COMBINED MICROWAVE-ASSISTED EXTRACTION AND GEL-PERMEATION CHROMATOGRAPHY AS SAMPLE CLEAN-UP FOR FISH TISSUE AND BLUBBER OF MARINE MAMMALS

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Introduction

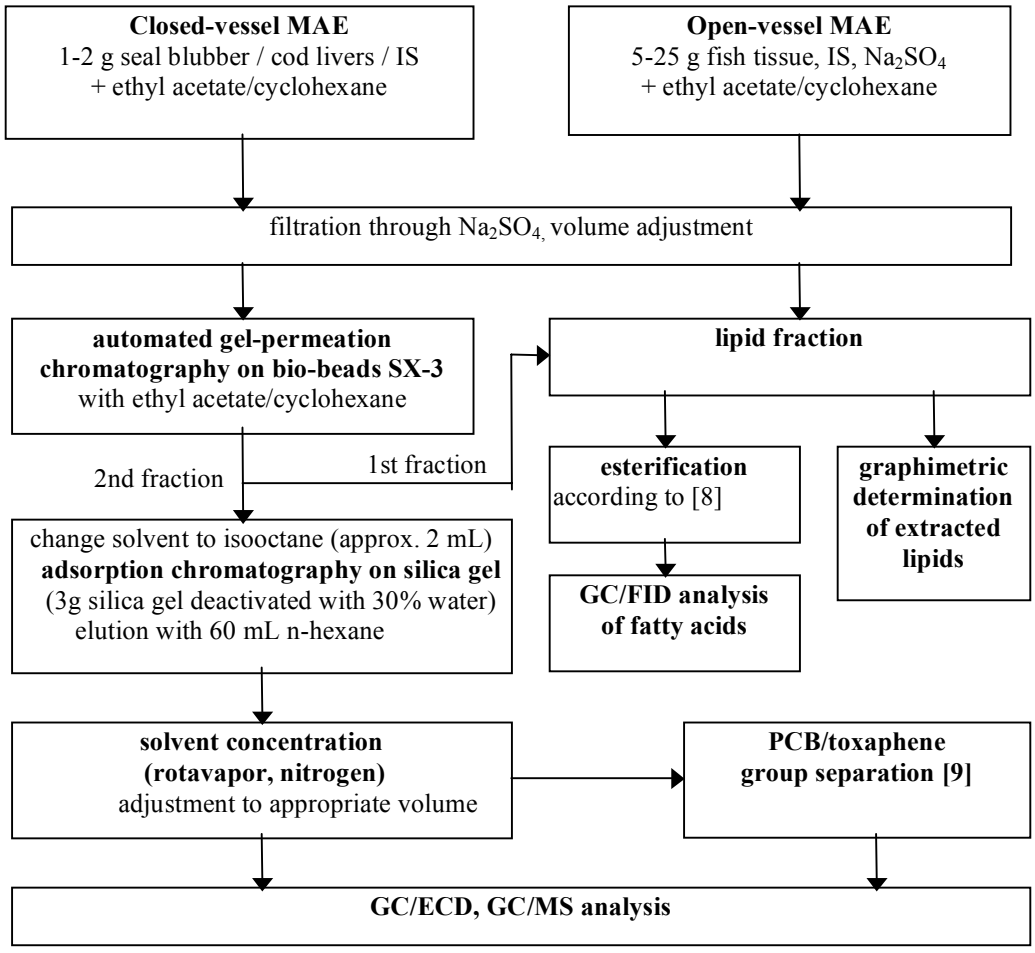
Recent method developments in analytical chemistry focus on faster sample clean-up techniques. For example, microwave-assisted extraction (MAE) has been suggested to replace the Soxhlet technique for the extraction of organochlorines from environmental samples. The major advantage of MAE is a more even heating of the sample in contrast to conventional heating which shows a temperature gradient from the flask to the center of the solution.
Sample clean-up procedures usually consist of several steps, and exchange of solvent between these steps is a source for the loss of volatile components. Consequently, two clean-up steps using the same solvent are desirable. The best conditions are those yielding (i) quantitative recovery and (ii) the easiest combination with follow-up clean-up steps.
Recently, we simplified sample clean-up techniques for lipid containing samples by the combination of microwave-assisted extraction (MAE) and gel-permeation chromatography (GPC) [1]. Instead of a mixture of n-hexane and acetone which is the most common extraction solvent in MAE [2][3], we applied ethyl acetate and cyclohexane (1:1, v:v) which is also the solvent of the GPC. No solvent exchange is necessary between the two steps, and this limits the loss of volatile substances and fastened the sample clean-up. Very good results were obtained for organochlorine determination in blubber of seals, cod livers, and eggs of birds [1][4].
Two different MAE techniques exist, i. e. extraction in closed vessels and extraction in open vessels and refluxing of the solvent. Both techniques have advantages and disadvantages in terms of handling. Here we present some of the theoretical background of the method.

Material and Methods

Closed-vessel MAE was performed with an MLS 1200 mega apparatus (Microwave Laboratory Systems, Leutkirch, Germany). A detailed description of the system was recently presented [5]. Six samples can be extracted in parallel in closed vessels. In brief, accurately weighed samples and the solvent were placed into 70 mL quartz vessels mounted and sealed in the digestion system. Focused open-vessel MAE was performed with the Soxwave 100 (Prolabo, France). More details can be extracted from references [4][6].
Automated gel-permeation-chromatography was carried out using an Autoprep 1002 (ABC, USA) with 50 g bio beads S-X3. Silica gel clean-up was performed as earlier described. GC/ECD and GC/MS analysis were performed with our lab standard methods [7]. Scheme 1 shows the complete sample clean-up technique suggested for lipid-containing samples.

Scheme 1: Sample clean-up for the quantitative determination of organochlorines

including combined microwave-assisted extraction (MAE) and gel-permeation chromatography with the same solvent



Results

Requirement for the adsorption of microwave energy is a sufficient dielectric constant of the sample and particularly the solvent which is usually present at a big surplus. A sufficient heating of the sample requires a sufficient boiling point of the solvent (open vessel MAE). Lipophilic substances are only quantitatively extracted with non-polar solvents. On the other hand, water in the sample matrix requires a sufficient water solubility of the solvent for an entire penetration of the sample. If this is not guaranteed, some of the target compounds may not be extracted. This was observed during the extraction of cod livers with the non-polar solvent n-hexane in combination with the microwave transformer Weflon.

The use of the solvent mixture ethyl acetate/cyclohexane has several advantages. It has a comparably high boiling point and forms a suitable azeotrop of an almost equimolar mixture. It has a sufficient dielectric constant and can be heated directly without addition of a microwave transformer [5][10]. Furthermore, it behaves ambivalent since it behaves non-polar, but accepts some water (see Table 1).

Table 1: Physical properties of different solvents

Solvent	boiling point [°C]	polarity (elotrop order)	dielectric constant	water uptake- in weight-%
Acetone	56.2	0.56	21.3	no limit
Cyclohexane	81.4	0.04	2.0	0.01
Dichloromethane	40	0.42	9.1	0.2
Diethylether	34.5	0.38	4.3	1.3
Ethyl acetate	77.2	0.58	6.0	7.7
n-Hexane	69	0	1.9	0.01
Water	100	-	80.3	(no limit)

Open-vessel microwave-assisted extraction.

This technique is similar to the Soxhlet method. The solvent is refluxed in a solvent condenser and temperatures around the boiling point of the solvent are obtained. Open vessel MAE was applied for the extraction of fish tissues (tissue of mackerel, cod, and flounder) [6] and even for the extraction of entire, partly lyophilized eggs of penguins and skuas [4].
Water in the sample is co-extracted and forms a mixture with ethyl acetate and cyclohexane (a ternary azeotrop). The system contained a tap to exit recondensed solvent. Above the tab, there is a solvent reservoir, which functions as a water trap. The distillate is collected in this water trap, and water (lower layer) separates from upper layer (ethyl acetate/cyclohexane) [4]. By this, water is selectively removed from the sample. The removal of water lasts approximately for 10 min. During this period, the extraction solvent (and also the sample) is getting less polar as water is separated from the sample flask. Therefore, the efficiency of the MAE increases with the extraction time. With the removal of the water, the number of dipoles in the sample is decreased and the microwave power has to be increased to maintain the refluxing of the solvent.

Closed vessel microwave-assisted extraction

Samples with high lipid content are efficiently extracted and high recoveries within a short time [1]. Water-containing samples can be divided in two classes:
(i) Samples with a water content up to 30 % (e. g. cod livers) can be extracted after addition of Na_2SO_4. Sodium sulfate partly binds water and less water is added to the solvent (see above).
(ii) Samples with higher water content are not extracted quantitatively within one extraction step. Therefore, these samples should be lyophilized before the extraction.

Discussion
Closed-vessel MAE is preferred in the case of samples with high lipid content and relatively high organochlorine levels because the sample weight is limited. Using closed-vessel MAE,

temperatures above the boiling point of the solvent are achieved, and the combination of heat and pressure yields a fast and quantitative extraction of the samples by using only 8 mL solvent. Excellent recovery rates were obtained for seal blubber and cod livers and no decomposition of labile organochlorines was observed [1][5]. MAE is based on the irradiation of dipoles, and the water content of the sample plays a particular role for the MAE conditions in terms of power and time. The higher the water content the less the power is necessary to achieve boiling of the solvent. Consequently, extraction conditions must be brought in line for every matrix. However, modern MAE instruments are not only programmable in power increments but also the temperature. More stable conditions with aqueous samples are obtained after mixing the sample with anhydrous sodium sulfate which binds water and reduces in this way the absorption of energy in comparison to free water (see above) [10]. Ethyl acetate and cyclohexane are well-suited for MAE. Comparably high amounts of water are soluble in ethyl acetate, and co-extracted water must be separated by filtration of the MAE extract through sodium sulfate prior to GPC. After this, MAE in combination with GPC is a strong tool for the sample clean-up of samples with 2-100% of fat. Due to the different methods, GC/ECD, GC/MS, and GC/FID, internal standards are not explicitly mentioned in Scheme 1. As mentioned above, after filtration through sodium sulfate and volume adjustment, the solution obtained after MAE can directly be subjected to gel-permeation chromatography. Independent on the method (open- or closed-vessel MAE) high recovery rates of organochlorines (PCBs and chloropesticides) were obtained. Due to the high efficiency for the extraction of organochlorines and also lipids, we also checked the lipid composition of the extracts. We found that the method yielded the same spectrum of fatty acids as the classic method of Bligh and Dyer [11][12]. Furthermore, the composition of the fatty acids determined after methylation was still the same using the first fraction of the GPC eluate which is usually discarded [6]. Consequently, the extraction method does not only allow for a quantitative determination of organochlorines but can used in combina-tion with further techniques enhancing so the information obtained from one sample extract.

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