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DETERMINATION OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN IN GOAT MILK AND TISSUES BY GLASS CAPILLARY GAS CHROMATOGRAPHY AND MEDIUM RESOLUTION MASS FRAGMENTOGRAPHY

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SUMMARY

An analytical method has been developed for the study of the elimination of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) by lactation and its determination in various tissues of goat. The method is based on the alkaline hydrolysis of milk, liver, fat, muscle, blood, faeces and urine samples, extraction with *n*-hexane, treatment with sulphuric acid-saturated silica gel, chromatographic clean-up on silica gel and alumina micro-columns, and glass capillary gas chromatography–medium resolution mass fragmentography (resolution 2000). 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin is used as the internal standard, the concentration of TCDD being determined from the calibration curve calculated from the peak heights of TCDD and the internal standard in the mass fragmentogram of the ion *m/e* 321.9.

The minimum detectable level of TCDD varied from 0.5 to 1 ppt* in milk (20 g), 1 ppt in muscle (10 g), 1 to 2 ppt in liver (10 g) and 2 to 5 ppt in fat (1 g). The TCDD level in the control animals was below the detection limit. The recovery of TCDD varied between 73 and 100%. The reproducibility of the analyses expressed as the relative standard deviation, was 5.1% at the 500-ppt level and 3.6% at the 20-ppt level. The reproducibility of the gas chromatographic–mass fragmentographic analysis was 2.4% (50–100 pg).

* Throughout this article, the American trillion (10^{12}) is meant

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INTRODUCTION

Analytical methods for chlorinated dibenzo-*p*-dioxins and dibenzofurans have recently been widely discussed[1–4]. Of these compounds, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been studied the most. The most versatile method for analysing these compounds has proved to be gas chromatography–mass spectrometry (GC–MS) using a selective ion detection system.

Depending on the type of material to be analysed, it is possible using this technique to determine TCDD at levels of a few ppt[5,6]. ^{37}Cl-labelled TCDD has most frequently been used as the internal standard in such analyses. These compounds have been detected by monitoring the fragments *m/e* 320 and 322 for TCDD and *m/e* 328 for the internal standard.

The aim of this study was to develop a selective, sensitive and precise method for determining TCDD in goat milk and tissues at low ppt levels. The study forms part of a larger investigation in which the accumulation, elimination and possible toxic effects of TCDD have been studied[7,8].

EXPERIMENTAL

Materials

Milk, liver, muscle, fat, faeces, serum, cells and urine of control animals and animals fed a diet containing TCDD were stored in a deep-freeze (−20°C) until the analysis. The TCDD used as reference compound in this study was synthesized by Mr. P. Paasivuo (State Institute of Agricultural Chemistry, Helsinki, Finland[9]). The internal standard, 1,2,3,4-tetrachlorodibenzo-*p*-dioxin, was kindly provided by Professor C. Rappe, University of Umeå, Umeå, Sweden).

The adsorbents used in column chromatography were Kieselgel 60, 230–400 mesh (Merck, Darmstadt, G.F.R.) and alumina B, activity I (Woelm, Eschwege, G.F.R.). All the chemicals used in this study were at least *pro analysi* grade.

Instrumentation

Capillary columns were prepared from borosilicate glass (Duran 50) using a slight modification of the method described by Grob and Grob[10]. The columns were coated with SE-30, OV-101, OV-17, Carbowax 20M and free fatty acid phase (FFAP). The lengths of the columns varied from 8 to 30 m and the I.D. from 0.32 to 0.40 mm. A Carlo-Erba Fractovap 2300 gas chromatograph, equipped with an all-glass solid injector (Chrompack), was connected to a Jeol D 100 mass spectrometer. The glass capillary column was interfaced by means of a Pt/Ir capillary tube (30 cm × 0.30 mm O.D. × 0.15 mm I.D.) into the ion source without a helium separator.

Helium was used as the carrier gas. The injection port temperature was 250°C. The column temperature was initially 70°C (2 min) and then ballistically heated or programmed for 39°C/min to 250°C. The temperature of the interface was 250°C.

The electron energy of the mass spectrometer was 23.5 eV, the electron multiplier voltage 1.9–2.0 kV, the recorder input voltage 0.1–0.5 V, the ionisation current 300 μA and the ion source temperature 220°C. The ion monitor was adjusted to *m/e* 321.9 by means of the internal standard (IS). The resolution of the instrument was adjusted to 2000 with 10% valley (main slit 100 μm, β-slit 0.5 mm, collector slit 50 μm).

Clean-up procedure

The samples were homogenized (fat melted) before weighing, and 20 g of milk, 10 g of liver, 1 g of fat, 10 g of muscle, 12–15 g of serum and cells were taken for analysis. The samples were transferred to screw-cap culture tubes (25 × 200 mm) fitted with a PTFE seal. Then 20 ml of 30% sodium hydroxide and 10 ml of absolute ethanol containing 50 pg/ml (50 ppt) of the IS were added to each sample. Then 500–2500 pg of IS were added to liver (1 g) and fat samples in cases where the TCDD content was high.

The tubes were sealed, shaken and heated in a boiling water bath for 1 h. If two phases developed, 5 ml of methanol were added. The completely dissolved sample was cooled and extracted three times with 10 ml of *n*-hexane with vigorous shaking on a mechanical shaker for 15 min.

The *n*-hexane was evaporated to dryness, and 2–3 ml of *n*-hexane and *ca.* 1 g of silica gel saturated with concentrated sulphuric acid were added, mixed and left to stand until the *n*-hexane phase became clear (*ca.* 1 h). The *n*-hexane phase was removed and the silica gel washed twice with 1 ml of *n*-hexane.

The *n*-hexane solution was chromatographed on silica gel and alumina microcolumns (pasteur pipettes), as described by Buser *et al.*[11]. The eluate was carefully concentrated to 1 ml, transferred quantitatively with *n*-hexane into small tubes with a conical bottom and evaporated to dryness in a stream of carbon dioxide. The sample was then dissolved in isooctane to give a final dilution of 5–20 μl, depending on the TCDD content, ready for GC–MS. From 0.8 to 1.0 μl of the isooctane solution was transferred to the solid injector and the solvent evaporated off. The splitless technique was employed.

Relative calibration curve

An internal standardization method was used as indirect calibration in the quantitative analysis of TCDD. In order to determine the standard curve, similar amounts of the IS and varying amounts of TCDD were added to the blank samples. The standard samples were handled in the same way as described in the clean-up procedure and analysed by GC–MS.

The peak heights of IS and TCDD on the mass fragmentograms were measured and peak height ratios (TCDD/IS) calculated and plotted against the concentrations of TCDD (Fig. 1). The calibration curve was determined using independently prepared standard solutions. The precise relationship between the peak heights and the amount of TCDD was determined using regression models.

Reproducibility of GC–MS analysis

Random errors arising from the use of the GC–MS instrument and sample introduction were studied by consecutively analysing the same sample ten times (16 ppt TCDD/25 ppt IS) under the same experimental conditions (Table I).

Reproducibility of the clean-up procedure and sample handling

The relative error of the clean-up procedure was studied by preparing ten milk, liver and muscle samples each containing equal amounts of TCDD and IS. The relative standard deviation was taken as a measure of the reproducibility (Table II).

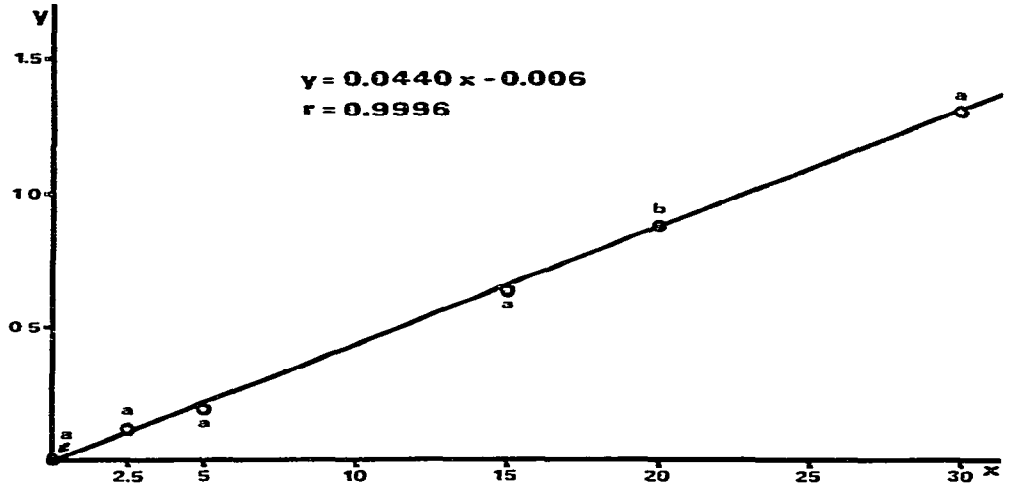

Fig 1. Standard curve (x = ppt TCDD, y = peak height ratio) for TCDD in milk (25 ppt IS/20 g sample). (a) Mean of three independently prepared samples. (b) Mean of ten independently prepared samples.

Recovery

Recovery of TCDD was studied at four concentration levels (Table III). Known amounts of TCDD were added to all the samples before the clean-up procedure. Half the samples received the IS before clean-up and half after. Recovery was also followed throughout the analyses by adding, from time to time, known amounts of TCDD to replicate samples as illustrated in Fig. 2 and Table IV.

Minimum detectable level

The minimum detectable level was determined by preparing a series of milk (fat, muscle, liver) samples containing a decreasing amount of TCDD and a constant

TABLE I
REPRODUCIBILITY OF GC–MASS FRAGMENTOGRAPHIC ANALYSIS

Run	*Peak height ratio (16 ppt TCDD/25 ppt IS)*
1	0.714
2	0 690
3	0 676
4	0 678
4	0 698
6	0 677
7	0.664
8	0 692
9	0 701
10	0 713
Mean	0.690
Standard deviation	0 016
Relative standard deviation (%)	2 4

TABLE II
REPRODUCIBILITY OF CLEAN-UP PROCEDURE, INCLUDING INSTRUMENTAL ERRORS

Tissue	*Number of observations*	*Concentration (ppt)*	*Peak height ratio*		*S D.*	*Rel S D (%)*
			Range	*Mean*		
Liver	10	20*	0 466–0 505	0 483	0.013	2.6
Liver	10	500**	1.160–1.373	1.243	0 064	5.1
Muscle	10	20*	0 465–0.527	0 488	0.017	3.6
Milk	10	20***	0 803–0 943	0 870	0 041	4 7

* IS 50 ppt
** IS 500 ppt.
*** IS 25 ppt

amount of IS. The samples were subjected to the clean-up procedure and analysed as described above. A detector response equal to two and a half times the noise level was accepted as the criterion for these determinations (Fig. 3).

TABLE III
RECOVERY

TCDD (ppt)	*IS (ppt)*	*Peak ratio*	*Mean*	*Recovery (%)*
2.5	25	0.118		
2.5	before	0.118	0.119	
2.5	clean-up	0 120		73.1
2.5	25	0.080		
2.5	after	0.096	0.087	
2 5	clean-up	0 085		
5	25	0 235		
5	before	0 211	0 221	
5	clean-up	0 218		86 0
5	25	0 192		
5	after	0.185	0.190	
5	clean-up	0.194		
15	25	0 673		
15	before	0 593	0.634	
15	clean-up	0 636		84.5
15	25	0 544		
15	after	0 551	0 536	
15	clean-up	0 513		
30	25	1 277		
30	before	1.305	1.309	
30	clean-up	1.345		78 1
30	25	1 005		
30	after	1 021	1.022	
30	clean-up	1 041		

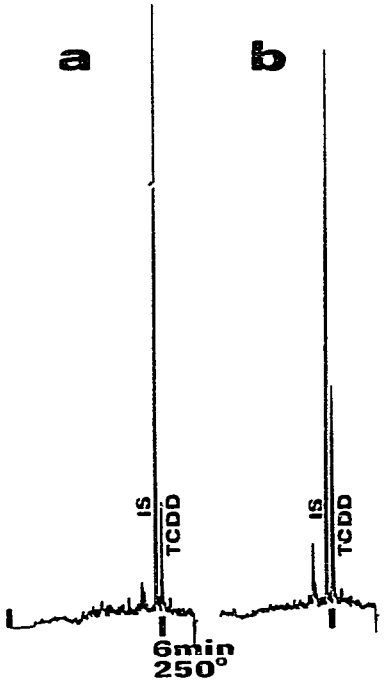

Fig 2. example of analysis results showing recovery of added TCDD (a) 3 8 ppt recovered; (b) 5 0 ppt added, 9.0 ppt recovered. Column, 20 m × 0.4 mm I.D. FFAP.

RESULTS AND DISCUSSION

In this study 1,2,3,4-tetrachlorodibenzo-*p*-dioxin was chosen as internal standard instead of ^{37}Cl-labelled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, because the multiple ion detection unit of our Jeol D 100 mass spectrometer was not able to follow very narrow peaks eluted from glass capillary columns if two or three fragments were measured simultaneously. It has also been reported[12] that the highest sensitivity can be reached by single ion measurement.

Comparison between the use of the 1,2,3,4-isomer and ^{37}Cl-labelled TCDD as the internal standard requires further study.

No difference was found in the behaviour of the IS and TCDD in different stages of the clean-up procedure described.

TABLE IV
CONTROL OF ANALYSIS METHOD

Test animal	*Recovered TCDD (ppt)*	*TCDD recovered, when 5 ppt added (ppt)*
2	3.8	9 0
3	3 8	8 2
4	2 8	7.8
6	3 6	8 2
7	3.7	8 3

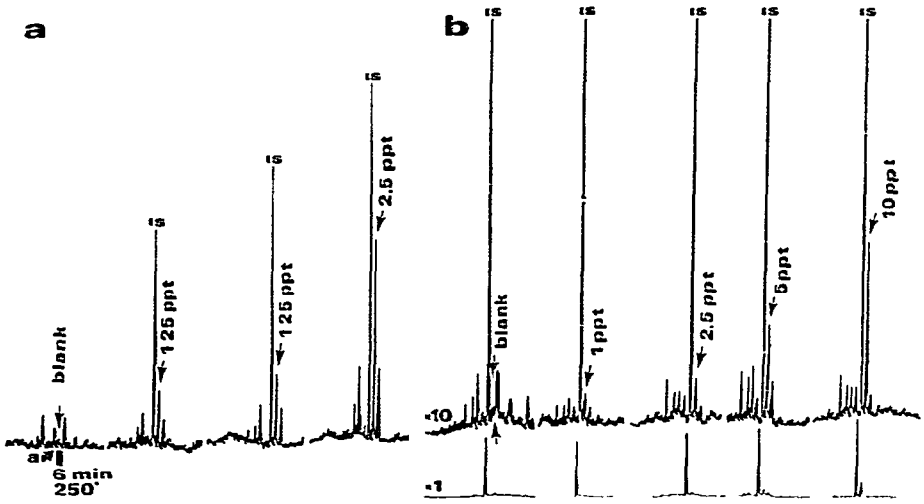

Fig. 3. Minimum detectable level of TCDD (a) in milk (IS 5 ppt); (b) in muscle (IS 50 ppt) Column, 8 m × 0 35 mm I.D. FFAP.

Of the liquid phases tested, Carbowax 20M and FFAP gave the best separation of TCDD from the IS and other possible interfering compounds. The non-polar phases SE-30 and OV-101 were not able to separate TCDD from the IS. The most suitable column for routine work was an 8 m × 0.35 mm I.D. FFAP column giving a separation number of 1.8 between TCDD and the IS. The small peak that is sometimes present, interfering with the IS (peak a in Fig. 3a), can be separated by using a longer column. The level of this interfering peak was never found to exceed 0.5 ppt and its effect on the results was negligible.

The fragment 321.9 was chosen for measurements because of its higher sensitivity and lower noise level in the mass fragmentograms obtained by measuring the ion 319.9. No interfering compounds above the detection level were found in the blank sample or in samples from control animals having the same retention time of TCDD in the mass fragmentograms.

The selectivity obtained by monitoring one ion was found to be sufficient in this study because the results were always compared with those from blank samples and samples from the control animals. Nevertheless, in the cases of environmental samples and samples of unknown origin the measurement of more than one ion should be taken into consideration.

A resolution of 2000 was found to be enough to separate TCDD and the IS from compounds that could overlap them at a resolution of 300–500. The separation was not improved by increasing the resolution of the mass spectrometer above 2000.

Both neutral and acid/base clean-up procedures have been reported in the literature[13,14]. In order to ensure that as much as possible of the TCDD is removed from biological material, alkaline hydrolysis was chosen in this study. Various hydrolysis temperatures and hydrolysis times were used. The results showed no differences between different treatments.

A cloudy precipitate sometimes appeared in the *n*-hexane extract. This can be

avoided by heating the test-tube for a short time at 50–60°C and carrying out extraction on the hot sample.

Two basic sources of error were taken into consideration when preparing the standard curve, namely the instrumental errors (stability of ion source, filament conditions, electron voltage, ion repeller voltage, magnetic drift, recording and amplifying system, sample introduction, etc.) and the errors arising as a result of sample handling.

The standard curve used in this study is shown in Fig. 1. The linear regression equation was calculated as $y = 0.0440x - 0.006$, $r = 0.9996$ ($n = 25$).

The high correlation coefficient indicates that the relationship between peak height ratio and the TCDD concentration is linear.

Repeated analysis of a single milk sample (16 ppt TCDD/25 ppt IS) gave the following results (Table I). The peak height ratio (TCDD/IS) varied from 0.664 to 0.714, with a mean value of 0.690 and a coefficient of variation of 2.4%. The results concerning the reproducibility of the clean-up procedure are shown in Table II. The values presented in this table included instrumental and sample-handling errors for milk, muscle and liver.

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