of Liège and consisting of a preliminary lipid digestion with a manually packed acidic silica column, followed by automated clean-up with PowerPrep™ equipped with a 3 column setup: standard ABN silica, alumina, and carbon column. “Short” and “fast standard methods” alternative approaches used the same column set and were suitable for low fat content samples (< 1 g) or they had to come after a preliminary acidic digestion with manually packed acidic silica column to have fat capacity of up to 7 g. They were designed from our routine automated method removing less crucial steps, namely column washing/conditionings and tubing washing. Silica, alumina and carbon column elution profiles were studied to assess minimum solvent amount for column elution. “Fast standard method” showed that also silica column initial conditioning could be skipped without affecting final recoveries and extract quality. These new approaches allowed from 30 to 50% saving in terms of solvent and time.

Another method, referred to as “Jumbo” as based on four column set including a jumbo acidic silica column, was developed as a fully automated alternative to our entire clean-up procedure including the manual and the automated steps. Jumbo method used 4 column set on the PowerPrep™: high capacity acidic silica column, mini ABN silica, mini alumina and carbon column, for a total fat capacity of up to 4 g and about 50% solvent and time saving in comparison to our whole clean-up procedure. After development, the method was tested on pork fat QC and vegetable oil to check the quality of the final extracts, and it demonstrated to be suitable for trace level analysis of dioxins and PCBs. From these preliminary tests it was also possible to understand that standard FMS alumina column is oversized as mini alumina columns, containing the half of sorbent amount, showed to be suitable for the scope.

Finally, a dedicated analytical method for the detection of Dechlorane compounds (six members of the Dechlorane family: Mirex, Dec 602, Dec 603, CP, DP syn, DP anti) was

presented. The use of ID and optimized PTV inlet parameters, as well as the use of GC-MS/MS Triple Quad instrument, enabled trace level detection (ppb level) for all target compounds. Some instrumental parameters for PTV inlet were set by means of the Experimental Design. The method was validated according to the applicable criteria reported in the EU Regulation for dioxin analysis and was applied to 88 samples from 9 different matrices, representing commonly consumed goods, with the idea of assessing daily dietary intake of Dechloranes. Levels in food and feed samples were found to be close to the method limits of quantitation, except for some samples, whose contamination appeared to be significantly higher. An estimation of the dietary intake showed that food was probably not the unique route of exposure. Further studies are necessary to understand contamination sources by Dechloranes and whether or not detected levels could be harmful to human. Reported levels can be integrated in a global study considering dust and other possible routes of exposure to investigate the contribute of each source to human exposure. The analytical method developed for Dechlorane detection at trace levels in food can be adapted, optimized and validated for Dechlorane measurements in different matrices.

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CURRICULUM

Personal details

Name and family name: **Chiara Calaprice**
Date and place of birth: 04/03/1983

Permanent address:
via Giacomo Matteotti 16, 70010 Adelfia (Bari) - Italy
Current address:
rue du Chataigner 9, 4100 Boncelles Seraing (Liege) - Belgium
Correspondence address:
Allèe du 6 Aout, 11 Bat. B6c-local R1, 4000 Liege - Belgium

Email: chiara.calaprice@poliba.it
chiara.calaprice@gmail.com

Phone number: +32 477 586 770
+39 328 42 19 573

Education

PhD in analytical chemistry in persistent organic pollutants (POPs) field

2013 – 4th April 2016	Polytechnic University of Bari and Interpolytechnic School (Italy) and University of Liège (Belgium) - Joint PhD in Environmental and Territory Safety and Control - Analytical Chemistry
2010 - 2011	University of Easter Piedmont, Alessandria (Italy) Master graduation in Chemistry
2009	University of Easter Piedmont, Novara (Italy) Post-graduation specialization course on materials for energy and environment
2002 - 2008	University of Bari (Italy) Bachelor degree in Chemistry

Research expertise

- Development of new sample clean-up techniques in dioxin analysis using automated systems (PowerPrep™ from FMS Inc., DEXTech™ from LCTech Gmbl)

- GC-MS/MS Triple Quad advanced user (Agilent)

- Development and validation of analytical methods for Persistent Organic Pollutants (POPs) detection in food and feed matrices with GC-MS/MS Triple Quadrupole instruments (Agilent)

- Multivariate analysis (PCA, cluster analysis) and modelling (MLR, PLS) with Matlab, Statistica and the Un-scrambler

- Experimental design (DoE)

- LC-MS/MS, LC-MS

- Development and validation of analytical procedures for pesticides detection in food matrices

Research stays

Feb 2014 - ongoing	University of Liège (Belgium) – joint agreement with Polytechnic University of Bari for the research in POP and dioxin analysis.
2nd - 22nd Dec 2015	Orebro University (Sweden) – sample preparation for low volume samples of blood. Supervisors: Dr. Ingrid Erickson and Prof. Bert Van Bavel
Feb – May 2011	Internship for master thesis: Eni Taranto Refinery (Italy) - Chemometrics, multivariate analysis for process optimization
Jul – Nov 2009	Internship for post-graduation course: Donegani Institute, Research Centre for Non-Conventional Energy of Eni S.p.A (Italy) - Organic synthesis, heterogeneous catalysis

Total list of publications

1 “Optimization of a new dioxin/PCB clean-up and fractionation procedure for an existing automated system”.

Calaprice C., Calvano C.D., Zambonin C., Focant J.-F., Organohalogen Compounds, Vol 77 (2015), 733-735

2 "Ultra-trace measurement of Dechloranes to investigate food as a route of human exposure" L'Homme B., Calaprice C., Calvano C. D., Zambonin C., Leardi R., Focant J.-F. ; Chemosphere, 139 (2015), 525–533

3 "Measurement of trace level Dechlorane flame retardants in food and feed by GC-MS/MS" L'Homme B., Calaprice C., Brasseur C., Calvano C.D., Zambonin C., Leardi R., Focant J.-F.; Organohalogen Compounds, Vol 76 (2016), 1460-1463

4 "Co-production of butyrate methyl ester and triacetylglycerol from tributyrin and methyl acetate" Battistel E., Calaprice C., Gualdi, E., Rebesco, E., Usai, E.M., Applied Catalysis A: General, 394 (2011), 149–157

Conferences

Oral presentations

1 "Fatty Matrices Cleaned-up with a Different Approach",

Invited speaker at "1st DEXTech™ User Meeting" – 21st and 22nd October at Governmental Lab Chemical and Veterinary Investigation Office (CVUA-MEL)- Muenster (Germany)

2 "Optimization of a new dioxin/PCB clean-up and fractionation procedure for an existing automated system"

At the 35th International Symposium on Halogenated Persistent Organic Pollutants – Dioxins 2015", 23rd – 28th August 2015 in Sao Paulo (Brasil).

3 "Ultra-trace measurement of Dechloranes to investigate food as a route of human exposure" (Otto Hutzinger award)

At the 35th International Symposium on Halogenated Persistent Organic Pollutants – Dioxins 2015", 23rd – 28th August 2015 in Sao Paulo (Brasil).

4 "Ultra-trace measurement of Dechloranes in food and feed"

At Massa 2015, conference on mass spectrometry by the mass spectrometry division of the Italian Chemical Society, 10th – 13th June 2015 in Alghero (Italy),

5 "Measurement of ultra-trace levels of Dechloranes in food and feed. Assessment of dietary intake"

At the 10th International Symposium on Recent Developments in POPs Analysis, organized by

Thermo Fischer on 29th and 30th April 2015 in Prague (Czech Republic)

6 "Measurement of trace level Dechlorane flame retardants in food and feed by GC-MS/MS"

At the 34th International Symposium on Halogenated Persistent Organic Pollutants – Dioxins 2014, 31st – 5th September 2014, Madrid (Spain)

Poster presentation

1 "Revisiting an existing dioxin/PCB clean-up and fractionation procedure to reduce solvent and time consumption"

At the 35th International Symposium on Halogenated Persistent Organic Pollutants – Dioxins 2015", 23rd – 28th August 2015 in Sao Paulo (Brasil).

2 "Study of the effects of heating on quality of soymilk proteins by means of MALDI-TOF MS"

At the "4th MS-J-Day - I giovani e la spettrometria di massa", 4th November 2013, Potenza (Italy)

Awards and scholarship

2015 Otto Hutzinger award: for outstanding student presentations at the annual Dioxin Symposium to acknowledge their scientific contribution to the field of halogenated persistent organic pollutants (http://www.dioxin20xx.org/award_winners.htm)

2015 Lerici scholarship: for Italian PhD student to carry out a research project in Sweden (research stay in December 2015)

2014 Scuola Interpolitecnica di Dottorato: additional grant for selected PhD students from the 3 Italian Polytechnic Universities Turin- Milan - Bari

2013 Fondo Giovani: 3 years grant for PhD students from the Italian Minister of University and Research.

2009 Ritorno al futuro: European scholarship for people from Puglia (Italy) to attend post-graduation courses in scientific field

Working and practical experience

Feb - Sept 2012 Laboratory technician at BonassisaLab s.r.l, Foggia (Italy) (www.bonassisa.it)

- LC-MS/MS detection of mycotoxins and pesticides in food
- Nutrition facts determination
- GC-FID detection of fatty acids in food matrices

Languages

Native language	Italian
B2 (certified)	English
B1	French

References

Prof. Jean François Focant

University of Liège (Belgium)
JF.Focant@ulg.ac.be
+32 (0)4 366 35 31