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Dioxin and polychlorinated biphenyl analysis: Automation and improvement of clean-up established by example of spices

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To analyze polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDDs/PCDFs) and polychlorinated biphenyls (PCBs) in spices by gas chromatography-mass spectrometry, a new clean-up method had to be developed owing to the high content of essential oils in the samples. A solid-phase extraction (SPE) column with activated silica endowed with sulfuric acid and sodium hydroxide was used. Under these conditions, clean-up was achieved using at least 5 – 7 g of pepper and even higher amounts of other spices. The automatized clean-up comprised three additional chromatographic steps after accelerated solvent extraction (ASE) followed by gel permeation: chromatography on a florisil SPE column, extract cleaning with the above-mentioned silica SPE column and chromatography with an activated charcoal column. On the basis of this automatized clean-up, a method that is more effective, rapid, simplified and economical than the available methods for PCDD/PCDF and PCB analysis is proposed. In model studies, the average recoveries for PCDDs/PCDFs ranged between 82.6% and 105.6% and for the PCBs between 71.3% and 113.3%.

Keywords: Automation / Dioxin-like polychlorinated biphenyls / Polychlorinated biphenyls / Polychlorinated dibenzo-p-dioxin/dibenzofurans / Spices

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1 Introduction

Polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins and -dibenzofurans (PCDDs/PCDFs) are ubiquitous pollutants in the environment of most industrialized countries [1]. PCDDs/PCDFs are mainly man-made compounds, which have been found as contaminants in several industrial chemicals. These undesirable substances are also produced in thermal processes [2], including the incineration of municipal waste [3]. Since 1989 [4] the production, the import, the export or the sale of PCBs in Germany is forbidden, but in our days PCBs are still found in the environment due to their long half-lives [5].

Owing to their persistence and lipophilic character, PCDDs/PCDFs and PCBs tend to accumulate in the food chain. As humans are at the top of the food chain, PCDD/PCDF and PCB congeners can also be found in human blood, milk and fat [6, 7]. The highest contribution to the total intake of PCDDs/PCDFs and PCBs through foods for consumers in Japan was from fish and shellfish followed by meat, eggs, milk and dairy products [8]. Hecht and Blüthgen [9] analyzed meat and meat products from Germany in a representative study for their PCDD/PCDF content and observed higher levels in meat products than in meat of the same farmed animal. Therefore, the question that spices could contaminate the meat products arose. Owing to the lack of data on the amount of PCDDs/PCDFs and PCBs in spices, a clean-up method had to be developed to remove essential oils from spices to an acceptable degree for subsequent analysis, especially regarding the contingently low concentrations of these compounds.

Another aim of this work was to reduce costs, amounts of solvent, man-power and time for each analysis by automating single clean-up steps for the PCDD/PCDF and PCB analysis in general.

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Abbreviations: ASE, accelerated solvent extraction; **GPC**, gel permeation chromatography; **HRMS**, high-resolution MS; **PCB**, polychlorinated biphenyl; **PCDD**, polychlorinated dibenzo-*p*-dioxin; **PCDF**, polychlorinated dibenzofuran

2 Material and methods

2.1 Material and reagents

The $^{13}C_{12}$-labeled PCDD/PCDF congeners (D 48, D 54, D 66, D 67, D 70, D 73, D 75, F 83, F 94, F 114, F 118, F 121, F 124, F 130, F 131, F 134 and F 135) and PCBs (PCB 28, PCB 52, PCB 77, PCB 81, PCB 101, PCB 105, PCB 114, PCB 118, PCB 123, PCB 126, PCB 138, PCB 153, PCB 156, PCB 157, PCB 167, PCB 169, PCB 180 and PCB 189) for the preparation of the internal standards and the deuterated PCDD/PCDF (D 48, F 69 and F 131) and PCB (PCB 77 and PCB 141) congeners for the recovery standards were obtained from Promochem (Wesel, Germany).

All solvents (n-hexane, toluene, cyclohexane, ethyl acetate and i-octane) had a purity of "picograde" and were also purchased from Promochem.

Florisil (0.150–0.250 mm) and silica gel 60 (0.063–0.200 mm) were purchased by Merck (Darmstadt, Germany). Sea sand was purchased from Aldrich (St. Louis, MO, USA; sand, white quartz −50+70 mesh). All three compounds were calcined for 12 h at 550°C.

During the clean-up, poly(acrylic acid), partial sodium salt-graft-poly(ethylene oxide) from Aldrich was used several times as a drying substance.

The gel permeation chromatography (GPC) column was filled with 60 g Bio-Beads S-X3 (200–400 mesh), a cross-linked divinylbenzene-styrene copolymer, from Bio-Rad Laboratories (Munich, Germany).

The following equipment for automation of the clean-up steps was used: an ASPEC XLi with a modified injection needle (a ventilated needle with a low pressure valve), an HPLC pump, a six-point valve and a switching valve (Gilson International, Middleton, WI, USA).

The LC glass column with an id of 12.5 mm was purchased from Latek, Eppelheim, Germany and filled with 250 mg activated charcoal (Supelclean ENVI-Carb from Supelco,MO, USA).

After use, the glassware containers were first cleaned in a dishwasher and then heated to 300°C for 12 h. Directly before use flasks and all other glass equipment were rinsed with n-hexane to prevent cross-contamination.

2.2 Standard solutions

The standard solution used for determining the mono- and di-ortho PCBs had a concentration of 8.4 ng/mL in i-octane; for determining the non-ortho PCBs, concentrations of 1.2 ng/mL were used. The PCDD/PCDF concentrations in the standard solution ranged from 0.1 to 0.6 ng/mL. All standards were dissolved in i-octane. Internal standard (50 μL of each standard solution) was added before sample extraction because spices contain less than 10% essential oils [10] (mean: 7.8%, median: 4.4%).

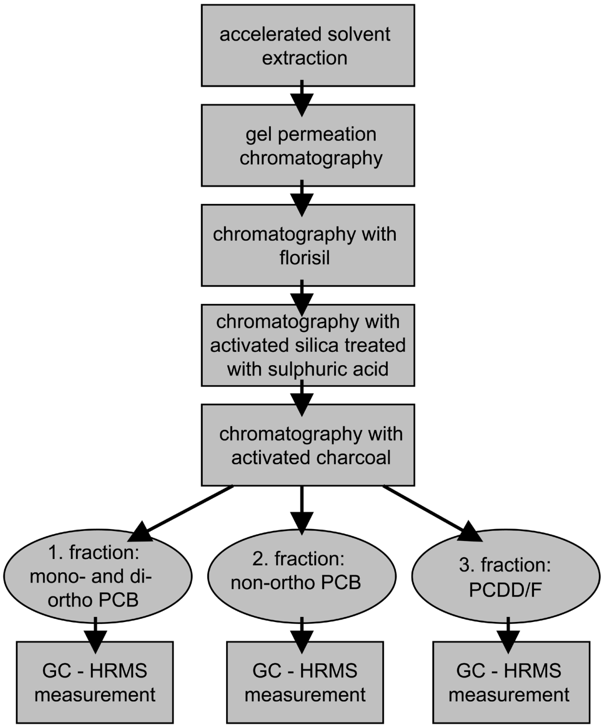

Figure 1. Flow chart to describe the whole clean-up procedure to analyze PCBs and PCDDs/PCDFs in spices.

2.3 Analytical procedures

The analytical procedure described by Karl et al. [11] was modified. Accelerated solvent extraction (ASE), GPC, chromatography with florisil followed by chromatography with activated silica/H_2SO_4-silica and a final chromatography on activated charcoal were used as clean-up steps before determining the analytes in the samples with GC-high-resolution MS (GC-HRMS) (Fig. 1).

2.4 ASE

The method for extracting PCDDs/PCDFs and PCBs was adapted from Jira [12]. Extraction cells were filled with dried sample material, drying substance and sea sand. Extractions were performed by ASE 200 from Dionex: For extraction, n-hexane was used at a pressure of 100 bar and a temperature of 100°C. The samples were extracted in two cycles with a static time of 10 min each. The solvent of the extraction was removed with nitrogen in a water bath at 40°C.

2.5 GPC

GPC was adapted from the method published by Jira [12] to remove higher molecular weight compounds from the sample (at most 1 g fat/injection). Only the wasting and collecting times were changed, to 0–28 and 28–38 min, respectively, for the clean-up of samples to analyze PCDDs/PCDFs and PCBs. For GPC, a LC glass column with an id of 25 mm was filled with 60 g Bio-Beads S-X3. The GPC (Abimed Gilson, Langenfeld, Germany) was carried out with a solvent mixture of cyclohexane : ethyl acetate (1 : 1) and a flow rate of 5 mL/min.

2.6 Clean-up with florisil

The samples were evaporated under pressure, carefully brought to dryness with nitrogen and dissolved in 1 mL toluene. Florisil, calcined for 12 h at 550°C, was deactivated with 4% water, and 3 g were filled into commercial disposable 8-mL SPE columns (id 12 mm). After conditioning of the columns with 10 mL toluene, the samples were applied and eluted with 60 mL toluene to remove more polar compounds.

2.7 Clean-up with activated and endowed silica

After evaporation of the solvent to near dryness with rotation evaporation and nitrogen, the samples were dissolved in 1 mL n-hexane. Commercially available 8-mL SPE cartridges with an id of 12 mm were filled, in the following order, with 0.3 g drying substance, 1 g 33% NaOH-silica, 0.25 g activated silica, 2.5 g 44% H_2SO_4-silica and 0.5 g 22% H_2SO_4-silica; all together about 5 g silica. After conditioning of the columns with 10 mL n-hexane, the sample extracts were put on the columns and eluted with 60 mL n-hexane. The basics of this method are described in [13].

2.8 Clean-up with activated charcoal

An LC glass column with an id of 12.5 mm was filled with 250 mg activated charcoal (Supelclean ENVI-Carb). For conditioning of the column, one blank run was made with a flow rate of 5 mL/min following program: (1) 20 mL solvent mixture 1 (n-hexane/toluene; 99 : 1); (2) 20 mL solvent mixture 2 (n-hexane/toluene; 75 : 25); (3) 60 mL toluene (back-flush mode); and (4) cleaning and conditioning of the column with toluene and solvent mixture 1 in both flow directions to use the column filling again for a new sample. Samples were concentrated to a volume of 1 mL and fractioned with the mentioned program. In the first fraction (20 mL n-hexane/toluene; 99 : 1) the di- and mono-ortho-substituted PCBs, and in the second fraction (20 mL n-hexane/toluene; 75 : 25) the non-ortho-substituted PCBs were collected. In the third fraction (60 mL toluene) the PCDDs/Fs were collected in back-flush mode because they have the characteristic to stick on activated charcoal. To prevent cross-contamination, the activated charcoal column was cleaned after each sample with solvent mixture 1 and with toluene in back-flush and in normal flow mode. Blank samples between normal samples could rule out possible cross-contamination.

2.9 GC-HRMS

The GS-HRMS system used was a Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) with an injection in split/splitless mode and combined with a VG Autospec Micromass (Manchester, UK) high-resolution mass spectrometer. The data interpretation was performed with Xcalibur software from Thermo Finnigan (Dreieich, Germany). A ZB-5ms column (id 0.25 mm, film thickness 0.25 µm, 5% polysilarylene, 95% polydimethylsiloxane) from Phenomenex (Torrance, CA, USA) with a length of 60 m was used with helium purity 5.0 as carrier gas. The GC program for the PCDD/PCDF measurement was as follows: 80°C (1 min) to 210°C (5 min) at 25°C/min, to 240°C (5 min) at 5°C/min and subsequently at 5°C/min to 320°C, then maintained for 10 min at 320°C. Owing to possible interactions of some native PCB peaks, another GC program was compiled to divide PCBs: 70°C (2 min) to 180°C (0 min) at 30°C/min, to 290°C (0 min) at 5°C/min and subsequently at 20°C/min to 320°C, then maintained for 10 min at 320°C. PCDDs/PCDFs and PCBs were measured working in the electron impact (EI) positive ion mode with an electron energy of 35 eV, the source temperature was set at 250°C. Data was recorded in selected ion recording mode. The resolution of the mass spectrometer was tuned up to 7000 (10% valley definition). The limit of detection (LOD) was 0.03 ng WHO-PCDD/PCDF-TEQ/kg fresh material; the limit of quantification (LOQ) was 0.10 ng WHO-PCDD/PCDF-TEQ/kg fresh material.

3 Results

To achieve a simplification of the method, initially the column size used in the clean-up was miniaturized, and then single steps were automated. The successful miniaturization of the columns was the pre-condition for the automation of the three following clean-up parts. For chromatography on florisil and on silica treated with sulfuric acid, 8-mL

Table 1. Recovery (R) rates in% from the automation of the chromatography over florisil, tested with standards.

Florisil: R (%)	PCB153	PCB138	PCB77	2378-TCDF	2378-TCDD	1234678-HpCDF
Column 1	115	97	90	96	98	106
Column 2	118	101	98	98	95	102
Column 3	115	106	101	96	101	108
Column 4	107	100	100	95	101	110
Column 5	112	99	101	103	100	106
Column 6	113	102	98	96	100	102
Column 7	119	102	108	96	96	105
Column 8	114	109	103	96	100	107
Column 9	109	106	98	96	93	105
Column 10	111	101	106	96	97	106
X	**113.3**	**102.3**	**100.3**	**96.8**	**98.1**	**105.7**
n	10	10	10	10	10	10
SD	3.7	3.7	5.0	2.3	2.8	2.5
RSD	3.3	3.6	5.0	2.4	2.8	2.3

X, mean; *n*, no. of repetitions; TCDD, tetrachloro-dibenzo-p-dioxin; TCDF, tetrachloro-dibenzofuran; HpCDF, heptachloro-dibenzofuran

SPE cartridges were used, both filled with the respective column material before the procedure was started. With the commercial cartridges, the ASPEC XLi system could be used to carry out the two mentioned clean-up steps. To achieve this, some adjustments and structural alterations of the original equipment were still necessary. To use an elution volume of 60 mL, special Teflon funnels had to be added, which were connected by Teflon tubing. This tubing drained the eluate in 100- or 250-mL round-bottom flasks located in a rack below.

Before the automated chromatography on florisil could be used in practice, the recoveries had to be checked with standard PCDDPCD/F and PCB compounds. Average recoveries ranged between 96.8% and 113.3% (Table 1).

The analytical problems with the essential oils were eliminated by using commercially available 8-mL SPE cartridges, filled as described above. Most of the interfering substances in the essential oils are oxidized, sulfonized or transferred into an ionic form by the H_2SO_4-silica, and cannot be eluted with hexane. The clean-up method using H_2SO_4 columns was successfully validated using essential oils of pepper. Both the capacity of this clean-up step and the influence of this method on the recoveries of the organochlorine target compounds were checked, by applying and eluting different concentrations of pepper oil solutions from these columns.

From the GC-HRMS measurements (as a control of this single clean-up step), the recovery in the first fraction (di- and mono-ortho-substituted PCBs) was 114%, in second fraction (non-ortho-substituted PCBs) was 62%, and in the third (PCDDs/Fs) was 73% for a starting amount of 0.2 g of essential oil from pepper. In combination with other results, and including the losses during the other clean-up procedures, about 0.15 g pepper oil per injection (a recovery of 60–120% according to [10] and with regard to the Horwitz Curve, [14]) was cleaned up with acceptable recoveries for the non-ortho PCBs that were collected in the second fraction during the chromatography on activated charcoal. The reason for this limited capacity is that higher amounts of the essential oil in the sample lead to charring of the organic compounds and to some of the analytes being retained. Pepper contains about 4% essential oil [15]. About half of the components of the essential oil are removed from the samples during the processing without the clean-up with sulfuric acid columns. With the new clean-up method, including chromatography with sulfuric acid columns, it is possible to use 5–7 g pepper for one sample with no visible essential oil left after clean-up. Further experiments showed that higher initial weights of other spices, with few exceptions, can be applied.

A feature of the automation using chromatography with activated charcoal was the elution of PCDDs/PCDFs by reverse flow, which necessitated some changes in the standard equipment. Since the disposable columns could not be used in back-flush mode, a LC glass column filled with activated charcoal was used. This LC column was connected to an HPLC pump by a switching valve (Fig. 2), which determines the flow direction of the solvent. To detect and quantify PCBs and PCDDs/PCDFs with GC-HRMS, the samples had to be divided on the activated charcoal column into three fractions. Owing to the different solvent mixtures for the three fractions, the HPLC pump was connected to a six-point valve. By modification of the racks, it was possible to collect the different fractions. To use the injection needle as a fraction collector, the needle was ventilated and had a low-pressure valve.

Chromatography performed automatically on activated charcoal yielded average recoveries between 71.3% and 104.9% (Table 2). Up to 40 samples in series could be frac-

Table 2. Recovery (R) rates in% from the automation of the chromatography over activated charcoal tested with standards

Charcoal: R (%)	PCB153	PCB138	PCB77	2378-TCDF	2378-TCDD	1234678-HpCDF
Column 1	128	119	64			
Column 2	120	115	70	66	65	76
Column 3	114	123	69	89	90	93
Column 4	119	110	69	98	65	94
Column 5	78	100	68	96	86	94
Column 6	81	92	66			
Column 7	99	90	75			
Column 8	100	100	72			
Column 9	97	99	72			
Column 10	96	83	74			
Column 11	114	77	88			
Column 12	113	102	68	78	107	83
X	**104.9**	**100.8**	**71.3**	**85.4**	**82.6**	**88.0**
n	12	12	12	5	5	5
SD	15.6	14.1	6.2	13.4	17.9	8.2
RSD	14.9	14.0	8.6	15.7	21.7	9.3

X, mean; *n*, no. of repetitions; HpCDF, heptachloro-dibenzofuran

Figure 2. ASPEC XLi system with some adjustments and structural alterations to clean-up samples for the PCB and PCDD/PCDF analysis: SPE unit (left), and additional equipment for the chromatography with activated charcoal (right); a: injection needle with a low pressure valve and a ventilated needle; b: special and new built racks to collect the different fractions; c: special Teflon funnels connected to Teflon tubing; d: LC glass column filled with activated charcoal; e: switch valve to change the flow direction; f: HPLC pump; g: six-point valve connected to the HPLC pump for the different solvents.

tionated on one activated charcoal column filling. To determine the point at which the activated charcoal column could not longer separate the target compounds into three fractions within the fixed elution times, the recoveries of the second fraction (non-ortho PCBs) had to be observed, because the recoveries of this fraction were the first to decrease.

The quality of this newly developed clean-up procedure for spices was successfully tested with reference material (NIST SRM 1588a). Furthermore, every sample series was controlled with two blank samples.

4 Discussion

Many different clean-up methods for the PCDD/PCDF measurements are described in the U.S. Environmental Protection Agency method 1613 [16]. In addition, some clean-up methods have been described for detecting PCDDs/PCDFs and PCBs especially in human matrices such as blood, serum or milk [8, 7, 17–19]. Clean-up procedures for different food [9, 20, 21], feed [22, 23] or environmental [1, 24, 25] matrices have also been very well investigated. However, there are strong distinctions between the qualities of the individual methods. Often very fast clean-ups implicate high LODs and LOQs, or do not remove interfering compounds to an adequate degree. Further clean-up methods function with acceptable results, but cannot be adapted to another sample matrix. This was the case for the detection of PCDDs/PCDFs and PCBs in spices with the aim of achieving an LOD of less than 0.03 ng WHO-PCDD/PCDF-TEQ/kg fresh material and an LOQ of less than 0.10 ng WHO-PCDD/PCDF-TEQ/kg. However, the developed clean-up and the automation using the three individual clean-up procedures can easily be adapted to similar methods.

The automated clean-up system has advantages over the EPA method, *i.e.*, the reduction of costs, solvent amounts, man-power and time for one analysis. An important point is the very high degree of reproducibility using the ASPEC XLi system.

In addition to these improvements, further changes could be envisaged, *e.g.*, a further six-point valve could be connected to the system to use different SPE procedures in one sequence.

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