Experience in South Africa of combining bioanalysis and instrumental analysis of PCDD/Fs

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We outline the experiences and the challenges of optimizing two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GCxGC-TOF-MS) in conjunction with the H4IIE-*luc* bio-assay for analyzing polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in the South African context. Investigating such alternative analytical methods can assist countries with developing economies to meet their obligations under the Stockholm Convention.

Keywords: Bioanalysis; H4IIE-*luc* bioassay; Persistent organic pollutant (POP); Polychlorinated dibenzo-*p*-dioxin (PCDD); Polychlorinated dibenzofuran (PCDF); Soil; South Africa; Stockholm Convention; Time-of-flight mass spectrometry (TOF-MS); Two-dimensional gas chromatography (GCxGC)

1. Introduction

Persistent organic pollutants (POPs), as defined in the Stockholm Convention (SC), are chemicals that are persistent, geographically widely distributed *via* long-range transport, bio-accumulative, and able to cause adverse health and environmental effects [1]. One group of POPs, which are among the most toxic chemicals known to man, causing pleotropic toxic effects in animals {e.g., wasting syndrome, developmental toxicity, changes in lipid metabolism, thyroid function and immunological effects [2]} include polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs). PCDD/Fs have never been intentionally produced, but are formed as unintentional by-products during thermal, chemical and industrial processes. Although PCDD/Fs are produced naturally, the main sources stem from anthropogenic activity.

To analyze the 17 most toxic PCDD/F congeners requires highly sensitive, selective analytical methods [3]. Currently, the only accepted method for the instrumental analysis of PCDD/Fs is

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gas chromatography/high-resolution mass spectrometry (GC-HRMS) [4,5]. Developing countries often do not have access to the full scope of analytical technology that can be found in laboratories of developed countries. GC-HRMS is expensive and requires highly-trained, skilled operators and specialized laboratory infrastructure, which is not available in all countries (e.g., in South Africa, there is no GC-HRMS equipment available for the routine analysis of POPs in the environment).

For certain classes of POPs, only GC-HRMS provides the sensitivity and selectivity required for the determination of individual congeners [4–7], as is the case with PCDD/Fs. This has led to a situation in which samples to be analyzed for PCDD/Fs have to be sent to overseas laboratories. This is not only time consuming, but can also lead to situations where members of the population and biota may experience risk while analytical results are awaited. Also, large amounts of money are spent on permits for samples, transportation, currency exchange and the greater relative cost of overseas human resources, making such analyses very expensive.

A limitation of GC-HRMS is that it relies on selected ion monitoring (SIM) to achieve the limits of quantification (LOQs) necessary for the analysis of certain POPs [8]. The disadvantage of SIM is that it only addresses a selection of compounds targeted for analysis. From a developing nation perspective, it would be advantageous to be able to screen samples for a broader range of compounds (including POPs) simultaneously, which would be less expensive and quicker, as only samples that need further confirmatory analyses need be shipped overseas while preliminary action to reduce exposures or emissions can be locally effected.

The National Metrology Institute of South Africa (NMISA) and the North-West University (NWU), in collaboration with several other institutions, have been implementing methods to screen samples for several classes of POPs. These methods not only screen for a variety of potentially harmful compounds, but also accurately quantify POPs at the concentrations required by statutory organizations in first world countries [9–11]. Such methodologies must be affordable, simple and robust, because, in addition to limited funding, in many developing countries, there is also a shortage of qualified personnel who can routinely conduct these analyses.

To address these challenges, the method implemented was to combine a bioanalytical screening technique with comprehensive, two-dimensional (^{2}D) gas chromatography coupled with time-of-flight mass spectrometry (GCxGC-TOF-MS). GCxGC-TOF-MS provides greater selectivity and sensitivity compared to one-dimensional GC-MS (^{1}D-GC-MS) [12]. The increased selectivity is provided by the increase in chromatographic capacity of the ^{2}D system and the increased sensitivity from the focusing effect of the modulator [4,13–17]. TOF-MS gives the acquisition rate necessary for accurate quantitation with ^{2}D-GC and also provides the full range mass spectra necessary for sample screening for a broad range of analytes in one analytical run [10]. As has been shown by others [10,11], GCxGC-TOF-MS has sufficient LOQs to allow for quantification of PCDD/Fs at environmentally and toxicologically relevant concentrations, so it can be used in regulatory monitoring that is mandated by both the EPA and the EU and it is ideal for sample screening before confirmatory analysis.

Bioanalytical techniques include amongst others, reporter gene bio-assays that are defined as a gene with a measurable phenotype distinguishable from background or endogenous proteins [18]. Dioxin-like chemicals share a common mode of toxicity by binding to the cytoplasmic aryl hydrocarbon receptor (AhR), which is the operating principle of the *in-vitro* H4IIE-*luc* reporter gene bio-assay [19]. Rat hepatoma cells were stably transfected with the firefly luciferase (*luc*) reporter gene under transcriptional control of the dioxin responsive element (DRE) [20–22]. When an AhR ligand (any dioxin-like compound) binds to the receptor, transcription of the reporter gene, luciferase, is initiated [22]. A luminescent signal proportional to AhR-active compounds in the sample is produced once luciferin, salts and ATP are added. The concentration of dioxin-like chemicals is determined by comparing its signal to that of the positive control, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and is reported as bioassay equivalents (BEQ) [19,22,23]. When used in a screening mode, a threshold BEQ can be established. If the threshold is exceeded, subsequent instrumental analysis can identify and quantify the congeners.

Since information is obtained on the overall potency specific to the class of compounds of interest, any compound with the same mode of action is assessed by the bio-assay and therefore provides little to no information on the concentrations of individual compounds responsible for the effects. Bio-assays therefore normally have a screening role when combined with instrumental analytical techniques. Three advantages of screening with a bio-assay are that it provides information on cumulative biological effects of multiple toxic chemicals, it allows ranking according to toxic potential of samples, and it has a reduced cost compared to instrumental analysis [19,20].

We describe the experiences and the challenges of implementing an approach that combines instrumental and bioanalytical approaches in South Africa. GCxGC-TOF-MS and H4IIE-*luc*, which have both been applied successfully in determining PCDD/F concentrations in previous studies [10,11,24,25], were applied in concert as an integrated method for determining PCDD/Fs in the South African environment.

2. Challenges arising from the use of laboratories abroad

The original approach followed for dioxin analysis was that bioanalytical studies were conducted at the NWU, Potchefstroom, South Africa, after preparing the extracts locally. Samples with a BEQ above a pre-determined level where then sent abroad for extraction and analysis. However, this approach was not feasible for large projects due to funding limitations.

Since labor is cheaper locally, the second approach was to send locally-extracted sediment samples to European laboratories for GC-HRMS, congener-specific quantification. This can lead to a ''black-box'' effect where analytical problems (e.g., low recoveries and calibration curves outside sample ranges) went unnoticed. There followed a period attempting to align the extraction and clean-up procedure used by the instrumental laboratory, spiking samples with their internal standards (ISs). However, the time delay resulting from this method-development process led to aging of samples and extracts, sometimes making the data irrelevant to a specific project and unsuitable for refereed publications. Also, the associated costs of transport and analysis were too high to fit within the budgets of research projects.

Another aspect, where delays played a role, was the slow response times experienced from sending samples abroad during an ecological emergency in 2008. At that time, there were sudden mass crocodile mortalities [26] threatening one of the largest naturally-occurring Nile crocodile populations in Southern Africa. It was crucial to determine if POPs could have been playing a significant role in these mass mortalities. However, due to delays, the laboratory data from the first mass deaths were only being received as the second seasonal incidence in 2009 occurred, once again highlighting the need for locally-available analytical capacity, largely independent of overseas facilities.

3. Experimental

3.1. Project outline

Since development of PCDD/F analysis had been on-going at two different institutions within South Africa, it was decided to pool resources and to develop an integrated South African approach to PCDD/F analysis. However, the approach had to work within budgetary, technical and instrumental constraints. The experience gained during this process is illustrated, using a case study, highlighting problems that occurred when initially developing a complex extraction and analysis procedure with limited funds and a lack of experienced personnel. The procedures followed during the bioanalytical and instrumental analysis are detailed below.

3.2. Site selection

Sediment and soil samples were collected from diverse areas of South Africa, such that they covered various land uses and anthropogenic impacts, theoretically representing a spectrum of PCDD/F sources. Sediment was collected from major rivers throughout South Africa (Fig. 1), while soil was collected mainly from industrialized regions that included coal-fired power stations, iron smelting, and petrochemicals manufacture (Fig. 1) and from agricultural and less-developed areas. Sampling procedures were followed as outlined in US EPA Method 1613 [9]. Samples were collected with pre-cleaned stainless-steel equipment, stored in glass containers, frozen immediately after sampling and kept at −20°C until extraction.

3.3. Extraction and clean-up procedures

Prior to analysis, soil was air dried, homogenized and sieved (0.5 mm). Soils and sediments were extracted and underwent clean-up procedures at the NWU according the US EPA methods [9,27–30] for instrumental and biological analysis.

For instrumental analysis, 40 g of soil was mixed with an equal amount of Na_2SO_4 and spiked with 10 μL of $^{13}C_{12}$-labeled IS (100 ng/mL, EPA-1613CSL) and extracted with a mixture of high-purity hexane and dichloromethane (DCM) in an accelerated solvent-extraction (ASE) apparatus [31]. Prior to clean-up, extracts were spiked with 10 μL of EPA-1613 CSS clean-up standard ($^{37}Cl_4$-2,3,7,8-TCDD). Extracts were treated with activated copper to remove sulfur, evaporated to reduce the volume, and then underwent gel permeation chromatography (GPC) and acid digestion with sulfuric acid, followed by sodium chloride and potassium hydroxide washes to remove co-eluting substances [e.g., polycyclic aromatic hydrocarbons (PAHs) and lipids]. Thereafter, samples were filtered through pre-cleaned glass wool covered with Na_2SO_4 to remove residual water and evaporated to a volume of 0.5 mL in iso-octane. This was the final volume used for instrumental analysis. Due to the large mass of sample used, the usual 10–25-μL reconstitution volume could not be used. An IS (1 μL, EPA-1613 ISS) was added to each extract before injection and analysis by GCxGC-TOF-MS.

For the H4IIE bio-assay, the same extraction was followed using 20 g of soil without the use of ISs, as ISs cannot be used in bio-assay samples, since the native and labeled PCDD/Fs will bind to the AhR receptor without bias.

3.4. H4IIE-luc bio-assay

The H4IIE-*luc* cells are rat-hepatoma cells stably transfected with a firefly luciferase gene under control of the dioxin-responsive element developed at the Michigan

Figure 1. A map of South Africa indicating the areas where sediment and soil samples were collected and the cities where the participating laboratories are located.

State University. The H4IIE-*luc* bio-assay method was adapted from the procedure described by Whyte et al. [19]. In short, H4IIE-*luc* cells were cultured at 37°C under an atmosphere of 5% CO_2, >90% humidity in fetal bovine serum supplemented Dulbecco's Modified Eagle's medium. Cells were plated into 96-well micro plates at a concentration of 50,000 cells/well, pre-incubated overnight and treated 24 h after plating with a dilution of either 2,3,7,8-TCDD or sample extract. After 72 h, cells were washed with phosphate-buffered saline, and incubated for 10 min with *LucLite* reagent at 37°C. Luciferase activity was measured [22] with a microplate-scanning luminometer (Microplate Reader FLX 800, Bio-Tek Instruments, Inc.).

3.5. Instrumental analysis

The GCxGC-TOF-MS system (LECO Pegasus 4D, LECO Africa, Pretoria, South Africa) was equipped with an Agilent GC and autosampler, a secondary oven and a dual-stage modulator. The GC parameters, including the multi-step temperature program and MS method, are summarized in Table 1. The detection system was tuned based on the 414 ion from the conventional perfluorotributylamine (PFTBA) mass calibrant. This is different from the standard tuning procedure and is an attempt to improve the signal intensity in the higher mass range [17]. All instrument functions and data processing were managed with the *LECO ChromaTOF* software (version 4.24). Quantitation was performed by measuring peak-area ratios (native/labeled material) and then using the calibration curve or the relative response factor (RRF).

Method viability for instrumental analysis was established by comparing results obtained by GCxGC-TOF-MS with those obtained by GC-HRMS for split samples [11]. A prime consideration in method development was the

Table 1. GCxGC-TOFMS method parameters for Rxi-XLB column set

First-dimension column	Rxi-XLB (30 m × 0.25 mm id × 0.25 μm df)
Second-dimension column	Rtx-200 (2.0 m × 0.18 mm id × 0.20 μm df)
Carrier gas	Helium
Injection mode	Splitless
Injection volume	2 μL
Solvent	Iso-octane
Flow mode	Constant flow
Flow rate	1.0 ml/min
Inlet purge time	60 s
Inlet purge flow	20 ml/min
Inlet total flow	21 ml/min
Inlet temperature	250°C
Oven-equilibration time	0.5 min
^{1}D-column temperatures	80°C for 1 min, ramp at 20°C/min to 220°C, no hold, at 2°C/min to 240°C, no hold, at 1°C/min to 250°C, no hold, at 5°C/min to 260°C, no hold, at 1°C/min to 270°C, no hold, at 5°C/min to 310°C, hold for 2 min
^{2}D-column temperatures	100°C for 1 min, ramp at 20°C/min^{-1} to 240°C, no hold, at 2°C/min to 260°C, no hold, at 1°C/min to 270°C, no hold, at 5°C/min to 280°C, no hold, at 1°C/min to 290°C, no hold, at 5°C/min to 330°C, hold for 2 min
Transfer-line temperature	270°C
Modulator-temperature offset	30°C
Modulation period	4 s
Hot pulse time	1.0 s
Cool time between stages	1.0 s
Acquisition delay	600 s
Start mass	100 amu
End mass	520 amu
Acquisition rate	50 spectra/s
Detector voltage	1 950 V
Electron energy	−70 V
Mass-defect setting	−40 mu/100u
Ion-source temperature	250°C

accurate determination of small concentrations of 2,3,7,8-TCDD. Using the EPA-1613 CVS standard calibration set (0.5–200 pg/μL), a calibration curve was constructed for the 17 congeners. The 2,3,7,8-TCDD calibration curve obtained was linear ($r^2 = 0.99$; slope and intercept: +0.01x and +0.00085) and the average response factor (aveRF) was 1.06.

The capability of the method to achieve the required level of quantitation was investigated as follows: the low-level standard (CS1) was analyzed, and the signal/noise (S/N) ratio for the *m/z* 322 ion for 2,3,7,8-TCDD was calculated to be 20, which is well above the concentration (>10) set by US EPA Methods 1613 and 8290A [9,6]. Even for the least concentration standard (0.5 pg/μL), the chromatographic peak for the *m/z* 322 ion was easily discernible and could be accurately quantified. The limit of detection (LOD) for 2,3,7,8-TCDD was 322 fg on column for spiked sediment samples.

4. Results and discussion

Due to inherent differences between conditions, both environmental and socio-economic, the exposure profiles of POPs and other emerging pollutants in South Africa differ from those in the northern hemisphere (greater concentrations of DDT and lesser concentrations of PCDD/Fs), so different approaches are required. Also, high analytical costs have a detrimental effect on environmental research in Africa. Virtually all available funding is used on targeting chemicals currently listed as POPs and very little is done on candidate POPs. Thus, as a continent, Africa has little influence at the negotiation and decision-making level of the SC on candidate POPs due to the lack of analytical infrastructure and data [32].

Also, South Africa, as other developing countries, is not in a position to address emergencies concerning POPs in the environment (e.g., the case of the crocodiles) or food. These shortcomings could lead to negative influence on human and environmental health and on trade and industry. This emphasizes the need to develop a local analytical capability that will employ regionally relevant methods and generate internationally acceptable results.

4.1. H4IIE-luc bio-assay results

In this study, the H4IIE-*luc* bio-assay indicated that only 22% of sediment (LOD = 103 ng BEQ_{20}; n = 96) and 58% of soil (LOD = 120 ng BEQ_{20}; n = 66) samples

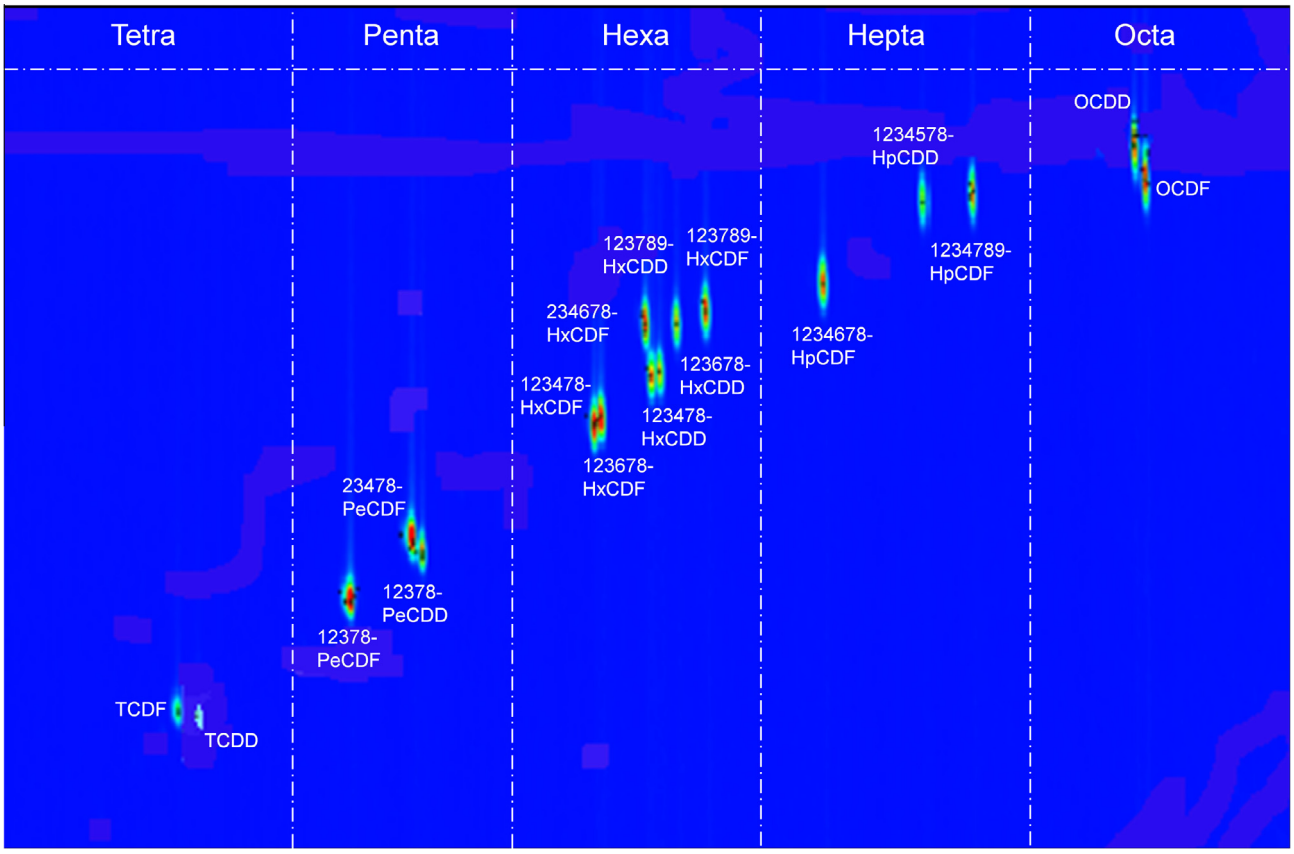

Figure 2. Separation of all 17 toxic congeners of PCDD/Fs on the GCxGC-TOF-MS system used during this study (displayed masses of major ions include 306, 332, 340, 356, 374, 390, 408, 426, 444 and 460).

analyzed had detectable concentrations of dioxin-like chemicals.

BEQ_{20} refers to the relative potency of the sample extract that elicited a 20% response of the TCDD positive control. BEQ_{20}, and not BEQ_{50}, is reported, because it was on average the highest response elicited [33].

For sediment, the sites that were impacted by industry contributed more than 80% to the total number of sites testing positive for the presence of dioxin-like activity (at above LOD), while residential and agricultural sites contributed less than 10%.

For soil, the greatest concentrations were observed in industrialized areas with agricultural and residential areas having concentrations at or near the LOD.

Sediment and soil samples that tested positive and six samples that tested negative for the presence of dioxin-like chemicals were analyzed using GCxGC-TOF-MS. Samples that tested negative were included to ensure that false negatives were not being obtained through the H4IIE-*luc* bio-assay.

4.2. GCxGC-TOF-MS results

In this study, samples were analyzed only for PCDD/Fs and not for dioxin-like PCBs. GCxGC-TOF-MS was used to separate and to quantify 17 toxic PCDD/F congeners (Fig. 2). Preliminary studies using real-world samples indicated that the extraction procedure followed was inadequate for complex samples. Interferences arose from closely-eluting compounds with similar RTs (including PAHs and halogenated aliphatics) that could mask the PCDD/Fs (Fig. 3). Previous studies have shown the concentration of dioxin-like chemicals in South African sediments and soils to be relatively low, often close to the LOD for GC-HRMS [23,34].

An additional issue experienced during the extraction sequence was the loss of IS. This loss was not constant throughout the sample set, which indicated a problem arising during the extraction procedure, rather than with the IS itself or the addition thereof.

A suspected problem area was during the GPC clean-up process, since the sample had to be split to compensate for the high viscosity and high level of suspended solids, after filtration. During injection, approximately 20% of the sample is lost. However, matrix-specific effects may also contribute to the IS loss. These challenges are currently under investigation.

Because of the extraction problems described above, recoveries were not calculated and the rest of the results

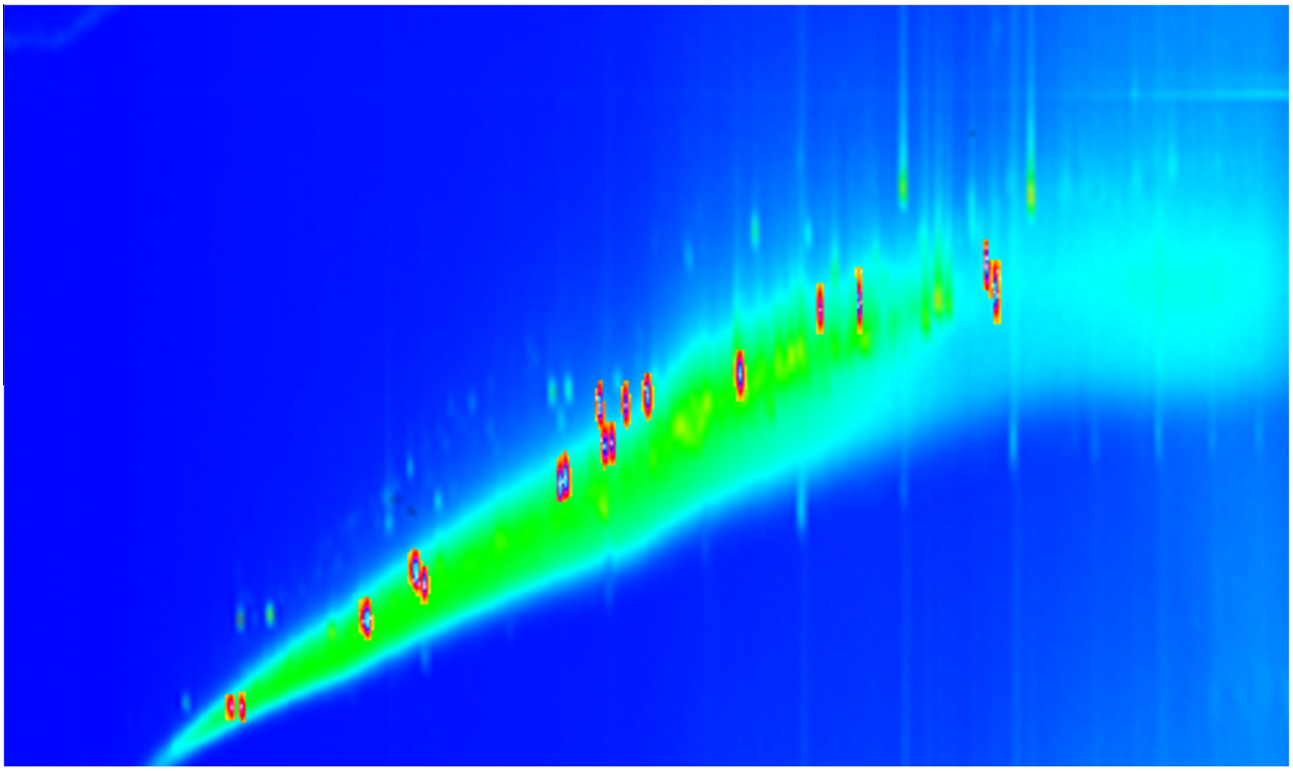

Figure 3. A typical total-ion chromatogram of the sediment and soil samples analyzed. The green band represents a multitude of organic compounds and the red area superimposed on the chromatogram represents the area where the PCDD/Fs occur.

were handled qualitatively rather than quantitatively. The samples that tested positive for the presence of dioxin-like compounds with the H4IIE-*luc* bio-assay were compared to the GCxGC-TOF-MS analysis of the same samples (Fig. 4). During this study the H4IIE assay was used as a screening tool to identify samples with a high AhR-activity. Although this activity could be caused by various dioxin-like chemicals, the assumption was made that a high AhR activity would correspond to an increased likelihood for the presence of PCDD/Fs.

Some of the samples were classified as false positive with H4IIE-*luc* (meaning below instrumental LOD) – 23% for soil and 41% for sediment. False-positive results were not only found at the lowest BEQ_{20} but were spread throughout the BEQ_{20} range (Fig. 4).

As stated earlier, the H4IIE-*luc* bio-assay is not PCDD/F specific. The assay will report the combined effects of all compounds capable of binding to the AhR. These compounds include PAHs, dioxin-like PCBs, flavonoids and other structurally-related compounds [35,36]. In the South African environment, PAHs are the most prevalent AhR inducers, with maximum concentrations previously measured up to 9000 times greater than PCDD/Fs [34], so, although samples did not contain concentrations of PCDD/Fs greater than the LOD, strong inducers of the AhR could have affected the H4IIE bio-assay.

With comprehensive clean-up techniques, interfering compounds could be reduced to a level that would exclude false positives. We are currently working on this issue. When working close to the LOD, every aspect of analytical work must be optimized, and increased baseline stability becomes crucial. This stability is reliant upon clean extracts with minimal interfering sample constituents present [4], emphasizing the need for a reproducible extraction and clean-up method.

4.3. Implementation

The primary objective of this investigation was to develop a combined system of methods for the analysis of dioxin-like chemicals. The H4IIE-*luc* bio-assay proved to be a useful screening tool, reducing the need for instrumental analysis by more than 50%. Previous studies using GC-HRMS as an instrumental analysis tool combined with extraction at the NWU indicated low recoveries with values often less than the LOD (data not shown). The issue of low recoveries could not be resolved, due to problems with method development when done in two different laboratories continents apart, despite samples, extracts and information going back and forth several times. When the analysis using GCxGC-TOF-MS was performed at the South African laboratory (NMISA), it indicated that the problem was

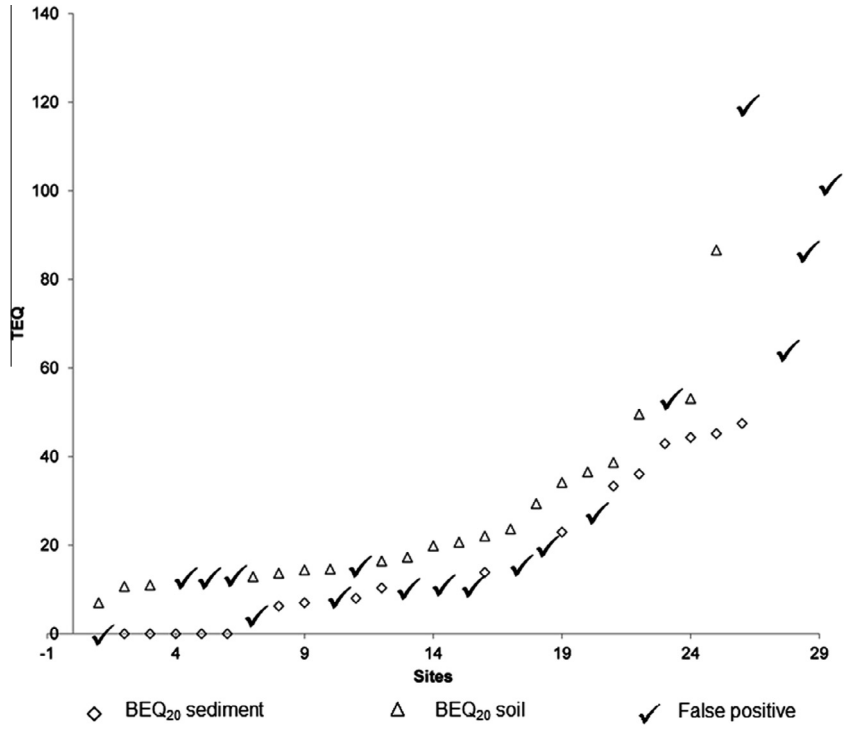

Figure 4. False positives obtained for PCDD/Fs from the H4IIE bio-assay.

associated with the clean-up procedure of the original method. Although acid-digestion treatments should be strong enough to remove PAHs [37,38], the large concentrations of these compounds in the original samples rendered this technique unsuccessful (another indication why samples from other regions may not be compatible with sample extraction and clean-up protocols normally covered in developed countries where the leading laboratories are located). Increased AhR-response was very likely due to organic compounds such as PAHs still present in the extracts.

The following steps are being implemented to address the existing challenges:

(1) to determine concentrations close to the LOD effectively, clean-up procedures should at least include three separation steps, using silica, alumina and carbon column fractions [39];
(2) at present, further sample-extraction and clean-up procedures using the Total Rapid Prep system (TRP-2) system from Fluid Management Systems (FMS) are used to resolve this issue; and,
(3) when conducting the analysis of these compounds within South Africa, problems with the level of standards used were also noticed. The concentrations initially spiked were too large and, during the course of this investigation, an intermediate level was found between detectability and masking.

5. Conclusion

These experiences and identified challenges were invaluable in determining and developing the current capacity for PCDD/F analysis in South Africa. Environmentally-relevant PCDD/F concentrations can be determined by combining the H4IIE-*luc* bio-assay and GCxGC-TOF-MS, demonstrating the first dioxin-analysis capability in South Africa.

GCxGC-TOF-MS is a viable tool for PCDD/F screening and quantitation, suitable for environmental applications where individual PCDD/F concentrations are greater than 1 ng/kg. Although the technique is ideal for application in developing countries where GC-HRMS is not available, and can be used to minimize costs by selecting only positive samples for further overseas analysis by GC-HRMS, experienced analysts are required. GCxGC-TOF-MS also provides full range mass spectra for all sample components, thus allowing for identification of non-target analytes with due consideration of the

sample-preparation steps employed. The combination of these methods can be seen as a cheaper, time-efficient approach suitable for developing economies and will be a very effective method once the sample-preparation issues have been resolved.

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