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Integrating gel permeation chromatography clean-up in the analysis of metabolites of polychlorinated biphenyls, dibenzo-*p*-dioxins and dibenzofurans extracted from a microsomal assay A comparison of different mobile phases

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Abstract

The metabolism of polychlorinated biphenyls, dibenzo-*p*-dioxins and dibenzofurans (PCBs, PCDDs, PCDFs) can be studied well with a cytochrome P450 containing microsomal assay. In the present study, the residues of microsomal assays were extracted with organic solvents to determine the metabolites of the studied compounds with GC–ECD and GC–MS. Extracts of microsomal assays contained a matrix which interferes with these type of measurements. The matrix was hard to remove completely with solid phase adsorption chromatography. To overcome the problem of the interfering compounds, clean-up properties of a gel permeation chromatography system were studied for this specific matrix. The clean-up was firstly studied with model compounds and two different mobile phases. Thereupon the most appropriate mobile phase was applied to the extracts of a microsomal assay. Acetone and cyclohexane–dichloromethane (CH–DCM, 1:1, v/v) were compared as mobile phases. The elution profiles of several lipids and some organohalogen compounds (OHCs) were determined. The mixture CH–DCM yielded the best separation between lipids and the OHCs. The results were discussed with a theoretical evaluation on the basis of interactions between solute, solvent and stationary phase. Recoveries were assessed for the GPC procedure and appeared to be good (98–100% for the column itself and 80–100% for the sample transfer from vial to column). The GPC was integrated in the clean-up of the extract of a microsomal PCDF metabolism assay yielding satisfactory results.

Keywords: Clean-up methods; Mobile phase composition; Polychlorinated biphenyls; Dibenzo-*p*-dioxins; Dibenzofurans

1. Introduction

For the clean-up of hydroxylated metabolites of halogenated benzenes, polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) obtained from bile or generated in a microsomal assay (in vitro), various methods have been applied ranging from thin layer chromatography [1], commercial solid phase [2], to HPLC-C_{18} column [3,4]. In a previous study, a

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combination of a Florisil column followed by an alumina column yielded satisfactory results for determination by GC with mass-spectrometric detection (GC–MS), using single ion monitoring (SIM), or GC with electron capture detection (GC–ECD) [5]. For accurate metabolite description and unambiguous identification it is necessary to obtain full scans with GC–MS. To overcome the low sensitivity of the MS in the full scan mode, samples have to be concentrated extensively. In our experience, the extracts of the microsomal assays appeared to be too contaminated with some high-molecular-mass compounds. Problems occurring most often were drifting retention times in the GC-run. In addition, mass spectrum interferences occurred due to the high ratio between the high-molecular-mass compounds and the compounds under investigation.

Since the interferences behave similarly to the metabolites on both solid phases, no improvement is to be expected from another clean-up based on chemical affinity. A better solution may be gel permeation chromatography (GPC). The separation of compounds by GPC is based on a complex of chemical interaction (adsorption to the stationary phase and partitioning into the mobile phase) and mechanic retention in the stationary phase on basis of molecular size [6–11]. When using Biobeads S-X3 as stationary phase and poorly solvating mobile phases like cyclohexane–dichloromethane (CH–DCM, 1:1) or CH–ethyl acetate (1:1) [8] both size exclusion and adsorption will occur. The stationary phase interaction is especially prevalent for chlorinated aromatics, alcohols, amines and amides [6,9]. The interactions with the solid phase are based on the interaction of the π-electron donating characteristics of the compounds with the π-electron accepting characteristics of the polystyrene stationary phase [10,11]. Other interactions are based on dipole–dipole interactions and the weak Lewis-base character of polystyrene [11].

GPC has been applied before in the clean-up of metabolites of PCBs, PCDDs and PCDFs. Klasson-Wehler [6] demonstrated satisfactory clean-up of PCB metabolites after extraction from dosed mouse tissues, when using GPC. Kuroki et al. [12,13] applied GPC to the clean-up of PCDF metabolites. Also clean-up characteristics of GPC proved to be satisfactory for compounds like tris(chlorophenyl)methanol [14].

The purpose of this study is to determine the applicability of GPC in the clean-up of extracts from microsomal assays. Firstly the most appropriate mobile phase was determined with model compounds. The elution point and fraction volume for metabolites of PCBs, PCBs and PCDDs were determined using a column filled with Bio Beads S-X3 and different mobile phases. In the literature, either hexane–DCM (H–DCM, 1:1) [6,15] or CH–DCM (1:1) [12,13] were suggested as the mobile phase. Hexane has been suggested because it was cheaper, more volatile and offered an increased separation. The mixture CH–DCM, however, was used for the specific compounds we are interested in [12,13]. Therefore, we decided to test the latter eluent mixture on separation and clean-up characteristics. Acetone was also tested since one of the steps in metabolites analysis is methylation. This is done in an acetone solution with methyl iodide and K_2CO_3 [16]. It would be convenient, and solvent economising, to do this step after GPC clean-up, in the collected fraction without any evaporation losses. In addition, Biobeads S-X3 swells less in acetone due to the hydrophobic stationary phase interaction with the polar solvent [11]. This will lower the size exlusion limit (SEL), which in turn can lead to improved separation. In order to explain our observations we investigated, in some more detail, the theoretical aspects of separation in systems described (presented in the discussion). The next step was to integrate GPC in the clean-up of a microsomal assay and thereby determine applicability. To that end, microsomal assays were conducted with 2,3,6,8-TCDF. The effects of the clean-up were studied by comparing full scans on GC–MS after only a Florisil clean-up [5] and after subsequent GPC, Florisil and alumina clean-up.

2. Experimental

2.1. Chemicals and reagents

The 2,2′,6,6′-tetrachloro-4,4′-$(OH)_2$-biphenyl (TCB-$(OH)_2$, Aldrich Chemicals, Milwaukee, WI, USA) was methylated to 2,2′,6,6′-tetrachloro-4,4′-$(OCH_3)_2$-biphenyl (TCB-$(OCH_3)_2$) according to Tulp et al. [16]. 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TCDD) was synthesised according to Poh-

land and Yang [17], 2,2′,4,5′-tetrachlorobiphenyl (2,2′,4,5′-TCB) was obtained from Promochem (Wesel, Germany), 2,2′,3,3′-TCB was obtained from Ultra Scientific (North Kingstown, RI, USA), 1,2,7,8-TCDD was obtained from Accu standard, (New Haven, CT, USA), 3-SO_2CH_3-2,2′4,5,5′,6′-hexachlorobiphenyl (MSF-HxCB) was a kind gift from Prof. Dr. Å. Bergman (Stockholm, Sweden), 2,3,6,8-TCDF, 7-OCH_3-2,3,6,8-TCDF, 9-OCH_3-2,3,6,8-TCDF, 3-OCH_3-2,4,6,8-TCDF and 4-OCH_3-1,3,6,7-TCDF were a kind gift from Dr. H. Kuroki (Fukuoka, Japan). Acetone (P.A. quality) was obtained from Jansen Chimica (Geel, Belgium), other solvents (glass distilled) were purchased from Rathburn (UK). The mesenteric adipose tissue was taken from around the kidney of a cow. Arachid oil was obtained from a local pharmacist. Milk fat from a grey seal (*Halichoerus grypus*) was obtained by extracting milk with acetone–hexane. Bovine serum albumin (BSA, fraction V) was obtain from Sigma (St Louis, MO, USA). Bio Beads S-X3 were obtained from Bio-Rad (Hercules, CA, USA).

2.2. GPC-system

A Gilson automatic clean-up system was used. The 231-401 injection system was applied, consisting of the 401 diluter module (volume: 5 ml) and 231 sample injector module, using rack code 24 as sample holder tray. The 302 piston pump module with 10 SC pump-head and 804C manometric module was used as a pump (pressure: 2 bar, upper limit: 6 bar, lower limit: 0). The column was a 250×25 mm I.D. glass column. The fraction collector (Model 201) with code 10 collection rack was coupled to the system. Bio Beads S-X3 were used as the stationary phase, swollen in the solvent used. The column was filled with either 48 g (acetone) or 30 g (CH–DCM). The injection volume was 2.3 ml and the flow-rate 2.5 ml/min. Automated injection cannot guarantee 100% sample application without risking the introduction of air onto the column. Conically tapered sample vials were specially designed to reduce the dead volume (±100 μl) of uninjected material, while still being safe from introducing air. The programmable sampler enabled us to apply different injection regimes. With a total sample volume of 2.4 ml (2.3 ml injection volume+100 μl dead volume), injecting once would theoretically result in applying 96% of the total sample on column. Rinsing the remaining sample volume (100 μl) once with one injection volume (2.3 ml) and applying this as well in a subsequent run, would result in a theoretical transfer yield of >99%. Some samples can have lipid loads in excess of the maximum capacity for the column (100 μg/ml sample for acetone, empirically assessed and 1 g/ml sample for CH–DCM, [12,13]). For such cases, a different injection regime was developed. The samples were diluted to 4.7 ml (twice the injection volume+100 μl dead volume). These samples were applied as two consecutive runs of 2.3 ml injection volume, followed by dilution and application of the 100-μl dead volume as a third run (theoretical transfer yield >99%). Recovery standards were added to the collected fractions before concentrating. Transfer yields were assessed by measuring the remains in the sample vials and summing these results with the recovery data.

2.3. GC–MS/ECD

GC-measurements were performed on a Hewlett-Packard 5980 series II GC with either ECD or the Hewlett-Packard 5970 MSD using a 30 m×0.25 mm DB-5 column; temperature program: 80°C, 1 min hold, 20°C/min to 195°C, 1 min hold, 2°C/min to 225°C, 30°C/min to 300°C, 15 min hold. The GC–ECD was equipped with an A200S autoinjecting system (Carlo Erba), injecting 1 μl cold on column. The GC–MS was injected manually with volumes of 1 to 2 μl cold on column. Both the full scan mode (100–400 *m/z*, 1 cycle/s) for qualitative purposes and the SIM mode (1 cycle/s) for quantitative purposes were used.

Quantitation on the GC–ECD was performed with a calibration line covering 10–125% of the starting concentrations. Linearity of the calibration was checked. Only when samples could be quantitated in the linear area, quantitation was approved and used. Initially, samples on GC–ECD were injected in triplicate. However, the contribution of the deviation in injection to the total standard deviation of the experiment was minor, therefore it was decided to continue with single injections. Since the MS was linear in response over the entire range used, quantitation on the GC–MS was performed with only one quantitation solution. Deviation between injections

of one sample was 3% typically. Detection limit (S/N ratio: 3) was 5 pg/μl for 2,3,6,8-TCDF and 13 pg/μl for 4-OCH_3-1,3,6,7-TCDF.

2.4. Spectrophotometer

A Shimadzu UV 160A was used with quartz cuvets. Each sample expected to contain TCB-$(OCH_3)_2$ was scanned between 200 and 300 nm. TCB-$(OCH_3)_2$ had a typical absorbance profile with peaks at 231 and 263 nm and a valley at 239 nm.

2.5. Experimental set-up

The experimental design for different mobile phases and model compounds is given in Table 1. Model lipids were dissolved in the used mobile phase in a final sample volume of 2.4 ml. BSA was only tested for acetone. Eluting fractions were collected in preweighed vials and lipid elution profiles were determined gravimetrically. For organohalogen compounds (OHCs), standard solutions (1 ml trimethylpentane+1.4 ml CH–DCM) were injected in the GPC system and pertinent fractions were collected. For elution profiles of the OHCs, the fractions were concentrated and analyzed by GC–ECD. By these two methods, differences in elution volume of model compounds for both mobile phases were assessed. On the basis of these results a mobile phase was selected and the influence of lipid loading on elution and recoveries were assessed (for measuring methods, see Table 1). For recovery experiments, 1,2,7,8-TCDD and 2,2′,3,3′-TCB were added as recovery standards before concentrating with a vigreux apparatus.

3. Results

3.1. Separation

The elution profiles of the lipids and the OHCs are given in Fig. 1A and Fig. 1B. The adipose tissue is given in mg/ml. The OHCs are expressed in per-

Table 1
Experimental design: mobile phases, model lipids and model OHCs

Mobile phase	Model compound	Method of measuring
Elution order under different mobile phases		
Acetone	Mesenterial lipid (250 mg) Bovine serum albumin (25 mg)	Gravimetrically
	1,2,3,4-TCDD (1.10 μg) TCB-$(OCH_3)_2$ (1.04 μg)	GC–ECD
CH–DCM (1:1)	Mesenterial lipid (0.2 g) Milk lipid (0.8 g) Arachide oil (1.0 g)	Gravimetrically
	1,2,3,4-TCDD (1.10 μg) TCB-$(OCH_3)_2$ (1.04 μg) MSF-HxCB (0.39 μg) 2,2′,4,5′-TCB (2.20μg)	GC–ECD
Influence of lipid loading on elution		
CH–DCM (1:1)	Arachide oil (0, 0.3, 0.7, 1.2 g) TCB-$(OCH_3)_2$ (27.6 μg)	Spectrophotometrically
Recovery of OHCs with different sampling regimes		
CH–DCM (1:1)	1,2,3,4-TCDD (1.10 μg) TCB-$(OCH_3)_2$ (1.04 μg) MSF-HxCB (0.39 μg) 2,2′,4,5′-TCB (2.20μg)	GC–MSD

Total injected amounts of compounds are given in brackets.

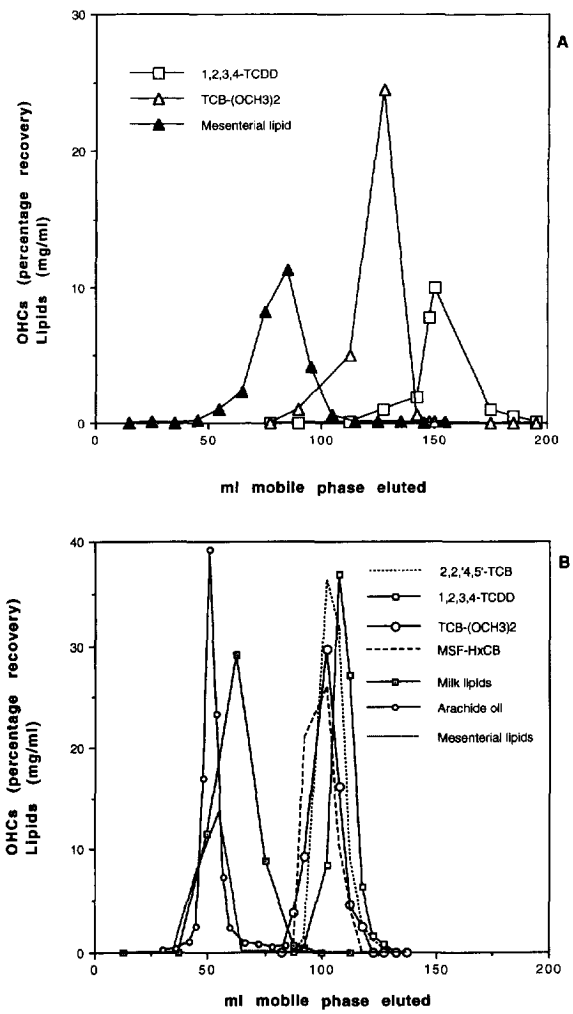

Fig. 1. Elution profile of lipids (in mg/ml) and OHCs (in % recovery) on a 250 mm long GPC-column, filled with Bio Beads S-X3. (A) Mobile phase is acetone, (B) mobile phase is cyclohexane–dichloromethane (1:1, v:v). In each case the data point of the accumulated amount of the fraction was placed at the last ml of that fraction.

centages of recovery. With acetone as mobile phase, the bulk of the mesenterial lipid eluted in the 40–110 ml fraction with the peak maximum around 75 ml. BSA behaved similarly to the mesenterial lipid (data not shown for clarity). At this very point (75 ml) also TCB-$(OCH_3)_2$ started to elute. The 1,2,3,4-TCDD peak started to elute after 100 ml, thus overlapping slightly with the lipid peak (Fig. 1A). Using CH–DCM as mobile phase, a significantly improved separation was achieved. Mesenterial lipid and arachide oil had completely eluted after 75 ml. The milk lipids had a broader peak with a small tail lasting up until 90 ml (Fig. 1B). The TCB-$(OCH_3)_2$ started to elute after 85 ml (1% of injected amount). The other OHCs started to elute at 90 ml (Fig. 1B). Elution of all OHCs was completed after 130 ml. It is interesting to note the narrow band in which all compounds eluted compared to using acetone as mobile phase.

3.2. Lipid loading

CH–DCM offered satisfactory separation characteristics and was used as mobile phase in further assessment of the system. The influence of lipid loading on the elution profile of TCB-$(OCH_3)_2$ was investigated. Several arachide oil loadings mixed with TCB-$(OCH_3)_2$ were applied. Although each lipid loading was only applied once, the results from each mixture tended to a consistent, small delay of elution of TCB-$(OCH_3)_2$ over the pure standard. The peak of elution shifted from 90 to 93 ml (Table 2).

3.3. Recovery

On the basis of the elution pattern, a fractionation point had to be chosen. Since additional solid phase columns are applied after the GPC step in the clean-up of our samples [5], it was decided to choose for maximum recovery rather than optimum separation. Therefore, the fractionation point was taken at 80 ml (or 32 min). Recoveries of several OHCs were assessed with two different sampling regimes. The regime with a sample volume of 2.4 ml (injecting once then subsequent rinsing and applying the rinsate as a second run) resulted in recoveries ranging from 90% for MSF-HxCB up to 98% for TCB-$(OCH_3)_2$

Table 2
Elution peak of TCB-$(OCH_3)_2$ under the influence of different arachide oil loadings

Amount of arachide oil (g)	Peak of elution (ml eluted)
0	90
0.3	93
0.7	93
1.2	93

Table 3
Recoveries of standard solutions of organohalogen compounds on a GPC column filled with Biobeads S-X3

Compound	Recovery (%)				Mass balance	
	Σ (one injection + one rinse)		Σ (two injections + one rinse)		Mean	S.D.
	Mean	S.D.	Mean	S.D.		
2,2',4,5'-TCB[a]	97.1	1.1	89.1	4.9	101.6	5.2
1,2,3,4-TCDD[b]	95.0	5.3	89.5	5.3	98.7	4.2
TCB-$(OCH_3)_2$[b]	97.8	5.4	87.6	3.3	99.3	5.0
MSF-HxCB[b]	90.8	3.3	87.0	8.6	b.d.[d]	

Recoveries were for applying the solutions with a one injection-one rinse injection regime or a two injections-one rinse injection regime (see text). In the second case, remains in the sample vial were analysed as well to assess the mass balance. Means and standard deviations (S.D.) of recovery after completion of all programmed runs are given ($n=4$ in all cases)

[a] Recovery standard: 2,2',3,3'-TCB.

[b] Recovery standard: 1,2,7,8-TCDD.

[c] Mass balance = Σ(two injections + one rinse) + remains in sample vial.

[d] Remains in sample vial below detection limit so unable to calculate mass balance.

(Table 3). The regime with a sample volume of 4.7 ml (injecting twice in consecutive runs, then rinsing once) resulted in somewhat lower recoveries (around 89%, Table 3). To complete the mass balance of the analytes for the second regime, the residual uninjected solutions in the sample vials were analyzed as well. Values near 100% were achieved for the mass balance (remains in sample vial and collected fractions) of nearly all OHCs (Table 3). These results indicated that the column did not retain any OHCs but that transfer from sample vial to the column could show some flaws.

3.4. Application

In Fig. 2A and Fig. 2B, two total ion chromatograms (TIC) of masses from 100–400 m/z are given of an extract of a microsomal assay (containing 1 mg protein) with 2,3,6,8-TCDF. In both cases, the arrow indicates the same unknown methylated hydroxy-metabolite (?-OCH_3-2,3,6,8-TCDF). Identification was not possible due to our lack of relevant standards. The sample of Fig. 2A has only had a Florisil column clean-up [5]. The finale volume of the extract was 500 μl. The second one has had the full procedure (GPC, Florisil, alumina, [5]) and was concentrated to 25 μl. The differences in height of the surrounding peaks (Fig. 2A and Fig. 2B) demonstrate clearly that the clean-up has been improved substantially. In the case of Fig. 2A, the metabolite cannot even be seen as a clear peak above the background. In Fig. 2B, this peak is much more pronounced.

In Fig. 3A and Fig. 3B, the mass spectrum of

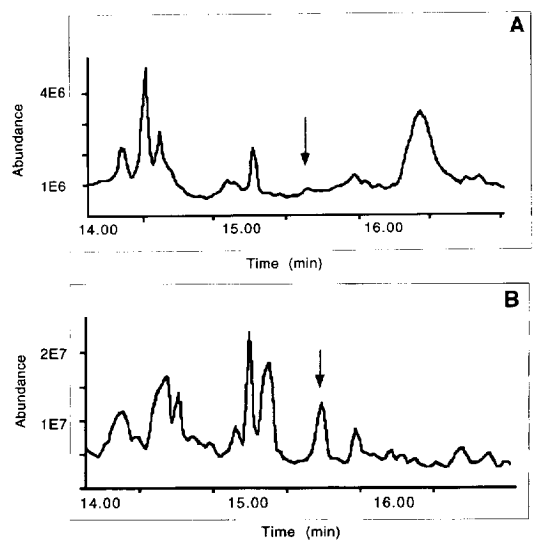

Fig. 2. Total ion chromatograms (TIC) of two full scans of extracts of microsomal assays with 2,3,6,8-TCDF. Analyses were performed on a HP 5970 MSD. (A) TIC of an extract which only has had a Florisil clean-up procedure; final volume 500 μl. (B) TIC of an extract which has had a GPC, Florisil, alumina clean-up procedure; final volume 25 μl. The arrow indicates the same unknown metabolite (?-OCH_3-2,3,6,8-TCDF).

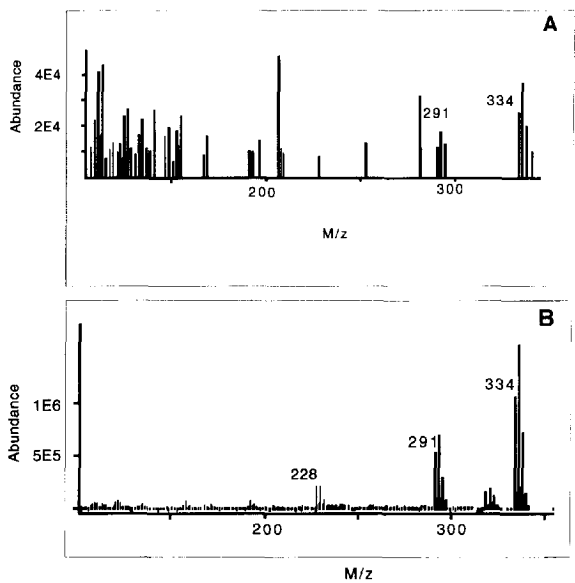

Fig. 3. Fragmentation pattern of the ?-OCH_3-2,3,6,8-TCDF which is indicated in Fig. 2A,B with the arrow. (A) Fragmentation pattern of ?-OCH_3-2,3,6,8-TCDF, after a Florisil clean-up procedure. (B) Fragmentation pattern of ?-OCH_3-2,3,6,8-TCDF, after a GPC, Florisil, alumina clean-up procedure. Numbers above the ion clusters relate to the first ion of each fragment.

?-OCH_3-2,3,6,8-TCDF is given. In Fig. 3A two dominant fragments can be seen with a tetra-chlorine pattern: the $[M]^+$ and the $[M-43]^+$. The remaining fragments show no chlorine pattern. The high background which was observed in Fig. 2A, was also encountered in the fragmentation pattern. The expected ^{13}C-ions were not visible. Most likely the intensities of these ions were too low, due to the high dilution (sample volume: 500 μl). In Fig. 3B, the mass spectrum is much clearer. Dominant peaks with a tetra-chlorine pattern can be seen at $[M]^+$, $[M-15]^+$ and $[M-43]^+$. A peak with a tri-chlorine pattern can be seen at $[M-106]^+$. These all correspond to the most abundant fragments in an electron impact fragmentation of OCH_3-TCDFs [1,2,18]. The metabolite had a different retention than 7-OCH_3-2,3,6,8-TCDF, 9-OCH_3-2,3,6,8-TCDF or 3-OCH_3-2,4,6,8-TCDF. The other peaks apparently belong to the background but they have a negligible intensity. In the case of the sample of Fig. 3A, TCB-$(OH)_2$ was added to the extraction procedure as a recovery standard. The fragmentation pattern of TCB-$(OCH_3)_2$ was identical to the standard of this compound (data not shown).

4. Discussion

In this study, different mobile phases were tested for a GPC column filled with Bio Beads S-X3. Acetone was compared to CH–DCM, because it may be more conveniently integrated in a procedure of analyzing metabolites of PCBs, PCDDs and PCDFs. In addition, a better size separation could be expected.

4.1. Separation

The lipids as well as the OHCs eluted faster when using CH–DCM as mobile phase than when using acetone. In addition, a better separation between desired (OHCs) and unwanted compounds (lipids) was observed with CH–DCM. This seems contradictory at first glance.

Why all compounds elute faster when using CH–DCM as mobile phase can be explained by the following formula [11,19]:

$$V_e = V_0 + K_{sec,i}V_p \quad (1)$$

where V_e = volume of elution (ml), V_0 = interstitial solvent volume between the gel beads (ml), $K_{sec,i}$ = partitioning coefficient of solute i over V_0 and V_p, V_p = solvent volume of the pores within the beads (ml). V_0 is influenced by the degree of swelling [11,20]. The more the beads swell in the mobile phase the smaller V_0 is. In the present study, we observed that one g of Bio Beads S-X3 swelled to 2.6 ml in acetone whereas in CH–DCM, it swelled to 4.3 ml (yielding a smaller V_0). Hence with a smaller V_0, all compounds will tend to elute faster in CH–DCM. Only compounds with a high $K_{sec,i}$ will have V_e's comparable to acetone.

The reduced separation in acetone as compared to CH–DCM had best be explained by three factors: reduced V_p, effects on $K_{sec,i}$ and chemical interactions. The sum of V_0 and V_p is constant [11,20]. Since V_0 is bigger in acetone, V_p will be smaller. Conse-

quently, separation between excluded and non excluded compounds will be worse.

Separation in acetone is also negatively influenced by effects on $K_{sec,i}$. $K_{sec,i}$ ranges from 0 for compounds which are completely excluded to 1 for compounds which permeate the pores completely [11]. The molecular mass range over which $K_{sec,i}$ operates is determined by the SEL. The SEL (in molecular weight, MW) is influenced by the degree of swelling [11,21]. A rough estimation of the SEL of not fully swollen S-X3 can be obtained by correlating the SEL of various S-X beads and their volume in benzene (as given [21], omitting the values for S-X1). This yields the emperical relationship:

$$\text{SEL} = 721(\text{volume}) - 1341, \quad R^2 = 0.93 \qquad (2)$$

where SEL = size exclusion limit (MW units), volume = (ml of S-X/g of S-X in benzene). It can be derived that the SEL is about 500 for acetone and about 1800 for CH–DCM. For acetone, the lipids with their MW-range of 600–1500 [7] are excluded. Their V_e is determined by V_0. The $K_{sec,i}$ of the OHCs will be close to 0 because their MW is close to 500. As a consequence, V_e is close to V_0 (Eq. (1)). Separation is therefore poor. For CH–DCM, the $K_{sec,i}$ for the OHCs will be closer to 1 and V_e will be determined by both V_0 and V_p (Eq. (1)). The lipids have a $K_{sec,i}$ close to 0 and V_e is mainly determined by V_0, thus improving separation.

The third factor influencing separation is chemical interaction. This follows from Eq. (3) [11].

$$\ln K_i = (V_{m,i}/RT)(\delta_m + \delta_s - 2\delta_i)(\delta_m - \delta_s) + \ln (n_s/n_m) \qquad (3)$$

K_i = capacity factor of the column, $V_{m,i}$ = molar volume of solute i (ml/mol), R = gas constant (J mol^{-1} K^{-1}), T = absolute temperature (K), δ_m = solubility parameter of mobile phase (J/cm^3)$^{1/2}$, δ_s = solubility parameter of stationary phase, δ_i = solubility parameter of solute, n_s = total amount of stationary phase (moles), n_m = total amount of mobile phase (moles). The first term is influenced by both chemical interactions and $V_{m,i}$. The second term is equivalent to Eq. (4) and therefore influenced by $V_{m,i}$ solely [11]:

$$K_{sec,i} = (C_{i,s})(C_{i,m})^{-1} \qquad (4)$$

with $C_{i,s}$ = concentration of solute i in stationary phase, $C_{i,m}$ = concentration of solute i in mobile phase. The term $(\delta_m - \delta_s)$ (Eq. (3)) has to be used with great care. The δ is composed of dispersive, dipolar and proton accepting/donating bonding type of interactions. When studying an interaction between two compounds, some of these bonding types may not be relevant or may even further increase deviations from theory. The term $(\delta_m - \delta_s)$ can be estimated from the degree of swelling. This gives a better indication of the degree of interaction and thereby the actually active and relevant bonding types of the theoretical δ's. In principle, the δ_m has to be equal to δ_s to obtain full swelling [11,23,24]. In our case, acetone has a large theoretical δ_m [19.2 (J/cm^3)$^{1/2}$]. Bio beads have an almost equal δ_s [18.2–18.8 (J/cm^3)$^{1/2}$] but show a reduced swelling [20]. An explanation could be that the basic character of acetone has a repelling interaction with the basic stationary phase. So the proton accepting bonding activity should be subtracted from the theoretical δ_m, thereby yielding a negative $(\delta_m - \delta_s)$ [11,22]. Then due to the first term, K_i becomes also dependent of δ_i, δ_m and δ_s.

Filling in Eq. (3), TCB-$(OCH_3)_2$ can be expected to have a lower δ_i than the planar 1,2,3,4-TCDD (Table 4). The heat of evaporation of the former will be lower due to the non planar structure and the high

Table 4
Molecular volumes of the used OHCs

OHC	Molec. vol.[a]	δ[b]
TCB-$(OCH_3)_2$	264.25	
MSF-HxCB	289.50	
2,2′,4,5′-TCB	212.57	
1,2,3,4-TCDD	220.21	22.4

Molecular volumes were calculated with SAVOL after minimisation with semi-emperical AM1 calculations in MOPAC. The following Van Der Waals radii (Å) were used: H: 1.01 (aromatic), 1.20 (aliphatic), C: 1.77 (arom.), 1.70 (aliph.), Cl: 1.77, O: 1.50, S: 1.85.

[a] Å^3, calculated in SAVOL after structure minimisation in MOPAC.

[b] (J cm^{-3})$^{1/2}$ [25].

V_m. The term $(\delta_m - \delta_s)$ is negative (Eq. (3)). Most likely the term $2\delta_i < (\delta_m + \delta_s)$ and hence $(\delta_m + \delta_s - 2d_i)$ is positive. K_i will be reduced as compared to a K_i which is derived from Eq. (1) (only based on $K_{sec,i}$). Consequently separation between lipids and TCB-$(OCH_3)_2$ is reduced.

For 1,2,3,4-TCDD, $d_i > \delta_s$ and the term $(\delta_m + \delta_s - 2d_i)$ is negative ([11], Table 4). The term $(\delta_m - \delta_s)$ is also negative (Eq. (3)). Then the K_i is increased as compared to the K_i from Eq. (1). Separation from the lipids is improved. Based on these data and the theory, the combination of S-X3 and acetone seems suitable for samples with a low lipid loading (100 μg/ml maximally) and desired compounds with a high δ_i.

For CH–DCM, almost full swelling of S-X3 was achieved resulting in $(\delta_m - \delta_s)$ being close to zero. Then K_i is determined by $K_{sec,i}$ and $V_{m,i}$ (Eqs. (1,4)). Looking at Fig. 1B and Table 4, K_i seems mostly determined by $V_{m,i}$. Differences in chemical interaction seem to play a minor role.

Although separation with CH–DCM is better than with acetone, one can still notice some tailing of lipids in the OHC fraction. The first suggestion to overcome this problem would be column length increase to enhance separation. Lipid tailing, however, is an inherent problem to GPC. Several authors have observed this phenomenon as well [7,13–15] and used additional clean-up methods. In the present study, other solid phase clean-up steps (Florisil and alumina) are available to purify the microsomal assay extract [5].

4.2. Lipid loading

The influence of lipid loading was studied on the elution of TCB-$(OCH_3)_2$. The peak of elution tended to shift to a later elution with increasing lipid loading. This is comparable to others [7]. The difference in elution maximum between Fig. 1B and Table 2 (viz. 100 an 93 ml) is most likely caused by differences in measuring methods (see Table 1). Since the criterion for the choice of the applied fractionation point was recovery rather than separation, this means that the fractionation point does not have to be adapted with increasing lipid loads. Recovery will be good anyhow.

4.3. Recovery

The designed conically tapered vials lead to satisfactory recoveries of 90% or higher (Table 3). The MSF-HxCB recovery tends to be lower than the other OHCs. Possibly, this is due to 1,2,7,8-TCDD being used as a recovery standard, rather than another compound more similar to MSF-HxCB. The complete mass balance of collected fraction and remains in the vial (Table 3) indicate that the column itself does not lead to any losses. Deviations from the theoretical recovery are therefore due to transfer losses caused by irregularities of the injection system.

4.4. Application

Applying the extensive clean-up procedure to a microsomal assay yields an extract which can be concentrated to at least 25 μl. The fragmentation pattern of metabolites can be determined with full scans. This enables description and identification not only on retention time, chlorine pattern and ratio of fragments determined with SIMs but also by the absence of fragments one would not expect. The ?-OCH_3-2,3,6,8-TCDF, for instance, as shown in Fig. 3B, shows a mass spectrum as can be expected from OCH_3-TCDFs [1,2,18,24]. On the basis of comparison with retention times of the standards available, ?-OCH_3-2,3,6,8-TCDF could not be 7-OCH_3-2,3,6,8-TCDF, 9-OCH_3-2,3,6,8-TCDF or 3-OCH_3-2,4,6,8-TCDF. One would expect the hydroxylation to occur at the 3 or 4 position in the PCDF molecule [2,3]. This would lead to either 4-OCH_3-2,3,6,8-TCDF or 4-OCH_3-2,3,7,8-TCDF (including NIH-shift). The fragment abundance ratio for the unknown metabolite was: $[M]^+$:$[M-15]^+$:$[M-43]^+$:$[M-106]^+$ = 100:15:50:21 which is different from the ratio reported in the literature for 4-OCH_3-2,3,7,8-TCDF (ratio: 54:100:25:33 [2]), tentatively suggesting the identity of the unknown metabolite to be 4-OCH_3-2,3,6,8-TCDF. One should keep in mind, however, that fragmentation is much depending on the MS instrumental conditions: compare e.g. the fragmentation of 4-OCH3-2,3,7,8-TCDF of several authors [2,3,24]. We ourselves e.g. observed a fragmentation pattern for 7-OCH_3-

2,3,6,8-TCDF (ratio $[M]^+:[M-15]^-:[M-43]^+ = 62:100:24$) quite different from the one reported in the literature (100:13:57 [24]).

5. Conclusion

Of the two mobile phases studied, CH–DCM (1:1) offers the best separation between lipids and the OHCs studied. Differences in separation between the mobile phases could be explained by differences in the interaction of the mobile phase and the stationary phase. In the case of acetone lipids were probably entirely size excluded. They eluted later, however due to an increase of V_0. For OHCs, K_i was markedly influenced by chemical interactions of solute, mobile phase and stationary phase with each other. The mobile phase CH–DCM behaves more like an ideal size exclusion system which is less influenced by chemical interactions.

In addition, recoveries are satisfactory and clean-up characteristics are markedly improved by integrating GPC into the overall clean-up procedure of the extract of a microsomal PCDF metabolism assay. The extract can be concentrated to a low a volume as 25 μl. Full scans with GC–MS yield well interpretable fragmentation patterns, characteristic of the compounds to be studied.

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