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Procedures for analysing phenolic metabolites of polychlorinated dibenzofurans, -dibenzo-*p*-dioxins and -biphenyls extracted from a microsomal assay: optimising solid-phase adsorption clean-up and derivatisation methods

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Abstract

A microsomal assay with the enzyme cytochrome P450 offers a good experimental model to study the principles of the metabolism of polychlorinated dibenzofurans, -dibenzo-*p*-dioxins and -biphenyls (PCDFs, PCDDs, PCBs). To study the phenolic metabolites on a gas chromatograph with mass spectrometric or electron-capture detection, some clean-up is necessary to remove coextracted phospholipids and other interferences. In this study, two solid-phase clean-up columns were studied: Florisil and alumina B, as well as a destructive column: an acidic/basic impregnated silica column (ABSC). The elution profile and recovery of a methoxytetrachlorodibenzofuran (methoxy-TCDF) and a dimethoxytetrachlorobiphenyl (dimethoxy-TCB) were determined using pentane or dichloromethane as eluent. For comparison, a TCB and a TCDD were assessed as well. Both Florisil and alumina B yielded satisfactory results for all compounds. The ABSC showed low recoveries for the dimethoxy-TCB. Next both solid-phases were applied on the extracts from microsomal assays with PCDFs and PCDDs. A successive combination of Florisil and alumina B resulted in samples sufficiently purified to allow for low concentration analysis. In addition attention was focused on several derivatisation methods. Methylation with methyl iodide and diazomethane and acetylation in an aqueous phase and an organic phase were studied. For the purpose of the present study the method with methyl iodide suited best.

Keywords: Sample preparation; Derivatization, GC; Microsomal assay; Polychlorinated dibenzofurans; Polychlorinated dibenzo-*p*-dioxins; Polychlorinated biphenyls; Phenolic compounds

1. Introduction

The metabolism of polychlorinated dibenzofurans, dibenzo-*p*-dioxins and polyhalogenated biphenyls (PCDFs, PCDDs, PHBs) can be studied well with an *in vitro* microsomal assay containing cytochrome

P450 (CYP) [1–4]. The microsomal assays are extracted with organic solvents to isolate the metabolites and to study the metabolism kinetics of the studied compounds by gas chromatography with mass spectrometric (GC–MS) or electron-capture detection (GC–ECD). The typical metabolites of such an assay are mono- or dihydroxylated forms of the parent compound with possible chlorine shifts

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(NIH-shift) or even chlorine abstraction [5]. The extracts appeared to contain a matrix which disturbs this type of measurements. For the clean-up of the PCDFs, PCDDs and PCBs numerous methods are available ranging from solid-phase adsorption chromatography to more complex clean-up methods like gel permeation chromatography, HPLC and carbon chromatography [6,7]. For the specific clean-up of their phenolic metabolites from a microsomal assay, various methods have been applied. In some cases the extracts of microsomal incubations received no clean-up [5,8,9]. Several authors used thin-layer chromatography (TLC) or HPLC (C_{18} column) [3,4,10–13]. Some open column solid-phase adsorption methods have also been studied. Materials like silica [9] or Florisil [14] have been used for organohalogen compounds (OHCs) and their methylated phenolic compounds. Destructive methods like treatment with concentrated sulphuric acid have also been reported [10].

One aim of this study was to optimise the clean-up procedure for these metabolites after extraction from a microsomal metabolism assay. It was decided to use solid-phase adsorption techniques since they offer a simple, easily manageable system [7]. The microsomes are endoplasmic reticulum and consist for about 36% of protein and 64% of lipids [15]. These lipids are mainly phosphoglycerides which have a phosphatidyl group, a basic amine group and two lipid chains. Two other important lipid types are sphingolipids which are comparable to phosphoglycerides in their ionic behaviour, and steroids [15]. Since the interfering compounds are extracted from the aqueous phase at $\text{pH} \leq 7.6$, extracted compounds are all neutral and expected to behave acidic in a basic environment.

On the basis of its matrix characteristics alumina B was selected because of its basic character [6]. Florisil (an acidic type solid-phase [6]) was selected because it had been used previously for methoxy-PCDFs [14]. An acidic/basic impregnated silica column (ABSC) was selected in order to compare the former two materials with a destructive one. Firstly elution behaviour of model OHCs was studied on the solid-phase columns using either dichloromethane (DCM) or pentane as eluent. Four compounds were selected to evaluate the elution behaviour: 2,2',6,6'-tetrachloro-4,4'-(OCH_3)₂-biphenyl [$\text{TCB}-(\text{OCH}_3)_2$] and 7- OCH_3 -2,3,6,8-TCDF (7- OCH_3 -TCDF) were

chosen as representatives of derivatised phenolic metabolites thereby assessing the behaviour of two different dipole moments (DP) as well. Two compounds (2,2',4,5'-TCB and 1,2,3,4-TCDD) were chosen as control compounds.

Normally OHCs with phenolic groups are derivatised to improve their chromatographic characteristics on solid-phase and in GC. Derivatisation is necessary to overcome the high reactivity of the phenolic group with the solid-phase and the injection system, pre-column and stationary phase of the GC [6]. Therefore, the efficiency of four derivatisation methods was evaluated. Two methods of methylation and two methods of acetylation were studied. Factors taken into account were: recovery, identification and quantitation potential from fragmentation patterns and convenience of application.

Finally, microsomal assays were performed with PCDFs and 1,2,3,4-TCDD and extracted with organic solvents. The effects of the clean-up were studied by comparing chromatograms of extracts which were passed over one or more solid-phases.

2. Experimental

2.1. Chemicals and reagents

Florisil (grain size: 0.150–0.250 mm), diisopropyl ether (analytical-reagent grade), acetic acid anhydride (95% pure) were obtained from Merck. Alumina: Alumina B Super I was obtained from ICN Biomedicals, Eschwege, Germany, 3-Cl-4-OH-biphenyl (MCB-OH) from Fluka. 2,3,6,8-TCDF, 1,3,6,7-TCDF, 7- OCH_3 -TCDF and 4- OCH_3 -1,3,6,7-TCDF were a kind gift from Dr. H. Kuroki, Daiichi College of Pharmaceutical Sciences, Fukuoka, Japan. All other chemicals were of the highest quality available [16]. A standard mixture solution (SMS) was made from 2,2',4,5'-TCB, 1,2,3,4-TCDD, $\text{TCB}-(\text{OCH}_3)_2$ and 7- OCH_3 -TCDF in trimethylpentane (TMP). An internal standard solution (IS) consisted of 2,2',3,3'-TCB, 1,2,7,8-TCDD and 4- OCH_3 -1,3,6,7-TCDF in TMP.

2.2. Florisil

Florisil was selected because it has been used for the PCDF metabolites we are interested in [14]. A

second advantage was that matrix compounds might irreversibly bind to the solid-phase [6]. It was activated by heating at 600°C for 14 h [17]. Next, it was stored in a water-free environment until use. Prior to use, it was deactivated with 10% (w/w) water. A 5 ml Gilson pipet tip was filled with glass wool and 0.8 g of deactivated Florisil. After rinsing with 8 ml of pentane, 1 ml of SMS was applied and subsequently eluted with different volumes of pentane and/or DCM. All fractions were collected. After addition of the IS, the samples were concentrated. To assess the reproducibility of activation, a second batch was activated and the elution of the OHCs was checked.

2.3. Alumina

Alumina B was chosen to represent a basic solid-phase interacting with the acidic groups of the extracted lipids. In addition this type of solid-phase has scarcely been used in literature on OCH₃-PCDFs and hence it would be interesting to describe the behaviour of the metabolites on this type of column. The alumina was activated by heating at 120°C for 12 h, and stored in an anhydrous environment. Prior to use, it was deactivated with 10% (w/w) water. A glass tube (diameter 6 mm) was filled with 2 g of alumina and washed firstly with 10 ml of DCM and then with 10 ml of pentane prior to applying the sample (all according to Kannan et al. [18]). One ml of SMS was applied to the alumina column and subsequently eluted with different volumes of pentane or DCM. All fractions were collected. After addition of IS, the samples were concentrated and measured.

2.4. ABSC

Twenty-two g of H₂SO₄ (98%) was mixed with 78 g of activated silica. Thirty-three g of 1 M NaOH was mixed with 67 g of activated silica. A glass tube (diameter 6 mm) was filled with either 0.5 g of acidic silica or 0.5 g of basic silica or both, separated by 0.5 cm activated silica (the acidic silica on top). The SMS was applied to the column and eluted with 20 ml of pentane. When both acidic and basic silica were present, a second fraction was eluted with 20 ml of pentane. All fractions were collected. After addition of IS, the samples were concentrated.

2.5. Methylation

2.5.1. Methyl iodide method

Methylation was performed according to Tulp et al. [19,20]. One ml of TCB-(OH)₂ in ethanol was dissolved in 50 ml of acetone, 2.6 g K₂CO₃ and 2 ml methyl iodide were added. The mixture was refluxed under stirring for at least 6 h. Next, 1 ml of TMP was added and the mixture was evaporated using a water bath and vigreux. The remaining TMP was filtered over 0.1 g silica (25% water). The filter was rinsed with pentane which was collected.

2.5.2. Diazomethane method

Diazomethane was prepared according to Vogel [21]. Subsequently 1 ml of a solution of diazomethane in diethylether was added to 1 ml TCB-(OH)₂ in TMP. After incubation overnight, the samples were concentrated and measured.

2.6. Acetylation

Aqueous phase method: 6 ml of NaKHPO₄ buffer (0.1 M, pH 7.60), 1 ml 1 M NaOH, 1 ml of acetic acid anhydride and 100 μl of a solution of MCB-OH in ethanol were mixed. The mixture was stirred for 5 h at 37°C. Next, the mixture was extracted with three aliquots of 10 ml DCM. All three aliquots were combined.

Organic phase method: 100 μl of a solution of PCB-OH in TMP was added to 3 ml hexane, 1.2 ml triethylamine (0.1 M in hexane) and 150 μl acetic acid anhydride. The mixture was shaken for 45 min. The excess of acetic acid anhydride was removed by extracting with 4 ml of NaKHPO₄ buffer (0.1 M, pH 6.0).

2.7. GC-MS/ECD

GC measurements were performed on a HP 5980 series II GC coupled to either an ECD system or the HP 5970 mass-selective detector using a 30 m×0.25 mm I.D. DB-5 column. The GC-ECD system was equipped with an A200S autoinjecting system (Carlo Erba), injecting 1 μl cold on column. The GC-MS system was injected manually with volumes of 1 to 2 μl cold on column. Either the full scan mode or the single ion monitoring (SIM) mode was used (ionisation energy: 70 eV) [16].

2.8. Quantitation

Quantitation by GC–ECD was performed with a calibration line covering 10–125% of the starting concentrations. Linearity of the calibration was checked. The maximum acceptable deviation was set at 10%. Only when samples could be quantitated in the linear area, quantitation was approved and used [16]. Since MS was linear in response over the entire range used, quantitation by GC–MS was performed with only one quantitation solution. Deviation between injections of one sample was typically 3% [16].

2.9. Semiempirical calculations

DP was calculated by a semiempirical quantum mechanical modelling method (AM1 Hamiltonians, [22], MOPAC 7.0). Initially, molecular structures were drawn and geometrically optimised in SYBYL 6.1. Next the structures were transferred to MOPAC 7.0 where the heat of formation was calculated together with geometrical structure, molecular orbitals and DP (Table 1).

2.10. Microsomal assay

Microsomal assays were performed with either microsomes from β -naphthoflavone induced rats (2,3,6,8-TCDF and 1,3,6,7-TCDF assays) or isosafrole induced rats (1,2,3,4-TCDD) according to [23]. The TCDF assays were performed in 1 ml containing 1 mg microsomal protein. After 10 min incubation, the assays were stopped with methanol. TCB-(OH)₂

was added as internal standard and the assay was extracted with diisopropyl ether [4,23]. The 1,2,3,4-TCDD-assay was performed in 5 ml containing 5 mg microsomal protein. After 4 h of incubation, the assays were stopped with 1 ml 3 M HCl and hydrolysed at 80°C for 2 h. TCB-(OH)₂ was added as internal standard and the assay was extracted with DCM. When the extracts were measured by GC–ECD, the extracts were concentrated and transferred to the solid-phases. When the extracts were measured by GC–MS, the extracts were concentrated, methylated [19] and transferred to the solid-phases.

3. Results and discussion

3.1. Florisil column

SMSs were transferred to a prewashed Florisil column. The OHCs were eluted with pentane or hexane followed by DCM. 2,2',4,5'-TCB eluted readily with pentane as expected (Table 2). Oxygen containing OHCs could not as easily be rinsed from the Florisil column. For TCB-(OCH₃)₂ a high recovery could be attained after elution of 26 ml of pentane. TCDD and especially 7-OCH₃-TCDF appeared tightly bound to the solid-phase (Table 2).

The elution profile with hexane following DCM is given in Table 3. The SMS was applied to the Florisil column. Subsequently, 3 ml of hexane were passed through to imitate three rinse steps of the vial which contained the sample. Elution was continued with DCM. Most OHCs appeared to have eluted completely after a regime of 1 ml of TMP, 3 ml of hexane and 4 ml DCM (Table 3). However, after passing an additional 2 ml DCM, another substantial amount of 7-OCH₃-TCDF was recovered (13%, Table 3) indicating that 6 ml DCM should be applied for satisfactory recoveries. As compared to Burka et al. [14] who also used Florisil clean-up and DCM elution, more DCM was necessary to elute all OHCs indicating a higher activity of the Florisil used in the present study. The differences in eluent volume between pentane and DCM can readily be explained by the characteristics of the solvents. DCM has a more pronounced DP as well as an inducible DP [24,25]. Therefore it is the strongest eluent, resulting in lower eluent volumes required for complete

Table 1
Dipole moment (DP) of the used organohalogen compounds (OHCs)

OHC	DP ^a
2,2',4,5'-TCB	1.179
1,2,3,4-TCDD	2.409
TCB-(OCH ₃) ₂	1.809
7-OCH ₃ -TCDF	3.675

2,2',4,5'-tetrachlorobiphenyl = 2,2',4,5'-TCB; 1,2,3,4-tetrachlorodibenzo-*p*-dioxin = 1,2,3,4-TCDD; 2,2',6,6'-tetrachloro-4,4'-(OCH₃)₂-biphenyl = TCB-(OCH₃)₂; 7-OCH₃-2,3,6,8-tetrachlorodibenzofuran = 7-OCH₃-TCDF) as calculated with the semiempirical method AM1 in MOPAC (93.0) [22].

^a In Debye units.

Table 2
Recovery of the elution of a mixture of OHCs (SMS) from a Florisil column with subsequent fractions of pentane

Fraction ^d	OHCs							
	2,2',4,5'-TCB ^a		1,2,3,4-TCDD ^b		7-OCH ₃ -TCDF ^c		TCB-(OCH ₃) ₂ ^c	
	%	SD	%	SD	%	SD	%	SD
0–10	109	4.4	34	12.7	6	5.7	51	10.8
10–14	0.2	0.1	23	4.2	10	4.4	34	8.6
14–18	0		17	4.1	12	1.4	15	2.3
18–22	0		10	2.4	12	0.2	4	0.9
22–26	0		7	1.3	11	2.2	1	0.7
Σ	109		91		51		105	

For each compound, the different fractions were summed for mass balance control (given as Σ), $n=3$, mean recoveries are given with standard deviations (SD), abbreviations as in Table 1.

^a Internal standard: 2,2',3,3'-tetrachlorobiphenyl.

^b Internal standard: 1,2,7,8-tetrachlorodibenzo-*p*-dioxin.

^c Internal standard: 4-OCH₃-1,3,6,7-tetrachlorodibenzofuran.

^d In ml.

elution. The binding of OHCs to Florisil is based on molecular interactions involving DP and binding with active groups at the solid-phase surface [6]. The longer retention of 7-OCH₃-TCDF can be explained by its higher DP (Table 1).

Because of these findings, it was decided to use DCM in the remaining part of the present study. The following protocol was adopted for the clean-up of microsomal extracts with the Florisil column. Firstly the extract of the microsomal assay is concentrated to a volume of 1 ml TMP. TMP is normally added to an extract because it is not easily evaporated during concentration on a warm water bath. This prevents the extracts from running dry which may lead to excessive losses of OHCs. After transfer of the sample to the Florisil, the sample vial is rinsed 3

times with 1 ml of hexane. Pentane is not recommended for rinsing since it evaporates substantially during the action. Hexane has a comparable elution behaviour to pentane [24,25]. Finally the analytes are eluted from the column with 6 ml of DCM.

Florisil has to be activated by heating for at least 6 h at 420°C [17]. This relatively high temperature is necessary to cope with its highly variable water-absorbing behaviour. In this process much variation can occur. Steps to improve standardisation were utilisation of the same oven, higher activation temperature (600°C) and increasing the activation time from 6 to 14 h. The latter was applied to reduce the relative contribution of small deviations in activation time [7]. To check the reproducibility, a second

Table 3
Recovery of the elution of a mixture of OHCs from a Florisil column with an initial application of 1 ml SMS, 3 ml of hexane to imitate three rinse steps of the sample containing vessel after application and subsequent fractions of DCM

Fraction ^a	OHCs							
	2,2',4,5'-TCB		1,2,3,4-TCDD		7-OCH ₃ -TCDF		TCB-(OCH ₃) ₂	
	%	SD	%	SD	%	SD	%	SD
0–8	113	5.0	105	4.7	99	7.3	102	6.2
8–10	0.3	0.2	8	1.1	13	1.4	7	1.1
Σ	113		113		112		109	

The application, rinse step and first 4 ml DCM were considered as one fraction. For each compound, the different fractions were summed for mass balance control (given as Σ), internal standards as in Table 2, $n=3$.

^a In ml.

batch was activated and the elution of the OHCs was checked. Elution and recoveries were similar (data not shown). It is advisable to check the elution profile of each newly activated batch of Florisil [7].

3.2. Alumina column

The SMSs were transferred to a prewashed alumina column. Next, the compounds were eluted by either pentane or DCM. Table 4 shows the elution profiles of the OHCs. When using pentane all compounds were eluted almost completely after 40 ml. For this combination of alumina B and eluent, PCBs are recovered quantitatively after 17 ml pentane or hexane [18]. In a pilot study similar results were obtained (data not shown). Also 1,2,3,4-TCDD had completely eluted after 20 ml. The methoxy-compounds, however, required up to 40 ml to attain 100% recovery. The analytes of interest started to elute from the column in the first mls (pilot study, data not shown). Therefore it appeared impossible to divide this 40 ml into smaller fractions.

With DCM, less solvent was necessary to elute the OHCs (10 ml for the TCB and TCDD, 15 ml for the methoxy compounds, Table 4). Because this solid-phase has a basic character, it was decided not to saturate the basic character with the acidic DCM

[24,25]. The neutral alkanes are a better option. The following application strategy with the alumina column was adopted: application of the sample in a minimum volume of TMP, rinsing the sample vial three times with 0.5 ml of hexane, and elution of all compounds with 39 ml of pentane.

3.3. ABSC

SMSs were applied on a destructive ABSC and eluted with hexane (Table 5). As was to be expected both 2,2',4,5'-TCB and 1,2,3,4-TCDD eluted after 20 ml with recoveries of at least 84% [18]. From TCB-(OCH₃)₂, on the contrary, nothing could be recovered. Next, this compound was passed over a separate acidic silica and a separate basic silica column (Table 5). The reduced recovery in the combined system is clearly due to the acidic part of the column. Tulp et al. [10] also noticed degradation of OCH₃-PCBs after sulphuric acid treatment. Therefore it was decided not to use any acidic treatment with the derivatised compounds. These results could help to explain why it has been difficult to demonstrate phenolic metabolites from PCBs in environmental samples. ABSC and sulphuric acid treatment still belong to the commonly applied clean-up procedures.

Table 4

Recovery of the elution of SMS from an alumina column with subsequent fractions of pentane or DCM

	OHCs							
	2,2',4,5'-TCB		1,2,3,4-TCDD		7-OCH ₃ -TCDF		TCB-(OCH ₃) ₂	
	%	SD	%	SD	%	SD	%	SD
<i>Fraction pentane^a</i>								
0–40	112	4.9	114	0.6	104	1.5	115	1.5
40–50	0.3	0.5	0.3	0.4	0.2	0.3	0.3	0.5
50–55	0		0		0		0	
Σ	112		114		104		116	
<i>Fraction DCM^a</i>								
OHCs								
0–10	89	0.9	110	10.0	87	11.1	93	19.1
10–15	1	0.2	1	0.2	2	0.4	2	0.6
Σ	90		111		89		95	

For each compound, the different fractions were summed for mass balance control (given as Σ). Internal standards as in Table 2, *n*=3, abbreviations as in Table 1.

^a In ml.

Table 5
Recoveries of three OHCs from an acidic–basic impregnated silica column (ABSC), acidic silica or basic silica column eluted with pentane

Column	Fraction ^a	OHCs					
		2,2',4,5'-TCB		1,2,3,4-TCDD		TCB-(OCH ₃) ₂	
		%	SD	%	SD	%	SD
ABSC	0–20	93	5	83	6	0	0
	20–40	0.2	0.1	0.6	0.8	0	0
Acidic silica	0–20	n.a. ^b		n.a.		2.3	0.3
Basic silica	0–20	n.a.		n.a.		100	1

Internal standards as in Table 2 (except TCB-(OCH₃)₂; internal standard as 1,2,3,4-TCDD), *n*=3 in all cases (abbreviations as in Table 1).

^a In ml.

^b Not analysed.

3.4. Derivatisation

Four methods of derivatisation were tested. The methods were judged on basis of recovery, characteristics of the resulting fragmentation pattern and convenience of use. The results of the different methods of derivatisation are given in Table 6. Only two methods yield fully derivatised compounds: the methylation with methyl iodide and the acetylation in the aqueous phase.

3.4.1. Methylation

The fragmentation pattern of TCB-(OCH₃)₂ observed in the present study, did not completely follow the rules as set by Tulp et al. [19]. The expected [M]⁺ (95%) and [M–15]⁺ (M–CH₃, 100%) fragments were present as major peaks. In addition, however, an unexpected [M–30]⁺ (6%) could be seen. Most likely this is the fragment resulting from dissociation of two –CH₃ groups from the [M]⁺ fragment. In the fragmentation pattern of MCB-OCH₃, the [M]⁺ was the 100% peak and [M–15]⁺ reached an intensity of 75%. We cannot

explain the differences between methyl iodide and diazomethane recoveries. Other researchers using PCBs-OH as internal standard had mean recoveries of 86% after derivatisation with diazomethane and full sample clean up [26]. Sandau and Norstrom [27] found that diazomethane had a lower yield than an ion pair methylation method using methyl iodide. This is more in agreement with our results. Others [3] applied a triplicate addition of diazomethane on TCDF-OH with at least 1 h incubation time in between. In principle, this strategy should be useless. After the first addition, diazomethane is still present in such an excess that another addition should not lead to an increase in the yield of derivatised products. Taking personal safety, convenience [6] and yield into account, we conclude that methyl iodide is the better method for methylation.

3.4.2. Acetylation

The fragmentation pattern of the acetylated MCB-OH (MCB-OAc) was characterised by a [M]⁺ peak which was only 5% of the 100% peak [M–42]⁺. Other fragments were of negligible importance. The fragment [M–42]⁺ is most likely a McLafferty rearrangement in which –COCH₃ donates a H-atom to the positively charged aromatic ring (Fig. 1). Next, –COCH₂ is released and a keto group is formed on the aromatic ring. Also TCB-(OAc)₂ demonstrated a very pronounced [M–42]⁺ peak with a few other, but negligible fragments. From the two acetylation methods, only the aqueous phase method yielded satisfactory recoveries (Table 6). This method also yielded satisfactory results in our

Table 6
Recovery percentages of several derivatisation methods

Derivatisation	Method	Compound	Mean	SD
Methylation	Methyl iodide	TCB-(OH) ₂	96	12.0
	Diazomethane		39	7.0
Acetylation	Aqueous phase	MCB-OH	101	8.3
	Organic phase		67	8.9

2,2',6,6'-tetrachloro-4,4'-(OH)₂-biphenyl= TCB-(OH)₂; 3-monochloro-4-OH-biphenyl= MCB-OH, *n*=4 in all cases except acetylation in the aqueous phase; *n*=3.

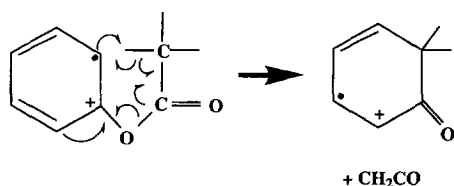


Fig. 1. MacLafferty rearrangement of acetylated phenol after electron impact ionisation.

laboratory with other compounds. The compound 1,2-dihydroxybenzene which is much more difficult to derivatise because of steric hindrance, was also completely derivatised. The organic phase method was not able to do so (unpublished data). Hence, of the two acetylation methods, the aqueous phase method in alkaline water appeared to function best.

The choice for methylation or acetylation depends on circumstances. Four aspects were considered: availability of original compounds, convenience of application, identification on the basis of both retention time and fragmentation pattern, characterisation of compounds for which no original standard is available and quantitation.

For PCBs, PCDDs and PCDFs, phenolic metabolites are usually synthesised in their methylated form and therefore only thus available [14,19,20,26,28]. Identification on the basis of retention time in combination with the specific fragmentation pattern usually gives no problems. The combination of methylation and electron impact MS is often used to obtain a characteristic fragmentogram. Fragmentation patterns of unidentifiable OCH_3 -PCBs and OCH_3 -PCDDs can even provide information on the position of the methoxy group. Unfortunately this is not valid for OCH_3 -PCDFs [19,20]. Exact quantitation of OCH_3 -PCDFs, for which no original standard is available remains impossible. In our case, the fragmentation patterns are usually characteristic and different from other available standards. Therefore it is difficult to choose an appropriate standard when trying to estimate the quantity of an unidentifiable compound. A reasonable approach is to monitor the characteristic fragments by SIM of the unidentifiable OCH_3 -PCDF and quantitate each fragment with the average fragment response of available OCH_3 -PCDFs. The obtained amounts per fragment can be averaged again to a final mean amount.

Acetylation is preferable when original phenolic standards are available and identification can be done by retention times. A major advantage is its high convenience. A second advantage seems to be the dominant MacLafferty rearrangement which generates only one dominating peak. This would enable the use of available isomers to quantitate compounds encountered in samples for which no standards are available. A disadvantage is the low intensity of the $[\text{M}]^+$ ion, reducing convenience of characterisation and identification. In conclusion, because of the availability of methylated standards of the phenolic PCDFs and because of the better possibility for characterisation, methylation is preferable in our case.

3.5. Application to microsomal assays

To test the clean-up, microsomal assays with 1,3,6,7-TCDF, 2,3,6,8-TCDF and 1,2,3,4-TCDD were performed. The extracts of the 1,2,3,4-TCDD assay were either methylated or not. Non-methylated extracts received a Florisil cleanup. Those samples were measured by GC-ECD. Also an alumina cleanup was applied on similar samples. In both cases, the GC-ECD chromatograms were apparently clean without any additional peaks (data not shown).

When the extracts from all three types of assay were methylated and measured by GC-MS, we observed a marked difference in clean-up. The GC-MS runs were performed in SIM mode (using $[\text{M}]^+$, $[\text{M}+2]^+$, $[\text{M}-15]^+$, $[\text{M}-13]^+$, $[\text{M}-43]^+$, $[\text{M}-41]^+$) for the OCH_3 -TCDFs. The combination of $[\text{X}]^+$ with $[\text{X}+2]^+$ was chosen to monitor the isotope ratio which can be expected from a tetrachlorine component: $[\text{M}]^+ : [\text{M}+2]^+ : [\text{M}+4]^+ : [\text{M}+6]^+ = 77:100:49:11$. The combination $[\text{M}]^+$, $[\text{M}-15]^+$, $[\text{M}-43]^+$ represents the major fragmentation of OCH_3 -TCDFs: $[\text{M}]^+$, $[\text{M}-\text{CH}_3]^+$, $[\text{M}-\text{COCH}_3]^+$ [14,19,20]. Another fragment to consider could be $[\text{M}-106]^+ = [\text{M}-\text{C}_2\text{ClO}_2\text{CH}_3]^+$ [14,16].

The SIMs of $[\text{M}]^+$ and $[\text{M}+2]^+$ (masses 333.9 and 335.9) of possible OCH_3 -TCDFs are shown after applying Florisil and alumina B clean-up (Fig. 2) and after Florisil clean-up only (Fig. 3). The SIMs of Fig. 2 could be used to assign metabolites using the following criteria: (i) peak not present in blank samples, (ii) peak present at all fragments, (iii)

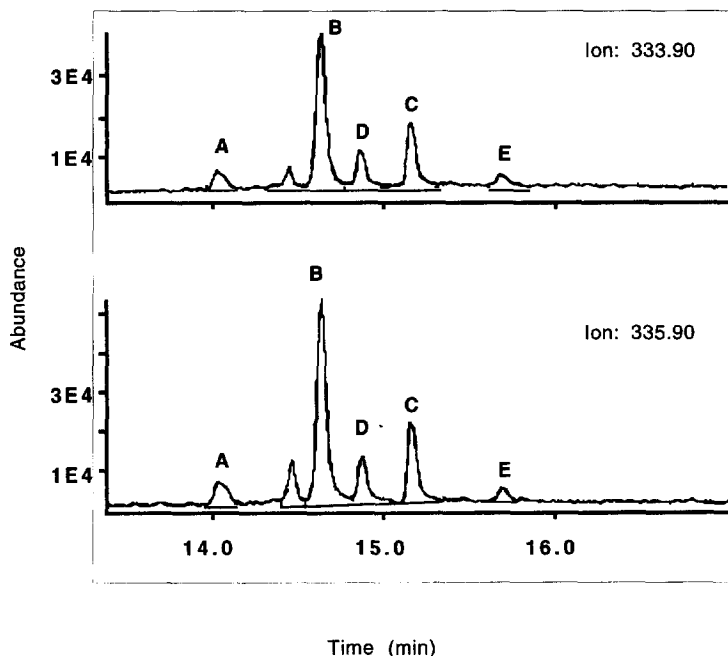


Fig. 2. Fragmentograms of $[M]^+$ and $[M+2]^+$ ions of methylated phenolic metabolites of 1,3,6,7-TCDF. A microsomal assay was performed and extracted with diisopropyl ether as described in Section 2.10. After methylation, the extract received a Florisil and an alumina B clean-up procedure; final volume 1 ml. Peaks A, B, C, D, E showed a consistent chlorine pattern for the $[M]^+$ (shown here), $[M-15]^+$ and the $[M-43]^+$ (both not shown) fragment and are therefore assigned to be metabolites of 1,3,6,7-TCDF. These peaks were not encountered in blank samples which were run with inactive CYP [23]. Analyses were performed on a HP 5970 EI MSD, ionisation energy: 70 eV.

proper tetrachlorine abundance ratio. The theoretical ratio is $[M]^+:[M+2]^+=77:100$. In general we consider ratios between 70:100 and 85:100 still acceptable. On this basis the peaks marked A to E (Fig. 2) were assigned to be metabolites. They could not be identified due to a lack of relevant standards. As compared to Fig. 2, the background in Fig. 3 (only Florisil clean-up) was high and irregular, hampering identification. Also more interfering peaks were present. Peaks A, B and C could represent correspondingly marked metabolites as in Fig. 2 although they did not fulfill the constraints of the tetrachlorine pattern.

After concentrating the extracts to a volume of 500 μ l, full scans were made for several other types of assays as well. Full scans with the MS (100–400 m/z) in the time range of the metabolites showed a decrease in total abundance between samples with only Florisil clean-up (Fig. 4) as compared to both Florisil and alumina clean-up (Fig. 5). A similar decrease in background can be seen for an assay

performed with 1,2,3,4-TCDD. TCB-(OH)₂ was added as an internal standard. The same sample is shown either after Florisil clean-up only (Fig. 6) or after a successive clean-up on both solid-phases (Fig. 7). The level of the total ion abundance is decreased from 10^7 to $<10^6$. TCB-(OCH₃)₂ as well as the metabolite (7-OCH₃-1,2,3,4-TCDD, [20]) are much more pronounced peaks in Fig. 7. The procedure of performing a microsomal assay, adding TCB-(OH)₂ as an internal standard, extraction, methylation and application to both solid-phases resulted in recoveries ranging from 70 to 90%.

4. Conclusions

In the current paper, methods for the clean-up of PCDF-OHs extracted from a microsomal assay have been evaluated. These involved chromatographic and derivatisation procedures. A satisfactory procedure could be established.

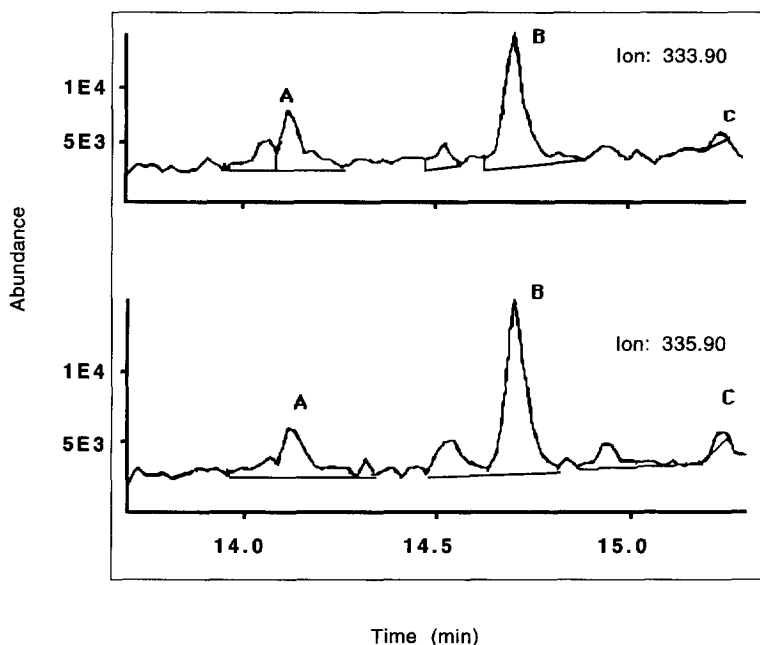


Fig. 3. Fragmentograms of $[M]^+$ and $[M+2]^+$ ions of methylated metabolites of 1,3,6,7-TCDF. The derivatised microsomal extracts (as in Fig. 2) received only a Florisil procedure; final volume 1 ml. Peaks A, B, C could presumably be metabolites since they were also encountered in the SIMs as shown in Fig. 2. They showed, however, no consistent chlorine pattern for the $[M]^+$, $[M-15]^+$ and the $[M-43]^+$ fragments (analysis as in Fig. 2, note the differences in time scale from Fig. 2).

Both Florisil and alumina columns showed full recovery of applied model OHCs. The elution scheme with the Florisil column was: sample (200–1000 μ l volume), 3×1 ml of hexane to rinse the sample vial, 6 ml of DCM, collecting all eluent. The elution scheme for alumina was: sample (200–1000 μ l volume), rinsing the sample vial with 3×0.5 ml of

hexane, next elution with 39 ml of pentane to elute all compounds. Methylated compounds like TCB- $(OCH_3)_2$ could not be recovered from a destructive ABSC. Therefore the use of destructive methods for methoxy-compounds is not recommended. Concerning methylation, methyl iodide offers a better recovery and more convenience for derivatising

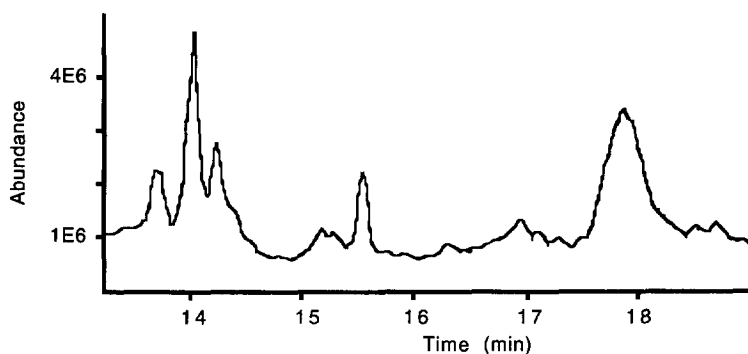


Fig. 4. Total ion chromatogram of a full scan of a diisopropyl ether extract of a microsomal assay with 2,3,6,8-TCDF using the same microsomal batch and protein concentration as in Figs. 2 and 3. After derivatisation, the extract only received a Florisil clean-up procedure; final volume 500 μ l (analysis as in Fig. 2).

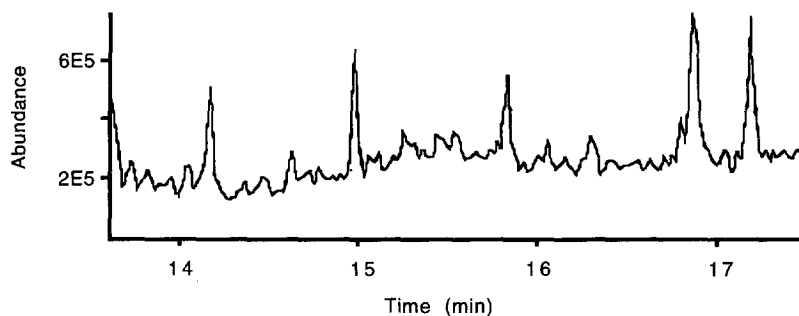


Fig. 5. Total ion chromatogram of a full scan of a diisopropyl ether extract of a microsomal assay with 1,3,6,7-TCDF using the same microsomal batch and protein concentration as in Figs. 2 and 3. The extract received a Florisil and alumina clean-up procedure; final volume 500 μ l (analysis as in Fig. 2).

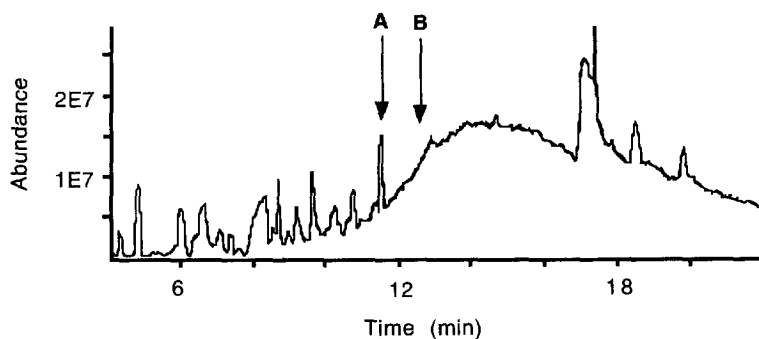


Fig. 6. Total ion chromatogram of a full scan of a DCM extract of a microsomal assay with 1,2,3,4-TCDD using an isosafrole induced microsomal batch and the same protein concentration as Figs. 2 and 3. The extract only received a Florisil clean-up procedure. Peak A: TCB-(OCH₃)₂, Peak B: 7-(OCH₃)-1,2,3,4-TCDD (identified with SIMs using the relevant principal fragments [19,20], analysis as in Fig. 2).

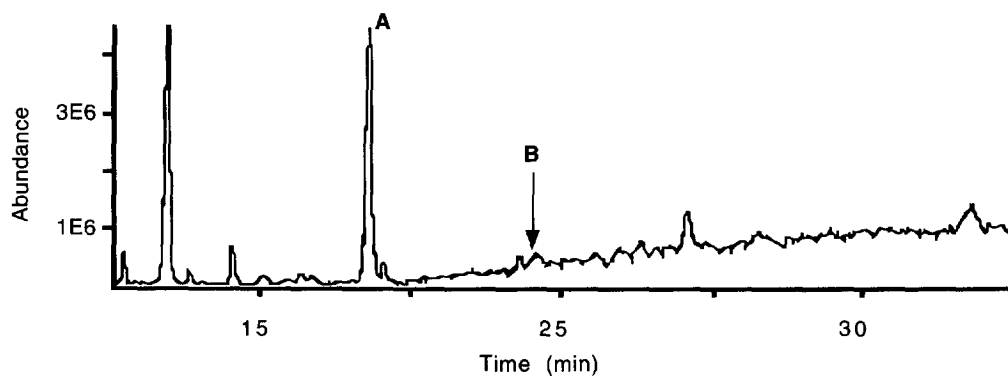


Fig. 7. Total ion chromatogram of a full scan of the same sample as in Fig. 6 but now after successive Florisil and alumina clean-up procedure. Peak A: TCB-(OCH₃)₂, Peak B: 7-(OCH₃)-1,2,3,4-TCDD (analysis as in Fig. 2). Differences in retention times were caused by differences in temperature program.

PCB-OHs, PCDD-OHs and PCDF-OHs than diazomethane. Acetylation in an alkaline aqueous environment with acetic anhydride is also well applicable with high recoveries and high convenience. In the clean-up of a microsomal assay, utilizing Florisil was sufficient to be able to measure OHCs in high concentrations, e.g., when only the decrease of the PCDF, PCDD or PCB is followed. For monitoring of metabolites which are present in low amounts, a sequence of Florisil and alumina clean-up is necessary to obtain a sufficiently low background and a reduced number of interfering peaks.

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