Advantages of the solvent mixture ethyl acetate/cyclohexane (1:1, v:v) for microwave-assisted extraction and accelerated solvent extraction in view of quantitative determination of organochlorines in fish tissue

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Introduction

After homogenisation, the extraction is the first step in the sample clean-up procedure for the determination of organochlorines (PCBs, HCHs, HCB, DDT, compounds of technical toxaphene - CTTs, chlordane) in environmental samples. In modern analytical laboratories the classic Soxhlet extraction is more and more replaced by faster, less solvent and time consuming techniques, such as microwave-assisted extraction (MAE) [1-4] and accelerated solvent extraction (ASE) [5-6]. The most common MAE technique is the closed-vessel MAE (CV-MAE) under pressure and high temperature. An alternative to CV-MAE is the focused open-vessel MAE (FOV-MAE) which operates at atmospheric pressure and refluxing [1-4]. ASE is an automated extraction technique, which uses hot solvents and high pressure for the extraction.

The solvent mixture we used for both ASE and MAE was ethyl acetate/cyclohexane (1:1, v:v) [3]. Nonpolar solvents like cyclohexane or n-hexane are preferable for extraction of organochlorines due to reduced coextraction effects, but they cannot be heated directly by microwaves [2,7]. Addition of ethyl acetate allows a direct heating of the solvent and also allows a penetration into the pores of a wet sample matrix. Another advantage is, that after volume adjustment of the extract, a direct performance of gel-permeation chromatography (GPC) with *bio-beads SX-3* is enabled, as GPC uses the same solvent mixture [3], and a time consuming source of error (solvent exchange) is avoided. The composition of the azeotrop is 54:46 [8], this means that evaporation does not change the solvent composition significantly.

MAE with ethyl acetate/cyclohexane (1:1, v:v) in combination with GPC proved to be well suited for the determination of organochlorines in seal blubber, cod livers, and partly lyophilised eggs with a water content up to 30% and a fat content from 5 to > 90% [3-4]. Now, this solvent mixture is applied for ASE of cod livers (30% water) and MAE of cod livers and fresh fish filet (70% water) without drying before, and the advantages of each technique are discussed.

Material and Methods

Chemicals and organochlorine standards. Standard solutions of organochlorines (10 ng/µL each) were obtained from Promochem, Wesel (Germany) or Dr. Ehrenstorfer, Augsburg (Germany). The organochlorines were determined as Σ DDT (= sum of p,p'-DDT, p,p'-DDD, p,p'-DDE), Σ PCBs (= sum of PCB 52, 101, 138, 153, 180), Σ CTTs (= sum of B8-1413, B8-2229, B9-1679, B9-1025), and Σ chlordane (= sum of oxychlordane, trans-chlordane, cis- and trans-nonachlor).

Sample origin. Canned cod livers were purchased in a supermarket in Jena, Germany (1998). Mackerels (*Scomber scrombrus*) were from the German North Sea (1997). Fresh fish filet was homogenised and mixed with Na_2SO_4 (1:1, w:w), cod livers were only homogenised.

Microwave conditions. The extraction was performed after addition of the internal standard perdeuterated α-HCH (α-PDHCH) [9].
FOV-MAE was performed in a Soxwave 100 (Prolabo, France). The reservoir (15 mL) above the tap originally designed to evaporate the solvent, was used as a trap to separate coextracted water. The procedure was recently described in detail [4]. CV-MAE was performed in an MLS 1200 mega apparatus (MLS, Leutkirch, Germany) by application of a microwave program with seven extraction cycles [2,3]. After the extraction of fish filet, coextracted water was separated manually by use of a pasteur pipette, 10 mL fresh solvent were added, and the samples were extracted a second time. Cod livers were extracted once without separation of water in both MAE systems, respectively. After volume adjustment of the extracts to 20 mL or 10 mL, 9 mL were directly subjected to GPC, 1 mL was used for gravimetric determination of fat.

Sample lyophilisation. The samples were frozen at -24°C, then lyophilised for 24 h in a Beta 1-8k apparatus (Christ, Osterode, Germany) at -30°C and vacuum (0.37 mbar). The temperature of the plates was 25°C.

ASE conditions. Cod livers were mixed with Na_2SO_4 (1:4, w:w) and extracted in a Dionex ASE 200 (Dionex, USA). The ASE conditions for the solvent ethyl acetate/cyclohexane (1:1, v:v) were optimised by determination of the fat content, which was compared to the fat content obtained with the method of Dionex [10]. Agreement in fat content was achieved with the following conditions: temperature 125°C, pressure 10 MPa, heat-up 6 min, 2 static cycles à 10 min, flush volume 60%, purge 1 MPa nitrogen for 120 sec. The extracts were adjusted to 50 mL, then subjected to GPC.

Gel-permeation chromatography conditions. Gel-permeation chromatographic separation of extracted fat from organochlorines was obtained with *bio beads S-X3* in combination with an Autoprep 1002 (ABC, Analytical Biochemistry Columbia, USA) system. Ethyl acetate and cyclohexane (1:1, v:v) were used as the solvent [3].

Adsorption chromatography on deactivated silica. The GPC eluate was condensed in a rotavapor, and after removal of the more polar ethyl acetate and solvent exchange to isooctane, adsorption chromatography on silica was performed on 3 g silica (deactivated with 30% water) as recently described [3,4]. Aliquots of the eluate were subjected to GC/ECD analysis or subjected to PCB/CTT group separation.

PCB/CTT group separation. For the determination of the CTTs, the final solution was fractionated on 8 g silica gel as described by Krock et al. [11]. PCBs were quantitatively eluted with 48 mL n-hexane, and CTTs and chlordane were eluted with a more polar solvent in a second fraction [12]. The eluates were condensed in a rotavapor and blown down in a nitrogen flow. Aliquots were subjected to GC/ECD.
GC/ECD conditions. The GC/ECD analyses were performed with an HP 5890 (Hewlett-Packard) gas chromatograph equipped with two capillary columns and two ^{63}Ni ECDs. The injector

(splitless) and detector temperatures were 250°C and 300°C. Helium was used as carrier gas at constant flow of 1.3 mL/min. Nitrogen was used as make-up gas. The capillary columns CP-Sil 2 and CP-Sil 8/20% C18 (both: length 50 m, 0.25 mm internal diameter, 0.25 µm film thickness) were from Chrompack, Middelburg, The Netherlands. After injection at 60°C (1.5 min) the GC oven temperature was ramped at 40°C/min to 150°C (5 min), then ramped at 2°C/min to 230°C, and finally ramped at 5°C/min to 270°C (15 min).

Results and Discussion

Determination of organochlorines in cod liver (approx. 50% fat, 30% water). Before ASE, the sample was mixed with Na_2SO_4 (1:4, w:w), with MAE, the samples were extracted directly. The extracts were muddy due to the remaining water content, for this they were filtered through Na_2SO_4 after the extraction. The reproducibility for each technique was very good with a standard deviation mostly < 5%, respectively. The levels for ASE were slightly higher than for the MAE techniques, except for chlordane (see Table 1).

Table 1: Organochlorine levels in cod livers [µg/kg wet weight]

	weight [g]	fat [%]	Σ DDT	Σ PCBs	Σ chlordane*	HCB
ASE (n=10)	5	45.5	595 ± 21	583 ± 22	66	27 ± 1
FOV-MAE (n=7)	2	44.0	548 ± 15	549 ± 17	63	25 ± 1
CV-MAE (n=5)	1	43.4	497 ± 32	512 ± 27	69	23 ± 1

* determined at the moment in only one sample, respectively

FOV-MAE allowed a separation of coextracted water during the extraction in the trap. For ASE, the water could not be separated, but it was bound with Na_2SO_4. For CV- MAE there was also no possibility to separate coextracted water, this means the solvent mixture is more polar due to its water content. This could be an explanation for lower organochlorine levels with CV-MAE. However, repeated extraction resulted in no further organochlorine extraction, and the higher levels for ASE may be partly due to slightly inhomogenous sample material.

Determination of organochlorines in fresh fish filet (approx. 3% fat, 73% water). The water content of the sample has no influence on the results, this was shown by extracting both, lyophilised and fresh fish filet with FOV-MAE and CV-MAE, respectively (see Table 2). There was no difference in the results of FOV- and CV-MAE for the lyophilised fish filet. Varying levels are attributed to deviations during sample clean-up and generally low levels.

Table 2: Organochlorine levels in fish filet (mackerel) [µg/kg wet weight]

	weight [g]	fat [%]	Σ DDT	Σ PCBs	Σ CTTs*	dieldrin
lyophilised (n=6)	1.3	3.5	13.9 ± 0.8	24 ± 2	2.8	2.1 ± 0.1
FOV-MAE fresh (n=8)	10	3.4	11.6 ± 1.3	27 ± 3	2.6	2.7 ± 0.5
CV-MAE fresh (n=8)	1.6	3.7	10.7 ± 1.7	25 ± 4	2.4	2.6 ± 0.6

* determined at the moment in two samples, respectively

For FOV-MAE, the solvent served for removal of water out of the sample matrix. Ethyl acetate coextracts water, and after refluxing, solvent and water departed into two layers in the trap of the FOV-MAE system. Only the upper solvent layer redrained into the glass tube with the sample, and after the extraction, the water phase was removed. The status of water removal was controlled

during the extraction by measuring the temperature of the azeotrop. The less the water content, the more the temperature of the distillate increases and the more apolar the solvent gets. Only the pure extraction solvent enabled a quantitative extraction of organochlorines. For this reason, the extraction procedure for CV-MAE required two extraction steps: the first for coextracting water and manual removal of water after this step, the second for extracting organochlorines quantitatively with the pure solvent. Therefore CV-MAE is less convenient and more time consuming for samples with high water content.
In general, the recovery of α-PDHCH was > 75%. Furthermore, the complete sample clean-up method was validated with certified cod liver oil SRM 1588, and the certified values for PCBs, Σ DDT, and HCB were reached [4].

Conclusions
The solvent ethyl acetate/cyclohexane (1:1,v:v) is well suited for ASE, FOV-MAE and CV-MAE in combination with GPC without solvent exchange. The mixture allows a quantitative extraction even of water containing samples like cod liver (30% water) and fresh fish filet (73% water) without drying before and the time consuming step of lyophilisation is avoided.

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References
1. Lopez-Avila V, Young R, Beckert WF; *Anal. Chem*. **1994,** 66, 1097
2. Hummert K, Vetter W, Luckas B; *Chromatographia* **1996**, 42, 300
3. Vetter W, Weichbrodt M, Hummert K, Glotz D, Luckas B; *Chemosphere* **1998**, 37, 2439
4. Weichbrodt M, Vetter W, Scholz E, Luckas B, Reinhardt K; *Int. J. Environ. Anal. Chem*. **1999**, in press
5. Höfler F, Ezzell J, Richter B; *LaborPraxis* **1995**, 3, 62
6. Richter BE, Jones BA, Ezzell JL, Porter N, Avdalovic N, Pohl C; *Anal.Chem*. **1996**, 68, 1033
7. Kingston HM, Jassie LB (eds.); Introduction to Microwave Sample Preparation - Theory and Practice, American Chemical Society, Library of Congress Cataloging-in-Publication Data, Washington DC, USA, **1988**, ISBN 0-8412-1450-6
8. Handbook of Chemistry and Physics, 56th edition **1976**, CRC Press, Cleveland, Ohio
9. Vetter W, Luckas B; *J. High Resol. Chromatogr*. **1995**, 18, 643
10. Application Note 321, Dionex Corporation, Sunnyvale, CA, USA
11. Krock B, Vetter W, Luckas B; *Chemosphere* **1997**, 35, 1519
12. Klobes U; doctoral thesis, University of Jena, **1999**