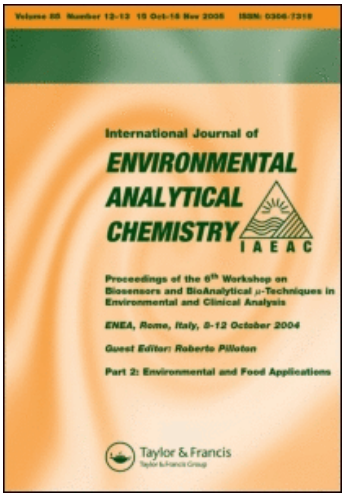

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Analysis of Chlorinated Biphenyl Congeners in Human Umbilical Cord: Some Approaches in the Development and Performance Evaluation of an Analytical Method

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ANALYSIS OF CHLORINATED BIPHENYL CONGENERS IN HUMAN UMBILICAL CORD: SOME APPROACHES IN THE DEVELOPMENT AND PERFORMANCE EVALUATION OF AN ANALYTICAL METHOD

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A sensitive GC-ECD method has been developed for the determination of chlorinated biphenyl (CB) congeners in human umbilical cord tissues. The process of developing this method, from a method aimed primarily at high levels of CBs in seal blubber, is described. Small factorial experiment designs were found to be very useful during method development.

The use of control samples and control charts as a method development tool is demonstrated. An initial estimate of the precision of the method was made from four duplicate measurements of a control sample, with a concentration of CBs ranging from 0.5 to 10 ng per sample extract. The precision was found to be 3–10% with a single exception of 17% (CB-138). The precision of the lipid determination step was 6%.

KEY WORDS: Chlorinated biphenyls, CB congeners, human umbilical cord, method development, control charts, quality assurance, analytical method, statistical analysis, experimental design.

INTRODUCTION

The presence of polychlorinated biphenyls (PCBs) in the biosphere has been extensively studied during the last 30 years, both in terms of *total PCBs* (Aroclor® equivalents), and more recently as individual chlorinated biphenyl (CB) congeners and the sum, ΣCB, of these[1].

The load of PCBs and other persistent organochlorine pollutants in humans have mostly been based on studies using blood and/or human milk as the matrix of interest[2]. The rationale behind this sampling strategy is, that sampling can be done without major inconvenience to the experimental persons. In human milk, the lipid content is on an average 2–4%[2], and most analytical methods for this matrix include a lipid determination in order to report lipid-based results. The lipid content of blood (whole blood, serum or plasma) is low[3,4], and CB congeners are most often reported on a wet-weight basis, that is, in ng/ml sample.

At NERI, a method was earlier developed for the determination of a number of CB congeners, including the coplanar CB congeners, in different tissue samples from marine mammals[5]. The laboratory was engaged to measure individual CB congeners and extractable lipids in human umbilical cord samples from the Faroe Islands where mercury exposures are high[6].

The determination of CB congeners in human umbilical cords has apparently not been reported previously. For this type of tissue, a low content of lipids and of CB congeners is expected. The relatively small amount of sample (1–5 g) that is available for the analyses, required a major revision and refinement of the analytical method.

The determination of total extractable lipids in methods for organochlorine residue analyses is usually done by gravimetry. During this step, where the organic solvent is evaporated entirely, the lipids in the sample extract can act as *keeper* of the analytes. The absence of a 'keeper effect' caused by lipids may affect the recovery of the CB congeners. Hence the gravimetric lipid determination can be a critical part of the method, when small samples of low lipid content are analysed. The method development therefore has to focus on natural cord samples, and to comprise the important factors in the method, for instance the method used for determination of extractable lipids and the extraction solvents used. Then the possible effects arising from each factor, can be studied on the laboratory recovery and recovery of 'endogenous' CB congeners in the samples.

The aim of the present study was to optimize an analytical methodology, hitherto used for high CB congener levels in biological samples, in order to enable identification and quantification of CB congeners present in very low concentrations in the human umbilical cord samples.

MATERIALS AND METHODS, CHEMICAL PART

Eleven CB congeners were included in the method development study (Table 1). Ten of these were recommended for marine monitoring by the International Council for the Exploration of the Sea, for use in marine monitoring. CB-149 was included for quality assurance of the GC separation. Additional congeners were used as internal standards and recovery surrogate standards in the analyses.

The method for measurement of CB congeners in seal tissue samples

The protocol for analysis of relatively large marine mammal tissue samples was described earlier[5]. The tissue samples were homogenized frozen in a Waring blender, and subsamples were extracted by CH_2Cl_2/methanol (2:1) in a 150 ml glass filter fitted extraction funnel by Ultra-Turrax blender. Extractable lipids were determined gravimetricable on a subsample of the extract. The residue was extracted with acidified saline water, and further purified by open column chromatography. For CB analyses of small samples, the purification steps have later been replaced by column clean-up on a 1 cm × 20 cm multi-layer column packed as follows (from the bottom): 5 g basic alumina deactivated with 3% w/w of hexane-extracted water/1 g of silica/5 g silica containing 40% H_sSO_4 (w/w)/1 cm of anhydrous Na_2SO_4[7]. All samples were spiked with recovery surrogate standards prior to extraction and clean-up. Laboratory recovery was calculated relative to an archived spike sample, which was prepared by adding an identical amount of recovery surrogate standards and internal standards to a 1 ml volumetric flask. The content of this flask was analysed with the samples.

The CB congeners were measured by dual-column high resolution capillary gas chromatography with electron capture detection (GC-ECD) and splitless injection[8]. The gas chromatograph was calibrated in the range from 5 pg to 400 pg. GC analysis yielded two results for each CB congener. If the difference between these results was less than

Table 1 Reference compounds for gas chromatographic analysis of human umbilical cord samples, identified by their IUPAC number and molecular structure.

CB (IUPAC No.)	*Molecular structure*	*Function*
CB-3	4-$C_{12}H_9Cl$	laboratory recovery
CB-28	2,4,4'-$C_{12}H_7Cl_3$	*
CB-31	2,4',5-$C_{12}H_7Cl_3$	*
CB-40	2,2'3,3'-$C_{12}H_6Cl_4$	laboratory recovery
CB-52	2,2'5,5'-$C_{12}H_6Cl_4$	
CB-53	2,2',5,6'-$C_{12}H_6Cl_4$	internal standard
CB-101	2,2'4,5,5'-$C_{12}H_5Cl_5$	
CB-105	2,3,3',4,4'-$C_{12}H_5Cl_5$	
CB-118	2,3',4,4',5-$C_{12}H_5Cl_5$	
CB-138	2,2',3,4,4',5'-$C_{12}H_4Cl_6$	
CB-149	2,2',3,4',5',6-$C_{12}H_4Cl_6$	GC separation
CB-153	2,2',4,4',5,5'-$C_{12}H_4Cl_6$	
CB-155	2,2'4,4'6,6'-$C_{12}H_4Cl_6$	internal standard
CB-156	2,3,3',4,4',5-$C_{12}H_4Cl_6$	
CB-180	2,2',3,4,4',5,5',-$C_{12}H_3Cl_7$	
CB-198	2,2',3,3',4,5,5',6-$C_{12}H_2Cl_8$	laboratory recovery
OCN**	$C_{10}Cl_8$	internal standard

* CB-31/28 can only be separated on the DB 5 columns, and only when a new column is being used. Carrier gas is being changed to hydrogen at present to overcome this problem. CB-31 and CB-28 can thus not be quantified in the cord samples.
** Octachloronaphthalene.

10%, the average value was reported. If the results deviated more than 10%, the lower value was reported, unless a blank value was present on one of the two columns. If a blank value was present on one column, the result from the other column was used.

Samples were processed in batches containing seven samples, one process blank and two aliquots of the internal reference sample (an seal oil, prepared from seal blubber). A mackerel oil, CRM-350 from the European Community Bureau of Reference (BCR), that was certified[9], was used as external reference sample[10].

METHOD DEVELOPMENT

Method development was in three parts. First, some preliminary qualitative and semi-quantitative experiments were made concerning homogenization, improvement of the detection limit of the GC method, and analysis of a few cord and blank samples. The results from these analyses revealed several problems about the analytical method, some of which were solved easily, while others required due consideration. Among the last type of problems, the solutions were sought by careful design and analysis of two quantitative experiments. In parallel with the quantitative experiments, the quality assurance of the umbilical cord method was introduced by establishment of control charts and by using a certified reference material.

Initial qualitative experiments

During the initial phase of the project, a Retsch ball mill and a Waring blender were tested for homogenization of the samples, but the cord sample material did not become

homogeneous by any of these methods. Finally wet extraction, i.e. mixing the sample and extraction solvent by an Ultra Turrax mixer, was tested.

The gas chromatographic method was optimized by preparing new calibration standards for the low levels of CB congeners in the umbilical cord samples. Stock solutions of the CB congeners were prepared from pure crystalline standards by weighing 5–10 mg of the standard into 10 ml iso-octane in a volumetric flask. From the stock solutions, a mixture of the analytes was prepared in iso-octane. The calibration standard mixtures for the gas chromatograph were made from this mixture, by dilution by volume with iso-octane containing internal standards. The GC-ECD method was not optimized further.

In the original CB method used at NERI, gravimetric lipid determination was done on an aliquot of the sample extract. This procedure was checked by preparing a solution of seal oil in n-hexane which contained an amount of lipid per 5 ml aliquot that was similar to the expected lipid content of a cord sample (about 20 mg). 5 ml aliquots of this lipid solution were taken, and the lipid content determined gravimetrically.

Following the initial method improvements, some human umbilical cord samples and blank samples were analysed. From these analyses it was concluded that several problems were present in the analytical method used, and subsequently two quantitative method development experiments were carried out.

Quantitative experiments

Experiment A. The influence of three factors, which were considered the most important for the analytical results, was studied. The experiment was carried out as a complete 2^3 factorial design without replicates, yielding 8 samples to be analysed (Table 2). The factors and the levels chosen were:

Table 2 Design and chemical results of Experiment A: 2^3 factorial with a single human umbilical cord sample.*

Exp No.	*Exp. ID*	*Factor levels – = low + = high*			*Amount (g)*	*Lipids (%)*	*Laboratory recovery (%)*		*CB congeners (ng/ml extract)*			
		A	*B*	*C*			*CB-3*	*CB-198*	*118*	*138*	*153*	*180*
8	(1)	–	–	–	–	–	1.7	83.7	{0.07}	nd	0.23	nd
4	a	+	–	–	–	–	1.2	84.8	{0.09}	0.20	0.10	nd
5	b	–	+	–	–	–	1.5	103.5	nd	0.035	0.06	nd
3	ab	+	+	–	–	–	1.6	91.8	nd	0.04	0.03	nd
1	c	–	–	+	2.7328	[0.135]	9.4	30.5	0.36	0.90	0.55	0.27
7	ac	+	–	+	2.0493	[0.063]	8.0	24.0	0.38	1.21	1.48	0.62
6	bc	–	+	+	1.9072	0.57	8.8	101.7	0.18	0.36	0.33	0.17
2	abc	+	+	+	3.0413	0.237	40.3	97.5	0.25	0.69	0.62	0.37

*Factors: A = concentration of internal standard ("–" = 5 ng/ml, "+" = 20 ng/ml), B = extraction solvent ("–" = CH_2Cl_2/methanol, 2:1, "+" = n-hexane acetone 1:1) and C = sample type ("–" = blank sample, "+" = cord sample). In samples 1 and 7, the lipid values were not reliable and the CB results are adjusted for laboratory recovery (CB-198). Values in { } were below the estimated limit of detection, but were used during preliminary ANOVA calculations. Lipid values in [ ] are unreliable.

Factor A: concentration of internal standard (5 ng/ml or 20 ng/ml in the final 1 ml sample extract).
Factor B: extraction solvent (original solvent mixture (dichloromethane/methanol, 2:1) or new solvent mixture n-hexane acetone, 1:1)
Factor C: sample type (blank sample or cord sample).

Factor A describes the possible contamination of the sample extracts by contaminants in the mixture of internal standards. Factor B describes both the possible contamination from the extraction solvent mixtures and differences in extraction efficiency between the two solvent mixtures.

A single human umbilical cord sample was used as sample matrix for the experiment, and this sample was assumed to be homogeneous. The sample was cut into four pieces. These four samples and four process blank samples were spiked with laboratory recovery surrogate standards, and the samples were subsequently extracted in accordance with the experimental plan of Table 2. The samples were processed in random order and in a single analytical run. The lipid content was measured using the entire sample extract. The GC analyses of the samples were carried out in randomized order.

Experiment B. Experiment B was a study on the possible influence of the method used for gravimetric lipid determination on the recovery of the CBs. The only factor studied was the amount of sample extract used for lipid determination (Table 3). When only a part of the extract was used for gravimetric lipid determination, the remaining part of the sample extract was used for subsequent PCB analyses, and this part had then not been evaporated to dryness. Using the entire extract for lipid determination, and thus evaporating the entire extract to dryness, may affect the recovery of the CB congeners in analyses of samples with low lipid content.

Factor D: amount of sample extract used for lipid determination (10% or 100% of the sample extract).

A single umbilical cord sample was divided into three parts of equal size, named I, II and III. Each of these three samples was spiked with laboratory recovery surrogate

Table 3 Design and chemical results of Experiment B: Two procedures for gravimetric lipid determination in three subsamples of a human umbilical cord sample.

Expt. ID No.	*Sub-sample*	*M_{sample} (g)*	*Level of factor D: + = 100% − = 10%*	*m_{lipid} (mg)*	*Lipid (%)*	*Laboratory recovery (%)*		*CB (ng/g wet weight)*		
						CB-3	*CB-198*	*153*	*138*	*180*
5	I	3.2299	−	0.7	0.33	111	90	0.27	0.19	0.17
2			+	5.4	0.43	45	100	0.30	0.19	0.19
6	II	3.2050	+	4.6	0.29	63	96	0.32	0.15	0.20
1			−	0.6	0.37	83	90	0.32	0.22	0.18
7	III	2.947	+	4.5	0.31	18	97	0.35	0.25	0.22
4			−	1.2	0.82	67	86	0.30	0.24	0.21

*M_{sample} is the weight of the subsample taken into analysis and m_{lipid} is the weight of the extracted organic material (lipids) from each subsample. Experimental ID No. 3 was a process blank sample. Blank values in per cent of sample value: CB-153 (30%), CB-138 (0%) and CB-180 (48%).

standards, and extracted by acetone/n-hexane (1:1). The volume of each sample extract was adjusted to 100 ml and the extract was divided into two parts of equal volume (labelled for instance IA and IB), yielding for subsamples in total. For three of the subsamples, the gravimetric lipid determination was done on the entire sample extract. For the other three subsamples the lipid determination was done on a 10% (5 ml) aliquot. The experimental plan is given in Table 3. The samples were processed in random order and in a single analytical run. The GC-ECD analyses of the samples were randomized.

Establishment of control charts

The levels of CBs in the seal oil, previously used as internal control sample, were far too high compared to the human umbilical cord samples. Hence, a sample that was stable, homogeneous and had CB congener levels and lipid content comparable to those of the cord samples, was needed for internal laboratory quality control. It was decided to use a certified mackerel oil sample, CRM-350, as internal control sample, but to use only 25 mg aliquots. In an aliquot of this size, the amount of lipids per control sample would be similar to that expected for the cord samples. The CB congener level per control sample extract would be about 2–10 times higher as that expected in the cord samples (range 0.6 ng/ml–8.0 ng/ml control sample extract). As only two 25 mg subsamples were used as control samples for each analytical run, a single sample of the reference material CRM-350 would be sufficient for several hundreds of internal control samples.

Sample homogeneity for CRM-350 was demonstrated down to 1.6 g aliquots, but certification analyses indicated that subsamples of down to 100 mg could be used[9]. A 25 mg aliquot of the reference material is below the amount recommended when using the certificate values, and thus the use of the certificate values may yield only an indication of the bias of the method. However, the use of a certified material as internal quality control sample is still advantageous, as this sample combines the internal and the external reference sample in one. The most important limitation of using CRM-350 as control sample in the present method is, that the sample matrix is quite different from the samples analysed, in terms of lipid content and possibly also in the types of lipids and other matrix components present.

Two quality control samples were added to each analytical run, after the preliminary qualitative method development and the first quantitative experiment studies had been evaluated, in order to determine the variance components in the method. The use of duplicate control samples had been used earlier[10], by inspiration of Taylor[11], and it was decided to continue with this protocol. The advantage of this procedure is that the estimated variance-between-samples can be monitored during batch-wise analyses of samples, even though only single analyses are made of the human umbilical cord samples because of the limited amount of sample available and the low levels of CBs in the samples.

MATERIALS AND METHODS, STATISTICAL PART

Experiment A

This experiment was a complete 2^3 factorial design in the factors A (concentration of internal standard), B (extraction solvent) and C (sample type), analysed without replicates. The effects of the single factors were mainly additive, and it was thus

reasonable to assume that two- and three-factor interactions could be ignored. The statistical model for analysis of data from the experiment was

$$Y_{ijk} = \mu + A_i + B_j + C_k + E_{ijk} \quad (1)$$

where A, B and C were the main effects of the three factors and E was random error. The analyses of variance of Experiment A were based on the results in ng/ml extract, and the data were thus not adjusted to the weight of the sample. The reason for doing so was that CB congeners and blank values in some cases occurred at similar concentrations. The four subsamples of the cord had identical weights.

Some of the data sets were unbalanced, as blank values were not detected for all CBs. In these cases the model was analysed by a stepwise regression analysis[12]. By this stepwise procedure, insignificant factors could be omitted from the model, and the residual variance with more degrees of freedom could be calculated.

Experiment B

This experiment had one factor with two levels and was carried out in three blocks, assuming cord homogeneity.

The statistical model for analysis of data from the experiment was

$$Y_{ij} = \mu + D_i + Block_j + E_{ij} \quad (2)$$

where *Block* corresponded to the subsamples (j = I, II, III), and D was the main effect of the factor (100% or 10% of the sample extract used for gravimetric lipid determination). It is reasonable to assume that there is no interaction between the factor and the blocks. After the analysis by the above model, non-significant effects were excluded, and the data were analysed by a reduced model in which a residual variance with more degrees of freedom was obtained.

Analysis of variance (ANOVA) was performed for CB-153, CB-138 and CB-180, given in ng/g wet weight. These three major CB congeners were selected for the statistical analyses because they were detected in most samples. Furthermore, the parameters lipid content and laboratory recovery (CB-3 and CB-198) were analysed.

Establishment of control charts

Control charts were established, based on ANOVA of the results obtained from analyses of the control sample. An X-chart for the average values of the duplicate measurements of the internal reference sample, and a Z-chart for the difference between the two measurements were established, for each variable (CB-101, CB-118, CB-149, CB-153, CB-138, CB-180, CB-170, p,p'-DDE, lipid content (%) and laboratory recovery).

The statistical model for analysis of the control sample data was

$$Y_{ij} = \mu + B_i + E_{ij} \quad (3)$$

where μ was the mean, B was the random contribution from batches and E was the residual variation of the method. The variance of B was $V(B) = \sigma_B^2$ and the residual variance was $V(E) = \sigma_E^2$. ANOVA of n sets of measurements consisting of r replicates

yields variation between batches, s_B^2, which estimates $r\sigma_B^2 + \sigma_E^2$ (with n-1 degrees of freedom), and the variation within batches, s_E^2, which estimates the residual variance $\hat{\sigma}_E^2$(with n(r-1) degrees of freedom).

The standard deviation of the mean of m measurements in a batch is estimated by

$$s_{\bar{Y}}^2 = \hat{\sigma}_B^2 + \frac{1}{m}\hat{\sigma}_E^2 = \frac{1}{2}s_B^2 \tag{4}$$

as m = r = 2 was used in this case.

The control limits for the X-chart are

$$\begin{aligned} &\textit{Warning: Average} \pm 2 \cdot s_{\bar{Y}} \\ &\textit{Action: Average} \pm 3 \cdot s_{\bar{Y}} \end{aligned} \tag{5}$$

The variance estimate of the difference between the two measurements of the reference sample from the same batch is

$$s_Z^2 = 2 \cdot \hat{\sigma}_E^2 = 2 \cdot s_E^2 \tag{6}$$

and the control limits of the Z-chart accordingly are

$$\begin{aligned} &\textit{Warning: } 0 \pm 2\sqrt{2} \cdot s_E \\ &\textit{Action: } 0 \pm 3\sqrt{2} \cdot s_E \end{aligned} \tag{7}$$

as the average of this chart is zero.

The estimated total variance (corresponding to the intermediary precision variance in the ISO 5725 standard[13] of *a single measurement* is $\hat{\sigma}^2_{\text{Total}} = \hat{\sigma}^2_B + \hat{\sigma}^2_E$, and $\hat{\sigma}^2_E = s^2_E$ is the estimated repeatability variance of the method. The estimated total variance corresponding to *the average of m measurements* from the same batch by the method is $\hat{\sigma}^2_{\text{Total, average}} = \hat{\sigma}^2_B + \frac{1}{m}\hat{\sigma}^2_E$.

RESULTS AND DISCUSSION

CB congeners that were detected in most cord samples and thus could form a basis statistical analysis of the models, have been selected for the Results and discussion section that follows. Generally, the CB congeners appeared in quite low concentrations in most samples. This was partly caused by splitting of the samples in the experimental designs. However, CB-28, CB-31 and CB-52 were not detected in any of the samples during method development.

Initial qualitative experiments

The wet extraction, where the sample was cut in small pieces, transferred to a vial and then mixed with the extraction solvent by an Ultra-Turrax blender, was found to work satisfactorily. This was implemented in the original CB method.

New calibration standard mixtures were prepared, corresponding to a calibrated range from 0.5 to 10 ng/ml, in five calibration levels with two levels placed at the low end of the calibrated range (0.5 and 1 ng/ml). A calibration curve is shown in Figure 1. The concentration of internal standard was adjusted after the conclusion of Experiment A, to 5 ng/ml.

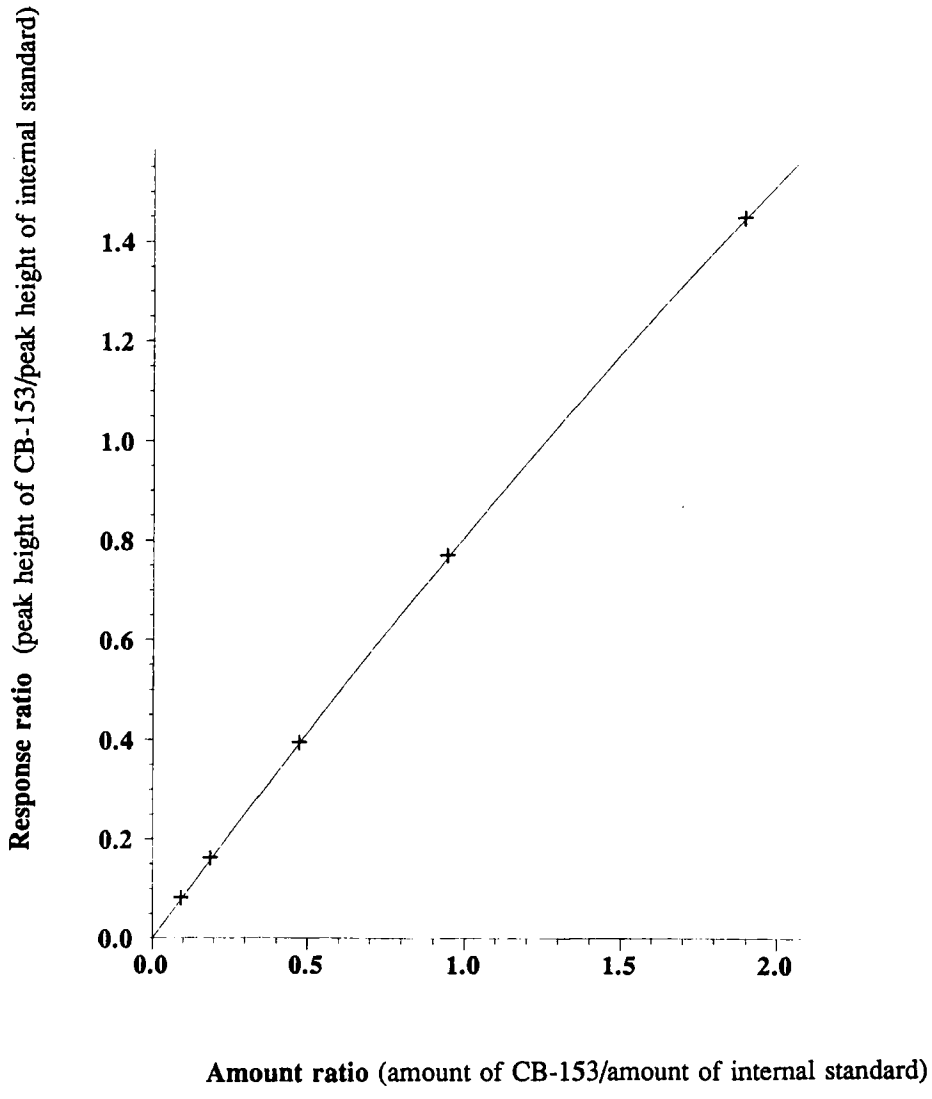

Figure 1 The calibration curve for CB-153, in the optimized gas chromatographic method. The calibrated range is 0.5–10 ng/ml. GC chromatographic column, DB-5; detector, ECD. Calibration curve fit: $Y = 0.034x^2 + 0.701 + 0.004$, where Y is the response ratio (height of CB-153/height of internal standard) and x is the amount ratio (amount of CB-153/amount of internal standard).

The relative standard deviation was 1%, when six replicate gravimetric determinations of 20 mg seal oil in 5 ml n-hexane (corresponding to a 10% aliquot of the total extract) were made. This variability was considered acceptable, and it was anticipated that an aliquot of the cord sample extracts could be used for gravimetric lipid determination.

No CB congeners could be detected in the samples due to background noise in the chromatograms, and CB-153 and CB-105 were detected in the blank samples. The origin of CB-153/CB-105 in the blank samples was traced to the internal standards, which were added to the samples prior to gas chromatographic analysis. The level of internal

standards for GC was reduced significantly prior to the next experiments. The malfunction of the clean-up method was believed to be related to co-extracted water in the dichloromethane/methanol extracted samples. Any residual water in the sample extract would de-activate the clean-up column material, and thus hamper the clean-up process.

Experiment A

The results obtained from the chemical analyses in Experiment A are given in Table 2. Residual water was present in two of the samples, which were extracted with dichloromethane/methanol, despite filtration of the sample extract through anhydrous sodium sulphate. An extra filtration through anhydrous sodium sulphate was introduced, but unfortunately in this step an insufficient amount of solvent was used to elute the sample from the sodium sulphate. These two samples were partly lost during clean-up, which was evident from poor laboratory recovery (about 30%) of CB-198. The results obtained for these two samples were therefore adjusted for the laboratory recovery (CB-198) before statistical analysis. The absence of reliable lipid determinations for these two samples prevents the comparison of lipid determinations by the two extraction solvent mixtures.

The laboratory recovery was calculated for CB-198, which was added to all samples at about 2 ppb wet weight level, and was 93.8 ± 8.5% (N = 6). No apparent influence of the factors, that is, extraction solvent, internal standard level and sample type, was observed for the laboratory recovery.

The factor A was not statistically significant for any of the CBs, but the data (Table 2) show that a high concentration of internal standard corresponds to a high CB concentration, compared to the low concentration of internal standards. Some interfering compounds/CBs thus seem to be added to the sample extracts with the internal standards, and the concentration of the latter should therefore be kept as low as possible, keeping in mind the quantitative use of these compounds.

Factor B was statistically significant for CB-118. The data (Table 2) indicate factor B to be important for the remaining CBs too, although not statistically significant, with an indication of lower values in blank samples and cord samples with n-hexane/acetone than with dichloromethane/methanol. The data indicate that the effect is caused by introduction of less interference to the sample extracts rather than a smaller extraction efficiency of n-hexane/acetone.

Factor C was statistically significant for all CBs but CB-180. No blank values were observed for CB-180, and therefore the result could not be tested statistically for this particular CB. However, the presence of CB-180 in all four sample extracts (Table 2) indicates that C is a significant contributer in this case also. So all four CBs could be detected in higher concentration in sample extracts than in blank samples.

A regression analysis, based on recovery-adjusted data and non-adjusted data of CB-153, was performed in order to detect possible differences in the statistical results. However, no differences were observed between the results of the analyses of the two data set.

A preliminary estimate of the detection limit was calculated from the residual standard deviation of these analyses. The limit of detection was calculated as 3 times the residual standard deviation. For CB congeners without blank values, the detection limit was calculated as from the standard deviation of the four subsamples. The estimated detection limits are given in Table 4. The detection limits were in the range 0.24–0.85 ng/ml extract, corresponding to about 0.12–0.43 ng/g sample (2 g sample processed). These detection limits are considered to be somewhat higher than those of the method when used routinely.

Table 4 Experiment A: Statistical results for CB congeners in one sample of umbilical cord (ng/g wet weight), in a complete 2^3 factorial experiment*

CB	Significance of factors				Most reduced model	CB congeners in sample no. (ng/g wet (weight)				Estimated det. limit (ng/ml)
	Z_A	Z_B	Z_C	$F(n,m)_{0.95}$		1	2	6	7	
118	0.25	24.37	10.10	$F(1,4) = 7.71$	$Y = \mu + B + C + E$	0.13	0.08	(0.10)	0.18	0.24 (n = 4)
138	1.12	6.05	10.27	$F(1,5) = 6.61$	$Y = \mu + C + E$	0.33	(0.23)	(0.19)	0.59	0.85 (n = 5)
153	1.29	0.30	34.85	$F(1,5) = 6.61$	$Y = \mu + C + E$	0.20	0.20	0.17	0.72[1]	0.30 (n = 5)
180[2]	–	–	—	–	$Y = \mu + C + E$	(0.10)	(0.12)	(0.09)	0.30	0.59 (n = 3)

[1] Outliet.
[2] No blank value.
*The detection limit was estimated from the residual variance of the experiment. Values below the estimated method limit of detection are given in parentheses. The detection limit (in ng/ml) can be converted to ng/g by dividing by the weight of the sample (M). $F(n,m)_{0.95}$ is the 95% fractile of the F-distribution with n,m degrees of freedom. z = (variance originating from the factor, n degrees of freedom)/(residual variance, m degrees of freedom) follows this F-distribution. When $z > F(n,m)_{0.95}$, the factor is considered a significant contributor in the statistical model.

In this experiment, the gravimetric lipid determination for the cord samples was not successful although the method had worked with seal oil dissolved in n-hexane, in the preliminary experiment. There are several possible causes for this, for instance the different extraction solvents, where the dichloromethane/methanol mixture gave much residual water in the sample extracts, and the different sample matrix (a simple matrix such as seal oil and a complex matrix such as a cord sample). This problem was investigated further in Experiment B.

Experiment B

Results for the CB congeners and laboratory recovery of the six subsamples are given in Table 3. A preliminary ANOVA revealed that the laboratory recovery of CB-3 was significantly lower than that of CB-198. The laboratory recovery of CB-198 has previously been shown to represent the remaining CB congeners[5], as CB-3 is more volatile, and thus partly evaporated during evaporation of the extraction solvent in the gravimetric lipid determination. The laboratory recovery of CB-198 was used for the remaining calculations. CB-40 is being tested as a possible replacement of CB-3, as recommended by Duarte-Davidson and co-authors[14].

The laboratory recovery of CB-198 in the samples was significantly higher when gravimetric lipid determination was performed on the entire sample extract, relative to when determined on a 10% subsample. The average laboratory recovery of CB-198 was (93.0 ± 2.6)%. The effect of factor D on the laboratory recovery is +4.7% for the high value of D (entire sample extract used) and –4.7% for the low value of D (10% of sample extract used). The laboratory recovery was anticipated to be higher when D was low, as the sample extract used for analysis of the samples was not evaporated to dryness in any step of the procedure. Possibly more than 10% of the sample is actually drawn from the sample extract, resulting in a lower calculated laboratory recovery from analysis of the remaining the subsample (90%).

In the preliminary analyses of variance of the levels of CB congeners (wet weight data), factor D was not significant, and no significant difference was observed between

subsamples when compared to the residual variance. Thus, the cord sample used for this experiment can be considered as homogeneous. The final (reduced) model for analysis of the data and the results are given in Table 5. The repeatability standard deviation, given as coefficient of variation (CV) of the method estimated for a single measurement by the method, was in the range 9.1%–18.5% (Table 5).

One of the six measurements of the lipid percentage in this experiment (sample III) was higher than the other results (0.82%), and this difference was significant based on Dixon's Q-test[15]. This result was therefore characterized as an outlier and it was omitted from the analyses. The same result (0.31) was used for both methods in this particular sample (sample III), in order to obtain a fully balanced data set for the ANOVA. Factor D was not significant, nor was any significant differences observed between subsamples. In the estimate of the standard deviation, four degrees of freedom were used instead of five, due to the identical value of the two samples III. The results are given in Table 5. The average lipid content of the umbilical cord samples obtained by the present method was (0.34 ± 0.06)%. The repeatability standard deviation, corresponding to a single gravimetric lipid determination, was 17%. In a study of human blood samples, Mes *et al.*[4] obtained relative standard deviations of 21% in 25 ml blood samples, with an average lipid content of 0.22%, by a similar gravimetric method. This corresponds to measuring about 40 mg lipids, and thus our repeatability CVs are of similar size for the gravimetric measurement. Mes *et al.* concluded that blood samples of at least 50 ml, each containing about 80 mg lipids, should be the minimum blood sample size in order to obtain reliable gravimetric lipid determination. However, it may be concluded that the precision of the gravimetric lipid determination in the present method, used on cord samples, is acceptable. Unfortunately, knowledge of a possible systematic error (bias) of the method can not be established from these data.

Table 5 Experiment B: Statistical results. CB congeners in ng/g wet weight and extractable lipids in % of a single human umbilical cord sample. The repeatability CV of the method is calculated based on n = 6 replicates.

Parameter	*Model*	*Factor D*	*Average <Y>*	S_r	CV_r
CB-153 (ng/g)	$Y = \mu + E$	+, –	0.309	0.028	9.1%
CB-138 (ng/g)	$Y = \mu + E$	+, –	0.206	0.038	18.5%
CB-180 (ng/g)	$Y = \mu + E$	+, –	0.193	0.020	10.5%
CB-198 (%)	$Y = \mu + D + E$	–	88.3[1]	2.34[2]	2.7%
		+	97.7[2]	2.50[2]	2.6%
Lipids (%)	$Y = \mu + E$	+, –	0.34	0.06	17.0%

[1] The average value of the laboratory recovery is 93.0%.
[2] The pooled standard deviation, S_{pool}, is 2.42 (CV = 2.6%).

Control charts—ANOVA results

So far only four batches of samples have been analysed. Normally a longer series of batches is required for establishing control charts. The precision may thus be somewhat underestimated.

The results of the ANOVA calculations for the control samples are given in Table 6. The estimated standard deviation between batches is higher than the standard deviation within batches in most cases.

The precision CV of a single measurement by the method ranged from 3.4% (CB-149) to 9% (CB-118), with the exception of CB-138, which had a CV of 17%. The increased variability for CB-138 may be due to interference from CB-163[16]. The influence of the interference from CB-163 and other compounds on the result for CB-138 is obviously strongly dependent on the gas chromatographic separation. The CV of CB-153 (highest concentration) is comparable to that of CB-52 (lowest concentration), although the average concentration of CB-153 is about five times higher than that of CB-52. The concentrations of CB-153 and CB-52 were 6.8 ng/ml and 1.4 ng/ml, respectively, in the extract of a control sample. The CVs are comparable to those obtained in the cord sample of Experiment B, although they are obtained from analyses of a less complex sample matrix (CRM-350).

Among the laboratory recovery surrogate standards, the CV of CB-3 was 27%, while CB-198 had a CV of 6.2%. The large variance of CB-3 is believed to be due to partial evaporation of this compound during the gravimetric lipid determination of the entire sample extract. The total CV of the lipid determination is 6.1%, which is about one-third of the CV obtained for the cord sample in Experiment B. The precision of the gravimetric lipid determination thus seem to be matrix dependent, and may possibly depend on some unidentified co-extracted compounds that are present in the cord sample matrix but not in the CRM-350 (mackerel oil) matrix.

Example of preliminary control charts that were established from the four batches of duplicate measurements (Table 6) are shown in Figures 2–4.

Optimized method

The analytical details of the optimized method are given briefly below, and will be given in full later[7]. Each sample was spiked with approx. 5 ng of each laboratory recovery surrogate standard. The samples were extracted with n-hexane acetone (1:1 by volume) with an Ultra-Turrax blender. The extract was dried, and total extractable lipids were

Table 6 ANOVa of data from analyses of four batches of samples, containing two control samples CRM-350. Preliminary values of the variance limits of the control charts, and an estimate of the repeatability and precision variance of the method, when single samples are analysed. The laboratory recovery is given in % for the three recovery surrogate standards.

	ANOVA of the control samples (4 batches of 2 samples)			*Variance limits for X- and Z-chart*		*Estimated relative variance of a single measurement by the method (CV)*	
Variable	$<Y>$ *(ng/g)*	$\hat{\sigma}_B$ *(ng/g)*	$\hat{\sigma}_E$ *(ng/g)*	$S_{<Y>}$ *(ng/g)*	S_Z *(ng/g)*	$\hat{\sigma}_{Total}$ *(%)*	$\hat{\sigma}_{Repeat}$ *(%)*
CB-52	59	3.2	3.8	4.2	5.4	8.5	6.5
CB-101	140	7.9	5.2	8.7	7.4	6.7	3.7
CB-149	150	3.7	3.5	4.5	5.0	3.4	2.3
CB-118	112	10	3.3	11	4.6	9.8	2.9
CB-153	270	17	4.4	17	6.2	6.3	1.6
CB-138	210	33	10	34	15	17	4.9
CB-180	58	2.1	1.4	2.4	2.0	4.4	2.4
CB-3 (%)	71	11	15	16	22	27	22
CB-198 (%)	90	5.0	2.3	5.3	3.3	6.2	2.6
CB-40 (%)	96	5.7	2.6	6.0	3.7	6.6	2.7
Lipids (%)	100	6.1	0.8	6.1	1.1	6.1	0.8

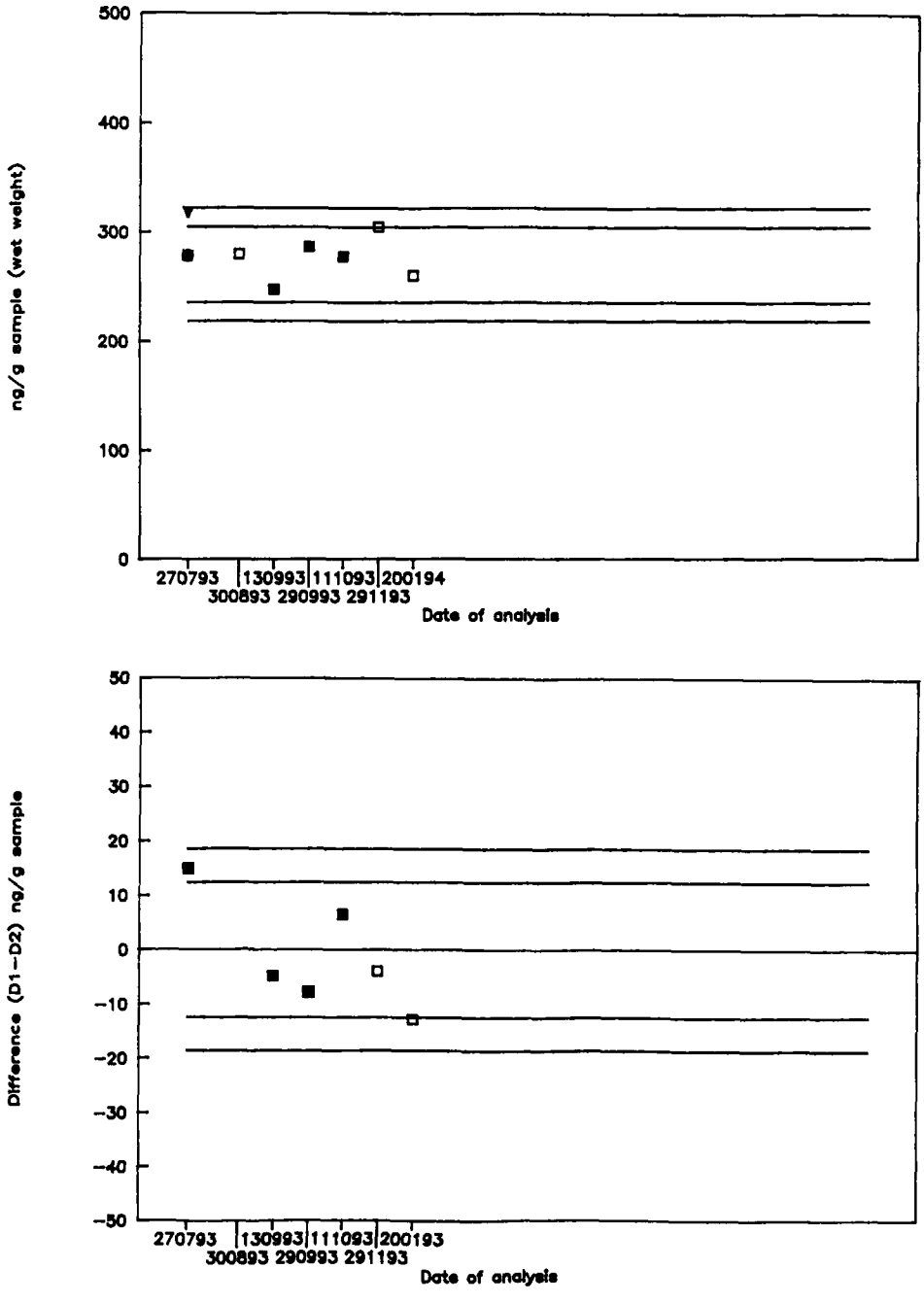

Figure 2 X-chart (upper) and Z-chart (lower) for CB-153 in the control sample CRM-350. The control limits are based on ANOVA of four duplicate measurements, given in Table 6 ■ data included in ANOVA; □, data not included in ANOVA; ▼,certificate value of CB-153 in the sample.

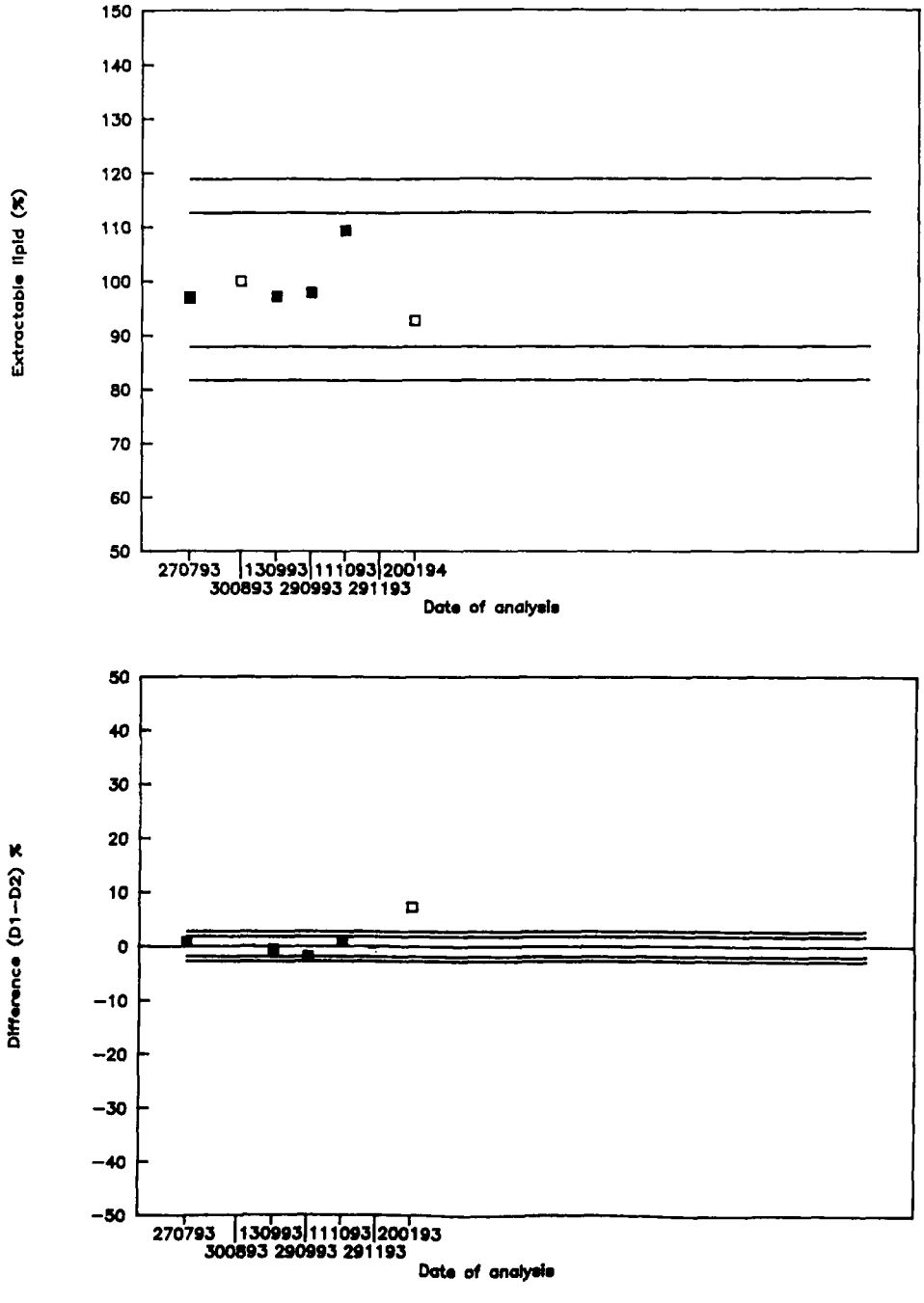

Figure 3 X-chart (upper) and Z-chart (lower) for the gravimetric lipid determination. ■, data included in ANOVA; □, data not included in ANOVA. See also legend to Figure 2.

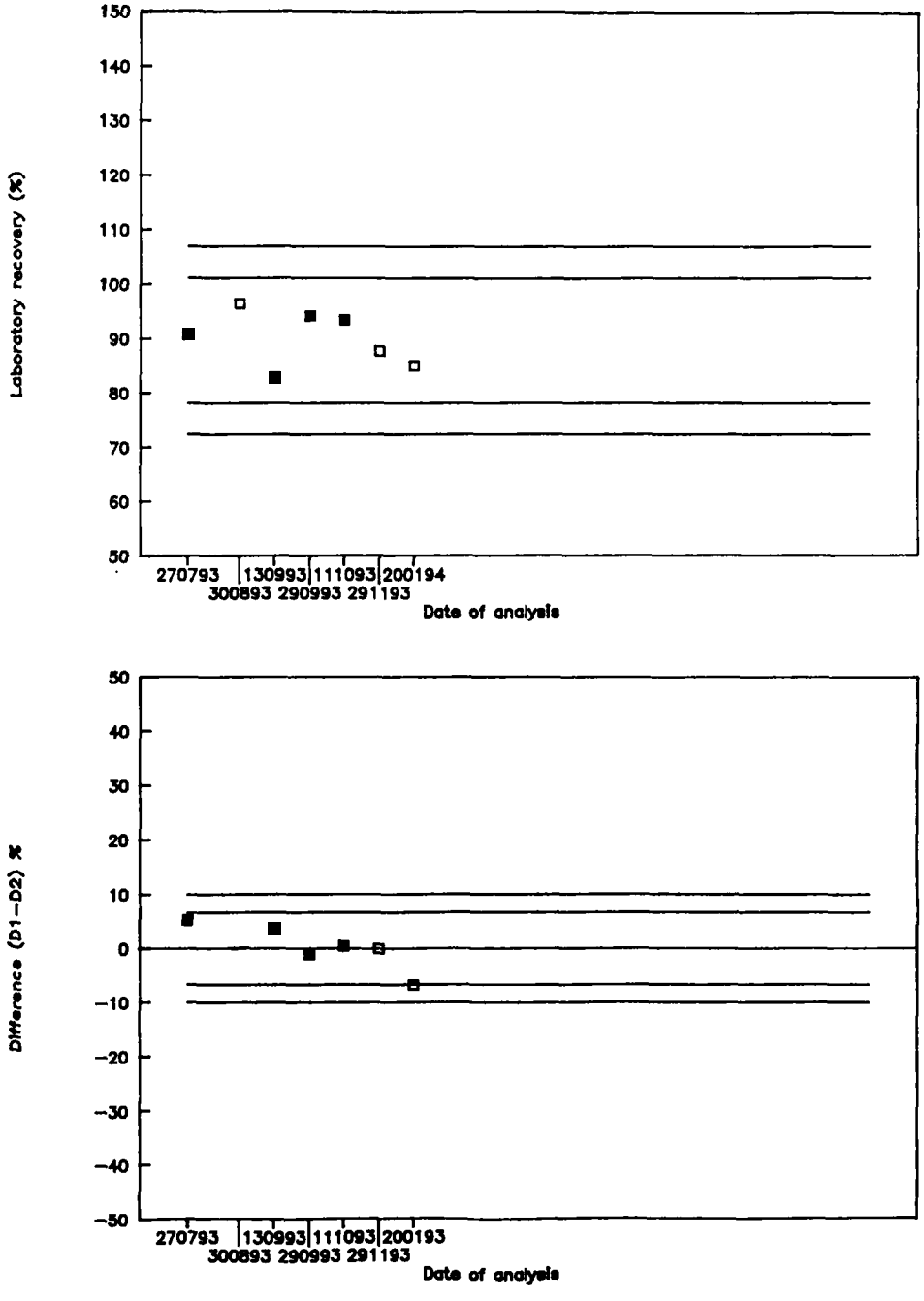

Figure 4 X-chart (upper) and Z-chart (lower) for the laboratory recovery (CB-198) in the control sample. ■, data included in ANOVA; □, data not included in ANOVA. See also legend to Figure 2.

determined gravimetrically on the entire extract. The extract was cleaned-up by open column chromatography on a multilayer column, and internal standards were added. Every batch of samples (15) included a process blank and a duplicate measurement of the internal reference material. The samples were analysed by dual-column capillary GC-ECD and quantified by internal standard methods. The gas chromatographic procedure was calibrated on five levels (0.5, 1.0, 2.5, 5.0 and 10 ng/ml), and a second order fit $ax^2 + bx + c$ was used for calculation of the calibration curve. Examples of chromatograms of a human umbilical cord sample, a process blank sample and a calibration standard, obtained by the optimized method, are given in Figure 5.

CONCLUSIONS

The present paper describes the method development performed in order improve a current method used for CB congener analyses in marine mammal samples, to enable trace-level CB congener analyses in human umbilical cord samples. The major problem is the small amount of sample available, combined with the low lipid and CB congener content. The analytical method has been optimized and quality assurance has been implemented in the measurements, including the gravimetric lipid determination.

The GC-ECD method was optimized by implementing a suitable calibrated range and by reducing the analytical blank values. The detection limit of the method has not been finally assessed as yet.

Difficulties encountered during the initial method development included: the presence of CBs as impurities in the internal standards, poor clean-up caused by water in the sample extracts, unstable lipid determination. Small factorial experiment designs were found to be very useful during method development.

The improvements of the analytical method enabled detection and quantification of some CBs in human umbilical cords. The most abundant CBs (CB-153, CB-138 and CB-180) could be detected in the human umbilical cord samples, but at quite low levels.

In our laboratory, the gravimetric determination of lipids in human umbilical cord samples should be performed on the entire sample extract. This method also yields the highest laboratory recovery.

A certified mackerel oil sample was chosen as control sample for the project, and was analysed in duplicate within each batch of samples. The precision CVs were established based on the control sample results, and preliminary precision CVs were between 3–10% for CB congeners, lipid content and laboratory recovery. Control charts were prepared based on the precision data obtained: One chart for the average of the two measurements and another chart for the difference between the measurements. The mackerel oil will be used until a more suitable control sample matrix is available for this type of analyses.

The control sample matrix (mackerel oil) is much less complex than the cord sample matrix. Precision CVs obtained from this relatively small number of experiments, indicate that CVs of the CBs are similar in the two types of matrices, while the CV of the gravimetric lipid determination step is 3-fold higher in the cord sample than in the mackerel oil.

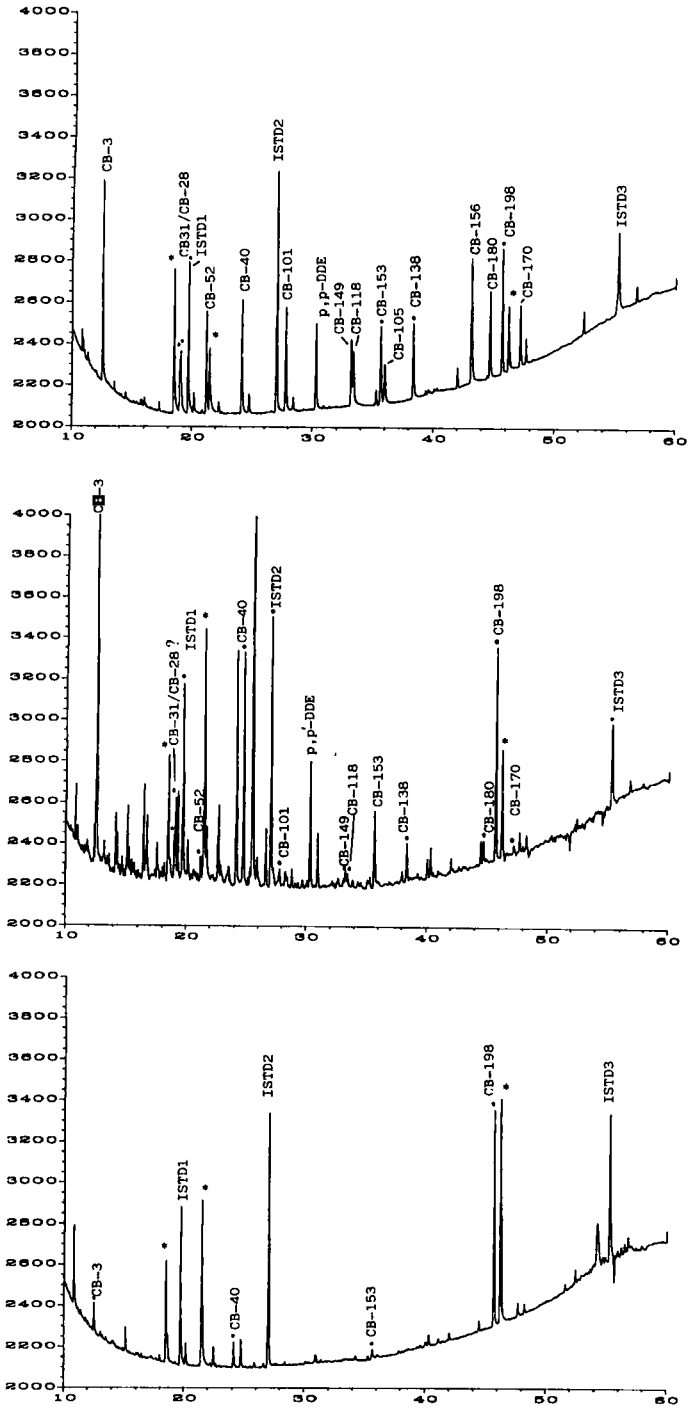

Figure 5 GC-ECD chromatograms of a 2 ng/ml calibration standard (A), a human umbilical cord sample (B) and a blank sample (C) obtained by the optimized method. GC column, DB-5.

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