The effect of different sample preparatory protocols on the induction of the aryl hydrocarbon receptor (AhR) in the H4IIE-*luc* reporter gene bio-assay

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Dei gratia

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Summary

Concern on a global scale gave rise to the founding of the Stockholm Convention on persistent organic pollutants (POPs) with a view to restrict the use and production of these toxic chemicals. As a signatory, South Africa is legally bound to abide to the Convention's objectives, including participating in relevant research and monitoring. However, developing countries such as South Africa have limited information concerning POPs, partially because these countries do not have sufficient analytical capabilities, and thus method development and refinement are necessary. One group of POPs consisting of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs), collectively referred to as dioxins, are of particular concern due to their high toxicity and persistence. Additionally, the analysis of dioxins is recognised as one of the most analytically challenging of its kind. This study investigated the effect of different preparatory protocols on the semi-quantification of dioxins using the H4IIE-*luc*-reporter gene assay. The protocols evaluated were either Soxhlet or pressurised liquid extraction (PLE) combined with a manual acid digestion, gel permeation chromatography (GPC) and Florisil fractionation clean-up procedure as well as the automated Total Rapid Prep™(TRP) system which makes use of a PLE combined with a multi-layer silica, alumina and carbon column clean-up procedure. To evaluate the protocols, an eight point matrix matched calibration curve, two soil samples and a certified reference material (CRM) were used. The extracts were semi-quantified by the H4IIE-*luc* bio-assay. During the course of the assay, the appropriateness of different standards was investigated, and a mixed standard containing all 17 toxic PCDD/Fs was chosen for quantification. During the data review process, higher bio-assay equivalent (BEQ) values were obtained from PLE compared to Soxhlet extraction, while no statistically significant difference (Kruskal-Wallis ANOVA: $p > 0.05$) was found between the assay quantifications for the different preparatory techniques. However, the results of the H4IIE bio-assay were larger than the expected values. The identity of the chemicals that were in all likelihood responsible for the higher response was investigated through instrumental analysis using comprehensive two-dimensional gas chromatography coupled to time of flight mass spectrometry (GCxGC-TOFMS). Instrumental results indicated a high level of PAHs in the extracts, which could lead to super induction of the aryl hydrocarbon receptor (AhR) and therefore, to a positive bias in the results. Instrumental

screening proved that all selected preparatory protocols were inadequate at removing interfering compounds and not sufficiently selective for PCDD/Fs, although the TRP was more successful in removing interferences. The high matrix interference hindered peak identification. Additionally, as indicated by instrumental analysis, the weak recovery of PCDD/Fs could be ascribed to high evaporation temperatures. The effect of different reference standards in the H4IIE bio-assay used during semi-quantification needs further investigation; similarly, the optimisation of extraction, evaporation and clean-up protocols and the use of different GCxGC-TOFMS column combinations aimed at more efficient separation needs to be investigated.

The assistance of the National Metrology Institute (funded through the Department of Trade and Industry) towards this research is hereby acknowledged.

Keywords: PCDD, PCDF, South Africa, clean-up protocols, H4IIE-*luc* bio-assay, GCxGC-TOFMS

Die effek van verskillende monstervoorbereidingsprotokolle op die induksie van arielkoolwaterstofreseptor (AkR) in die H4IIE-biosiftingstoets

Opsomming

Wêreldwye besorgdheid aangaande blywende organiese besoedelstowwe (BOBe) het aanleiding gegee tot die stigting van die Stockholmkonvensie. Hierdie konvensie is gerig daarop om die produksie en gebruik van dié tipe stowwe te beperk. As 'n ondertekenaar van die verdrag is Suid-Afrika wetlik daartoe verbind om die konvensie se doelwitte, waaronder toepaslike navorsings- en monteringsaktiwiteite, na te kom. Ontwikkelende lande soos Suid-Afrika het dikwels beperkte inligting oor BOBe, deels weens 'n gebrek aan die nodige analitiese vermoëns. Een groep BOBs, naamlik die dioksiene-insluitende poligechloreerde dibenzo-*para*-dioksiene (PCDDs), poligechloreerde dibensofurane (PCDFs) en dioksien-agtige poligechloreerde bifeniele (d-PCBs) is van besondere belang op grond van hulle groot toksisiteit en lang-blywendheid in die omgewing. Dioksienanalise is besonders uitdagend en daarom is hierdie studie begaan met die effek van verskillende voorbereidingstegnieke op die semi-kwantifisering van dioksiene in die H4IIE-biosiftingstoets. Die metodes wat geëvalueer is, is die Soxhlet en die hoëdrukvloeistofekstraksie (HVE) gekombineer met 'n suurwas, jelchromatografie en Florisilfraksionering, asook die geoutomatiseerde HVE gekombineer met multi-laagsilika-, alumina- en koolstofkolomme (Total Rapid Prep^{TM}). Die verskillende metodes is met mekaar vergelyk deur gebruik te maak van 'n agt-punt-ekstraksie-matriks-kalibrasiekromme, twee Suid-Afrikaanse grondmonsters en gesertifiseerde verwysingsmateriaal (GVM). Die ekstrakte is daarna met die H4IIE-biosiftingstoets gesemi-kwantifiseer. Tydens die kwantifiseringsproses is die geskiktheid van verskillende standaarde ondersoek, waarna besluit is om 'n standaardmengsel met al 17 PCDD/Fs te gebruik. Die HVE-ekstraksie het hoër bio-siftingsekwivalente opgelewer as die Soxhlet-ekstraksie. Geen statisties-betekenisvolle verskille (Kruskal-Wallis ANOVA, $p > 0.05$) is tussen die verskillende voorbereidingstegnieke gevind nie. Daarbenewens was die H4IIE resultate aansienlik groter as verwag. Die omvattende tweedimensionele gaschromatograaf tyd-van-vlug-massa-spektrometer (OTG-TVMS) is gebruik vas te stel wat die hoër reponse kon veroorsaak. Hoë vlakke van polisikliese aromatiese koolwaterstowwe (PAKe) is tydens die instrumentele analise gevind. Hierdie verbindings kan super-induksie van die AkR veroorsaak, wat moontlik kon aanleiding gee tot distorsie van die resultate. Met behulp van intrumentele sifting het dit duidelik geword dat die geselekteerde

voorbereidingsmetodes onvoldoende was om PCDD/Fs te isoleer, alhoewel die Total Rapid Prep stelsel meer suksesvol was. ‘n Groot hoeveelheid matriksbeïnvloeding het die piek-identifikasie tydens instumentele ondersoek bemoeilik. Addisionele instrumentele analise het swak herwinning van PCDD/Fs getoon, moontlik as gevolg van hoë ekstraksie-temperature. Verdere ondersoeke is nodig met betrekking tot die volgende aspekte van die studie: die effek van verskillende verwysingstandaarde vir die semi-kwantifisering van die H4IIE-biosiftingstoets, die optimisering van die ekstraksie, verdamping- en skoonmaakmetodes, en die gebruik van ander analitiese kolomme vir die OTG-TVMS analise om skeiding te verbeter.

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Sleutelwoorde: PCDD, PCDF, Suid-Afrika, skoonmaakprotokolle, H4IIE-biosiftingstoets en GCxGC-TVMS

Acronyms and abbreviations

ABN	acid-basic-neutral
AhR	aryl hydrocarbon receptor
AMP	adenosine monophosphate
ARNT	aryl hydrocarbon nuclear translocator
ASE	accelerated solvent extraction
ATCC	American Type Culture Collection
BC	blank control
BEQ	bio-analytical equivalents
CRM	certified reference materials
CV	coefficient of variation
DCM	dichloromethane
DDT	dichlorodiphenyltrichloroethane
df	film thickness
dl-PCBs	dioxin-like polychlorinated biphenyls
DMSO	dimethyl sulphoxide
DRE	dioxin response element
EA	European co-operation for accreditation
EC	effective concentrations
EGFR	epidermal growth factor receptor
EIAs	enzyme immunoassays
FBS	foetal bovine serum
FMS	fluid management systems
GCxGC-TOFMS	comprehensive two dimensional gas chromatography coupled to time of flight mass spectrometry
GPC	gel permeation chromatography
HEPA	high efficiency particulate air
HCB	Hexachlorobenzene
HPLC	high performance liquid chromatography
HRGC/HRMS	high resolution gas chromatography high-resolution mass spectrometry
HSP	heat shock protein
IAEA	International Atomic Energy Agency
id	internal diameter
LN2	liquid nitrogen
LOD	limit of detection
LOQ	limit of quantification
MAE	microwave-assisted extraction
MSW	municipal solid waste
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide
NIST	National Institute of Standards and Technology
PAHs	polyaromatic hydrocarbons
PBS	phosphate buffer saline
PCAs	Anisols
PCABs	Polychlorazobenzenes
PCABs	Polychlorazoxybenzenes
PCANs	Anthracenes
PCAOBs	Polychlorozoxybenzenes
PCBs	polychlorinated biphenyls
PCDD	polychlorinated dibenzo-*p*-dioxins
PCDF	polychlorinated dibenzofurans

Acronyms and abbreviations continued

PCDPT	Polychlorinated diphenyltoluens
PCFL	Fluorenes
PCNs	polychlorinated naphthalenes
PFE	pressurised fluid extraction
PFTBA	Perfluorotributylamine
PLE	pressurised liquid extraction
POPs	persistent organic pollutants
PPi	inorganic pyrophosphate
QA	quality assurance
QC	quality control
R^2	correlation coefficient
REP	relative potency
RLU	relative light units
SC	solvent control
SFE	supercritical fluid extraction
SPE	solid phase extraction
TEF	toxic equivalency factor
TEQ	tcdd toxic quotient
TRP	total rapid prep system
UNEP	United Nations Environmental Programme
US EPA	United States Environmental Protection Agency
UV	Ultraviolet

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1
Introduction

1.1 Global perspective on POPs

Persistent organic pollutants (POPs) are a group of chemically stable, toxic compounds consisting of pesticides, industrial chemicals, and by-products of combustion processes that are resistant to photolytic, chemical, and biological degradation. The common physical and chemical characteristics of these compounds allow POPs to bio-accumulate and bio-magnify in food webs, and also make it possible for them to be transported over long distances, thus leading to global distribution (Jones & de Voogt, 1999).

The Stockholm Convention on POPs, which was created in order to restrict the global use and production (intentional and unintentional) of POPs, came into force on 17 May 2004. The first 12 POPs on the list were categorized in three groups, namely pesticides (aldrin, chlordane, dichlorodiphenyltrichloroethane (DDT), dieldrin, endrin, heptachlor, mirex and toxaphene); industrial chemicals (polychlorinated biphenyls (PCBs) and hexachlorobenzene (HCB)) and unintentionally produced by-products (polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDD/PCDF)). However, HCB and PCBs, although produced intentionally, can be formed as by-products during industrial and thermal processes (Lui *et al*., 2012). To date, 10 more chemicals/chemical groups have been added to the list: (alpha- and beta-hexachlorocyclohexane, chlordecone, hexa-bromobiphenyl, penta-chlorobenzene, perfluorooctane sulfonic acid and its salts, technical endosulfan and its related isomers, tetra-, penta-, hexa-bromodiphenyl, commercial hepta-, octa-bromodiphenyl ether (Stockholm Convention, 2012) and more are under review. The Convention currently has 152 signatories and 178 parties listed (Stockholm Convention, 2012).

The Convention has five essential objectives that aim to assist the international community to protect human health and the environment from POPs (United Nations Environmental Programme; UNEP, 2011). These are to:

- Eliminate the release and use of the 21 POPs listed in the Convention. To achieve this, the Convention bans and restricts the use of these POPs, with specific exemptions for selected chemicals for a limited time to enable parties to replace these POPs with alternative technologies such as the use of DDT in malaria control.

- Secondly, to support the transition towards safer alternatives. Simply replacing POPs with other hazardous chemicals should be avoided. If all the signatories of the Stockholm Convention work together, the possibility of discovering new and effective alternatives through research and analysis worldwide is enhanced.
- To prevent further damage to human health and the environment, the Convention targets additional POPs for action. A POPs review committee was established to consider other chemicals for the POPs list. A structured evaluation process is followed using the best available scientific data to determine if the chemical is persistent, bio-accumulative, mobile and toxic.
- An essential aim is to clean up old stockpiles and equipment containing POPs. These need to be managed in a safe and environmentally friendly way so that the POPs are destroyed or irreversibly transformed. The Basal Convention works closely with the Stockholm Convention to assist with technical guidelines on the removal and destruction of POPs.
- The final aim, namely working together for a POPs-free future, needs worldwide consensus. Increasing public awareness and providing information on these pollutants is a major task, and to this end, the Safe Planet Campaign was established to assist with the dissemination of information aimed at raising awareness. Through art exhibitions, videos and working with celebrities, the Campaign helps to make people more conscious of issues pertaining to chemical management.

South Africa officially signed the treaty on 4 September 2002 (Bouwman, 2004), and this legally binds South Africa to abide to the objectives listed in the Convention. Developing countries like South Africa have limited information on POPs and data gaps still exist on some of the POPs, making it important to investigate and generate data for the country. Therefore, the refinement of analytical techniques used to analyse these chemicals is crucial.

1.2 The analysis of POPs in South Africa

With the exception of pesticides, there is at present a shortage in South Africa of analytical capabilities for POPs – both instrumentally and bio-analytically. In general, chemical analysis of POPs relies on highly sophisticated extraction and analytical technology. However, this technology is prohibitively expensive, which makes the need for cheaper, affordable, simple, rapid and sensitive analytical techniques much more pertinent. Indeed, the need for a cost-effective, high capacity screening method to select samples for further chemical analysis is commonly recognised in the scientific community (Chuang *et al.*, 2009).

The use of bio-detectors such as bio-assays is a growing area in the analysis of dioxin-like polychlorinated biphenyls (dl-PCBs), PCDDs and PCDFs (which will be collectively referred to as dioxins) (Behnisch *et al.*, 2001a). Whereas chemical analysis yields little information regarding the biological effect of complex mixtures, *in vitro* bio-assays serve as a screening system for the combined toxic effect of chemicals that operate through a similar mode of action (Hilscherova *et al.,* 2001). From previous international studies on dioxin analysis (Behnisch *et al.*, 2001a; Giesy *et al*, 2002), it is clear that bio-assays are quick, sensitive and relatively inexpensive, and may be a solution to South Africa's shortage of complex instrumentation and expertise. The H4IIE-*luc* reporter gene bio-assay (H4IIE bio-assay) has been used in South Africa before, confirming that the H4IIE bio-assay is complementary to the chemical analysis of dioxin and dioxin-like chemicals (Vosloo & Bouwman, 2005; Nieuwoudt *et al.*, 2009; Roos *et al.*, 2011).

Due to the complexity of dioxin analysis and the established use of the H4IIE bio-assay in South African studies, this study set out to investigate ways of refining sample preparatory protocols with a view to ensure that only dioxins are present in the final extract before analysis and thereby improve selectivity of the methods. Bio-assays and instrumental analysis, in the case of the H4IIE bio-assay, share the same sample preparation.

1.3 The importance of sample preparation

Sample preparation impacts on all the steps in an analysis (whether biological or instrumental analysis); it can interfere with the identification, confirmation and quantification of analytes (Chen *et al*., 2008). An entire analysis process can be invalidated due to poor sample treatment and badly prepared sample extracts. The aim of sample preparation methods is to convert a real matrix (such as sediment or blood serum) into a sample suitable for biological or instrumental analysis. An extraction and clean-up method should remove the analytes from the matrix using an appropriate solvent with the optimum yield and selectivity (Smith, 2003). Problems that are often encountered with dioxin analysis are that dioxin-like chemicals (i) are often present at ultra-trace levels within (ii) complex matrices. Therefore, it is crucial to ensure that the clean-up techniques used do remove as many interfering compounds as possible. Additionally, due to the ultra-trace levels of these compounds, the extracts need to be highly concentrated; this causes a corresponding amplification of matrix interferences (Harrison & Eduljee, 1999).

Although a wide variety of techniques for extraction and clean-up are available, these share a number of aims (Smith, 2003), namely:

- Removing potential interfering compounds from the sample by increasing the selectivity of the method used;
- Converting the analyte into a more suitable form for analysis;
- Increasing the concentration of the analyte, which leads to a corresponding increase in the sensitivity of the analysis;
- Designing a method that is robust and reproducible;
- Having the ability to use smaller initial sample sizes;
- Automation;
- Developing more environmentally friendly methods and using less solvent;
- Greater specificity and/or selectivity.

When using a bio-assay, it is crucial to remove as many interfering compounds as possible since the bio-assays sum the effects of all the compounds present in the extract to which the assay responds (Brown *et al*., 2002). The H4IIE-bio-assay used in this study makes use of the ability of the endogenous aryl hydrocarbon receptor (AhR) to bind to cyclic hydrocarbons (Tillet *et al*., 1991). However, there are a number of compounds that can react as AhR agonists (Table 1) and which can cause a false positive when the aim of a study focuses solely on the presence and toxicity of PCDD/Fs and dl-PCBs. Therefore, it is crucial to remove all other AhR active compounds besides the PCDD/Fs and dl-PCBs. This is achieved through the use of comprehensive clean-up techniques.

Table 1: Three compound classes that can potentially bind to the AhR (Hilscherova *et al*., 2000; Behnisch *et al.*, 2001a)

Class A	Hydrophobic aromatic compounds with a planar structure and with a molecule the correct size to fit into the binding site of the AhR Examples: Planar congeners of PCBs and PCDD/Fs, polychlorazobenzenes (PCABs), polychlorazoxybenzenes (PCAOBs), polychlorinated naphthalenes (PCNs) and high molecular mass polyaromatic hydrocarbons (PAHs)
Class B	Compounds with a specific stereochemical configuration Examples: polyhalogenated (chlorinated, brominated and fluorinated), mixed halogenated (chlorinated, brominated and fluorinated), and alkylated analogs of the previously listed class of compounds, polychlorinated xanthenes and xanthones (PCXE/PCXO), polychlorinated diphenyltoluenes (PCDPT), anisols (PCAs), anthracenes (PCANs) and fluorenes (PCFL).
Class C	Transient inducers and weak AhR ligands that deviate from the traditional criteria of planarity, aromaticity and hydrophobicity and are rapidly degraded by detoxification enzymes Examples: Natural compounds like indoles, hetrocyclic amines, some pesticides and drugs with various structures

1.4 Hypothesis, aim and objectives of the project

Hypothesis: Various extraction and clean-up techniques that target PCDD/Fs and dl-PCBs will cause variations in the induction of the AhR receptor within the H4IIE cell line.

The aim of the project was to evaluate the effectiveness of different clean-up protocols to select for only PCDD/Fs and dl-PCBs by using the H4IIE-*luc* reporter gene bio-assay.

The objectives of the project are:

- To evaluate the effectiveness of comparing clean-up protocols against four different sample types:
    1. Pre-cleaned sand spiked with appropriate internal standards
    2. Certified reference material
    3. Real soil collected in the Vaal Triangle
    4. Method blank
- The extraction and clean-up protocols that were compared (as described in section 2.3), included:
    - Accelerated solvent extraction (ASE) and an overnight percolating extraction (Soxhlet) both with a manual clean-up method consisting of an acid digestion, gel permeation chromatography (GPC) and solid phase extraction (SPE) using dual layer silica.
    - Pressurized liquid extraction (PLE) with an automated clean-up system using multi-layer silica, alumina and carbon columns.
    - Accelerated solvent extraction (ASE) and an overnight percolating extraction (Soxhlet), with no clean-up steps.
- The response of the H4IIE assay was verified by means of comprehensive two dimensional gas chromatography coupled to time of flight mass spectrometry (GCxGC-TOFMS).

2
Literature Review

Over the last 30 years, the number and range of publications on toxic substances have increased substantially, indicating the need for specialised extraction and clean-up techniques that can be used to analyse for these compounds. Dioxins, specifically, have been under scientific and legislative scrutiny due to their high toxicity (Whyte *et al*., 2004). Dioxins form part of the Stockholm Convention's original "dirty dozen", which means that the use and production of dioxins are globally restricted. In South Africa, there is limited information available on dioxins, which points to an urgent need for experimental data (Bouwman, 2004). Previous studies have indicated the presence of dioxins in the South African environment at very low levels (Vosloo & Bouwman, 2005; Roos *et al*., 2011). Due to trace levels present in matrices, the need exists to optimize extraction and clean-up protocols with a view to increase the sensitivity of dioxin analysis.

2.1 Dioxins

The focus of this study is on PCDD/Fs and dl-PCBs; these will be collectively referred to as dioxins while all other AhR agonists will be referred to as dioxin-like chemicals.

2.1.1 Physical and chemical characteristics

PCDDs and PCDFs are two series of planar tricyclic aromatic compounds with similar chemical properties (Buser, 1985; Rappe, 1994). Each compound consists of two benzene rings interconnected by oxygen atoms, where the PCDDs are joined by two oxygen bridges and PCDFs are connected by a carbon-carbon bond and oxygen bridge (Figure 2.1; McKay, 2002). These dioxins can have between one and eight chlorine atoms bound to the molecule (Fiedler, 2003). It is the position and number of chlorine atoms that allow for 75 congeners of PCDDs and 135 congeners of PCDFs, but only 7 PCDDs and 10 PCDFs are regarded as toxic (Fiedler, 2003).

Another group of compounds with dioxin-like activity are the PCBs. PCBs are aromatic compounds formed by two benzene rings bonded by a single carbon-carbon bond (Figure 2.1), where hydrogen atoms can be replaced by up to 10 chlorine atoms to form various PCB congeners. In total there are 209 possible PCB congeners, but only 12 are classified as toxic dioxin-like PCBs (Ulaszewska *et al*., 2011).

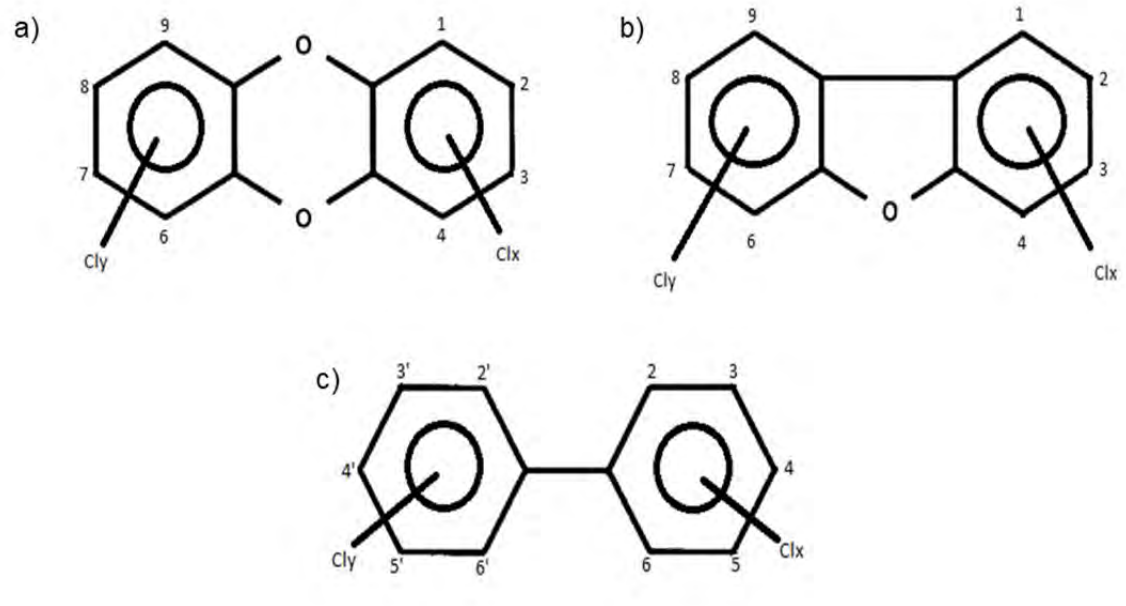


Figure 2.1: Chemical structure of (a) PCDDs, (b) PCDFs and (c) PCBs (McKay, 2002)

The physical and chemical structures of dioxin-like and non-dioxin-like PCBs are similar, but dl-PCBs lack *ortho* substitutions, where non-dioxin-like PCBs have chlorine atoms in one or more of the *ortho* positions. Non-dioxin-like PCBs bind with low affinity to the AhR, while the dl-PCBs bind with high affinity (Fischer *et al*., 1998). PCDD/Fs and dl-PCBs have low vapour pressures and water solubilities (Table 2.1) and high K_{ow} coefficients, indicating that they are freely soluble in non-polar organic solvents and biological lipids (Fiedler, 2003).

Table 2.1: Physical and chemical properties of PCDD/Fs (McKay, 2002; Fiedler 2003)

Compound	Vapour pressure (mmHg at 25°C)	Log K_{ow}	Solubility (mg/ℓ at 25°C)	Henry's constant
TCDD	8.1×10^{-7}	6.4	3.5×10^{-4}	1.4×10^{-3}
PeCDD	7.3×10^{-10}	6.6	1.2×10^{-4}	1.1×10^{-4}
HxCDD	5.9×10^{-11}	7.3	4.4×10^{-6}	1.8×10^{-3}
HpCDD	3.2×10^{-11}	8.0	2.4×10^{-6}	5.1×10^{-4}
OCDD	8.3×10^{-13}	8.2	7.4×10^{-8}	2.8×10^{-4}
TCDF	2.5×10^{-8}	6.2	4.2×10^{-4}	6.1×10^{-4}
PeCDF	2.7×10^{-9}	6.4	2.4×10^{-4}	2.0×10^{-4}
HxCDF	2.8×10^{-10}	7.0	1.3×10^{-5}	5.9×10^{-4}
HpCDF	9.9×10^{-11}	7.9	1.4×10^{-6}	5.8×10^{-4}
OCDF	3.8×10^{-12}	8.8	1.4×10^{-6}	4.0×10^{-5}
PCB 77	4.4×10^{-7}	6.6	1.8×10^{-1}	4.3×10^{-5}
PCB 81	-	6.3	-	-
PCB 105	6.5×10^{-6}	7.0	3.4×10^{-3}	$8.3 \times 10^{-4-}$
PCB 114	5.5×10^{-6}	7.0	1.6×10^{-2}	9.2×10^{-5}
PCB 118	9.0×10^{-6}	7.1	1.3×10^{-2}	2.9×10^{-4}
PCB 123	-	7.0	-	-

Compound	Vapour pressure (mmHg at 25°C)	Log K_{ow}	Solubility (mg/ℓ at 25°C)	Henry's constant
PCB 156	1.6×10^{-6}	7.6	5.3×10^{-3}	1.4×10^{-4}
PCB 157	-	7.6	-	-
PCB 167	5.8×10^{-7}	7.5	2.2×10^{-3}	6.9×10^{-5}
PCB 169	4.0×10^{-7}	7.4	1.2×10^{-2}	1.5×10^{-5}
PCB 189	1.3×10^{-7}	8.3	7.5×10^{-4}	5.1×10^{-5}

2.1.2 Sources

PCDD/Fs are formed unintentionally during industrial, chemical, thermal and natural processes under appropriate conditions when carbon, hydrogen and chlorine are present. This formation can occur in a matter of seconds in the vapour phase or on particle surfaces (soot/ash) between 200 - 400°C and 500 - 800°C (Stanmore, 2004). Dioxin sources can be divided into four categories: incineration, industrial, combustion and reservoirs (Figure 2.2).

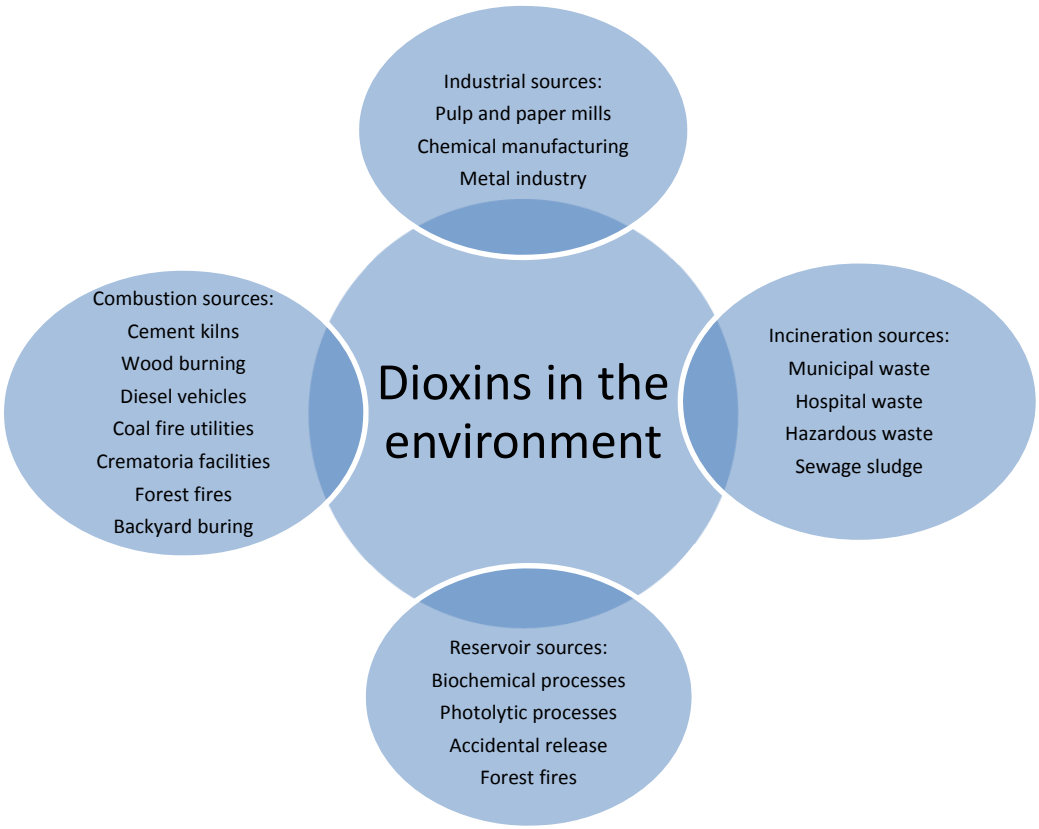


Figure 2.2: Dioxin sources impacting the environment (McKay, 2002; Kulkarni *et al*., 2008)

Municipal solid waste (MSW) incineration is one of the primary sources of dioxins within the incineration category. Backyard burning refers to the combustion of household waste that can include garden refuse. The low combustion temperatures and lack of oxygen during combustion can lead to the formation of products from incomplete combustion (Wevers *et al*., 2004). In South Africa, where open fires are often used for cooking and heating,

backyard burning could contribute significantly to dioxin levels (Nieuwoudt *et al*., 2009). Although the exact mechanism for the formation of dioxins in backyard burning has not yet been extrapolated, it can be assumed that the formation process of dioxins is to be similar to what occurs in MSW incineration. During the MSW process, dioxins are formed by surface catalytic processes, including formation from precursors and the *de novo* synthesis (Kulkarni *et al*., 2008). During formation from a precursor Cl_2 reacts with an organic molecule containing a ring structure (benzene or phenol) that is formed during incomplete combustion of waste. This reaction leads to the formation of chlorinated molecules like PCDD/Fs. *De novo* synthesis occurs at low temperatures of between 300 and 450°C where the oxidation and chlorination of carbon occur in the presence of oxygen (Tuppurainen *et al*., 1997).

Natural sources like volcano eruptions as well as forest and bush fires cause combustion-induced formation of dioxins. In studies conducted by Gullet *et al.* (2008) in the United States of America, two grass types and five forest types were collected and tested in an open burning facility. All of the collected plant material indicated dioxin formation, confirming that natural processes also have an impact on the levels of dioxins present in the environment. Therefore, in South Africa where bio-mass burning occurs either naturally or in controlled circumstances to reduce the risk of wide spread bush fires, this source could be of particular importance.

Although PCBs have been manufactured since the 1930s, their production and use were banned since the 1980s due to evidence of their toxicity (UNEP, 1999). Up until this time, PCBs were used in industrial applications as insulators, plasticisers, lubricants, dielectric fluids in capacitors and transformers and fire retardants (UNEP, 1999). Current emissions of PCBs are mainly due to unintentional formation as by-products during chemical and thermal processes, or due to spillage and burning of old stockpiles (McKay, 2002). Stockpiles include stored oil, old buildings (demolition sites), old fluff storage (waste from upholstery, padding and insulating material), paints and old electrical equipment (UNEP, 1999). Studies have indicated that current formation of dl-PCBs is similar to the formation of PCDD/Fs (Schoonenboom *et al*., 1995; Yasuhara *et al*., 2003). Additionally, reservoir sources such as soil, sediment and vegetation are of concern due to the potential of redistribution and circulation of these compounds in the environment (McKay, 2002; Fiedler, 2003).

Identifying all the possible sources that may give rise to the formation or release of dioxins is a continuous challenge. This challenge has led to the publication of the Standardized Toolkit for Identification and Quantification of Dioxins and Furans Releases (UNEP, 2005). This Toolkit was designed to aid countries in identifying possible sources of PCDD/F. The Toolkit

divides all the possible sources of release and/or formation into ten categories, making it easier to identify them. These ten categories are: waste incineration (municipal, hazardous, medical and sewage), ferrous and non-ferrous metal production (lead and aluminium production, as well as iron ore sintering), power generation and heating (domestic and household heating and cooking), production of mineral products (cement and lime production), transport (variety of engines), uncontrolled combustion processes (biomass burning), production and use of chemicals and consumer goods (pulp and paper production), miscellaneous (crematoria), disposal (waste dumps) and identification of potential hot-spots (such as production sites of chlorine) (UNEP, 2005). With the assistance of this Toolkit to identify possible sources of release or formation, South Africa will be able to comply with the Stockholm Convention's objectives to reduce and eliminate the listed POPs (Refer to section 1.1), in this way decreasing South Africa's contribution to the global levels of these compounds.

2.1.3 Global transport

Once dioxins are released, they are distributed between environmental compartments (atmospheric, aquatic and terrestrial). Due to their physical and chemical characteristics, dioxins partition between gaseous, particulate and liquid phases in these compartments. Dioxins are primarily bound to particulate and organic matter in soil, sediment and biota (fatty tissue); whereas in the atmosphere they exist in both the gaseous phase and bound to particles (Fiedler, 2003). When dioxins enter the gas phase, they volatilise from the environmental compartments and enter the atmosphere, where they can travel long distances before being re-deposited. This cycle of volatilisation and deposition (dry and wet) may occur numerous times, and is referred to as the grasshopper effect. The grasshopper effect results in global distribution of dioxins, which means that these chemicals can be found in areas where they were never produced (Jones & de Voogt, 1999; Moon *et al.*, 2005) – including, for example, polar regions. This is of particular concern since polar regions have low temperatures and long winter darkness, which limit evaporation and UV degradation thus trapping organic pollutants so that these conditions mean that polar regions act as long-term reservoirs (Corsolini *et al*., 2002).

2.1.4 Environmental fate

Dioxins can be deposited on plant or soil surfaces through wet and dry (chemicals bound to atmospheric particles) deposition, as well as through the diffuse transport of gaseous chemicals in the air to the surface of plants. In turn, animals are mainly exposed to dioxins by eating contaminated plants; these pollutants then accumulate in the fatty tissue of the organism (bio-accumulation). This is a constant cycle between the different compartments

and sources (Figure 2.3). An example in the aquatic environment is that dioxins can partition to organic matter and accumulate in sediments. Aquatic biota such as phytoplankton utilise the contaminated matter as a food source and are, in turn, preyed upon by zooplankton and fish; this gives rise to an increase in contaminants corresponding to trophic level (bio-magnification). Due to the increase along the trophic levels, top predators may accumulate high concentrations of contaminants, which give rise to detrimental effects like birth defects, deformities, and even death (Fiedler, 2003).

There are a variety of ways in which humans can be exposed to dioxins, namely through inhalation of contaminated air, accidental ingestion of contaminated soil, dermal absorption and ingestion of food. The main mode of exposure is through the intake of contaminated food, especially food of animal origin such as meat, fish, and dairy products (Charnley & Doull, 2005; Schecter *et al.*, 2006).

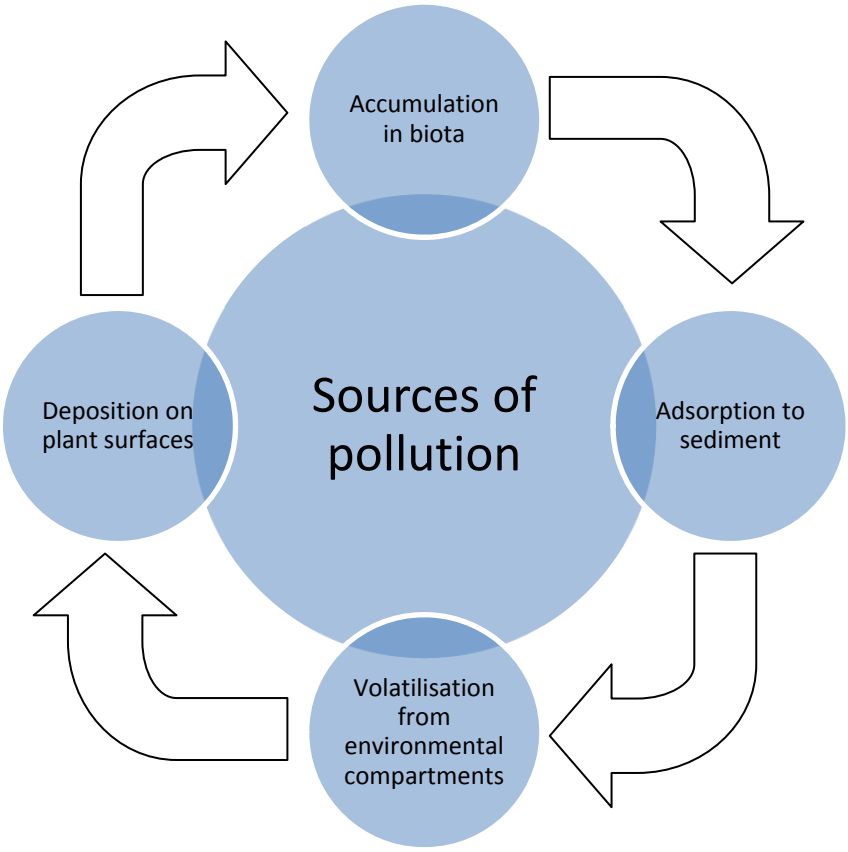


Figure 2.3: Life cycle of dioxins between sources and different compartments

2.1.5 Toxicity

Lateral chlorine atoms in the positions 2, 3, 7, and 8 on the dioxin molecule are the primary cause of the toxicity of dioxins. Dioxins of toxicological importance (Table 2.2) share a number of structural properties, including a rigid sterically accessible aromatic ring with lateral substitutions (McKinney, 1989). They have different degrees of toxicity and each

congener's toxicity is expressed in terms of the toxicity of the most toxic dioxin 2,3,7,8-tetrachloro dibenzo-*p*-dioxin (TCDD). This relative toxicity of these congeners are called toxic equivalency factors (TEF). These TEF values are used to calculate the toxic potential a mixture of these dioxins have on birds, mammals and fish (Van den Berg *et al*, 2006). A number of studies (Vanden Heuvel & Lucier, 1993; Schecter *et al.*, 2006) have shown that dioxins' main mode of toxicity is through the AhR pathway (Refer to section 2.2.1). A recent study has shown evidence that a group of dioxin-like chemicals (PAHs) elicite a super induction (above the maximum effect of 2,3,7,8-TCDD) of the AhR receptor in the H4IIE bio-asssay (Larsson *et al*., 2012). However, this effect needs further investigation.

The AhR receptor forms part of the basic helix-loop-helix family of transcriptional regulators and plays an important role in (Shimba *et al*., 1998; Randi *et al*., 2008):

- the development of major organs including the liver, heart, and kidneys,
- the maintenance of homeostatic functions, and
- it can affect cellular functions by activating cellular kinases.

Additionally, AhR binds its ligands and up-regulates (increases) a battery of xenobiotic metabolizing enzymes, including cytochrome P450 1A1 (Cyp1A1). This enzyme is responsible for the detoxification of polycyclic aromatic compounds through metabolising substrates into more soluble and therefore extractable products. However, this process can generate mutagenic metabolites (Brauze *et al*., 2006; Swedenborg & Pongratz, 2010). The AhR complex can up-regulate protein kinase cytoplasmic tyrosine kinase protein (c-Src), which in turn activates the epidermal growth factor receptor (EGFR). This leads to multiple signalling cascades which could influence cell proliferation, migration, protein secretion, differentiation and oncogenesis (Randi *et al*., 2008).

Table 2.2: IUPAC name and TEF values of the toxic PCDD/F and dioxin-like PCBs (Van den Berg *et al*, 2006)

Specific isomers	Compound name	WHO 2005 TEF (for mammals)
2,3,7,8-TCDD	2,3,7,8-Tetrachloro dibenzo-*p*-dioxin	1
1,2,3,7,8-PeCDD	1,2,3,7,8-Pentachloro dibenzo-*p*-dioxin	1
1,2,3,5,8,9-HxCDD	1,2,3,5,8,9-Hexachloro dibenzo-*p*-dioxin	0.1
1,2,3,4,6,7,8-HxCDD	1,2,3,4,6,7,8-Hexachloro dibenzo-*p*-dioxin	0.1
1,2,3,7,8,9-HxCDD	1,2,3,7,8,9-Hexachloro dibenzo-*p*-dioxin	0.1
1,2,3,4,6,7,8-HpCDD	1,2,3,4,6,7,8-Heptachloro dibenzo-*p*-dioxin	0.01
1,2,3,4,5,6,7,8,9-OCDD	1,2,3,4,5,6,7,8,9-Octachloro dibenzo-*p*-dioxin	0.1
2,3,7,8-TCDF	2,3,7,8-Tetrachloro dibenzofuran	0.03
1,2,3,7,8-PeCDF	1,2,3,7,8-Pentachloro dibenzofuran	0.3
2,3,4,7,8-PeCDF	2,3,4,7,8-Pentachloro dibenzofuran	0.1
1,2,3,4,7,8-HxCDF	1,2,3,4,7,8-Hexachloro dibenzofuran	0.1
1,2,3,6,7,8-HxCDF	1,2,3,6,7,8-Hexachloro dibenzofuran	0.1

Table 2.2 continued: IUPAC name and TEF values of the toxic PCDD/F and dioxin-like PCBs (Van den Berg et al, 2006)

Specific isomers	Compound name	WHO 2005 TEF (for mammals)
1,2,3,7,8,9-HxCDF	1,2,3,7,8,9-Hexachloro dibenzofuran	0.1
2,3,4,6,7,8-HxCDF	2,3,4,6,7,8-Hexachloro dibenzofuran	0.01
1,2,3,4,6,7,8-HpCDF	1,2,3,4,6,7,8-Heptachloro dibenzofuran	0.01
1,2,3,4,7,8,9-HpCDF	1,2,3,4,7,8,9-Heptachloro dibenzofuran	0.0001
1,2,3,4,5,6,7,8-OCDF	1,2,3,4,5,6,7,8-Octachloro dibenzofuran	0.0003
3,3',4,4'-TeCB	3,3',4,4'-Tetrachloro biphenyl (PCB 77)	0.1
3,4,4',5-TeCB	3,4,4',5-Tetrachloro biphenyl (PCB 81)	0.0003
3,3',4,4',5-PeCB	3,3',4,4',5-Pentachloro biphenyl (PCB 126)	0.0003
3,3',4,4',5,5'-HxCB	3,3',4,4',5,5'-Hexachloro biphenyl (PCB 169)	0.0003
2,3,3',4,4'-PeCB	2,3,3',4,4'-Pentachloro biphenyl (PCB 105)	0.0003
2,3,4,4',5-PeCB	2,3,4,4',5-Pentachloro biphenyl (PCB 114)	0.0003
2,3',4,4',5-PeCB	2,3',4,4',5-Pentachloro biphenyl (PCB 118)	0.0003
2',3,4,4',5-PeCB	2',3,4,4',5-Pentachloro biphenyl (PCB 123)	0.0003
2,3,3',4,4',5-HxCB	2,3,3',4,4',5-Hexachloro biphenyl (PCB 156)	0.0003
2,3,3',4,4',5'-HxCB	2,3,3',4,4',5'-Hexachloro biphenyl (PCB 157)	0.0003
2,3',4,4',5,5'-HxCB	2,3',4,4',5,5'-Hexachloro biphenyl (PCB 167)	0.0003
2,3,3',4,4',5,5'-HpCB	2,3,3',4,4',5,5'-Heptachloro biphenyl (PCB 189)	0.0003

In rodents, which have been the primary end point used to estimate human health risks, a spectrum of toxic responses has been observed. These include effects on the immune function, reproduction, teratogenicity, lipid and glucose metabolism; behavioural abnormalities and carcinogenic tumours on the liver and skin (Poland & Knutson, 1982; Vanden Heuvel & Lucier, 1993; Mann, 1997; Schecter *et al*., 2006). Based on these effects, concern was raised as to the probable human toxicity of dioxins. The United States Environmental Protection Agency (US EPA) characterized dioxins as "likely human carcinogens" in 2003, and the most toxic compound 2,3,7,8-TCDD as a known human carcinogen (US EPA, 2003). A skin condition known as chloracne was one of the first health effects recorded after dioxin exposure and comprises a persistent acne form which could last for months or even years after exposure (Mann, 1997; Schecter *et al*., 2006). Suspected human health effects attributed to dioxin and dioxin-like compounds are summarised in Table 2.3.

Table 2.3: Suspected human health effects attributed to dioxin and dioxin-like compounds (Schecter *et al.*, 2006)

Suspected health effects	Compounds responsible for health effect
Tumour prompter	2,3,7,8-TCDD
Cancer mortality	PCDD/F
Immune deficiency	PCBs (118, 138, 153, 180), PCDD/Fs
Reproductive abnormalities	PCBs, PCDD/Fs
Developmental abnormalities	PCDDs

Table 2.3 continued: Suspected human health effects attributed to dioxin and dioxin-like compounds (Schecter et al., 2006)

Suspected health effects	Compounds responsible for health effect
Central- and peripheral nervous system pathology	PCBs, PCDFs
Diabetes	2,3,7,8-TCDD
Decrease thyroid hormone levels	2,3,7,8-TCDD
Decreased pulmonary function and bronchitis	PCBs
Elevated serum cholesterol and triglycerides	2,3,7,8-TCDD
Death from cardiovascular disease	2,3,7,8-TCDD, PCDD/Fs
Death from ischemic heart disease	2,3,7,8-TCDD, PCDD/Fs
Liver damage	2,3,7,8-TCDD
Skin rashes	2,3,7,8-TCDD
Chloracne	2,3,7,8-TCDD
Pruritus	PCBs, PCDFs
Hypertrichosis	2,3,7,8-TCDD
Enamel hypo-mineralization	2,3,7,8-TCDD
Gum pigmentation	2,3,7,8-TCDD
Eyelid pathology	2,3,7,8-TCDD
Meibomian gland hyper secretion	PCBs
Hyper pigmented conjunctivae	PCBs
Nausea	2,3,7,8-TCDD
Vomiting	2,3,7,8-TCDD
Loss of appetite	2,3,7,8-TCDD
Headache	PCBs, PCDFs
Fatigue	PCBs, PCDFs
Change in serum testosterone	2,3,7,8-TCDD

2.2 Methods used to determine concentrations of dioxins

Dioxins have been analysed since the 1970s and the standard method for the analysis of dioxins is high resolution gas chromatography high-resolution mass spectrometry (HRGC/HRMS) (Hui *et al.*, 2007). However, due to the high cost of these analyses, a need exists for more cost-effective, affordable, simple, rapid and sensitive analysis techniques (Refer to section 1.2). The HRGC/HRMS can reliably identify and quantify the toxic dioxin congeners, but cannot take into account the additive, synergistic or antagonistic interactions of these chemicals. This deficiency gave rise to the concern that chemical analysis may under- or over-estimate the potential risk of dioxins (Hilscherova *et al.*, 2000).

Over the last decade, bio-detectors such as bio-assays, enzyme immunoassays (EIAs) and biomarkers have been used with increasing frequency for dioxin analysis. The use of bio-assays has a number of advantages, including that results can be gleaned for compounds for which there are no analytical techniques or standards; furthermore, an indication of the total biological activity of the extracts is possible, as well as sensitive and rapid analysis. In addition, bio-detectors assist in assessing the risk to the environment from complex

mixtures. The main disadvantage of many bio-assays is that they cannot identify the specific chemicals which elicit a response. Therefore, bio-assays cannot replace chemical analysis (Hilscherova *et al.*, 2000; Behnisch *et al.*, 2001a; Denison *et al*., 2004), but should be used in conjunction with more traditional instrumental analysis.

As previously described, the relative toxicity potency of a mixture of dioxin-like chemicals is assessed by the TEF approach which entails that the concentrations of the individual chemicals are multiplied by their specific TEF values (Table 2.2); and the sum of these values are expressed as toxic equivalent quotient (TEQs). The bio-assay reports data as bio-analytical equivalents (BEQs) (Baston & Denison, 2001) and is similar to TEQ derived from chemical analysis (Garrison *et al.*, 1996). One of the bio-assays used to determine the dioxin-like toxic potential of extracts is the H4IIE bio-assay.

2.2.1 Mechanism of the H4IIE bio-assay

In this study, the biological assay used is the reporter gene assay using the H4IIE rat hepatoma cells.

The H4IIE-*luc* cells were stably transfected with a firefly luciferase reporter gene. The bio-assay indirectly measures cytochrome P450 induction, which is an endpoint in the AhR mediated response (Hilscherova *et al.*, 2000). The luciferase gene was inserted down-stream of the P450 gene.

AhR receptors in the cytoplasm of the cell occur as a complex with 90-kDa heat shock proteins (Hsp90). When AhR agonists (such as dioxins) enter the cytoplasm of the cells, they bind to the AhR complex and Hsp90 dissociates from the complex. The AhR is translocated to the nucleus of the cell where it forms a heterodimer with the Ah nuclear translocator (ARNT) protein. The complex binds onto a specific DNA sequence known as the dioxin response element (DRE), which results in transcriptional activation of genes (Poland & Knutson, 1982; Giesy *et al.*, 2002; Whyte *et al.*, 2004). In the case of the H4IIE cells, the gene that is activated is the luciferase reporter gene (Figure 2.4).

$$\text{D-luciferin} + \text{ATP} + O_2 \xrightarrow[Mg^{2+}]{\text{Luciferan}} \text{Oxyluciferin} + \text{PPi} + \text{AMP} + CO_2 + \text{light}$$

When the substrate luciferin is added to the cells, a light-producing reaction is catalysed. Where d-luciferin together with adenosine triphosphate (ATP) and oxygen reacts with the substrate given (luciferin) to form oxyluciferin with inorganic pyrophosphate (PPi), adenosine monophosphate (AMP), CO_2 and light (Alam & Cook, 1990). The amount of light emitted is

directly proportional to the amount of dioxins present in the sample (Hilscherova *et al.*, 2000).

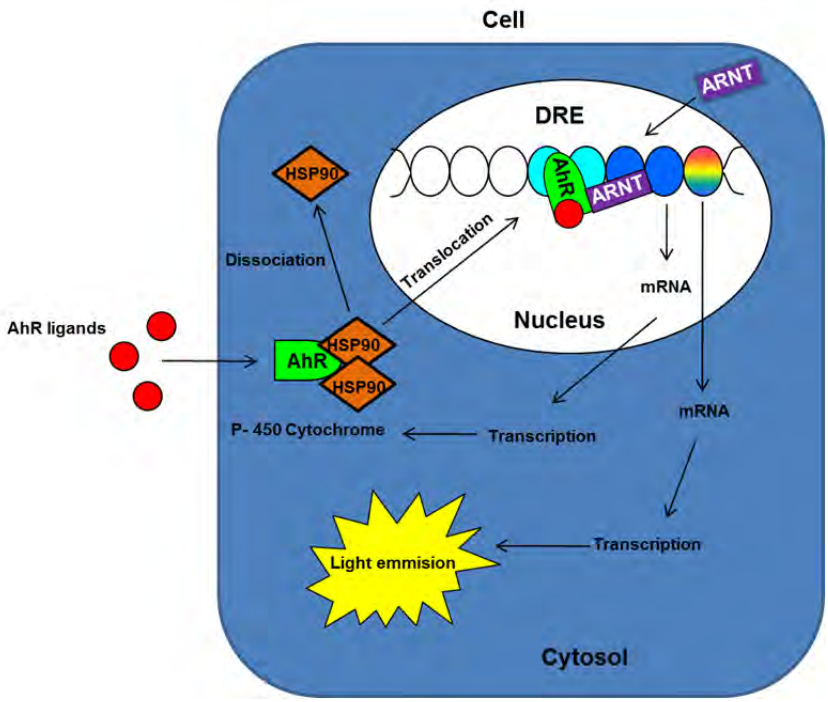


Figure 2.4: Mechanism of the H4IIE bio-assay (adapted from Hilscherova *et al*., 2000)

2.3 Extraction and clean-up methods used in the analysis of dioxin-like chemicals

Extraction and sample clean-up is one of the crucial steps during dioxin analysis (Refer to section 1.3). The extraction device invented by Professor von Soxhlet in 1987, where solvent recirculates while accumulating extracted analytes in a heated flask, is regarded as the reference method for the extraction of dioxins from solid samples (soil, sediment and fly ash) (Focant *et al*., 2004). One of the drawbacks of Soxhlet extraction is the high volume of solvent used, as well as the duration of the extraction process (12-24 hours). This situation lead to an increased demand for new techniques with reduced organic solvent consumption, automation and reduced sample preparation time (Björklund *et al.*, 2000). Methods such as pressurised liquid extraction (PLE), supercritical fluid extraction (SFE) and microwave-assisted extraction (MAE) were introduced. These different extraction methods are compared in Table 2.4.

Table 2.4: The advantages and disadvantages of four different extraction methods (Björklund et al., 2000; Focant *et al.*, 2004; Miyawaki *et al*., 2008)

Extraction process	Solvent usage	Duration	Advantages	Disadvantages
Soxhlet	High	6-24 hours	Extensive	Time consuming, high solvent use
PLE	Medium	10 min	Fast and extensive	Use of organic solvents, high capital cost
SFE	Medium	2 hours	Environmentally friendly, no concentrating	High capital cost, low extraction efficiency for dioxins
MAE	Low	10 min	Fast and extensive	Lack of selectivity

For purposes of this study, the reference method Soxhlet was compared to PLE. Much confusion exists regarding the name of the extraction technique that uses high temperature and pressure. The Dionex Corporation developed the accelerated solvent extraction (ASE) system. ASE is now a trade name for Dionex, which gave rise to the use of alternative names such as PLE and pressurised fluid extraction (PFE) (Björklund *et al*., 2000). The PLE and similar ASE techniques use less solvent; also, due to static-purge cycles, these minimise the cycling of the solvent and extraction time (Focant *et al.*, 2004; Fayez *et al.*, 2008). Furthermore, the high temperatures reduce the viscosity of the solvent, increasing the solvent's ability to wet the matrix and solubilize target analytes, and also assist in breaking down analyte matrix bonds. The elevated pressure ensures that the solvent remains in a liquid state, although the working temperature is above their atmospheric boiling points (Björklund *et al*., 2000). Previous studies have shown that the ASE and PLE are essentially equivalent to the Soxhlet reference method for dioxins in environmental matrices (Bautz *et al*., 1998; Richter *et al*., 1997; Hubert *et al*., 2000).

Prior to final analysis and quantification, an efficient clean-up method is needed to purify samples (Refer to section 1.3), for the H4IIE bio-assay as well as the GCxGC-TOFMS analyses. Dioxins are present in low levels in matrices, and most, if not all interferences must be removed to have a quality extract with only the analytes of interest remaining (Oleszek-Kudlak *et al*., 2007). There are a couple of methods one can use to assist with dioxin clean-up; among these are US EPA methods 1613 and 8290 (Figure 2.5). The conventional clean-up consists of an acid washing treatment and sequential columns of multi-layer silica, alumina and/or Florisil as well as carbon columns (US EPA, 1994a, b, c; Oleszek-Kudlak *et al*., 2007).

The acid wash treatment is employed to remove interfering compounds such as PAHs, and lipids. An alternative to the manual acid wash is the use of multi-layer silica (acid-basic-

neutral; ABN) to remove any lipids and other oxidisable compounds from the extract. The use of ABN silica allows the extract to return to neutral and ensures that all acid components are neutralized before entering the alumina column. The alumina column removes halogenated compounds such as pesticides and non-coplanar or "*ortho*-substituted" PCBs present in the extract. Florisil can be used to remove a variety of compounds, depending on the solvent used. In dioxin extraction it is used to remove polar compounds (de Souza Pereira, 2004). Carbon columns have a high affinity for planar aromatic molecules and this affinity is strengthened by electronegative substitutes such as chlorine and bromine. To elute the planar fraction from the carbon column, reverse-elution is necessary (Focant *et al*., 2004; Oleszek-Kudlak *et al*., 2007), while other compounds are irreversibly bound.

The high lipid content of some matrices may lead to the incomplete removal of lipids during the acid digestion, thus increasing the complexity of the clean-up procedure. To address this, the application of gel permeation chromatography (GPC) for the removal of lipids through size exclusion has been introduced. GPC can also be used to remove sulphur components, which negates the necessity of copper/tetrabutylammonium sulphite clean-up methods (US EPA, 1996; Brennan *et al*., 2009). However, this total clean-up procedure is time-consuming, uses large amounts of solvent and requires sample manipulation that increases the risk of cross contamination and possible loss of sample through many transfers, leading to the need for automated sample clean-up (Bautz *et al*., 1998; Focant *et al*., 2004).

In 1986, the first automated apparatus capable of sequential clean-up for dioxins was reported, where the clean-up process time required was reduced by 50% (Focant *et al*., 2004). Collaboration with Fluid Management Systems (FMS) yielded an instrument using silica, alumina and carbon columns which could clean-up more samples simultaneously. The fractionation procedure of the instrument allows for isolation of the ten PCDFs, seven PCDDs and 12 PCBs in the extract (Pirard *et al*., 2002; Focant *et al.*, 2004). Multi-layer silica (4 g acid, 2 g base and 1.5 g neutral), alumina (8 g) and PX-21 carbon (2 g) Teflon columns which are individually packed are used for the automated clean-up procedure (Figure 2.5). This system has been used in an array of studies, indicating reduced solvent usage, with minimal sample handling and reduction of blank levels (Abad *et* al., 2000; Focant *et al*., 2004; Fayez *et al*., 2008).

In this study a manual as well as an automated sample clean-up was evaluated (Figure 2.5). The manual method followed was modified from the US EPA method 8290 A and method 1613, consisting of an acid digestion, followed by a GPC and Florisil clean-up. The

automated method consisted of the FMS PowerPrep multi-column sample clean-up system with high capacity ABN silica, alumina and carbon columns.

Extraction	SFE	Soxhlet	MAE	PLE
Clean-up: acid wash	MANUAL	Liquid-liquid		TRP system with ABN silica, alumina and carbon columns
Clean-up: size exclusion		GPC		
Further clean-up		Florisil fractionation		

AUTOMATED

Figure 2.5: Summary of different extraction and clean-up protocols

2.4 Samples used in method validation

The use of certified reference materials (CRMs) for quality control purposes is well recognised by international, national and professional organisations and is aimed at evaluating analytical methods for a variety of compounds in various matrices, including dioxins in sediment. A CRM can be used to calibrate an apparatus; for method validation; for assessment of method and instrumental performance; and for establishing the traceability of measurement results. An ideal CRM is one which is matrix matched and analyte matched to the sample analysed (International Atomic Energy Agency; IAEA, 2003). CRMs are prepared in batches, and property values are assigned with uncertainty limits, which allows for the traceability of measurements to a defined reference (SI units) (IAEA, 2003). To ensure that the CRM is valid, the instructions for use of the CRM must be followed, otherwise the certified values are not applicable. The storage and drying instructions of the material must be closely adhered to and the shelf life should be noted. This allows an analytical laboratory to validate a method for an analyte to promote quality and confidence in measured results (IAEA, 2003; EA, 2003). CRMs were used in this study to evaluate the different clean-up methods on a highly contaminated soil sample with known concentrations of pollutants.

A matrix matched calibration curve is one of the most important tools used to determine the accuracy, precision, sensitivity and specificity of a method (Danzer & Currie, 1998). A matrix matched calibration curve takes the effect of the matrix into consideration and the possible analyte interactions with the matrix. A calibration curve is defined as a mathematical function which indicates the relationship between values derived from a measuring instrument (or assay) and the known values of a reference standard. Thus, it shows the relationship between instrument or bio-assay response and the concentration, leading to a linear response, which can be indicated by the equation $y = mx + c$, with y = instrument/ bio-assay

response, x = concentration of standard, m = slope of the response and c = y intercept (Danzer & Currie, 1998; Barwick, 2003).

The present study explored the effectiveness of different clean-up methods, using a matrix matched calibration curve (spiked with internal standard; refer to chapter 3), CRM and South African soil samples. The H4IIE bio-assay was used as a semi-quantitative tool, while the GCxGC-TOFMS was used to evaluate the effectiveness of the different extraction and clean-up method combinations.

3
Materials and methods

3.1 Sample types selected

Various sample types were extracted and subjected to various clean-up protocols in order to determine the effect of various clean-up techniques on the H4IIE assay. These sample types consisted of:

- pre-cleaned sea sand that was extracted for 24 hours using the Soxhlet apparatus with a 3:1 dichloromethane (DCM):hexane solvent mixture and dried overnight at 105°C, prior to spiking with internal standards (Refer to section 3.2) for the matrix matched calibration curve,
- a highly contaminated CRM which is a sediment from a New York/New Jersey waterway (1944; National Institute of Standards and Technology; NIST, USA).
- two South African soil samples collected from the Vaal Triangle during a previous study that had shown a significant response in the H4IIE bio-assay (BEQ values of 14.60 ngTEQ/kg and 53.12 ngTEQ/kg), and
- pre-cleaned sea sand with no added standard was used as a method blank to ensure that there was no contamination from the analytical process in the laboratory (US EPA, 2009).

Cleaned sea sand was the best available option for the “matrix matched” sample series, although soil samples contain organic material which would bind dioxin and dioxin-like compounds. The CRM was added to assist with this possible interference.

3.2 Internal standard concentration ranges

Previous studies indicated the optimum concentration range for 2,3,7,8-TCDD (reference compound) for the H4IIE bio-assay was: 120.00, 30.00, 7.50, 1.88, 0.47 and 0.12 pgTCDD/well (Giesy *et al*., 1997; Nieuwoudt *et al*., 2009). Taking these values as well as the limit of detection (LOD) for the GCxGC-TOFMS into account, an eight point calibration curve was constructed (Table 3.1) using Method 8290 matrix spiking solution (Cambridge Isotope Laboratories; Canada). The target concentration is the concentration of the final extract and the concentration on column/per well is the concentration injected/dosed with (target concentration multiplied by 2.5 µℓ). This calibration curve was used to generate both the matrix matched calibration curve as well as the non-matrix matched calibration curve of the H4IIE bio-assay.

Table 3.1: Target concentration range for the calibration curve (Cal) used in the H4IIE bio-assay and GCxGC-TOFMS analysis

Target concentration	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7	Cal 8
In final extract (TEQng/mℓ)	150	80	40	18	3	0.75	0.19	0.05
On column/per well (TEQpg/uℓ)	300	200	100	50	8	2	0.6	0.2

3.3 Extraction and clean-up protocols

Before commencing with sample preparation, extraction, clean-up and analysis all the glassware and laboratory equipment used were washed with phosphate free soap, and then rinsed with warm tap water followed by double distilled water (18.2 MΩ) and allowed to dry. Thereafter, the equipment was rinsed in triplicate with high performance liquid chromatography grade (HPLC) acetone and hexane (Burdick and Jackson) to remove both polar and non-polar compounds that could contaminate the sample (USEPA, 1994a). All the solvents used were HPLC grade (Burdick and Jackson), unless stated otherwise.

During and after extraction, samples were evaporated under a gentle flow of nitrogen gas at 50°C in a Turbo-vap®II (Caliper Lifescienes) or the FMS SuperVap concentrator. After the final concentration step, the extracts were reconstituted to 250 µℓ hexane, where 150 µℓ was pipetted into an amber vial for the H4IIE assay, and the remaining 100 µℓ was evaporated and reconstituted in nonane (Sigma-Aldrich) for GCxGC-TOFMS analysis.

3.3.1Extraction methods

Two extraction methods were used during this study: distillation extraction at ambient pressure and a temperature slightly below the boiling point of the solvent used – the well-known Soxhlet extraction method (Richter *et al.,* 1997; McCant *et* al., 1999; Focant *et al.*, 2004) and the newer, quicker process using high temperature and pressure during the extraction process. However, this latter procedure was done using two individual instruments: the accelerated solvent extractor (ASE) by Dionex and the pressurised liquid extractor (PLE) by Fluid Management Systems (FMS). The Soxhlet method can be seen as the reference extraction method for dioxins (Focant *et al*., 2004).

3.3.1.1 Pressurised liquid extraction using the ASE (Dionex)

PLE (ASE® 350) uses elevated temperature and pressure, in an enclosed vessel, to extract analytes of interest from the matrix (Refer to section 2.3) in a shorter time and using less solvent than the more traditional approach of the Soxhlet. Approximately, 10 g (Ontario Ministry of the Environment, 2004) of each sample was mixed with pre-cleaned anhydrous

sodium sulphate to remove traces of water (Na_2SO_4, Merck) and packed in a 100 mℓ stainless steel extraction cell between two 30 mm cellulose filters closing the vessel with its screw lids (Figure 3.1). For the matrix-matched calibration curve, the standard was added on top of the mixed sample before the top filter was inserted.

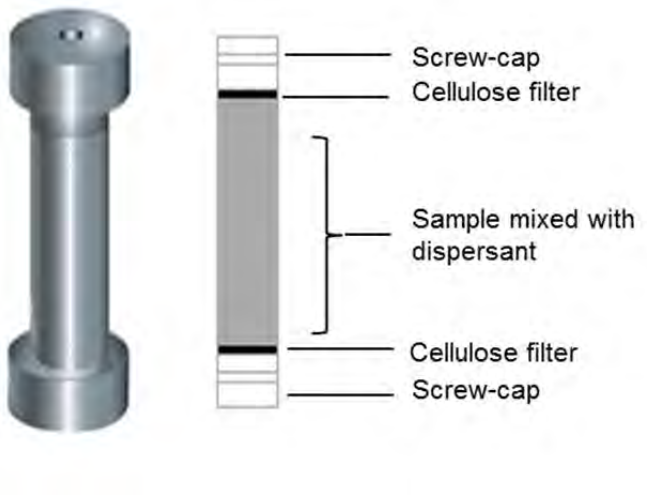


Figure 3.1: ASE extraction cell (Adapted from Richter *et al*., 1996)

The following extraction parameters were used: 100°C, 10 342 kPa, 10 minute static time, 5 minute heat time, and 300 seconds purge with nitrogen, solvent saver mode on "flow mode" at 1 mℓ/min with a solvent mixture of 3:1 DCM:hexane (McCant *et* al., 1999). Two extraction cycles were run for each sample and collected in a single pre-cleaned vial. The extracts were stored at 4°C until further sample processing.

3.3.1.2 Pressurised liquid extraction using the PLE (FMS)

The Total Rapid Prep system (TRP) consists of a pressurized liquid extraction system together with a Power Prep multi-column sample clean-up system that will be discussed in section 3.3.2.2 as part of the clean-up systems. The PLE cell was packed with a Na_2SO_4 plug of approximately 5 g at the bottom and top of the stainless steel cell, the sample (10 g) was mixed with pre-cleaned hydromatrix, inert diatomaceous earth used as packing material, (Varian) to fill the rest of the cell volume (Figure 3.2). As with the ASE extraction, standard was added to the top of the sample mix, before the final Na_2SO_4 was added, for samples used to generate the matrix matched calibration curve. The packed cell was placed in the PLE cell holder and secured (Figure 3.3). The heating blocks surrounding the cell were closed. The configuration of the heating blocks (Figure 3.3) allows for homogenous heating of the sample.

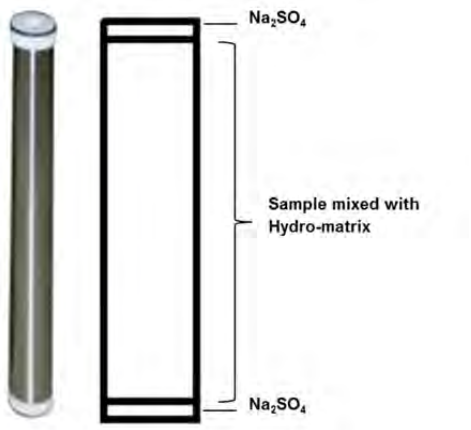


Figure 3.2: PLE extraction cell

The PLE section of the TRP is a fully automated system that is run through DMS 6000 software (Focant & Shirkhan, 2009). The extraction sequence consisted of 10 individual steps. During steps 1 and 2 the extraction cells were filled with the solvent mixture (3:1 DCM:hexane), before step 3 commenced with the pressurization of the cells to approximately 10 342 kPa. During steps 4 and 5 the pressure was maintained and the oven reached the set temperature of 120°C (Focant *et al*., 2009). After extraction, the oven cooled down to ambient temperature (step 6) and the extraction cells were flushed with solvent to ensure that the total extract had been removed (steps 7 and 8), followed by a nitrogen purge (step 9). After steps 1-9 had been completed, the programme automatically repeated itself for a two-cycle extraction. After the second cycle, the sequence was stopped with an end signal (step 10) (Focant *et al*., 2004). The total extract from both cycles was collected in a single pre-cleaned vial and evaporated to dryness.

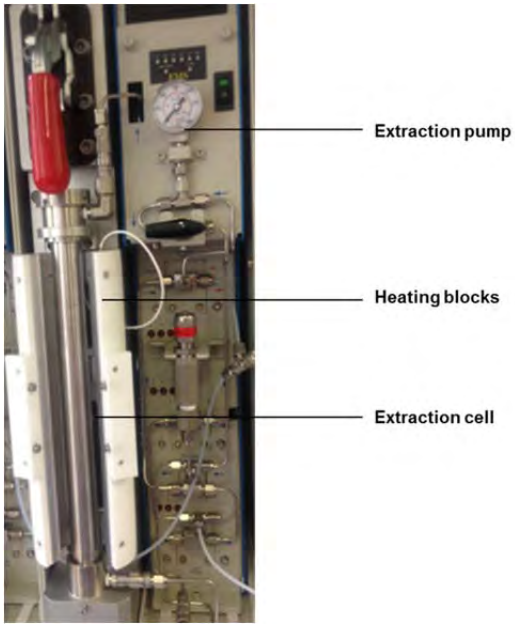


Figure 3.3: PLE extraction unit

3.3.1.3 Soxhlet extraction

Pre-cleaned glass wool was placed in the bottom of the Soxhlet apparatus to prevent the sample from filtering into the round-bottomed flask. Each sample (10 g) was mixed with equal volumes of pre-cleaned Na_2SO_4 and extracted for 24 hours with 400 mℓ, 3:1 DCM: hexane mixture (Kannan *et al.,* 2000). The standard for the matrix matched calibration was added on top of the sample in the Soxhlet (Figure 3.4). To ensure commensurate heating of the solvent, pre-cleaned boiling stones were placed in the round-bottomed flasks. After extraction, the extracts were transferred to pre-cleaned Turbo-vap®II (Caliper Lifescienes) flasks and evaporated under a gentle nitrogen gas stream to dryness to remove all solvent residues (Besselink *et al.*, 2004). The Turbo-vap®II water bath was set to 60°C (Lai *et al*., 2004).

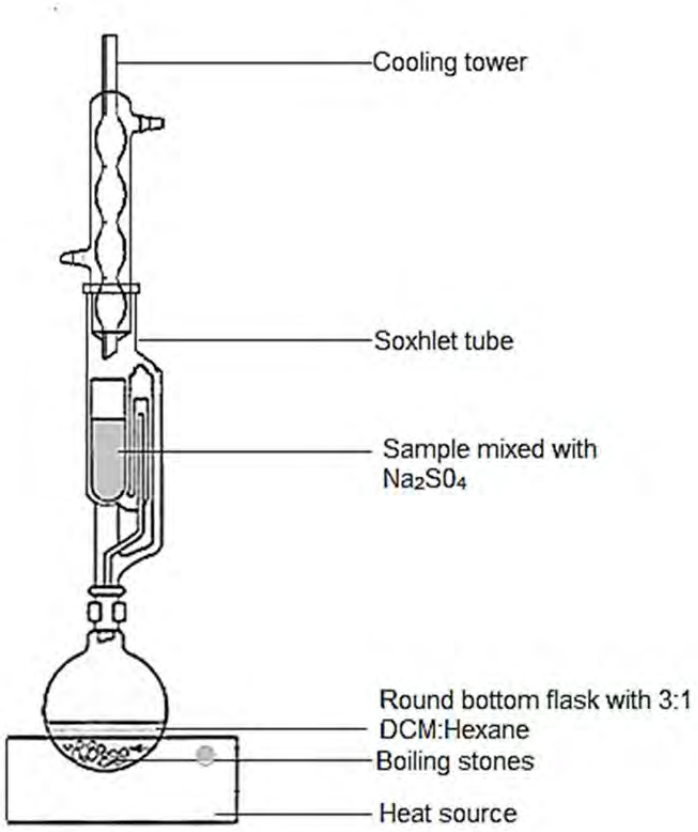


Figure 3.4: A graphical representation of Soxhlet extraction (Adapted from de Castro & Priego-Capote, 2010)

3.3.2 Clean-up methods

3.3.2.1 Manual clean-up method

For both the Soxhlet and the ASE extraction methods, the clean-up steps set out below were followed. Unlike the TRP system, these clean-up steps were performed manually.

Step 1: Acid digestion

An acid digestion was used in order to remove oxidizable interfering compounds that could bind to the AhR receptor such as PAHs (Whyte *et al.*, 2004). The extract was reconstituted to 10 mℓ hexane and added to 15 mℓ 98% sulphuric acid (H_2SO_4, Merck) in a pre-cleaned separation funnel. The mixture was shaken gently whilst venting regularly. The extract was allowed one hour to partition the acid and the organic phases. The acid (bottom phase) was removed, taking care not to lose any of the organic fraction, and fresh acid was added. This step was repeated until the acid was found to be clear of colour after mixing with the sample (3-5 times) (US EPA, 1996). After the final wash, 15 mℓ of 5% sodium chloride solution (NaCl, Fluka) was added to the extract to remove any traces of acid. After one hour, the NaCl was replaced with 15 mℓ potassium hydroxide solution (20% KOH, Sigma-Aldrich) to neutralize any remaining acid (US EPA, 2007) for no more than 15 minutes as KOH is a strong base and known to degrade PCDD/Fs (US EPA. 1994b). Thereafter, another NaCl wash ensured that all the KOH (Sigma-Aldrich) was removed from the extract. The extracts were evaporated to dryness at 50°C under a gentle flow of nitrogen gas.

Step 2: Gel Permeation Chromatography (GPC)

GPC is based on size exclusion, where analytes are separated based on their size or hydronamic volume. In this study, the GPC was used to remove lipids and sulphur, as well as any other compounds with similar sizes, present in the extracts. The lipids can alter the partitioning of chemicals to the cells (Whyte *et al*., 2004) and can cause interferences in GCxGC-TOFMS, while sulphur is toxic to the H4IIE cells (Hilscherova *et al*., 2000).

The system consists of the following: a Waters 717 plus auto-sampler, Waters 1515 isocratic HPLC pump, Waters 2487 dual λ absorbance detector and a Waters fraction collector III. Two Envirogel chromatographic columns connected in series (19 x 150 mm and 19 x 300 mm) were used for size exclusion chromatography.

A standard mixture containing corn oil (Sigma-Aldrich), phthalate (PESTANAL), perylene (PESTANAL), methoxychlor (PESTANAL) and sulphur (PESTANAL) was injected (2 mℓ) to calibrate the GPC and determined the retention time (Figure 3.5) of each compound (US EPA, 1994b).These standard injections (repeated 3x) were additionally used to determine the repeatability and resolution of the column set. For this study the extract was collected from 11.5 to 20.5 min (Figure 3.5), taking the elution times of corn oil and sulphur as well as

the dead time between the detector and the fraction collector into consideration. The system was set at a flow rate of 5 mℓ/min for 30 minutes, with DCM as the carrier solvent.

Before GPC, the evaporated extracts were reconstituted to 2 mℓ DCM and filtered through a 1 µm glass fibre filter (Sigma-Aldrich), fitted to a glass luer lock syringe, and into a recovery vial. The recovery vials were weighed empty, again with the extract, and after injection, to determine the mass fraction lost during the GPC process. Blank solvent (DCM) was run in between samples to assess and prevent carry-over from one sample to the next. During elution, the extract was collected into a turbo-vap flask and evaporated at 60ºC.

Step 3: SPE using dual layer silica

Dual Layer Superclean Florisil liquid chromatography silica (LC-Si, 2g/2g) SPE cartridges (Supelco) were used to remove all polar compounds present in the extracts (Refer to section 2.3) (Oleszek-Kudlak *et al*., 2007). The SPE cartridge was conditioned with 6 mℓ hexane, after which the extract was added (reconstituted to 8 mℓ in hexane). As determined with previous experiments.

The SPE cartridge was eluted by gravity flow with 12 mℓ of a 1:1 DCM:hexane mixture followed by 2 mℓ DCM to ensure that all compounds of interest were removed from the cartridge. The samples were evaporated under a gentle steam of nitrogen gas using a syringe nitrogen evaporation apparatus with a warm water bath (N-Vap).

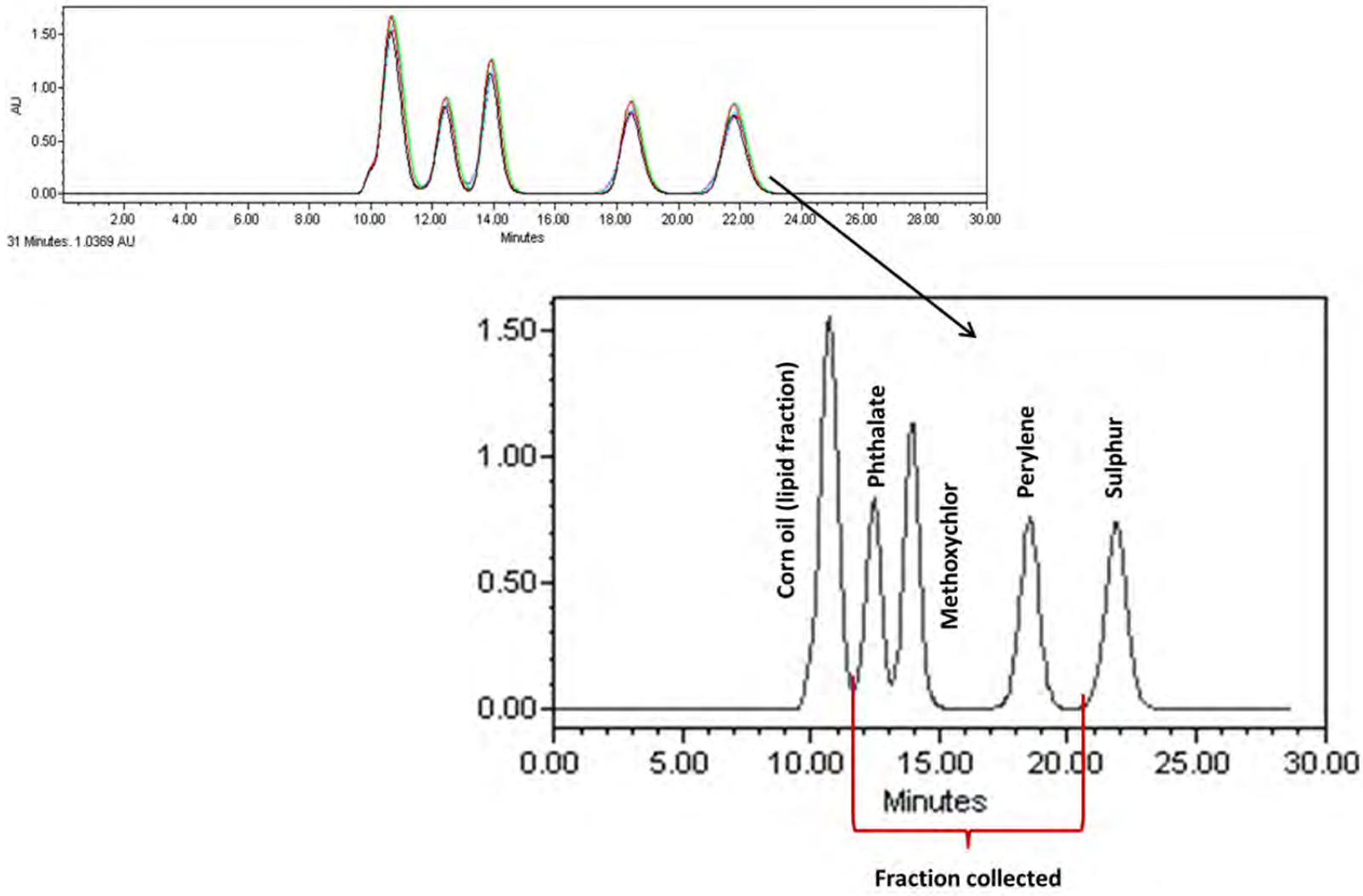


Figure 3.5: Results of calibration standards run on GPC indicating repeatability and the time fraction for collection

3.3.2.2 PowerPrep automated clean-up system

PowerPrep multi-column sample clean-up (FMS) makes use of DMS 6000 software to control the flow, volume and type of solvent at different stages during the clean-up process. The flow rates used were between 5 - 10 mℓ/min and the pressure did not exceed 172 kPa. A set of disposable, individually packed Teflon columns were used, which were manufactured at FMS and which consisted of high capacity ABN silica columns, alumina columns and PX-21 carbon columns (Refer to section 2.3; Focant & De Pauw, 2002). The flow, volume and type of solvent used and the process followed by the software are summarised in Table 3.2. After clean-up, the samples were transferred to turbo-vap flasks and evaporated using the PowerVap Concentrator from FMS at 60°C to dryness. All the elution steps were collected to evaluate possible carry-over. No carryover was detected.

Table 3.2: Summary of the program followed whilst using the PowerPrep automated clean-up system

Step	Flow (mℓ/ min)	Volume (mℓ)	Solvent	Column
1.Wet silica	10	50	Hexane	Silica
2.Flush bypass	10	10	Hexane	None
3.Wet alumina	10	30	Hexane	Alumina
4.Wet carbon	10	20	Hexane	Carbon
5.Condition silica	10	250	Hexane	Silica
6. Change to toluene	10	12	Toluene	None
7.Pre-elute	10	40	Toluene	Carbon
8.Change to 50% ethyl acetate (ETAC)/Toluene	10	12	50% ETAC/Toluene	Carbon
9.Pre-elute	10	10	50% ETAC/Toluene	Carbon
10.Change to 50% DCM:hexane	10	12	50% DCM:Hexane	None
11.Pre-elute	10	12	50% DCM:Hexane	Carbon
12.Change to hexane	10	12	Hexane	None
13.Pre-elute hexane	10	30	Hexane	Carbon
14.Add sample	5	14	None	Silica & Alumina
15.Elute silica	10	200	Hexane	Silica & Alumina
16.Change to 50% DCM:hexane	10	12	50% DCM:Hexane	None
17.Elute with 50% DCM:hexane	10	120	50% DCM:Hexane	Alumina & Carbon
18.Change to 50% ETAC/toluene	10	12	50% ETAC/Toluene	None
19.Elute with 50% ETAC/toluene	10	4	50% ETAC/Toluene	Carbon
20.Change to hexane	10	12	Hexane	None
21.Flush with hexane	10	10	Hexane	Carbon
22.Change to toluene	10	12	Toluene	None
23. Elute PCDD/Fs	5	75	Toluene	Carbon

3.3 Biological analysis of samples

3.3.1 Maintenance of the H4IIE bio-assay

Aseptic conditions were followed during routine maintenance of the cell culture. All areas, surfaces and equipment were cleaned with 70% ethanol (EtOH) prior to and after working with the cell culture. The incubator was fitted with a high efficiency particulate air (HEPA) filter that removes bacteria from air (Mitry & Hughes, 2011). The laminar flow cabinet was washed with 70% EtOH before and after use, and once a week the UV lamp was switched on for 24 hours to sterilise the interior of the laminar flow cabinet.

The H4IIE-*luc* cells were routinely maintained in tissue culture dishes (100/20 mm, LASEC) with Dulbecco's Modified Eagle's Medium (DMEM) without phenol red (Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich) and incubated in humidified air (95% air/5% CO_2) at 37°C. The cells were washed with phosphate buffered saline (PBS) when media was changed or before cells were passaged. The cells were passaged when confluent with 1.5 mℓ of trypsin (Highveld Biological) (Hilscherova *et al.*, 2001). These cells were a gift in 2004 from Prof JP Giesy, then from Michigan State University in the USA.

3.3.2 H4IIE bio-assay

The bio-assay procedure was modified from Tillit *et al.* (1991). On day one of the assay, the interior 60 wells of a sterile 96-well plate was seeded with 50 000 cells/well. The exterior 36 wells were filled with PBS to create a homogenous micro-environment (Figure 3.6). The plates were incubated for 24 h at 37°C in a 5% CO_2 incubator. Before commencing with a bio-assay the sample had to be diluted to form a dilution series to create a dose response curve (Whyte *et al*., 2004).

On day two, the cells were dosed in triplicate with 2.5 µℓ of the sample dilution series as well as a solvent control (SC) of hexane, to control for possible effects of the solvent, and blank control (BC) that consisted of cells and media only (Figure 3.6). On every second plate, a reference control standard (2,3,7,8-tetrachloro dibenzo-*p*-dioxin; TCDD) was dosed at a four times dilution series (120.00, 30.00, 7.50, 1.88, 0.47 and 0.12 pg TCDD/well). The extracts were dosed with a three times dilution series (Figure 3.6) (Hilscherova *et al*., 2003) except for the CRM extracts. Due to the high levels of toxins such as metals present in the CRM, these extracts were dosed with a four times dilution series since this extract could be cytotoxic to the H4IIE cells. The plates were incubated for 72 h after dosing at 37°C (Giesy *et al*., 1997).

On day five, the cells were visually inspected to determine if cytotoxicity occurred; the confluence of the cells was noted. The media was removed and the cells were washed with

PBS which contained Ca^{2+} and Mg^{2+}. The addition of Mg^{2+} and Ca^{2+} ensures a excess of these ions to preclude them as rate limiting factors during the light producing chemical reaction (Refer to section 2.2.1). The plates were stored at -80 °C for 30 minutes after the lysis buffer for cultured mammalian cells (Sigma-Aldrich) was added to ensure complete rupture/ lysis of the cell membranes. Thereafter, the plate was placed in a luminometer (Berthold multi mode micro plate reader, model-LB941) which automatically added 100 µℓ of the flash reagent (luciferin; 20 mM tricine, 1.07 mM $Mg(CO_3)_2Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM EDTA-disodium salt, 33.3 mM dithiothreitol, 270 µm coenzyme A, 530 µM ATP and 470 µM beetle luciferin; Villeneuve *et al*., 1999) to the wells. During the digestion of luciferin by the luciferase, luminescence was produced (Refer to section 2.2.1), expressed as relative light units (RLU). The amount of light emitted during this reaction is directly proportional to the amount of dioxin-like compounds the cells are exposed to (Nie *et al*., 2001).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Sample 1	Sample 1	Sample 1	Hexane	Sample 2	Sample 2	Sample 2	TCDD 1	TCDD 1	TCDD 1	
C		Sample 1:3	Sample 1:3	Sample 1:3	Hexane	Sample 2:3	Sample 2:3	Sample 2:3	TCDD 2	TCDD 2	TCDD 2	
D		Sample 1:9	Sample 1:9	Sample 1:9	Hexane	Sample 2:9	Sample 2:9	Sample 2:9	TCDD 3	TCDD 3	TCDD 3	
E		Sample 1:27	Sample 1:27	Sample 1:27	Blank control	Sample 2:27	Sample 2:27	Sample 2:27	TCDD 4	TCDD 4	TCDD 4	
F		Sample 1:81	Sample 1:81	Sample 1:81	Blank control	Sample 2:81	Sample 2:81	Sample 2:81	TCDD 5	TCDD 5	TCDD 5	
G		Sample 1:243	Sample 1:243	Sample 1:243	Blank control	Sample 2:243	Sample 2:243	Sample 2:243	TCDD 6	TCDD 6	TCDD 6	
H												

Figure 3.6: Layout of a 96-well plate used during H4IIE bio-assay (TCDD 1- 120 pg/well; TCDD 2 – 30 pg/well; TCDD 3 – 7.5 pg/well; TCDD 4 – 1.88 pg/well; TCDD 5 – 0.47 pg/well; TCDD 6 – 0.12 pg/well and the blue wells indicate wells with only PBS)

The triplicate RLU values measured were captured into Microsoft Excel (2010) and the mean, standard deviation and the coefficient of variation (CV) were calculated. As part of the quality assurance, the CV had to be less than 20%, since a higher CV indicated unreliable data (Whyte *et al.*, 2004). If the CV was higher, one of the RLU values was removed from the calculations. If this did not improve the CV, the assay was repeated for this sample. The percentage maximal induction (% TCDD max) relative to the reference standard was also calculated for each reference compound- and sample series.

Dose-response curves were plotted for both the reference mixture and the samples (Detailed method found in Sanderson and Giesy, 1998). For the reference plot, the logarithm of the reference mixture concentration per well was plotted on the x-axis and its corresponding percentage TCDD maximum (% TCDD max) on the y-axis. For the samples dose-response

curves, the logarithm of the volume of sample per well was plotted on the x-axis. Data points in the linear part of the curve were used to calculate the straight line equation (y = mx + c) to determine the slope, y-intercept and correlation coefficient (R^2). The amount of reference mixture needed to elucidate a 20%, 50% and 80% response from the cells, were calculated from the curves by using the corresponding x-value, providing the effective concentrations (EC). The ECs of the samples (EC_{20}, EC_{50} and EC_{80}) together with the ECs from the reference curve were used to calculate the relative potency values (REP) by dividing the $EC_{20\text{-}80\ TCDD}$ by the $EC_{20\text{-}80\ sample.}$ Thereafter the $REP_{20\text{-}80}$ was calculated for each sample. Due to unequal slopes and efficacies (maximal induction) of the responses, multiple point estimates ($REP_{20,\ 50\ \&\ 80}$) were used to characterise the responses and slopes (Villeneuve *et al.*, 2000).The REP_{20} was divided by the mass of the sample which was divided by 250 µℓ in which the sample was reconstituted to get the final value of ng/g BEQ value. The LOD for the H4IIE bio-assay was calculated by determining the average of EC_0 for all the 2,3,7,8-TCDD dose response curves. The EC_0 is seen as the point where TCDD no longer induces the Ah-receptor. The 95% confidence interval was added to the average for an ngTCDD/g LOD value (Thomsen *et al.,* 2003; Nieuwoudt *et al*., 2009).

3.3.3 Viability assay (MTT)

A viability assay was run parallel to the reporter gene assay, dosed according to a similar regime, to determine if the samples contained cytotoxic compounds. This was done in order to prevent false negatives, where low or LOD responses in the H4IIE assay might not necessarily be due to the presence or absence of agonists, but rather to cell toxicity. The single difference in the dosing regime was the addition of a series of wells containing cells and media that would be killed at the end of the experiment to have a control for wells with 100% cell mortality, i.e. to have a negative control. The wells that received hexane only served as the positive control, i.e. representing 100% viable cells.

In this study, the MTT (hydrogen acceptor 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) assay was used as the viability test. The yellow MTT solution is metabolized by the living cells, forming blue formazan – the viability of the cells can be determined by quantifying the formazan production spectrophotometrically (Vistica *et al*, 1991).

On day five, the MTT plates were rinsed using the same procedure as described in section 3.3.2. However, the cells used in the MTT test did not receive lysis buffer. The wells received 100 µℓ of MTT solution (0.5 mg/mℓ MTT in un-supplemented media) after which the plates were incubated at 37ºC for 30 minutes with 5% CO_2. After incubation, the cells were visually inspected to determine if intracellular formazan crystals had formed. If the crystals were present in the cells, the yellow MTT solution was discarded by inverting the plates and gently tapping

the solution out. The formed crystals were dissolved by adding 200 µℓ of dimethyl sulphoxide (DMSO) (Sigma-Aldrich) to the wells. The plates were left at room temperature for 15 min, before the content of the wells were mixed by repeat pipetting and the absorbance at 560 nm was measured (Berthold multi-mode micro plate reader) (Vistica *et al.*, 1991).

The absorbance of the methanol killed cells was subtracted from all other wells to account for the absorbance caused by dead cells that may be present. The resultant absorbance of the wells containing sample extracts or reference standard was expressed as % viability of the absorbance of the solvent control. If the percentage viability was below 80%, the extract was considered to have a cytotoxic effect where cytotoxicity could have affected the assay results.

3.4 Chemical analysis

The Pegasus 4D GCxGC-TOFMS (LECO Africa) was used to characterize and analyse the sample extracts. The system consists of a 7890 Agilent injector (split/splitless) connected to an Agilent 7890 GC with a secondary oven and a dual stage modulator. The liquid nitrogen (LN_2) was maintained by an AMI Model 186 liquid level controller, where the LN_2 was used to cool the nitrogen for the cold jets, and synthetic air was used for the hot jets. An uncoated 10 m silica retention column was placed in front of the analytical columns, which consisted of an Rxi-XLB and RTX-200 (Restek). The retention column was due to larger volume injection (2.5 uℓ), and helped with the refocusing of analytes on the analytical column. The columns were connected with a press-tight connector and the carrier gas used was chromatographic grade helium (99.99%) from Air Products. The system was tuned on a 69 ion from the conventional perfluorotributylamine (PFTBA) mass calibrant. The LECO Chroma-TOF software (Version 4.4) was used in data processing together with the NIST and custom made libraries. The parameters used for the chosen column set are summarised in Table 3.2 - 3.5. Previous studies indicated successful separation of dioxin-like compounds when using the Rxi-XLB (excellent efficiency, inertness, low bleed and high thermal stability) column in the first dimension coupled to a mid-polarity phase Rtx-200 in the second dimension (de Vos *et al.*, 2011). The LOD and LOQ were calculated from the reference standard for the 17 PCDD/Fs in this study. LOD= x-variable + (3 x standard error) and LOQ = x variable + (10 x standard error) (Armbruster *et al.,* 1994).

Table 3.2: GCxGC parameters used in the study

Column set	First dimension column: Rxi-XLB (30 m x 0.25 mm internal diameter (id) x 0.25 µm film thickness (df) Second dimension column: Rtx-200 (2.0 m x 0.18 mm id x 0.25 µm df)	**Inlet temperature**	250°C
Carrier gas	Helium	**Transfer line temperature**	270°C

Table 3.3: Temperature program used

Primary oven				Secondary oven			
#	Rate (°C/min)	Target temperature (°C)	Duration (min)	#	Rate (°C/min)	Target temperature (°C)	Duration (min)
1	-	110	8	**1**	-	130	8
2	10	230	0	**2**	10	250	0
3	1	300	5	**3**	1	320	5

Table 3.4: Modulator timing

#	Start	End	Modulation period (s)	Hot pulse time	Cool time
1	Start of run	End of run	5	1.20	1.30

Table 3.5: Mass spectrometer parameters

Detector voltage (V)	1850	**Acquisition rate (spectra/second)**	100
Electron energy (mu/100u)	-80	**Ion source**	250 °C

3.5 Statistical analysis

Statistical analysis was done using Microsoft Excel and Statistica (Version 10). The normality of the data was determined using the Lilliefors test, which uses the mean and variance from the data set (Razali & Wah, 2011) to determine distribution. Thereafter, parametric and non-parametric tests were used depending on the normality of the data set. Non-parametric statistics used included the Kruskal Wallis ANOVA that compares the medians of three or more different samples (Fowler *et al.*, 1998) whilst parametric regression analysis was used to estimate the relationship between variables for the different clean-up methods. A p value smaller than 0.05 was considered to be statistically significant.

4
Results and discussion

As previously described, four methods for the extraction and clean-up of dioxins in soil and sediment were evaluated for use, specifically in the H4IIE bio-assay. The methods evaluated were: (1) ASE and Soxhlet extraction in combination with a manual clean-up method consisting of an acid digestion, GPC and SPE using dual layer silica, (2) PLE with an automated clean-up system using multi-layer silica, alumina, and carbon columns, and (3) ASE and Soxhlet extraction with no clean-up steps. The abbreviations used to describe these methods throughout the rest of the chapter are summarised are Table 4.1.

Table 4.1: Abbreviations for the different extraction and clean-up methods

Method	Abbreviation
ASE with manual clean-up (acid digestion, GPC and SPE)	AGF
Soxhlet with manual clean-up (acid digestion, GPC and SPE)	SOX
PLE with automated clean-up (silica, alumina and carbon columns)	TRP
ASE with no clean-up	NC ASE
Soxhlet with no clean-up	NC SOX

The following results and discussion that compare the four different methods that were evaluated during this study are presented in two main sections. In section 4.1, the data from the H4IIE bio-assay is discussed, followed by the instrumental analysis results in section 4.2. The BEQ_{20} of the assay results was used, as the samples did not elicit a response above 50% for all samples.

4.1 Results of biological analysis

4.1.1 MTT viability assay results

The MTT viability assay was used to determine if the compounds with which the cells were dosed had led to reduced cell viability. The viability of cells was assessed spectrophotometrically by their ability to metabolise MTT to purple formazan (Vistica *et al.,* 1991*;* ATCC, 2011). When high viability was observed for the same sample and low luminescence was found in the H4IIE bio-assay, the response from the cell was attributed to low levels of the Ah-ligands and not to dead or dying cells. Absorbance values lower than the values of control cells indicate impeded viability, while higher absorbance levels compared to controls indicated an increase in cell proliferation (ATCC, 2011). For this study, values below 80% were

considered sufficiently significant to influence bio-assay results. There was only one sample NC SOX Cal 2 (Table 4.2) that was cytotoxic, but the reasons for this observation are not known. Due to a limited amount of CRM, no CRM was evaluated for NC SOX.

Table 4.2: Summary of the MTT viability assay results for the various extracts, expressed as a percentage of the extract exposed cells viability, against those of the control cells.

	Cell viability (%)				
Sample	**AGF**	**SOX**	**TRP**	**NC ASE**	**NC SOX**
Cal 1	87	108	120	92	104
Cal 2	85	94	92	88	29
Cal 3	112	92	99	94	178
Cal 4	118	142	95	91	133
Cal 5	100	99	100	87	74
Cal 6	96	94	86	98	156
Cal 7	142	126	117	110	106
Cal 8	89	102	156	117	123
Blank	65	100	100	85	112
CRM	97	102	100	96	-
Sample 1	116	108	89	100	79
Sample 2	103	104	105	95	115

Cal: Spiked calibration curve samples
Sample 1 and 2: Soil samples from Vaal Triangle

The wells containing soil samples and CRMs that underwent no clean-up (NC ASE & NC SOX) were of a yellow colour and showed signs of fungal growth. Microscopic investigation revealed no effect on the overall well confluence; however, the pure extract RLU's were lower than the first dilution which indicated an anomaly in cell growth although this was not quantifiable by the MTT assay. The yellow colour of the extracts could indicate the presence of sulphur, which would be toxic to the H4IIE cells (Hilscherova *et al.*, 2001; Hurst *et al*., 2004).

It is well known that sulphur can occur at high concentration in the sediments of lakes and rivers (Puacz *et al*., 2001; Seidel *et al.*, 2006). Due to the toxicity of sulphur to cells, a few techniques have been proposed to remove sulphur – including a copper wash, treatment with silver nitrate or fractionation with GPC. Sanctorum *et al.* (2007) reported a decrease in cell response because of copper treatment due to formation of toxic copper adducts in the extract. A more promising method for elemental sulphur removal is the use of silver nitrate (Hurst *et al*., 2004; Sanctorum *et al*., 2007). An added advantage to the use of silver nitrate is that this step does not need to be performed separately; it can be combined with the silica clean-up or added during extraction (Figure 3.1 and 3.2). For the current study, GPC was selected to remove sulphur from the samples. The GPC removes lipids and sulphur in a single clean-up, which means less handling of the sample (Oleszek-Kudlak *et al.,* 2007, Brennan *et al*., 2009). No fungal growth or yellow colouration was observed with samples that underwent clean-up. There was no statistically significant difference in cell viability among various clean-up procedures (Kruskal-Wallis Anova, $p > 0.05$; Figure 4.1).

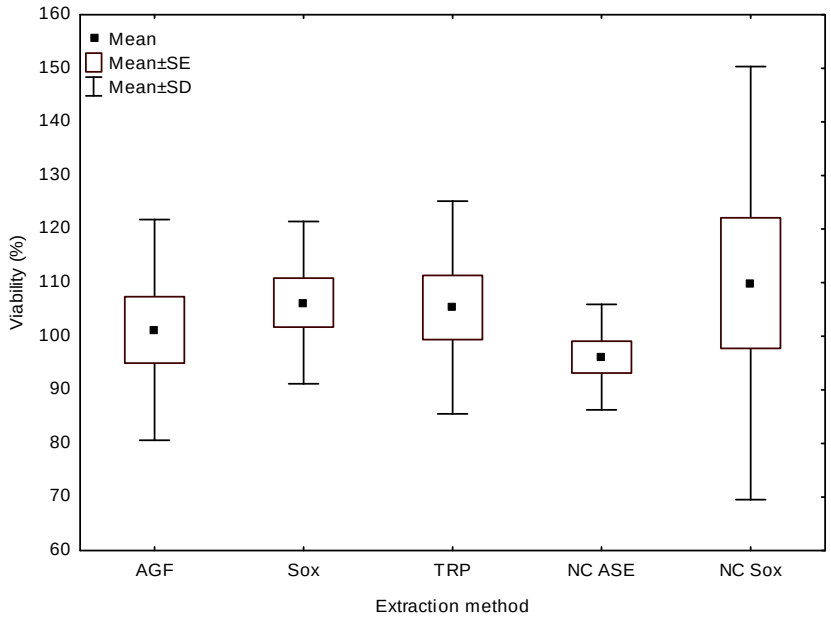


Figure 4.1: Kruskal-Wallis box plot of cell viability for the various extraction and clean-up techniques

4.1.2 H4IIE bio-assay results

4.1.2.1 Quality control and assurance

Quality control (QC) and assurance (QA) are of great importance as they ensure meaningful and scientifically correct data. QC and QA evaluate accuracy and precision of the method (Barnett, 1969). QC and QA methods were included in this study.

Method blanks were included to assess sample contamination from the extraction and clean-up procedures (US EPA, 2009) and were added to the sample set for each method tested. The method blank was the same pre-cleaned sand used in the matrix matched calibrations (AGF, SOX and TRP Cal 1 - 8). The method blank had an additional purpose, namely to assess the dioxin and PAH content of the pre-cleaned sand used in the calibrations. All the method blanks were below the LOD (0.03 ng BEQ/g), indicating that sample treatments as well as the pre-cleaned sand did not impact the H4IIE bio-assay results.

1. Quality control in the H4IIE bio-assay procedure

Quality control of the H4IIE assay included the use of solvent and blank controls, testing samples and standards in triplicate (Koh *et al*., 2004). In addition, the results had to fall within set limits for the coefficient of variation (CV) between repeats.

The solvent control determined if the dosing solvent contributes to the cell response, as these solvents (such as n-hexane) are often toxic to mammalian cells (Liu *et al*., 2012; Zapór *et al*.,

2002). The suggested method that one should use to correct for solvent control effects is to subtract the mean of the solvent control RLUs from the standard and sample RLUs (Besselink *et al*., 2004). However, this correction was not applied in this study since negative values where obtained. A blank control was included on each plate to ensure the cells dosed were viable and confluent without any solvent or sample interference (Windal *et al.,* 2005).

Dosing was performed in triplicate to evaluate cell response repeatability (Besselink *et al*., 2004; Windal *et al*., 2005). As part of the quality assurance, the CV of wells dosed in triplicate had to be less than 20%, because a higher CV indicates unreliable data (Whyte *et al.*, 2004), with low repeatability. If the CV was higher, one of the RLU values was removed from the calculations. If the CV was still above the 20% threshold, the assay for that sample was repeated.

The reference standard with known concentrations was also dosed in every second 96 well plate during the bio-assay.

During the evaluation of quality control procedures, a problem was identified within the H4IIE bio-assay results. The selected concentration range for the matrix matched calibration curve results (AGF, TRP and SOX Cal 1 - 8) was expected to elicit a response above the LOD (0.03 ng/g) in 30 of the 40 samples analysed. However, only 15 of the expected 30 samples had detectable dioxins levels.

To ensure the validity of the concentration range selected, the spiking solution (Matrix spiking solution, Method 8290) was analysed. For the pure analytical standard, Cal 1 – 5 elicited a measureable response, and therefore 25 of the 40 matrix matched calibration standards should have been above the LOD, assuming 100% recovery. To determine if the low levels of response could be ascribed to the reference standard (2,3,7,8-TCDD) or to poor recovery, the dose response curve obtained from the original spiking solution was used to semi-quantify the rest of the extracts. The outcome indicated 26 of the 40 matrix matched calibration curve correlating well with the expected results. The difference between the 2,3,7 8-TCDD as reference standard, compared to the original spiking solution, needs further investigation. A possible explanation for this finding is that the concentration of the 2,3,7,8-TCDD reference standard might have increased due to solvent evaporation during prolonged use of the same standard. The problem may have been prevented by preparing a new standard series on the day of dosing (Hilscherova *et al*., 2003).

The spiking solution referred to as the dioxin mixed standard was selected to semi-quantify the various extracts. This decision was based not only on the uncertainty of the TCDD reference standard's true concentration, but also on the nature of most of the environmental extracts.

Except for the two soil samples and the CRM, the Cal samples were created using the dioxin mixed standard. In light of the aims of this study — namely, to compare extraction and clean-up efficiencies using the H4IIE assay — it would be best to use a reference that is as close to the extracts' content as possible. In the case of the two sediment samples with the unknown dioxin composition, using a dioxin mixed reference is more environmentally relevant, since dioxins usually occur as a mixture of congeners rather than as a single compound (Behnisch *et al.*, 2001b). Although the specific dioxin congener composition of the sediment samples might be different to that of the dioxin mixed reference, for the purposes of this study, the same reference was used for all of the extracts. The use of a mixed standard can therefore account for additive, synergistic, and antagonistic effects that could occur on the cellular level when more than one compound is present in the sample extract (Roa & Unger, 1995; Behnisch *et al*., 2001b & Matsu *et al*., 2003).

Table 4.3 indicates the BEQ values obtained during the bio-assay with 2,3,7,8 -TCDD standard and the dioxin mixed standard used to semi-quantify the results from the H4IIE bio-assay. The mixed standard samples showed a much higher response which can probably be ascribed to synergistic interactions between chemicals and compounds present in environmental samples (Behnisch *et al*., 2001b). The chemical compounds present in the samples were investigated by means of instrumental analysis; the findings of these are discussed in section 4.2.3.

Table 4.3: Result from the H4IIE bio-assay using 2,3,7,8- TCDD (TS, ng TCDD-eq/g) and dioxin mixed standard (MS, ng dioxin-eq/g)

Sample	Expected concentration (ng/g)	AGF		TRP		SOX		NC ASE		NC SOX	
		TS	MS	TS	MS	TS	MS	TS	MS	TS	MS
Cal 1	3.762	0.11	13	0.55	91	0.05	25	0.63	307	0.16	22
Cal 2	3.261	0.06	11	0.09	26	0.11	29	0.37	220	0.85	35
Cal 3	1.022	<LOD	7.1	<LOD	10	0.05	25	0.40	28	0.12	1.5
Cal 4	0.461	<LOD	3.6	<LOD	6.8	<LOD	<LOD	0.19	76	<LOD	14
Cal 5	0.073	<LOD	<LOD	<LOD	1.1	<LOD	<LOD	<LOD	3.2	<LOQ	2.4
Cal 6	0.017	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.5	<LOD	2.6
Cal 7	0.004	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.2	<LOD	1.9
Cal 8	0.001	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
CRM	0.244	0.22	44	0.10	16	0.84	175	7.12	2242	NA	NA
Sample 1	Unknown	22	111	0.02	9.6	18	144.	535	619	0.09	177
Sample 2	Unknown	0.08	5.02	LOQ	2.1	0.06	1.9	8.6	19	0.19	98
Blank	Unknown	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

LOD – response below 20% TCDD or dioxin max, and/or below LOD of assay (0.032 ng/g)
LOQ – single response above 20% TCDD or dioxin max, not quantifiable

4.1.2.2 Comparing bio-assay results across the different preparatory methods

Sample preparation has a bearing on analysis because it interferes with the identification, confirmation and quantification of analytes (Smith, 2003; Chen *et al*., 2008). Due to the ultra-trace levels of dioxins in samples, this study investigated refining sample preparatory protocols in an attempt to ensure that only dioxins were present in the extracts. Two methods that do not include clean-up were selected for comparing the extraction techniques, PLE and Soxhlet, and three preparatory methods: AGF, TRP, and SOX were evaluated during the study (Table 4.1).

a) Comparison of the matrix matched calibration curve results

A matrix matched calibration curve takes into consideration the possible analyte interactions with the matrix (Barwick, 2003). A calibration curve is defined as a mathematical function, which indicates the relationship between values derived from a measuring instrument and the known values of a reference standard (Danzer & Currie, 1998; Barwick, 2003). A matrix matched calibration curve was used in this study to evaluate the different methods.

In an ideal situation, the response from the bio-assay should be the same for the two methods where no clean-up was used, and it should also be similar across the three methods where clean-up protocols were used. Since the matrix matched calibration was spiked using the same concentration range into the same matrix, it was expected to find the same results as those obtained for the methods where no clean-up was employed. In addition, one would expect the same answer from the three protocols where clean-up was employed (Refer to section 2.3).

To evaluate and compare the matrix matched calibration curve of the extraction and clean-up methods, a multiple regression analysis was performed. Since some of the points in the calibration range fell in the LOD range, only Cal 1 - 5 were selected for evaluation. The regression would indicate whether the linear relationship between the different methods tested was equivalent, because a regression analysis is used to define the relationship between x and y values (Fowler *et al*., 1998).

In this study, a regression analysis was used to define the linear relationship between the BEQ_{20} results found and the concentrations of the calibration curve. The linearity was determined by the coefficient of correlation (R^2; Figure 4.2 a, b). AGF samples had a R^2 value of 0.90, indicating a statistically significant linear relationship between the concentration and BEQ_{20} values ($p = 0.01$). Similar results can be seen for TRP ($R^2 = 0.90$, $p = 0.02$) and NC ASE ($R^2 = 0.92$, $p = 0.02$), thus indicating a linear relationship between

H4IIE bio-assay response and the concentration. For the SOX and the NC SOX samples, there a poor linear relationship (SOX R^2 = 0.50, p = 0.19 and NC SOX R^2 = 0.42, p = 0.23) was found. This could be ascribed to loss of the analytes of interest, and as such, this finding needs further investigation.

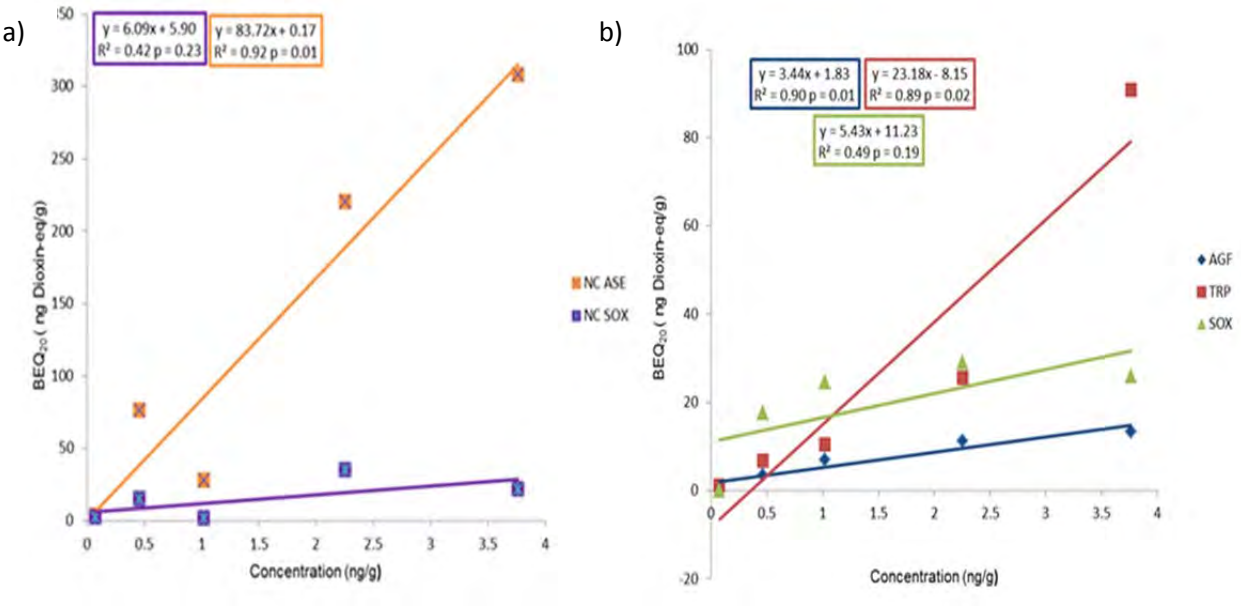


Figure 4.2: Regression analysis for each of the extraction methods used, plotting concentration against BEQ values (a: NC ASE and NC SOX; b: Clean-up methods AGF, TRP and SOX)

A linear response for the bio-assay is dependent on the linearity of the luminometer (Besselink *et al*., 2004). As an extra quality control measure, a concentration range of luciferase can be prepared and the activity measured with a view to ensure there is a linear response between the concentration of luciferase, and the amount of light emitted (Besselink *et al*., 2004). However, since a linear response was obtained from the standard curve, the linearity of the luminometer can be conferred.

The results from the regression indicate that the NC ASE and the TRP had the highest BEQ values compared to the other methods. The AGF, SOX, and NC SOX had similar values. Comparing the two no clean-up methods (NC SOX and NC ASE), different responses were found in the assay – the NC ASE had higher levels than the NC SOX (Figure 4.2 a). In literature, ASE and Soxhlet extraction are seen as equivalent (Bautz *et al*., 1998; Richter *et al*., 1997; Hubert *et al*., 2000). However, this was not the case in the current study; this finding could be ascribed to analyte loss during transfer steps. This situation was investigated further during instrumental analysis and will be discussed in section 4.2.

b) Non-parametric analysis of the CRMs and soil samples to evaluate the clean-up methods (AGF, TRP, and SOX)

A manual and an automated clean-up process and findings were compared in this study. The manual procedure is a time-consuming process which makes use of large volumes of solvent and requires sample manipulation between the different steps. These challenges gave rise to the development of automated sample clean-up procedures (Bautz *et al*., 1998; Focant *et al*., 2004). The manual and automated clean-up were evaluated to determine if the procedures are indeed as comparable as stated in the literature (Abad *et al*., 2000; Focant *et al*., 2004).

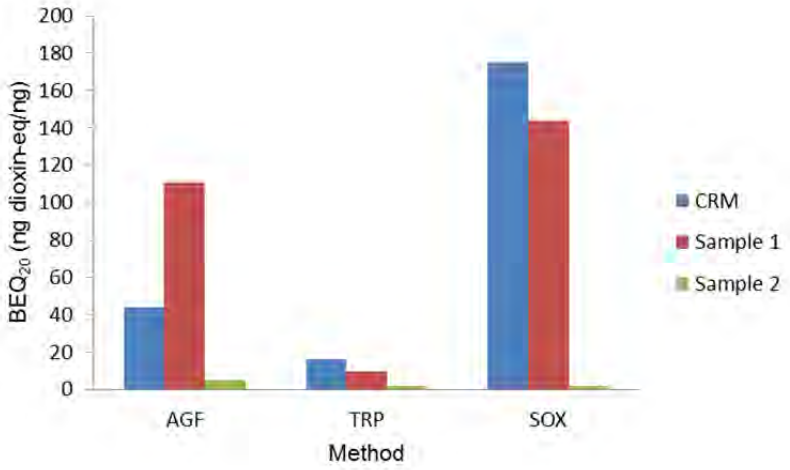


Figure 4.3: The BEQ_{20} values for the real soil samples and CRMs for the extraction methods with a clean-up procedure

From Figure 4.3 it is apparent that the manual and automated clean-up are not, in fact, comparable. The two manual clean-up methods (AGF and SOX) indicated a greater response than the TRP method. The same clean-up methods were used for AGF and SOX samples, indicating a possible loss from one step to the next or inadequate removal of interfering compounds. The H4IIE bio-assay cannot identify compounds in the extract to which the cells respond, and this is addressed by the instrumental analysis (section 4.2), which explains the different responses found for the different methods.

The data was not distributed normally as was indicated by the Lilliefors normality test (Refer to section 3.5), ($p < 0.05$; Razali & Wah, 2011), and therefore, non-parametric test statistics were used. To evaluate the clean-up methods, a Kruskal Wallis ANOVA was performed using the CRMs and soil samples, and the results are expressed in box plots (Figure 4.4). The Kruskal Wallis ANOVA indicated that there was no statistically significant difference in the H4IIE bio-assay results when using different clean-up methods.

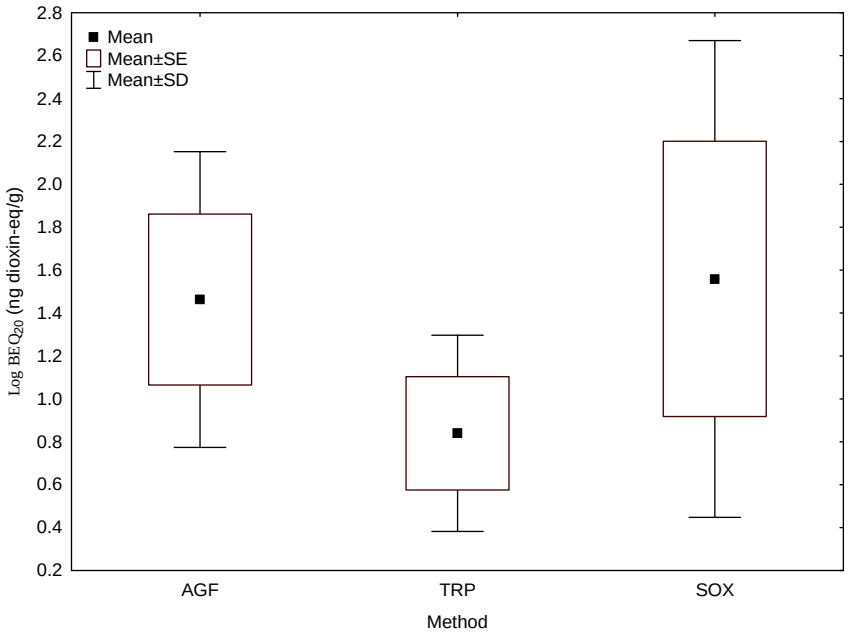


Figure 4.4: Kruskal Wallis box plot of the soil and CRMs extracts generated through the AGF, TRP, and SOX methods

The H4IIE-bio-assay can only be used as a screening tool, and cannot identify the compounds present in the extract which elicit the response. Therefore, the assay has to be used in combination with instrumental analysis (Behnisch *et al*., 2001a; Hilscherova *et al*., 2003*;* Hui *et al*., 2007). For this study, a GCxGC-TOFMS was used to evaluate the extracts, and also to identify those compounds which elicited a response from the H4IIE bio-assay.

4.2 Results of instrumental analysis results

The instrumental analysis results were obtained from a GCxGC-TOFMS. The analytical columns used were Rxi-XLB and Rtx-200. The Rxi-XLB has a low polarity column phase coupled to the mid-polarity phase Rtx-200 in the second dimension leading to effective separation of the analytes of interest.

4.2.1 Separation of the 17 PCDD/Fs on the GCxGC-TOFMS

Separation of the 17 PCDD/Fs (Figure 4.5) was obtained with the column set and parameters shown in section 3.5, and the LOQ/LOD for the individual congeners on the GCxGC-TOFMS are listed in Table 4.4. The LECO ChromaTOF software was used for data processing, although all peaks were manually reviewed. A library search was conducted using a custom library generated during a previous study (de Vos *et al*., 2011), as well as the NIST MS search (version 2) to indicate compounds of interest. The total ion chromatogram (TIC) of a chromatogram may have too many interfering compounds, making it difficult to detect target compounds. Therefore, a single ion or an extracted ion can be used to identify

the target analytes (McMaster, 2008). Extracted ions used for the dioxins were 306, 322, 340, 356, 374, 390, 460, 410, 424, 460, and 442.

Table 4.4: LOD and LOQ for the GCxGC-TOFMS for 17 PCDD/Fs

Compound	LOD (ng/mℓ)	LOQ (ng/mℓ)
2,3,7,8-TCDF	4.12	1.65
2,3,7,8-TCDD	4.65	1.86
1,2,3,7,8-PCDF	5.55	2.22
2,3,4,7,8-PCDF	4.97	1.99
1,2,3,7,8-PCDD	5.50	2.20
1,2,3,4,7,8-HxCDF	7.52	3.01
2,3,4,6,7,8-HxCDF	6.32	2.53
1,2,3,6,7,8-HxCDF	5.78	2.31
1,2,3,4,7,8-HxCDD	5.56	2.22
1,2,3,6,7,8-HxCDD	5.28	2.11
1,2,3,7,8,9-HxCDD	5.68	2.27
1,2,3,7,8,9-HxCDF	7.01	2.80
1,2,3,4,6,7,8-HpCDF	5.62	2.25
1,2,3,4,6,7,8-HpCDD	4.93	1.97
1,2,3,4,7,8,9-HpCDF	6.02	2.41
OCDD	5.75	2.30
OCDF	5.65	2.26

The H4IIE bio-assay responds to compounds which bind to the AhR, and ^{13}C-labelled analogs of the analytes will respond in the same way as the dioxins in the sample (Hilscherova *et al*., 2003), therefore ^{13}C-labels were not added prior to extraction. The samples were evaluated by the GCxGC-TOFMS and if PCDD/Fs were present, ^{13}C-labelled analogues could be added to quantify the results (Hilscherova *et al*., 2003). However, this was not done, because due to the low concentrations of target analytes in the samples, the dilution effect of adding ^{13}C-labels would have negatively impacted the detection of the compounds. Thus, the GCxGC-TOFMS was used only for identification of the compounds in the samples.

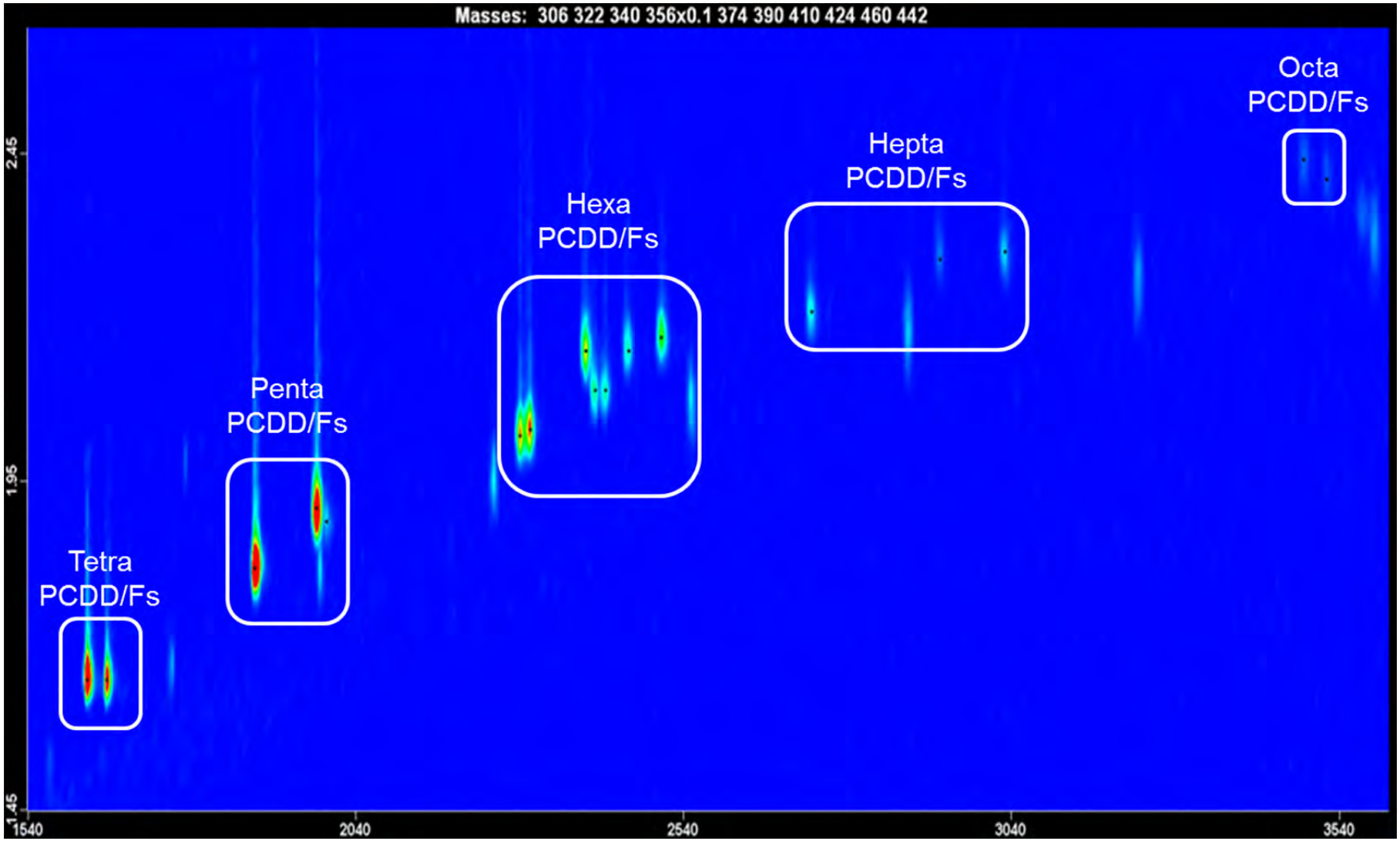


Figure 4.5: Mass extracted GCxGC-TOFMS contour plot, displaying the separation of the 17 toxic PCDD/F congeners (150 ng/mℓ, 2.5µℓ injection)

All the sample extracts were run on the GCxGC-TOFMS, except for those samples which had not undergone any clean-up. These extracts would contain significant interferences, and could cause analytical problems such as severe wrap-around and filament saturation (McMaster, 2008) as well as increased pressure that could lead to system leaks. The increase in matrix compound present would also cause increased system maintenance requirements such as replacement of the gold seal, liner, septa, and after multiple injections, also the syringe (McMaster, 2008; Scott, 2012). H4IIE bio-assay results could be directly compared to the GCxGC-TOFMS results, because the same volume of sample, namely 2.5 µℓ, was used in both methods.

When the analytical standard used to spike the matrix matched calibration was run on the system, points one to three (150 – 40 ng/mℓ) were above the LOD of the GCxGC-TOFMS. In the presence of matrix, only the first point (150 ng/mℓ) of the spiked extracted matrix matched calibration curve samples was above the LOD and it became increasingly more difficult to confirm peak identification of the lower concentrations (Figure 4.6), even with the power of two dimensional separation. Therefore, this study could not confirm whether the compounds were lost during the extraction procedure or if they were at levels within the noise of the chromatogram (Figure 4.6). Additionally, no dl-PCBs were found in the samples or CRM, indicating either loss of the analytes or masking of the analytes due to matrix interference.

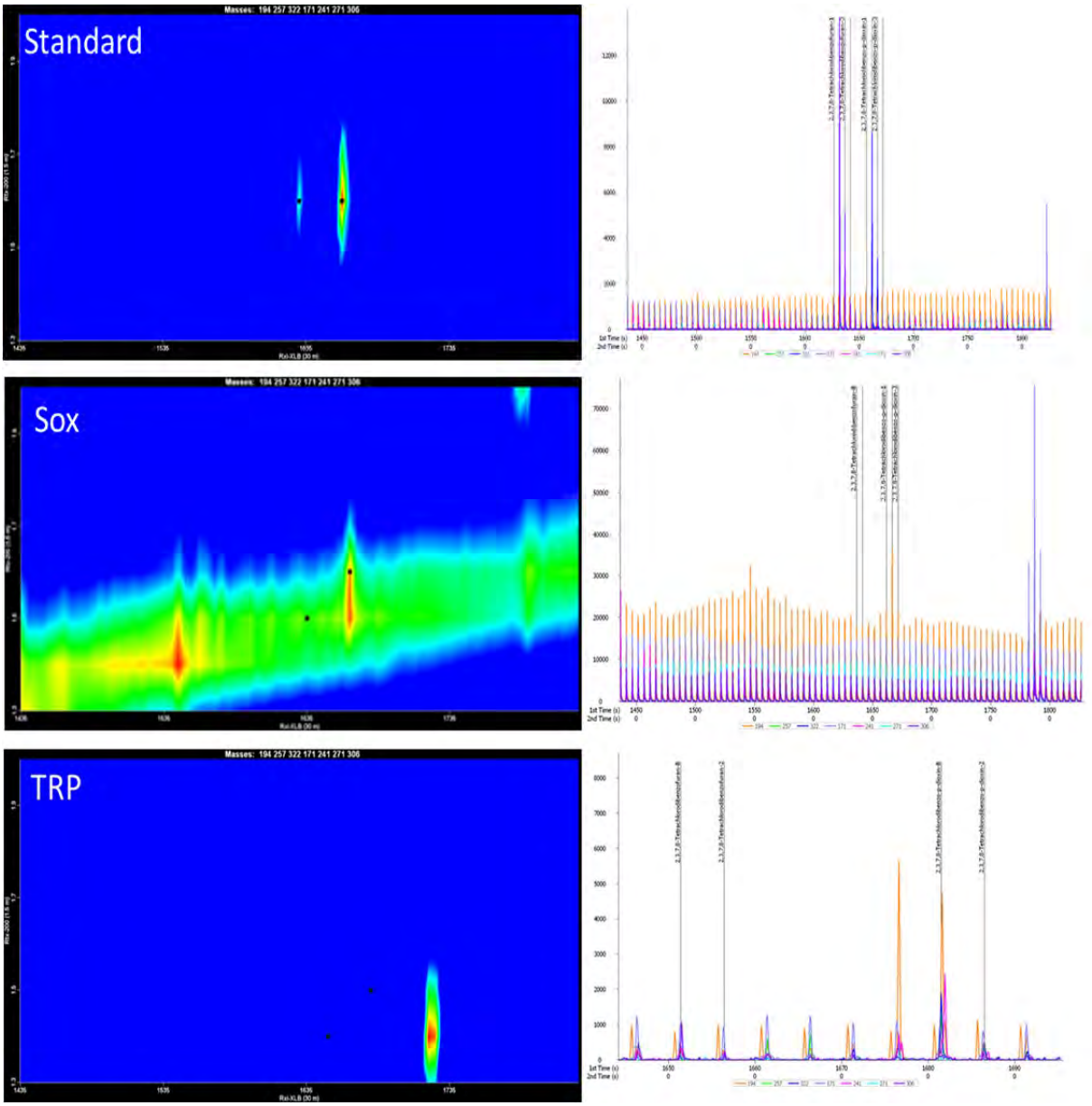


Figure 4.6: Extracted mass contour plot of 2,3,7,8-TCDD and 2,3,7,8-TCDF in standard (150 ng/mℓ), a SOX and TRP extraction (150 ng/mℓ) indicating the effect of matrix interference on analyte identification.

Upon closer inspection, PAHs were found in the analytical standards used for spiking as well as in the method blanks. However, the response from the H4IIE bio-assay was below the LOD, which may indicate that carry-over was present in the GCxGC-TOFMS analysis.

Although the extraction methods were selected in an attempt to ensure that only dioxins would be present in the sample, it became apparent after chemical analysis that the high response found in the H4IIE bio-assay was due to PAHs (section 4.3). Using the CRM values for the PAHs, the TEF approach was followed to determine if the bio-assay results can be ascribed only to the PAHs (section 4.2.3).

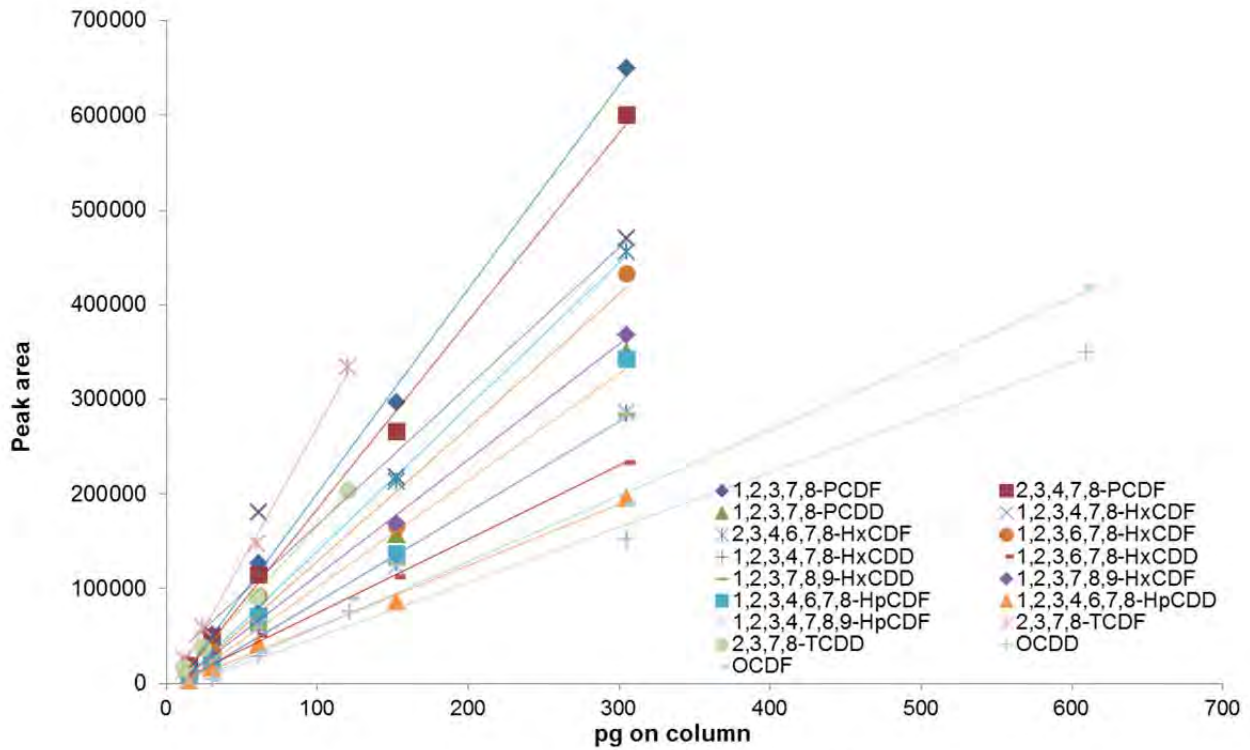
In order to properly understand the intensity of the peak reference, the signal-to-noise ratio (S/N) and peak area are briefly discussed. A signal-to-noise ratio is used to define the sensitivity of the detector for the minimum concentration that can be determined. Peaks become concealed if they are in the same range as the noise of the detector or the sample interferences. Therefore, the signal of the peak must be greater than the noise to allow for positive identification. Usually, the ratio of the signal size to that of the noise must be three times greater that of the noise to unambiguously identify the peak (Scott, 2012). The lower the S/N ratio, the greater the uncertainty associated with peak identification (Danielsson *et al*., 2002; Scott, 2012).

In general, when working within the linear range, the peak area is proportional to the amount of the compound present in the samples. However, this is only true for a linear relationship, and linearity must be confirmed before any deductions can be made. By fitting a straight line through the data and determining how good the fit is, the presence of a linear relationship is observed (as was discussed in section 2.4). The standard concentration range was run on the GCxGC-TOFMS, demonstrating an acceptable linear relationship for all 17 of the toxic PCDD/Fs ($R^2 > 0.9$) (Figure 4.7).

When the neat standard (used to spike the matrix matched calibration) was injected (three repeats) on the GCxGC-TOFMS, only points one to three (150 – 40 ng/mℓ) were detectable on the GCxGC-TOFMS. For the bio-assay, points one to five were detectable, confirming that the assay is more sensitive than chemical analysis. However, it must be noted that chemical analysis measures a single compound, whereas the bio-assay measures total toxic effect of the extract (Hilscherova *et al*., 2001). The peak area and S/N of the 17 PCDD/Fs found in Cal 1, 2 and 3 is found in supplementary data, whilst the range is indicated in table 4.5.

Table 4.5: Peak area and S/N of neat standard injected for Cal 1 - 3

Points on calibration curve	Peak area range	S/N
Cal 1 (150 ng/mℓ)	19 112 – 72 363	635 – 125
Cal 2 (80 ng/mℓ)	43 961 – 10 754	372 – 69
Cal 3 (40 ng/mℓ)	14 639 – 19 285	147 – 38



Compound	Linear equation	R^2	Compound	Linear equation	R^2	Compound	Linear equation	R^2
2,3,7,8-TCDF	y = 2837.3x - 11635	0.9969	2,3,4,6,7,8-HxCDF	y = 1525x - 12468	0.9994	1,2,3,4,6,7,8-HpCDF	y = 1130.2x - 11465	0.9883
2,3,7,8-TCDD	y = 1724.3x - 5916.4	0.998	1,2,3,6,7,8-HxCDF	y = 1409.8x - 11874	0.9835	1,2,3,4,6,7,8-HpCDD	y = 648.72x - 4965.4	0.9949
1,2,3,7,8-PCDF	y = 2157.7x - 14958	0.9983	1,2,3,7,8,9-HxCDF	y = 1224.3x - 8581.9	0.9982	1,2,3,4,7,8,9-HpCDF	y = 950.04x - 8603.2	0.9945
2,3,4,7,8-PCDF	y = 1986.1x - 14535	0.997	1,2,3,4,7,8-HxCDD	y = 953.67x - 9561.8	0.9972	OCDD	y = 579.41x - 9006.9	0.994
1,2,3,7,8-PCDD	y = 1158.4x - 8287.5	0.9976	1,2,3,6,7,8-HxCDD	y = 779.12x - 3907.4	0.997	OCDF	y = 696x - 10330	0.9959
1,2,3,4,7,8-HxCDF	y = 1460.9x + 21696	0.9473	1,2,3,7,8,9-HxCDD	y = 940.7x - 6803.1	0.9963			

Figure 4.7: Analysis of the linearity of the peak area response using varying concentrations of PCDD/Fs

For the extracted calibration curve, only the data from Cal 1 was used. The majority of the 17 PCDD/Fs were found in Cal 1. However, there was a low S/N and small peak area, indicating possible loss during extraction. The GCxGC-TOFMS analysis of the PCDD/Fs did not explain why the BEQ values of the assay were higher than the expected concentration (Table 4.3). This is discussed in section 4.2.3.

Only the TRP extract of Cal 1 contained all 17 PCDD/Fs of interest, while the AGF Cal 1 extract contained 12, and SOX Cal 1 extract 11 (supplementary data). The peak area and S/N of the extracted Cal 1 for AGF, TRP, and SOX are summarised in table 4.6. When comparing the peak area and S/N of Cal 1 (neat standard) in table 4.5 and table 4.6, it is evident that the peak area and S/N is much lower in the extracted Cal 1 for all the methods (AGF, TRP and SOX), thus indicating losses of the 17 PCDD/Fs. It is possible that there were too many transfer steps in the manual clean-up method (AGF & SOX), which were minimized in the automated clean-up. However, this explanation does not address the losses of PCDD/Fs using the TRP method.

Table 4.6: Peak area and S/N of extracted Cal 1 for AGF, TRP, and SOX

Method extracted Cal 1	Peak area range	S/N
TRP	18 216 – 1 335	129 – 15
AGF	31 753 – 2 660	196 – 8
SOX	5 113 – 558	71 – 13

The methods followed were scrutinised while consulting relevant literature to identify areas where losses could occur. From this investigation, the following areas for analyte loss were identified:

1. During the manual clean-up procedures, extracts were stored overnight at 4°C until the clean-up procedure was continued. The samples were stored in the glass ASE collection vessels, and in the glass turbo vap flasks. PCDD/Fs can irreversibly adsorb to active sites present on glass surfaces (US EPA, 2007). Chemically active sites on glass are caused by hydroxyl groups or heavy metal ions. These active sites can be removed/deactivated by treating glassware by treatment with hexamethyldisilazane prior to use (Scott, 2012).
2. A “keeper” was absent during the evaporation steps for the automated and manual clean-up methods. A keeper is a solvent, such as nonane or dodecane, that prevents the evaporation and degradation of compounds of interest. This is the case due to the higher boiling points of these solvents (Focant *et al*., 2002; Sjodin, 2007). The evaporation temperatures (60ºC) used (Lai *et al*., 2004) used in the course of the

study may have been too high, possibly causing the degradation or volatilisation of PCDD/Fs. Studies have indicated that lower chlorinated pollutants such as PCBs undergo faster degradation than their higher chlorinated homologues (Backe *et al.*, 2004; Umar *et al*., 2012). Temperatures reported in literature range from 35 - 65ºC (Dolezal *et al*., 1995; Ontario Ministry of the Environment, 2004) as well as nitrogen evaporation at ambient temperatures (Yoo *et al*., 2006), and evaporation without the addition of gas at ambient temperatures (Weber & Sakurai, 2001; Focant *et al*., 2002). This issue needs further investigation to determine which method would be most suitable for the evaporation of dioxins.

These losses will be investigated further; however, the additional extractions needed fall outside the scope of the current study.

4.2.2 Contour plots for the dioxins of the soil samples and CRMs (AGF, TRP, and SOX)

The two soil and CRM extracts generated from the various methods were injected to determine the presence of dioxin-like chemicals in the extract. The concentrations of the analytes in the CRM are listed in the supplementary data, for dioxins the concentrations of the individual PCDD/F analytes varied between 0.019 - 25 ng/g. In AGF and SOX extracts no PCDD/F could be identified, and in TRP extract only one (1,2,3,4,6,7,8-heptachloro dibenzo-*p*- dioxin). As seen in the contour plots and chromatograms (Figure 4.8), there was a high level of matrix interference in the extracts that could have obstructed the identification of the 17 PCDD/Fs as previously described in Dagan (2000). Comparing the contour plots of the different methods, it is apparent that the TRP (Figure 4.8; 3a) had much less matrix interference than AGF (Figure 4.8; 1a) and SOX (Figure 4.8; 2a) extracts.

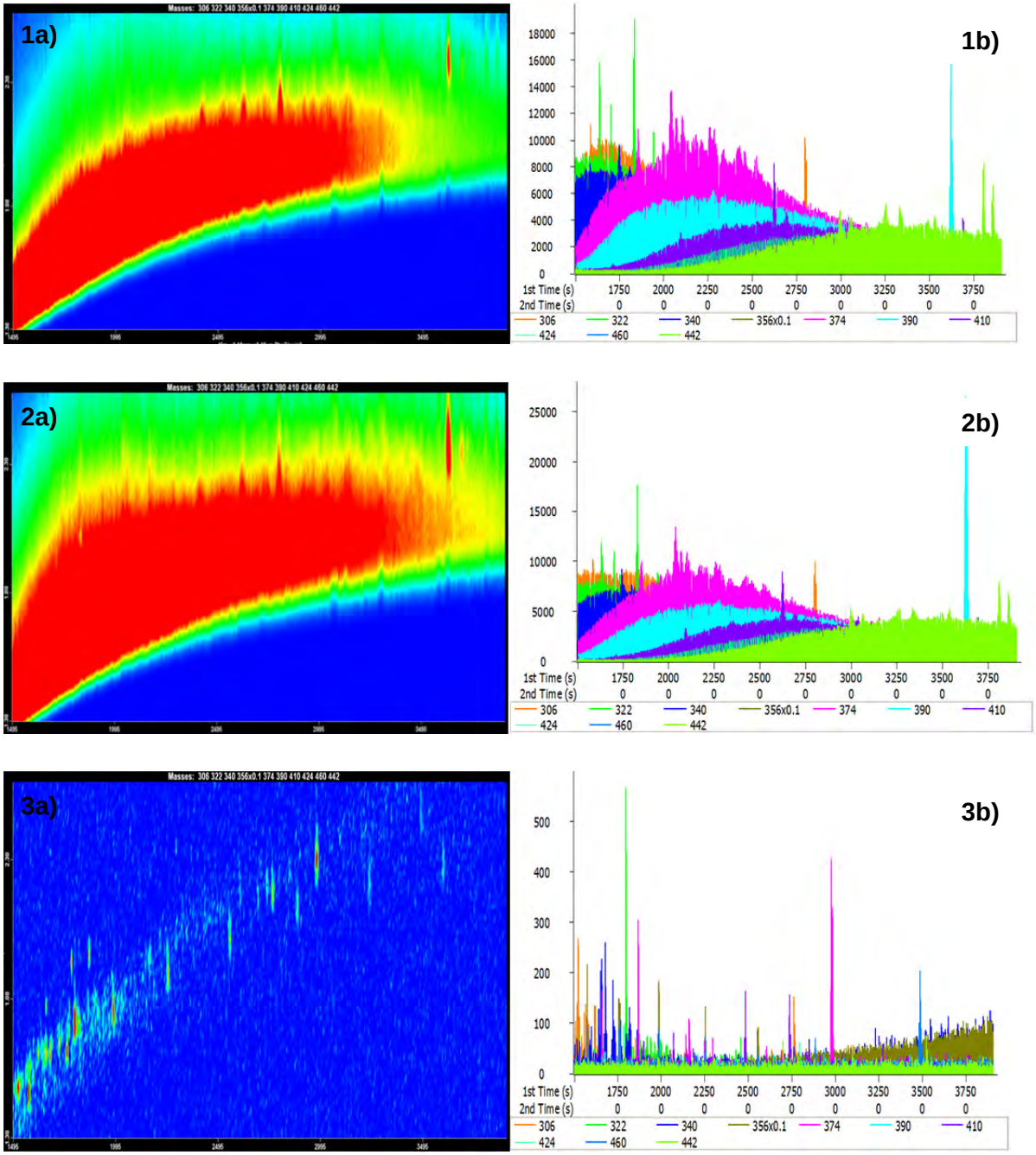


Figure 4.8: Contour plots and chromatograms for the 17 PCDD/Fs of the extracted soil samples and CRMs: 1a) AGF contour plot, 1b) AGF chromatogram; 2a) SOX contour plot, 2b) SOX chromatogram; 3a) TRP contour plot and 3b) TRP chromatogram.

4.2.3 Additional instrumental analysis

Due to the low amount of 17 PCDD/Fs found in the extracted Cal 1, soil samples and CRM, additional investigation was needed to explain the high response from the H4IIE bio-assay results (Table 4.3). A mixture of available PAH standards (15 US EPA priority PAHs) was injected (100 ng/mℓ) using the same analytical method as for the PCDD/Fs to allow for the potential identification of selected PAHs within the samples (Figure 4.9; extracted ions: 76, 101, 114, 126, 138, 139, 125, 153, 166, 178, 202, 228, 252, 276, and 278). This led to the identification of 13 of the 15 PAHs. The loss of the lower molecular mass PAHs, such as naphthalene, was likely due to the temperature and mass spectrometer programs that were optimised for PCDD/Fs.

Table 4.7: US EPA priority PAHs with TEF values (Collins *et al*., 1998)

Compound	TEF values
Naphthalene (2-ringed)	0.001
Acenaphthylene*(3-ringed)	0.001
Acenaphthene (3-ringed)	0.001
Fluorene*(3-ringed)	0.001
Phenanthrene*(3-ringed)	0.001
Anthracene*(3-ringed)	0.01
Fluoranthene*(4-ringed)	0.001
Pyrene*(4-ringed)	0.001
Benzo[a]anthracene*(4-ringed)	0.1
Chrysene*(4-ringed)	0.01
Benzo[b]fluoranthene*(5-ringed)	0.1
Benzo[k]fluoranthene*(5-ringed)	0.1
Benzo[a]pyrene*(5-ringed)	1
Dibenzo[a,h]anthracene*(6-ringed)	5
Benzo[ghi]perylene*(6-ringed)	0.01
Ideno[1, 2, 3-cd]pyrene (6-ringed)	0.1

*PAHs found in the standard injected

The standard used to spike the matrix matched calibration curve and which was used as the reference standard (MS) in the H4IIE bio-assay, was also injected. Seven PAHs were found in the analytical PCDD/F standard (supplementary data; figure 4.10), indicating possible contamination within the standard itself or from pipettes and/or solvents. This could possibly account for the high response found in the H4IIE bio-assay results (Table 4.3)

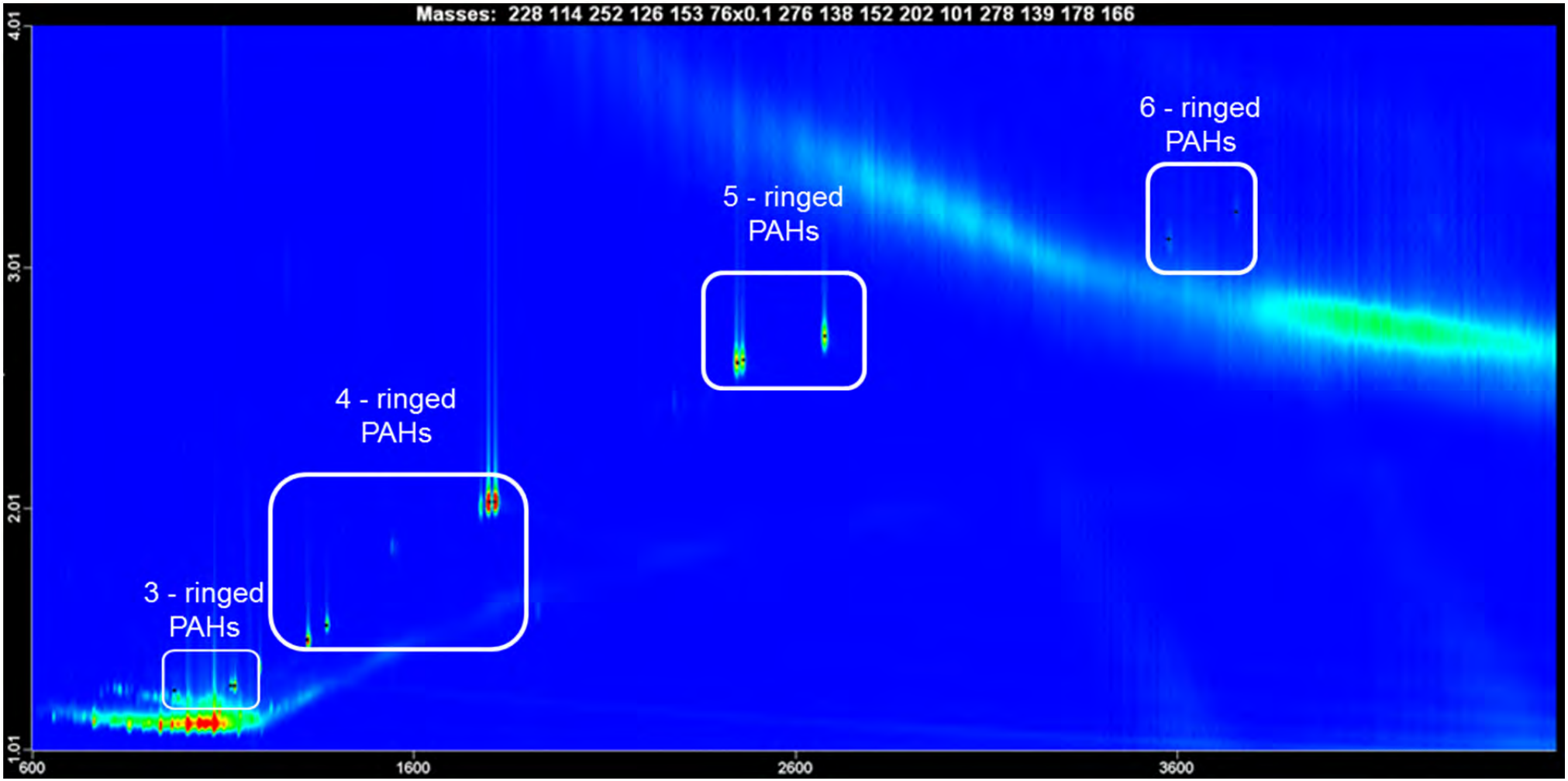


Figure 4.9: Contour plot of 13 priority PAHs found during GCxGC-TOFMS analysis of the analytical standard (100 ng/mℓ)

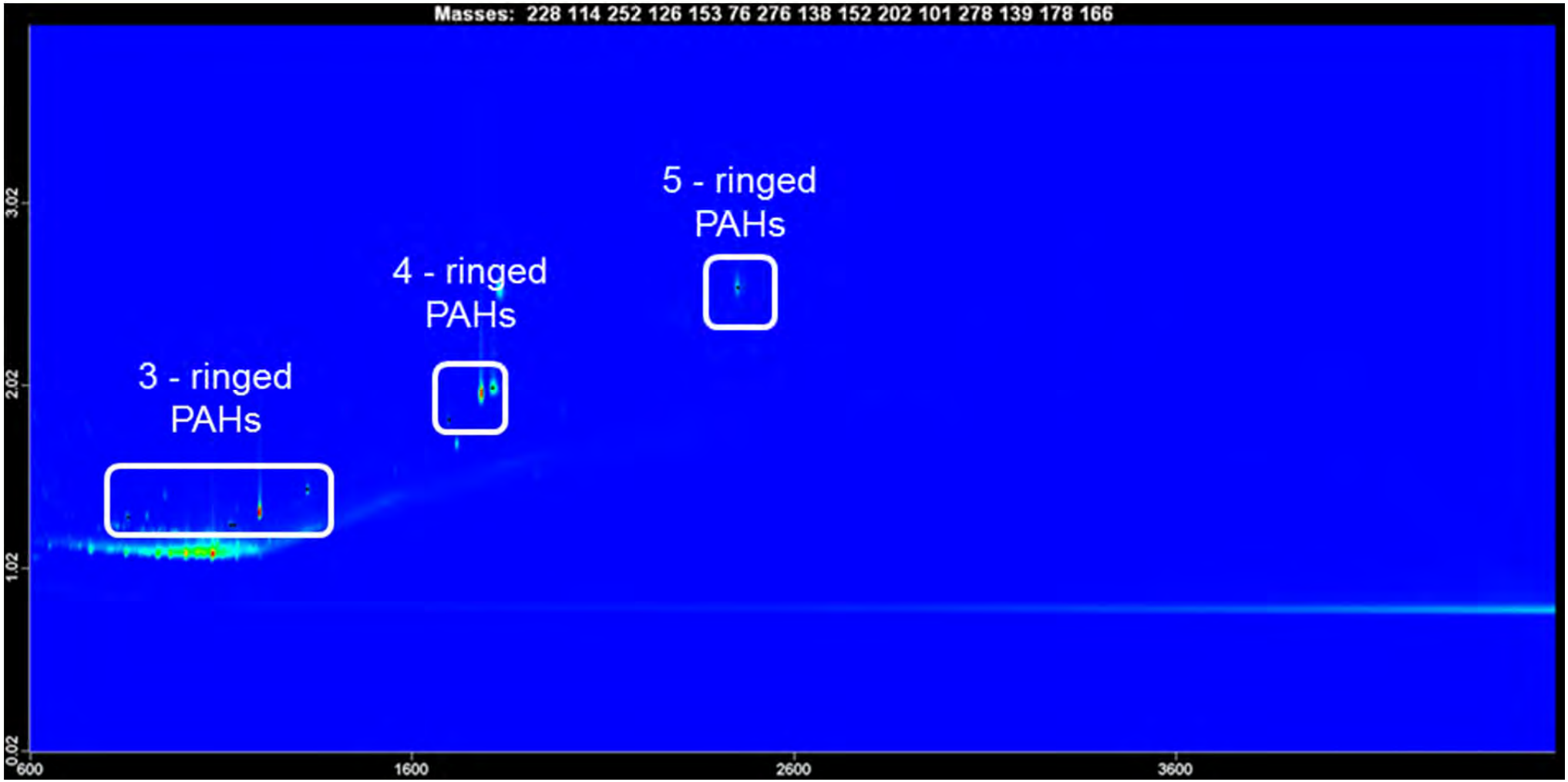


Figure 4.10: Contour plot of the PAHs found in the PCDD/F analytical standard (Matrix spiking solution, 150 ng/mℓ)

PAHs are known AhR agonists and various studies have indicated that the mechanism of toxicity of PAHs is similar to that of dioxins (Sanderson *et al*., 1996; Behnisch *et al*., 2001b; Villeneuve *et al*., 2002). As previously described, the AhR agonist (in this case the PAH) enters the cytoplasm of the cells, and binds to the AhR complex. The AhR is translocated to the nucleus of the cell, where it forms a heterodimer with the ARNT protein. The complex binds to the DRE, which results in transcriptional activation of genes.

There is evidence from early research (Poland & Knutson, 1982) that PAHs bind to the AhR receptor, competing for the cytosol binding area with high affinity (1/3 to 1/30 to that of 2,3,7,8-TCDD). Studies using the H4IIE bio-assay have also shown that PAHs, specifically dibenzo[a,h]anthracene, benzo[k]fluoranthene, benzo[a]pyrene, and ideno[1,2,3-cd]pyrene, can cause super-induction (levels above the maximum effect induced by TCDD) (Behnisch *et al*., 2003; Larsson *et al*., 2012). Not only does super-induction occur in these cases, but additive effects have also been observed for PAH mixes (Fent & Bätcher, 2000; Larsson *et al*., 2012). Additive effects of a mixture occur when the total toxicity of a mixture can be described by summing the toxicity of individual compounds, whereas in the case of synergism, the overall effect is greater than the sum of the individual effects (Larsson *et al*., 2012).

The study conducted by Larsson *et al.* (2012) used different PAH mixtures. During this study, PAHs exhibited additive effects. However, as the amount of PAHs increased in the mixtures, the observed effect was greater than expected, which could indicate synergistic effects. These effects of PAHs could explain the higher-than-expected values observed in the H4IIE bio-assay. As previously described, AhR agonist may result in the false positives found in the bio-assay and PAHs are class A agonists (Table 1) of the AhR.

The contour plots of the CRM samples (Figure 4.12) for the different methods revealed high levels of PAHs which were observed in all the samples. Of the 13 PAHs screened, AGF extracts had 11, SOX 12 and TRP 10 (supplementary data), which is a strong indication that the clean-up methods used were not successful in removing these interfering compounds. The peak areas and S/N levels were high, but comparing the three methods, the TRP had the lowest and SOX had the highest response (Figure 4.11). This finding was comparable to the response found in the assay results (TRP CRM: 16 ng dioxin-eq/g; AGF CRM: 44 ng dioxin-eq/g; and SOX CRM: 175 ng dioxin-eq/g). The GCxGC-TOFMS results for the two soil samples indicated similar results, where the TRP had the lowest response and the SOX the highest response. This corresponds well with the H4IIE bio-assay results, which

has the same pattern. By using the concentrations of the PAHs in the CRM and the TEF values in table 4.7, a TEQ value was obtained for the PAHs present in the CRM (7.6×10^3 ng/g). This value is greater than the BEQ values from the assay, suggesting that at least some of the PAHs were removed during the clean-up procedure.

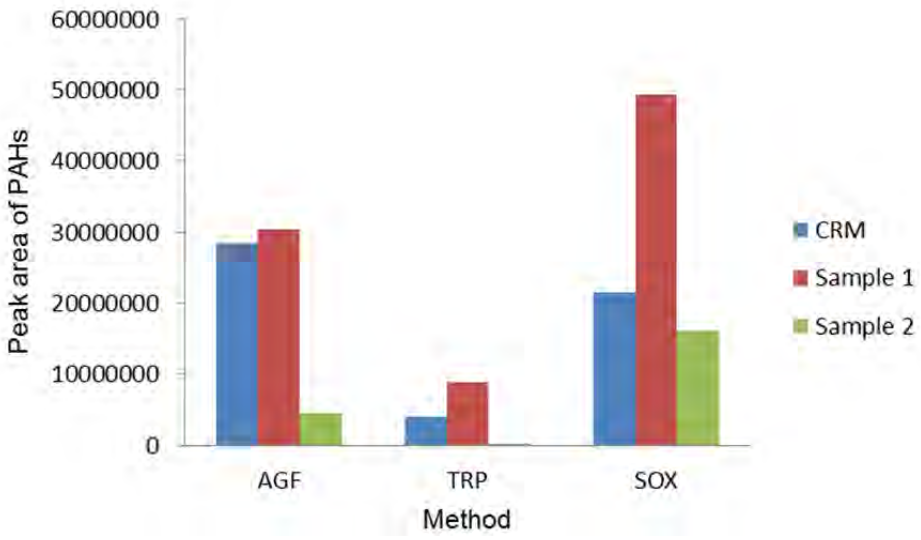
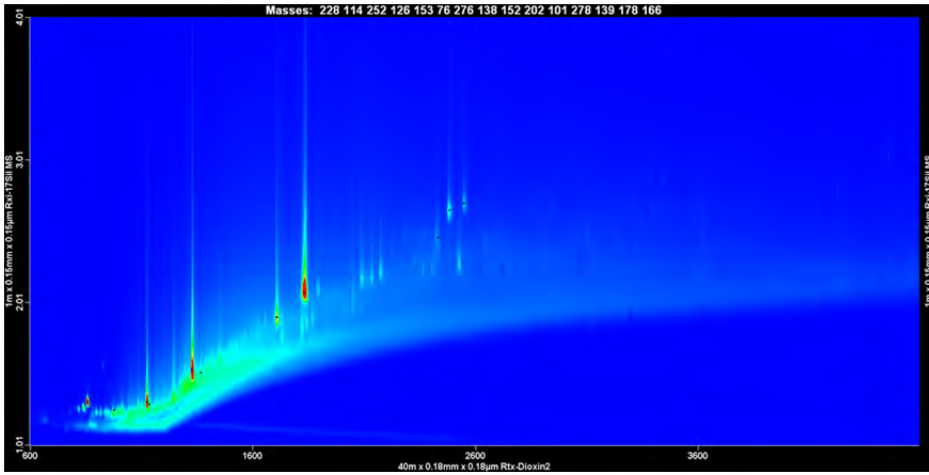
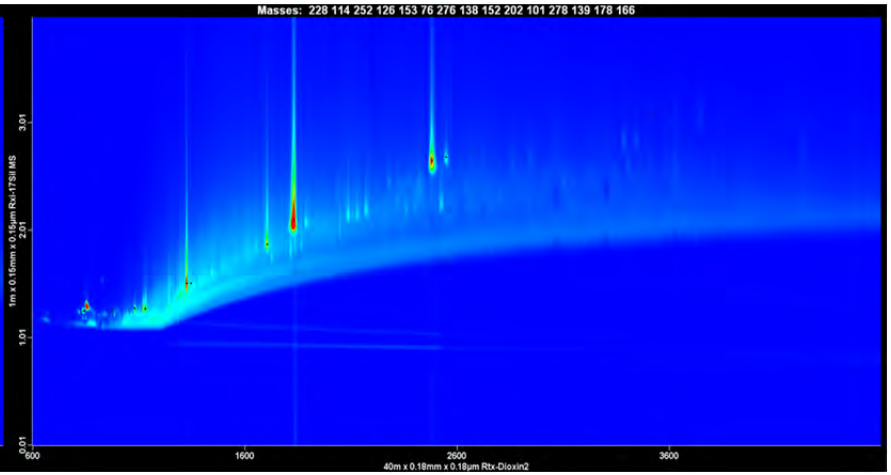


Figure 4.11: Comparison of the different clean-up methods using the peak area generated from the PAHs

a)



b)



c)

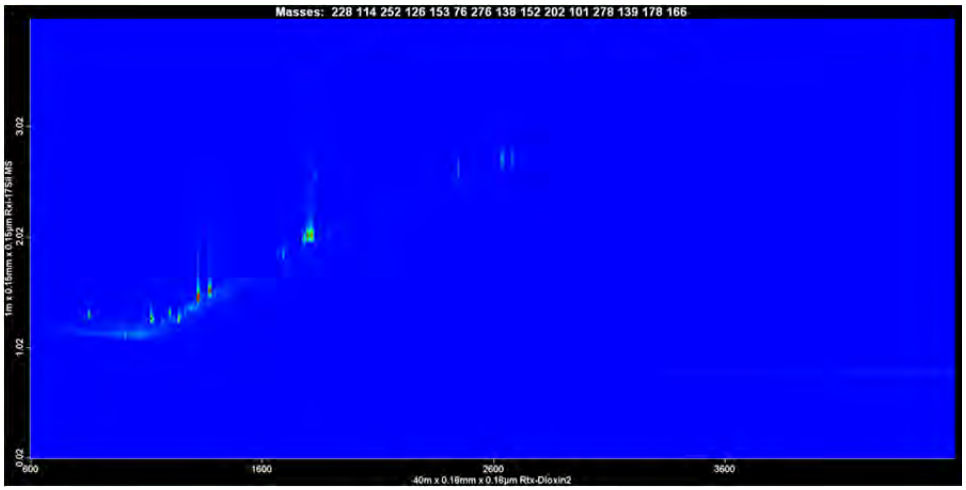


Figure 4.12: Contour plot of the CRM samples of a) AGF b) SOX and c) TRP for PAHs

4.3 Associations between instrumental and bio-analysis

When comparing the GCxGC-TOFMS results to the H4IIE bio-assay results, it becomes evident that the H4IIE bio-assay's response can be ascribed to the PAHs present in the samples rather than that to PCDD/Fs. This explains why the response was higher than expected in spiked samples, which thus indicates possible synergistic effects. The clean-up methods were selected to retain only dioxins and to remove interfering compounds such as PAHs; but this was not the case. The acid digestion used in the manual clean-up method did not remove the PAHs. In total, a three to five hour acid wash was used in this study, whilst the literature indicates that a one hour acid wash should be sufficient to remove 99% of the PAHs present. However, reported studies noted a dioxin-like response from acid breakdown products. Only after a 10 hour acid wash, all PAH and PAH derivatives were destroyed and no dioxin-like response was observed (Villeneuve *et al*., 2002; Xu *et al*., 2004). This extended acid wash protocol should be included in future investigations in sample clean-ups.

Less matrix interference was observed in sample clean-up using the automated clean-up method, but PAHs were still present in the extract – which could have led to competitive binding between the PAHs and dioxins for the AhR receptor. More optimal results will be yielded if one were to remove all PAHs and other interfering compounds from the extract. The addition of another high capacity acidic silica column to the set of three columns (ABN silica, alumina and carbon) used on the TRP may reduce PAHs sufficiently. Furthermore, additional acid digestion could prove sufficient to remove the PAHs still present in the sample.

The study indicated that the manual and automated clean-up methods were not able to fully remove interfering compounds from the samples, and require further refinement to yield only dioxins in the extract. The H4IIE bio-assay is capable of screening samples for dioxin and dioxin-like chemicals, and is comparable to chemical analysis.

5
Conclusion and recommendations

Developing countries like South Africa have limited information on dioxins. These data gaps necessitate the establishment of extraction and analytical techniques in SA for the analysis of these chemicals. This study made a positive contribution to the data generated for South Africa, as it highlighted problem areas in the current analytical methodologies. This knowledge of the shortcomings could prevent unnecessary expense in future analytical work as well as assist with method development by highlighting potential shortfalls.

5.1 Conclusions

During data analysis of the H4IIE bio-assay, a problem with the 2,3,7,8-TCDD reference standard was identified. When 2,3,4,8-TCDD was used as the reference standard in the quantification process only 15 of the expected 40 matrix matched calibration curve samples were above the LOD. To ensure validity of the concentration range, the spiking matrix solution was analysed, resulting in 25 of the 30 samples to be above the LOD. The spiking solution was used to semi-quantify the assay, resulting in 26 of the 40 matrix matched calibration curve samples being above the LOD. However, a higher response than expected was found in the bio-assay results for all the methods evaluated and this was further investigated with the instrumental analysis.

For the regression analysis of the matrix matched calibration curve samples, only Cal 1-5 could be used, as Cal 6 - 8 were below LOD. Results from the regression analysis indicated that the extraction methods, PLE and Soxhlet, were not equivalent to each other because the slope for NC ASE was substantially higher than that of the NC SOX. The NC ASE showed a higher BEQ value than the NC SOX.

No statistically significant difference was found between the clean-up methods (Kruskal Wallis ANOVA; $p > 0.05$). A histogram of the BEQ results from the clean-up methods demonstrated that the TRP had the lowest and the SOX the highest response from the H4IIE bio-assay. The method blanks indicated no contamination by the extraction process.

Instrumental analysis indicated low levels of PCDD/Fs in the extracted matrix matched calibration curves. The PCDD/Fs of Cal 1-3 of the non-matrix matched analytical standard were identified with the GCxGC-TOFMS, and was expected due to the LOD levels (Table

4.4) found for the 17 PCDD/Fs in the GCxGC-TOFMS analysis. However, in the extracted samples, only Cal 1 had the majority of the spiked PCDD/F analytes. This demonstrated a high amount of analyte loss during the extraction and clean-up procedures. The loss of dioxins from the samples might be due to high evaporation temperatures combined with the lack of a solvent keeper. For the CRM and soil samples, only one analyte was found – in the TRP CRM extract – while no dl-PCBs were identified in any of the samples. Matrix interference played a significant role, and prevented the positive identification of peaks in the GCxGC-TOFMS chromatogram.

Additional instrumental analysis revealed that PAHs were more abundant than PCDD/Fs in the samples, likely by orders of magnitude. PAHs are a class A agonist (Table 1), and can result in false positives in the bio-assay. Additionally, PAHs were found in the matrix match spiking solution, indicating possible contamination from laboratory glassware and solvents used during the study. Chemical analysis indicated PAHs in all the samples which were evaluated. When plotting the peak areas found for the PAHs on a histogram and comparing this to the histogram of the BEQ values form the H4IIE bio-assay, the two plots were found to be very similar, indicating the response elicited from the H4IIE bio-assay was probably due to the PAHs in the samples. Although the TRP clean-up method had less PAHs in the CRMs and soil samples than the AGF and SOX, not all the PAHs were removed during the TRP clean-up procedure.

After evaluating the H4IIE bio-assay data in relation to the GCxGC-TOFMS data, it was clear that the methods selected did not perform as expected. The extraction methods (PLE and Soxhlet) were not nearly as equal as stated in the literature. The clean-up protocols were not selective or specific enough to target only dioxins. Analytes were lost in the automated and manual clean-up methods.

The hypothesis of the study was proven wrong as the various extraction and clean-up methods did not lead to a variation in the induction of the AhR within the H4IIE cell line. Furthermore, no statistically significant difference was found between the clean-up protocols performed during this study. Additionally, the effectiveness of the different clean-up protocols to select only for PCDD/Fs and dl-PCBs was inadequate, and this was the case due to loss of dioxins and the presence of interfering compounds (PAHs) in the final extracts. Future studies need to focus on optimising extraction and clean-up methods for dioxin analysis.

5.2 Recommendations

A mixed dioxin standard used as a reference standard in the H4IIE bio-assay should be evaluated to take competitive binding and synergism into consideration. Before using the standard for the bio-assay, it should be tested through instrumental analysis to ensure that all the compounds are present, and also to ensure that no contamination takes place with other possible agonists such as PAHs. If possible, the standard should be evaluated the same day as the bio-assay, in order to ensure that no degradation or contamination occurs through repeated use.

The loss of dioxins from the samples could be ascribed to evaporation temperatures that were too high, and also to the absence of a solvent keeper. These problems will have to be evaluated in future studies by determining the loss that occurs in neat standards that have been concentrated at varying evaporation temperatures and conditions.

The extraction methods used (PLE and Soxhlet) should be re-evaluated and compared to ensure they are indeed comparable. A pre-tested standard should be used to spike clean matrix and the extraction procedure should be followed without the use of clean-up protocols.

The clean-up protocols used were not selective or specific to target only dioxins in the extracts. The removal of PAHs from the sample extracts for both the manual and automated methods should be examined further. An acid digestion time course study is advised in the manual clean-up method, to determine what the optimum number of rinses is required for the removal of PAHs. For the automated method, other column combination sets should be investigated, such as the use of high capacity acid silica in combination with acid-basic-neutral silica columns.

With a view to decrease the effect of matrix interference on the GCxGC-TOFMS, other column sets may be tried to separate the matrix constituents from the compounds of interest more efficiently. If it is possible to distinguish between the matrix and the analytes, the GCxGC-TOFMS will be able to identify the analytes of interest at lower concentrations, even if matrix is present. This was attempted with a Dioxin 2 (Restek) as the primary column and an Rtx-17Sil MS (Restek) as the secondary column. There was not sufficient available time to repeat all samples on this column set, thus only preliminary results are included in the recommendations, and not discussed in previous chapters, as this needs to be evaluated. This column set efficiently separated individual PCDD/Fs (Figure 5.1) and PAHs (Figure 5.2) whilst separating PCDD/Fs and PAHs from one another as well as the matrix interferences

(Figure 5.3). To evaluate whether this column set would be effective for investigating an extracted soil sample, an AGF sample from a previous study was injected. Separation for the PCDD/Fs and PAHs from the matrix, as well as from each other can be achieved as seen in figure 5.3. This method will have to be evaluated in further studies.

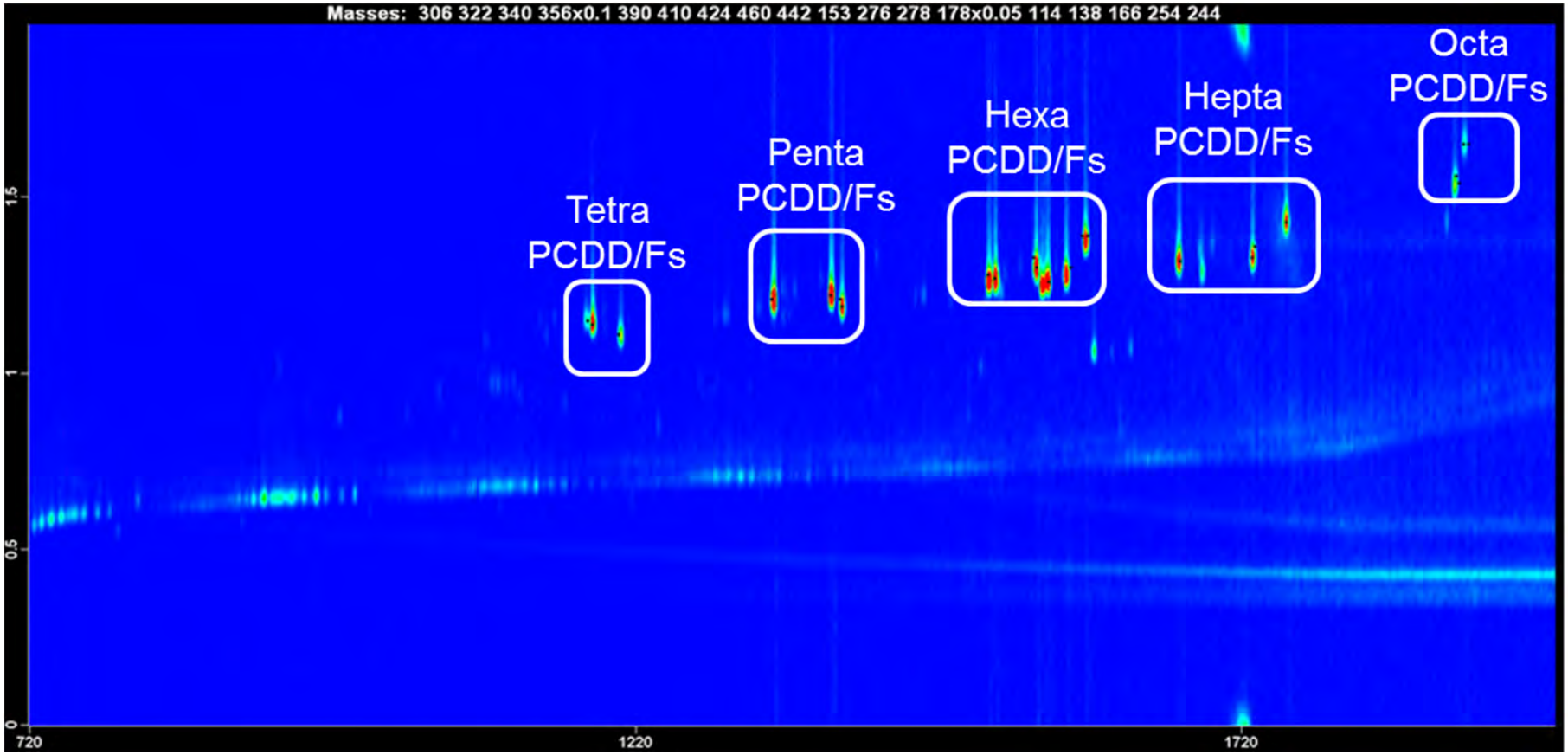


Figure 5.1: Contour plot of separation found for the 17 PCDD/Fs on the proposed column set (1: TCDD/Fs, 2: HpCDD/Fs; 3: HxCDD/Fs and 4: PxCDD/Fs and 5: OCDD/Fs; 100 ng/mℓ)

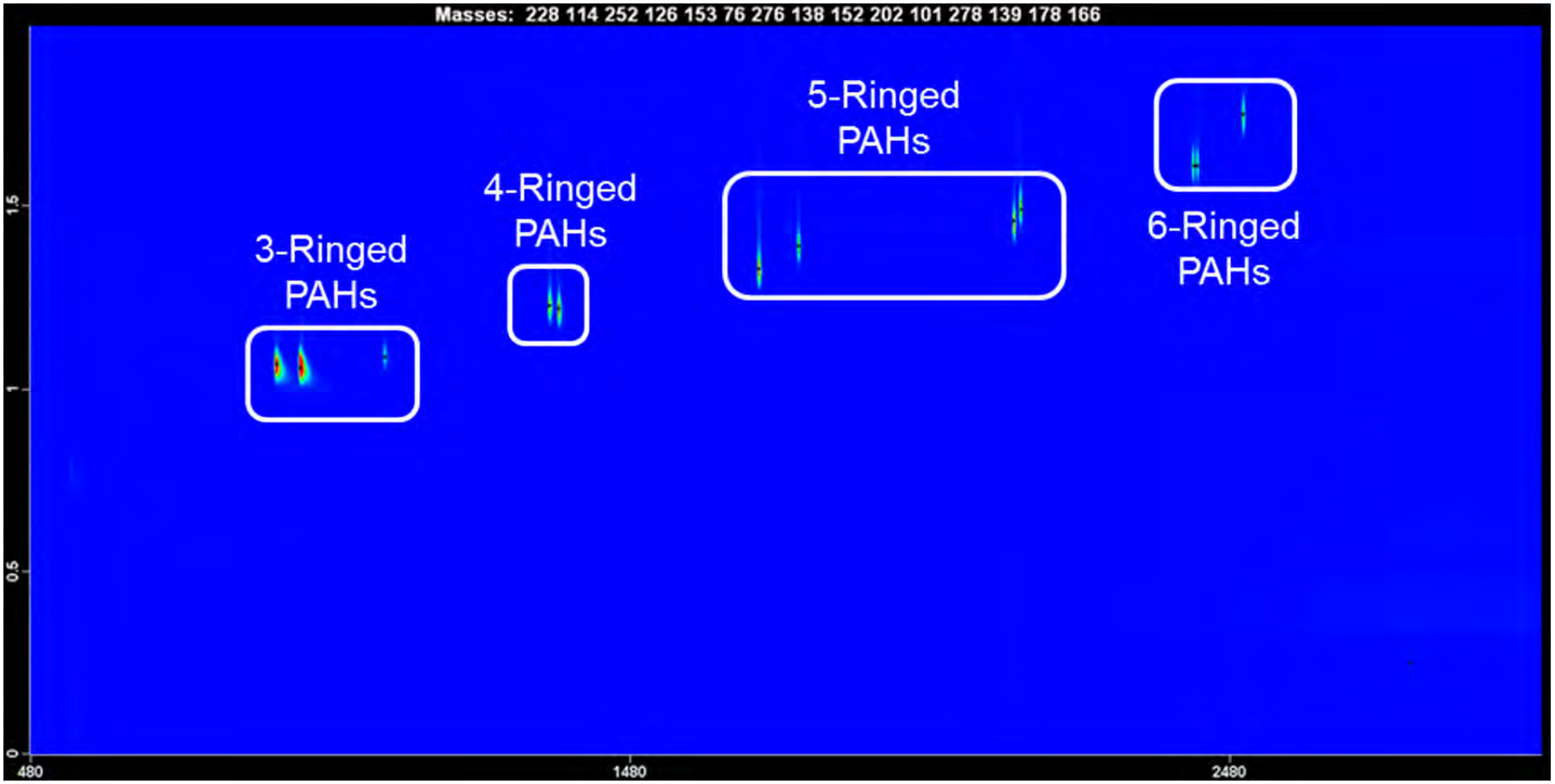


Figure 5.2: Contour plot of the separation found for the 13 priority PAHs on the proposed column set (1: 3-ringed PAHs; 2: 4-ringed PAHs; 3: 5 ringed PAHs and 4: 6-ringed PAHs; 100 ng/mℓ)

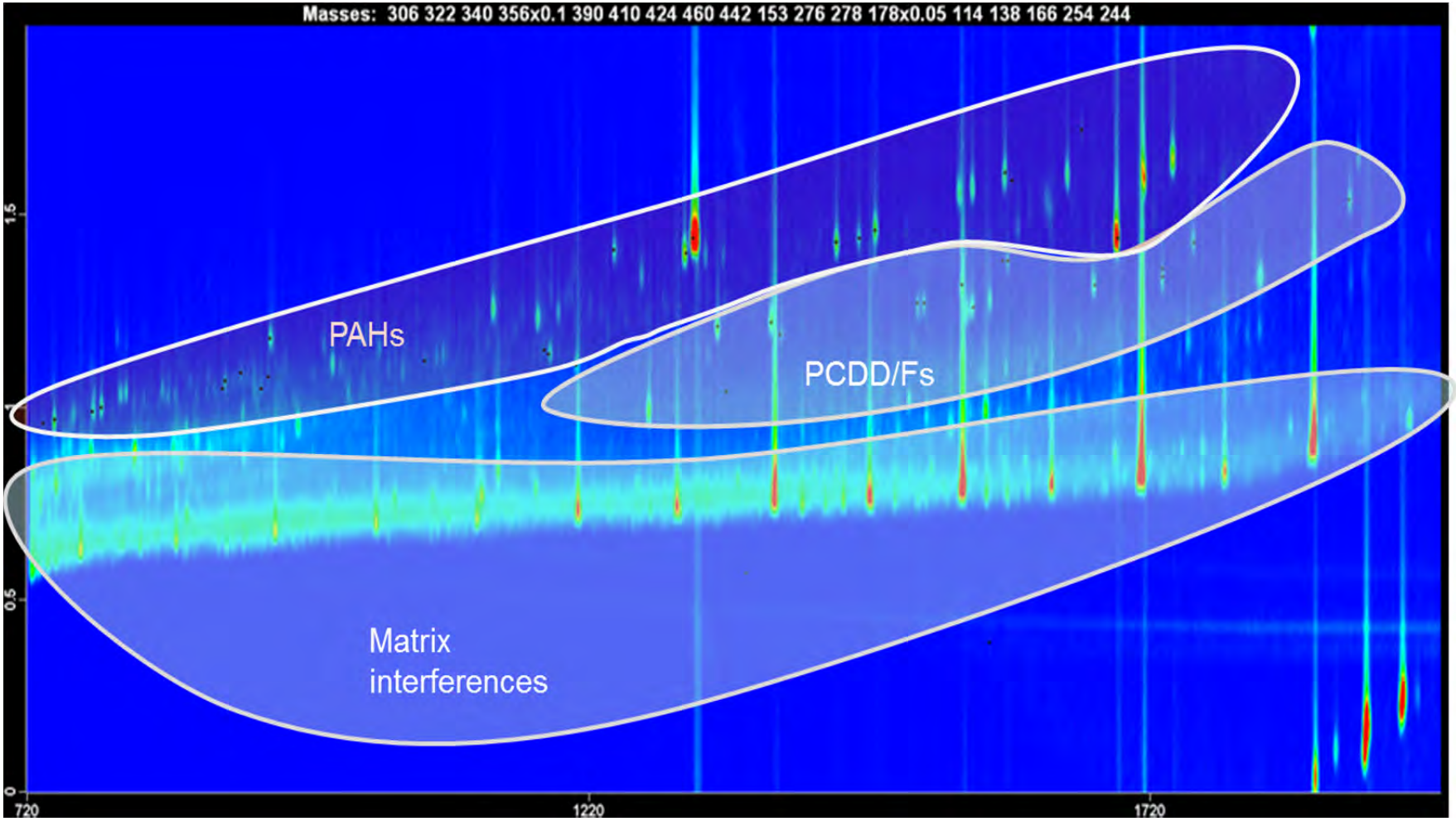


Figure 5.3: Contour plot of the separation found on the proposed column set for a soil sample where matrix interference, PAHs and PCDD/Fs are separated from one another

6
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Supplementary data

Table S1: Peak area and signal to noise of PCDD/Fs found in samples

Table S2: Peak area and signal to noise of PAHs found in samples

Table S3: Dioxins (PCDDFs and dl-PCB) and PAHs present in CRM

Table S4: Certified values for dioxins (PCDD/Fs and dl-PBS) in matrix spiking solution

Table S1: Peak area and signal to noise of PCDD/Fs present in samples

Name of compound	Peak area	Signal to noise (S/N)	1st Dimension time (s)	2nd Dimension time (s)	Similarity	Unique mass
Neat standard Cal 1 (150 ng/ml)						
2,3,7,8-Tetrachlorodibenzofuran	42132	517	1630	1.65	883	306
2,3,7,8-Tetrachlorodibenzo-p-dioxin	25831	319	1660	1.65	819	322
1,2,3,7,8-Pentachlorodibenzofuran	72363	635	1885	1.82	885	340
2,3,4,7,8-Pentachlorodibenzofuran	62675	567	1980	1.91	868	340
1,2,3,7,8-Pentachlorodibenzodioxin	36234	300	1995	1.89	662	356
1,2,3,4,7,8-Hexachlorodibenzofuran	35425	294	2290	2.02	783	374
2,3,4,6,7,8-Hexachlorodibenzofuran	41460	332	2305	2.15	787	374
1,2,3,6,7,8-Hexachlorodibenzofuran	34904	259	2390	2.15	787	374
1,2,3,4,7,8-Hexachlorodibenzodioxin	20388	136	2405	2.09	729	390
1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin	19122	140	2420	2.09	725	390
1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin	21173	135	2455	2.15	729	390
1,2,3,7,8,9-Hexachlorodibenzofuran	26547	186	2505	2.17	760	374
1,2,3,4,6,7,8-Heptachlorodibenzofuran	22430	125	2735	2.21	700	410
1,2,3,4,6,7,8-Heptachlorodibenzodioxin	11905	91	2930	2.29	650	424
1,2,3,4,7,8,9-Heptachlorodibenzofuran	15512	96	3030	2.3	676	410
Octachlorodibenzo-p-dioxin	18315	91	3485	2.44	700	460
Octachlorodibenzofuran	22233	126	3520	2.41	700	442
Neat standard Cal 2 (80 ng/ml)						
2,3,7,8-Tetrachlorodibenzofuran	25670	268	1630	1.65	883	306
2,3,7,8-Tetrachlorodibenzo-p-dioxin	13658	133	1660	1.65	819	320
1,2,3,7,8-Pentachlorodibenzofuran	43961	372	1885	1.82	885	340
2,3,4,7,8-Pentachlorodibenzofuran	37804	331	1980	1.9	868	340
1,2,3,7,8-Pentachlorodibenzodioxin	21348	166	1995	1.89	662	356
1,2,3,4,7,8-Hexachlorodibenzofuran	29721	211	2290	2.02	783	374
2,3,4,6,7,8-Hexachlorodibenzofuran	27121	205	2305	2.04	791	374
1,2,3,6,7,8-Hexachlorodibenzofuran	19890	158	2390	2.15	787	374
1,2,3,4,7,8-Hexachlorodibenzodioxin	12057	94	2405	2.08	729	390
1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin	11843	91	2420	2.09	725	390
1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin	10754	67	2455	2.16	729	392
1,2,3,7,8,9-Hexachlorodibenzofuran	13450	99	2505	2.18	760	374
1,2,3,4,6,7,8-Heptachlorodibenzofuran	12217	89	2735	2.21	700	410
1,2,3,4,6,7,8-Heptachlorodibenzodioxin	6716.3	58	2930	2.29	650	426
1,2,3,4,7,8,9-Heptachlorodibenzofuran	9003.1	47	3030	2.31	676	408
Octachlorodibenzo-p-dioxin	9890.1	48	3485	2.44	700	458
Octachlorodibenzofuran	12458	78	3520	2.41	700	444
Neat standard Cal 3 (40 ng/ml)						
2,3,7,8-Tetrachlorodibenzofuran	9337.5	76	1635	1.65	883	306
2,3,7,8-Tetrachlorodibenzo-p-	5128.9	38	1660	1.65	819	322

dioxin						
1,2,3,7,8-Pentachlorodibenzofuran	17249	147	1890	1.82	885	340
2,3,4,7,8-Pentachlorodibenzofuran	14639	120	1980	1.9	868	340
1,2,3,7,8-Pentachlorodibenzodioxin	7784.8	62	2000	1.89	662	356
1,2,3,4,7,8-Hexachlorodibenzofuran	10395	74	2290	2.02	783	374
2,3,4,6,7,8-Hexachlorodibenzofuran	8871.5	81	2305	2.02	791	374
1,2,3,6,7,8-Hexachlorodibenzofuran	5649.2	62	2390	2.15	787	374
1,2,3,4,7,8-Hexachlorodibenzodioxin	4871.3	43	2405	2.08	729	390
1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin	3747.3	41	2420	2.08	725	390
1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin	4661.5	37	2455	2.15	729	390
1,2,3,7,8,9-Hexachlorodibenzofuran	1928.5	19	2505	2.16	760	372
1,2,3,4,6,7,8-Heptachlorodibenzofuran	2793.3	40	2735	2.22	700	408
1,2,3,4,6,7,8-Heptachlorodibenzodioxin	1329.3	19	2930	2.29	650	426
1,2,3,4,7,8,9-Heptachlorodibenzofuran	1510.4	24	3030	2.3	676	408
Octachlorodibenzo-p-dioxin	4053.6	25	3485	2.45	700	460
Octachlorodibenzofuran	1558.9	28	3520	2.42	700	442
		Extracted Cal 1 AGF				
2,3,7,8-Tetrachlorodibenzofuran	6842.3	80	1630	1.69	883	306
1,2,3,7,8-Pentachlorodibenzofuran	31753	196	1890	1.85	885	340
2,3,4,7,8-Pentachlorodibenzofuran	26137	158	1980	1.94	868	340
1,2,3,7,8-Pentachlorodibenzodioxin	5355.4	36	1995	1.93	662	354
2,3,4,6,7,8-Hexachlorodibenzofuran	16468	100	2305	2.07	791	376
1,2,3,6,7,8-Hexachlorodibenzofuran	13819	80	2390	2.19	787	374
1,2,3,4,7,8-Hexachlorodibenzodioxin	4872.1	75	2405	2.12	729	390
1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin	9242.5	57	2420	2.13	725	390
1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin	4222.8	61	2455	2.2	729	390
1,2,3,7,8,9-Hexachlorodibenzofuran	4799	8	2500	2.24	760	374
1,2,3,4,6,7,8-Heptachlorodibenzofuran	2774.6	38	2735	2.26	700	410
1,2,3,4,7,8,9-Heptachlorodibenzofuran	2660.9	18	3030	2.33	676	406
		Extracted Cal 1 TRP				
2,3,7,8-Tetrachlorodibenzofuran	1780.2	27	1630	1.69	883	306
2,3,7,8-Tetrachlorodibenzo-p-dioxin	2386	42	1660	1.67	819	320
1,2,3,7,8-Pentachlorodibenzofuran	18177	129	1890	1.85	885	340
2,3,4,7,8-Pentachlorodibenzofuran	4510.8	53	1980	1.92	868	340
1,2,3,7,8-Pentachlorodibenzodioxin	6067.4	49	1995	1.92	662	356
1,2,3,4,7,8-Hexachlorodibenzofuran	13968	110	2290	2.06	783	374
2,3,4,6,7,8-Hexachlorodibenzofuran	18216	110	2305	2.06	791	374
1,2,3,6,7,8-Hexachlorodibenzofuran	1335.4	24	2390	2.18	787	374
1,2,3,4,7,8-Hexachlorodibenzodioxin	5297.5	42	2405	2.11	729	390
1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin	4966.6	35	2420	2.12	725	392

1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin	3719.4	34	2455	2.19	729	390
1,2,3,7,8,9-Hexachlorodibenzofuran	7621.7	51	2505	2.2	760	374
1,2,3,4,6,7,8-Heptachlorodibenzofuran	8115.2	60	2735	2.26	700	410
1,2,3,4,6,7,8-Heptachlorodibenzodioxin	2417.1	27	2930	2.34	650	426
1,2,3,4,7,8,9-Heptachlorodibenzofuran	4809.8	40	3030	2.34	676	410
Octachlorodibenzo-p-dioxin	3370.8	15	3485	2.47	700	332
Octachlorodibenzofuran	7427.7	44	3520	2.45	700	442
Extracted Cal 1 SOX						
1,2,3,7,8-Pentachlorodibenzofuran	3039.2	26	1885	1.86	885	342
2,3,4,7,8-Pentachlorodibenzofuran	4170.6	72	1980	1.93	868	340
1,2,3,7,8-Pentachlorodibenzodioxin	1943.4	13	1995	1.94	661	230
1,2,3,4,7,8-Hexachlorodibenzofuran	5113.6	493	2285	2.04	783	119
2,3,4,6,7,8-Hexachlorodibenzofuran	3926.9	71	2305	2.06	791	374
1,2,3,4,7,8-Hexachlorodibenzodioxin	4453.1	51	2405	2.12	729	390
1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin	2641.9	41	2455	2.18	729	390
1,2,3,4,6,7,8-Heptachlorodibenzofuran	3721.5	39	2735	2.24	700	408
1,2,3,4,6,7,8-Heptachlorodibenzodioxin	1168.6	24	2930	2.34	650	424
1,2,3,4,7,8,9-Heptachlorodibenzofuran	3096.4	35	3030	2.33	676	408
Octachlorodibenzofuran	558.22	20	3520	2.46	700	444
TRP CRM						
1,2,3,4,6,7,8-Heptachlorodibenzofuran	1165	20	2735	2.29	700	410

Table S2: Peak area and signal to noise of PAHs in samples

Compound name	Peak area	S/N	1st Dimension time (s)	2nd Dimension time (s)	Similarity	Unique mass
Neat PAH standard (100 ng/ml)						
Fluorene	138439	585	970	1.26	845	166
Phenanthrene	369155	2436	1120	1.28	922	178
Anthracene	860153	5987	1130	1.28	883	178
Fluoranthene	1069852	5147	1320	1.47	892	202
Pyrene	503571	2809	1370	1.53	883	202
Benz[a]anthracene	1299185	5467	1795	2.04	934	228
Chrysene	1197971	4698	1810	2.04	777	228
Benzo[b]fluoranthene	860445	1886	2445	2.62	893	252
Benzo[k]fluoranthene	670469	1545	2460	2.63	929	252
Benzo[a]pyrene	828117	1710	2675	2.73	892	252
Dibenz[a,h]anthracene	113634	249	3575	3.13	832	278
Benzo[ghi]perylene	53178	136	3750	3.24	739	276
Mixed dioxin standard (Method 8290) (150 ng/ml)						
Acenapthylene	17835	604	855	1.3	891	152
Phenanthrene	148082	715	1125	1.26	922	178
Anthracene	42779	477	1135	1.26	883	178
Fluoranthene	233538	2268	1325	1.45	892	202
Benz[a]anthracene	43709	279	1695	1.83	934	228
Chrysene	591582	2098	1810	2.01	777	228
Benzo[b]fluoranthene	299770	953	2450	2.56	893	252
AGF Cal 1						
Phenanthrene	469561	1609	1120	1.29	922	178
Anthracene	44351	253	1135	1.27	883	178
Fluoranthene	170856	1063	1325	1.47	892	202
Pyrene	58559	403	1375	1.53	883	202
Benz[a]anthracene	50040	287	1695	1.86	934	228
Chrysene	467617	1290	1810	2.05	777	228
Benzo[b]fluoranthene	170788	373	2450	2.61	893	252

		TRP Cal 1				
Phenanthrene	75930	597	1120	1.28	922	178
Fluoranthene	34162	584	1325	1.46	892	202
Pyrene	30126	431	1375	1.52	883	202
Chrysene	28616	184	1810	2.02	777	228
		SOX Cal 1				
Fluorene	27882	337	970	1.15	845	107
Phenanthrene	175082	699	1120	1.28	922	178
Anthracene	26010	161	1135	1.27	883	178
Fluoranthene	130690	1078	1325	1.46	892	202
Pyrene	24608	233	1375	1.52	883	202
Benz[a]anthracene	4323.5	47708	1780	2.02	934	167
Chrysene	144597	619	1810	2.03	777	228
Benzo[b]fluoranthene	3499.2	33	2460	2.58	929	252
		AGF CRM				
Acenapthylene	3148164	19503	855	1.32	888	152
Fluorene	5052901	4124	975	1.26	845	166
Phenanthrene	saturated(76160405)	4618	1125	1.32	922	178
Anthracene	9435466	9264	1135	1.3	883	178
Fluoranthene	1.07E+08	14954	1325	1.54	892	101
Pyrene	4500056	8613	1365	1.52	883	220
Benz[a]anthracene	19365918	10539	1705	1.91	934	228
Chrysene	72288569	211	1830	2.08	777	188
Benzo[b]fluoranthene	949078	967	2430	4.47	893	284
Benzo[k]fluoranthene	9849843	3706	2480	2.66	929	252
Benzo[a]pyrene	5506635	2449	2545	2.71	892	252
		TRP CRM				
Acenapthylene	211757	12358	855	1.32	886	152
Phenanthrene	5771839	8149	1120	1.29	922	88
Fluoranthene	14392502	30719	1325	1.47	892	101
Pyrene	8524747	47323	1375	1.54	883	202
Benz[a]anthracene	2744125	19263	1795	2.03	934	228
Chrysene	4349531	18105	1815	2.04	777	228
Benzo[b]fluoranthene	923261	3610	2445	2.63	893	252
Benzo[k]fluoranthene	1463534	4924	2635	2.7	929	252
Benzo[a]pyrene	835999	2523	2675	2.72	892	252
Benzo[ghi]perylene	515921	1221	3755	3.26	739	276
		SOX CRM				
Acenapthylene	3869646	1126	840	1.26	901	152
Fluorene	2579049	1087	975	1.27	845	166
Phenanthrene	7934583	5379	1080	1.29	922	178
Anthracene	26707037	11175	1130	1.28	883	178
Fluoranthene	78835885	21118	1325	1.52	892	202
Pyrene	4713110	2083	1345	1.52	883	202
Benz[a]anthracene	19908934	7026	1705	1.88	934	228
Chrysene	45444052	780	1830	2.11	554	202
Benzo[b]fluoranthene	63251271	11014	2480	2.66	893	252
Benzo[k]fluoranthene	2916964	64	2545	2.69	929	174
Benzo[a]pyrene	868457	645	2660	2.73	892	270
Dibenz[a,h]anthracene	605578	318	3515	3.10	832	278
		AGF Sample 1				
Acenapthylene	2911418	45188	855	1.32	887	152
Fluorene	2428942	13430	990	1.26	845	166
Phenanthrene	11474898	31789	1120	1.29	922	178
Anthracene	34839355	46428	1130	1.29	883	178
Fluoranthene	70264554	12580	1325	1.49	892	100
Pyrene	1024042	2017	1370	1.54	883	202
Benz[a]anthracene	35079899	3979	1690	1.85	934	246
Benzo[b]fluoranthene	55995590	613	2455	2.67	893	246
Benzo[k]fluoranthene	22440835	26253	2520	2.71	929	252
Benzo[a]pyrene	856440	1234	2640	2.71	892	252
Benzo[ghi]perylene	9633.3	19	3750	3.1	739	306
		TRP Sample 1				
Acenapthylene	259619	12328	855	1.3	891	152
Phenanthrene	123811	1778	1120	1.27	922	178
Anthracene	670178	5097	1130	1.26	883	178
Fluoranthene	6074907	45670	1325	1.45	892	202
Pyrene	14449428	122775	1370	1.52	883	202
Benz[a]anthracene	9797029	63111	1795	2.01	934	228
Chrysene	23606214	93559	1805	2.03	777	228
Benzo[b]fluoranthene	26239510	78972	2445	2.63	893	252
Benzo[k]fluoranthene	3613318	13644	2510	2.63	929	252
Benzo[a]pyrene	13304493	41468	2675	2.70	892	252
Dibenz[a,h]anthracene	541776	1908	3575	3.07	832	278

Benzo[ghi]perylene	7231205	21057	3755	3.22	739	276
		SOX Sample 1				
Acenapthylene	5793259	37785	850	1.31	878	152
Fluorene	11405068	19886	1000	1.26	845	166
Phenanthrene	6368217	12119	1080	1.28	922	178
Anthracene	saturated(45433700)	8803	1130	1.28	883	89
Fluoranthene	1.06E+08	2845	1325	1.48	892	150
Pyrene	4259269	8883	1370	1.52	883	202
Benz[a]anthracene	45645488	533	1695	1.90	934	186
Chrysene	8550075	1074	1805	**2.02**	777	244
Benzo[b]fluoranthene	3.57E+08	650	2460	2.73	893	227
Benzo[k]fluoranthene	31820302	4187	2520	2.67	929	126
Benzo[a]pyrene	11977882	10960	2640	2.69	892	252
Dibenz[a,h]anthracene	4540970	4366	3480	3.08	832	278
Benzo[ghi]perylene	3647276	8174	3700	3.08	739	278
		AGF Sample 2				
Acenapthylene	276415	6421	855	1.31	889	152
Fluorene	114738	306	970	1.26	845	166
Phenanthrene	435289	1085	1115	1.28	922	178
Anthracene	2023208	5021	1130	1.28	883	178
Fluoranthene	11123535	38261	1325	1.47	892	202
Pyrene	257162	825	1360	1.46	883	220
Benz[a]anthracene	2985943	7719	1690	1.86	934	228
Chrysene	26716159	38730	1810	2.05	777	228
Benzo[b]fluoranthene	9654348	16354	2450	2.62	893	252
Benzo[k]fluoranthene	691048	1155	2515	2.68	929	252
2Benzo[a]pyrene	138112	252	2635	2.70	892	252
Dibenz[a,h]anthracene	275680	340	3345	2.83	832	277
		TRP Sample 2				
Acenapthylene	15968	983	855	1.2	883	152
Phenanthrene	103913	701	1125	1.26	922	178
Fluoranthene	263613	3711	1325	1.45	892	202
Pyrene	257236	3299	1375	1.51	883	202
Benz[a]anthracene	23960	194	1695	1.82	934	228
Chrysene	442900	1855	1815	2.00	777	228
Benzo[b]fluoranthene	185077	711	2445	2.57	893	252
Benzo[k]fluoranthene	274145	1332	2635	2.66	929	252
Benzo[a]pyrene	81585	307	2680	2.65	892	252
Dibenz[a,h]anthracene	877.19	17	3580	3.05	832	278
Benzo[ghi]perylene	113196	363	3755	3.19	739	276
		SOX Sample 2				
Acenapthylene	1067047	14494	855	1.20	897	152
Fluorene	888673	2601	1000	1.26	845	166
Phenanthrene	1194941	4138	1120	1.28	922	178
Anthracene	8676479	24270	1130	1.27	883	178
Fluoranthene	44185546	12998	1320	1.47	892	101
Pyrene	570476	1475	1370	1.52	883	202
Benz[a]anthracene	8565020	21200	1690	1.85	934	228
Chrysene	86425696	5120	1805	2.05	777	200
Benzo[b]fluoranthene	53971642	6642	2450	2.62	893	126
Benzo[k]fluoranthene	1994134	2661	2515	2.66	929	252
Benzo[a]pyrene	914547	1201	2635	2.67	892	252
Dibenz[a,h]anthracene	317853	468	3580	3.07	832	278
Benzo[ghi]perylene	173851	783	3700	3.03	739	278

Table S3: Certified values for dioxins (PCDD/Fs and dl-PBS) and PAHs present in CRM

Dioxins	
Compound name	Concentration (pg/kg)
2,3,7,8-Tetrachlorodibenzo-p-dioxin	1.3×10^5
1,2,3,7,8-Pentachlorodibenzo-p-dioxin	1.9×10^4
1,2,3,4,7,8-Hexachlorodibenzo-p-dioxin	2.6×10^4
1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin	5.6×10^4
1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin	5.3×10^3
1,2,3,4,6,7,8-Heptachlorodibenzo-p-dioxin	8.0×10^5
Octachlorodibenzo-p-dioxin	5.8×10^6
2,3,7,8-Tetrachlorodibenzofuran	3.9×10^4
1,2,3,7,8-Pentachlorodibenzofuran	4.5×10^4
2,3,4,7,8-Pentachlorodibenzofuran	4.5×10^4
1,2,3,4,7,8-Hexachlorodibenzofuran	2.2×10^5
1,2,3,6,7,8-Hexachlorodibenzofuran	9.0×10^4
2,3,4,6,7,8-Hexachlorodibenzofuran	5.4×10^4

1,2,3,4,6,7,8-Heptachlorodibenzofuran	1.0 x 10^6
1,2,3,4,7,8,9-Heptachlorodibenzofuran	4.0 x 10^4
Octachlorodibenzofuran	1.0 x 10^6
PCB 105	2.5 x 10^7
PCB 118	5.8 x 10^7
PCB 156	6.5 x 10^6
PAHs	
Compound name	**Concentration (pg/kg)**
Fluorene	4.8 x10^8
Phenanthrene	5.3 x10^9
Anthracene	9.0 x 10^8
Fluoranthene	8.9 x 10^9
Pyrene	9.7 x 10 ^{9}
Compound name	**Concentration (pg/kg)**
Benz[a]anthracene	4.7 x 10^9
Chrysene	4.9 x 10^9
Benzo[b]fluoranthene	3.9 x10^9
Benzo[k]fluoranthene	2.3 x 10^9
Benzo[a]pyrene	4.3 x 10^9
Dibenz[a,h]anthracene	4.2 x 10^8
Benzo[ghi]perylene	2.8 x 10^9

Table S4: Certified values for dioxins (PCDD/Fs and dl-PBS) in matrix spiking solution

Method 8290 Matrix spiking solution in nonane	**Purity (%)**	**Prepared concentration (ng/ml)**
2,3,7,8-Tetrachlorodibenzo-p-dioxin	99	100
2,3,7,8-Tetrachlorodibenzofuran	99	100
1,2,3,7,8-Pentachlorodibenzo-p-dioxin	99	250
1,2,3,7,8-Pentachlorodibenzofuran	98	250
2,3,4,7,8-Pentachlorodibenzofuran	98	250
1,2,3,4,7,8-Hexachlorodibenzo-p-dioxin	98	250
1,2,3,4,7,8-Hexachlorodibenzofuran	99	250
1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin	98	250
1,2,3,6,7,8-Hexachlorodibenzofuran	99	250
1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin	99	250
1,2,3,7,8,9-Hexachlorodibenzofuran	98	250
2,3,4,6,7,8-Hexachlorodibenzofuran	98	250
1,2,3,4,6,7,8-Heptachlorodibenzo-p-dioxin	99	250
1,2,3,4,6,7,8-Heptachlorodibenzofuran	99	250
1,2,3,4,7,8,9-Heptachlorodibenzofuran	99	250
Octachlorodibenzo-p-dioxin	99	500
Octachlorodibenzofuran	99	500