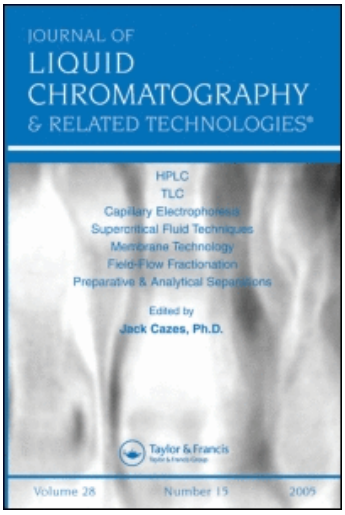

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USE OF SUPERCRITICAL FLUID EXTRACTION-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN THE DETERMINATION OF POLYNUCLEAR AROMATIC HYDROCARBONS FROM SMOKED AND BROILED FISH

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ABSTRACT

Polynuclear aromatic hydrocarbons (PAHs) from smoked and broiled fish were isolated by supercritical fluid extraction (SFE), and quantitatively determined by reversed phase liquid chromatographic (RP-HPLC) methods. The SFE-method was tested first with inert matrix. As expected, the extraction of PAHs from quartz sand gave good recoveries ranging from 80 to 100 % with R.S.D. of 3-11 %. The extraction of fish material showed that methanol had to be used as a modifier, because larger PAH molecules were weakly extracted with pure CO_2. The use of methanol resulted in, however, that lipophilic compounds were co-eluting in greater amounts, interfering the analysis of PAHs by HPLC - UV. That is why the extracts were purified using solid phase extraction (SPE) prior to

chromatographic determination. The extraction of one sample was completed in 20 minutes, while several hours are consumed in the conventional solvent extraction methods. The sensitivity of the method used is in ppb-range, which corresponds well with the concentration of PAHs in smoked fish products.

INTRODUCTION

The polynuclear aromatic hydrocarbons (PAHs) are formed in the inefficient combustion of organic matter. They contaminate foods through direct deposition from the atmosphere as environmental contaminants, or they are produced during cooking, baking or smoking of foods.

Some of the PAHs, such as benzo(a)pyrene, benzo(b)fluoranthene and dibenzo(a,h)anthracene, have been shown to have carcinogenic or mutagenic activity, the most effective being those with 5 or 6 fused rings. Fortunately, the most abundant PAHs in foods are smaller compounds thus being less harmful.[1-3]

Supercritical fluid extraction (SFE) has shown great potential in offering shorter extraction times with higher recoveries and low consumption of organic solvents comparing with traditional liquid extraction methods. The most commonly used fluid is carbon dioxide, CO_2, mainly because of its mild supercritical conditions (t_c= 31.1 °C, p_c= 7.38 MPa).[4]

The extraction and analysis of PAH-compounds have been the most popular application of supercritical fluid techniques in the field of environmental analysis.[5-10] SFE-methods have been developed for extracting PAHs from samples such as soil, marine sediments, aqueous media, and air samples.[9-13] One of the most difficult points in optimizing SFE of PAHs has shown to be the effect of matrix.[9,14]

As a matrix in SFE, fish tissue is rarely used, thus almost unknown. Nam et al. have published some papers dealing with the SFE of PAHs from spiked fish tissues[15] and pesticide residues from river fish.[16] Lee et al. have developed a method for a rapid determination of PCBs from fresh fish;[17] and a few papers about SFE of fish lipids have been published.[18-20]

In this study, we have developed a fast SFE-method for isolating PAHs from fish tissue. PAHs extracted were quantitatively determined by using liquid chromatographic methods. Samples compared were two distinct Baltic Herring (*Clupea harengus*) products: smoked and broiled fish samples.

MATERIALS AND METHODS

Preparation of Samples

Samples of fresh, smoked and broiled Baltic Herring products were purchased from local markets in January, 1995. The bones of broiled fish, the bones and the skin of the smoked fish were removed, and the edible parts of fish were homogenized and lyophilized. The fresh Baltic Herring fillets were homogenized as such. Prior to lyophilization, some fresh Baltic Herring samples were spiked with the synthetic mixture of PAH standard.

Standards

PAH-standards used were naphthalene (N) (E. Merck, Darmstadt, Germany); fluoranthene (F), pyrene (Py), (Aldrich-Europe, Beerse, Belgium); phenanthrene (Phen), anthracene (An), perylene (Per), benzo[a]pyrene (BaP), benzo[a]anthracene (BaA), fluorene (Fl) and chrysene (Ch) (Sigma Chemical Co., St. Louis, MO, USA). Standard stock solutions (1 mg/mL) were prepared in HPLC-grade trichloromethane (E. Merck, Darmstadt, Germany).

SFE-Procedure

Freeze-dried fish sample (1.000 g) and quartz sand (1.00 g) were mixed thoroughly and filled tightly into the extraction cartridge (volume 2.5 mL). In the extraction, ISCO SFX 220 SFE-equipment (ISCO Inc. Lincoln, NE, USA) with dual syringe pumps and restrictor heating device was used. The other pump was in the connection with a CO_2 -cylinder containing helium head pressure (99.998 %; OY AGA AB, Helsinki, Finland) and the other one with a reservoir containing HPLC-grade methanol (Lab-Scan Ltd., Dublin, Ireland). Before extraction, the cartridge filled was held about 10 min. at the extraction temperature (70 °C).

The other extraction conditions were as follows: pressure 350 atm (1 atm = 0.101325 MPa), fluid volume (measured as liquid at the head of the pump) 20 mL, and the fluid flow rate controlled by a piece of fused silica capillary (30 cm x 50 μm i.d., Polymicro Technologies Inc., Phoenix, AZ, USA) 1.5 - 1.7 mL/min. The analytes were collected by inserting the outlet of the restrictor into a tube containing 3 mL of hexane/dichloromethane (3:1). The collection

solvent was held at 5-10 °C in order to diminish restrictor plugging during the extraction.[21] The SFE-extracts were evaporated to dryness under nitrogen and diluted into 1 mL of HPLC-grade hexane (E. Merck, Darmstadt, Germany).

Cleanup of Extracts

The extracts were purified by scaling down the method described by Perfetti et al.[22] Instead of using commercial solid phase extraction (SPE) cartridges, the simple cleanup columns were prepared by packing 1.0 g alumina (Aluminum oxide 90, E. Merck, Darmstadt, Germany) and 0.8 g Silica Gel 60 (E. Merck, Darmstadt, Germany) into separate Pasteur pipettes. Alumina column was placed above silica column, and PAHs were eluted through the columns with 2 x 1.5 mL hexane/dichloromethane (3:1). The eluate was evaporated under nitrogen to about 1 mL after adding 2 mL of acetonitrile into tube. The eluate was further purified by elution through octadecyl SPE-cartridge (Bond Elut C18, 500 mg) with 2 mL acetonitrile. The sample was not further concentrated to avoid loss of more volatile PAHs with steam of acetonitrile. Instead, 1 μg naphthalene was added into the eluate in order to standardize the sample volume. Naphthalene was chosen, because it was not found in the fish samples.

Chromatographic determination

The purified extracts were analyzed by using a high-pressure binary gradient LC-system LC-6A with SCL-6A system controller unit and SPD-6AV spectrophotometric detector at 254 nm. (Shimadzu Co, Kyoto, Japan). The separation of PAHs was achieved with LiChrospher 100 RP-18 column (125 x 4 mm, 5 μm, E. Merck, Darmstadt, Germany) in 24 minutes when stepwise gradient from 50 % to 97.5 % acetonitrile in water and flow rate of 0.8 mL/min was used. In the extracts, PAHs were identified by using co-injection. The quantitative results were calculated by applying external standardization, the compounds quantified are listed in materials section.

RESULTS AND DISCUSSION

The HPLC-chromatograms of the synthetic mixture of PAH standards and PAHs from different Baltic Herring samples are shown in Fig. 1. The chromatogram A shows the resolution of the standard mixture. The detection limit with UV-detection used varied between 2 and 20 ng/mL, for anthracene

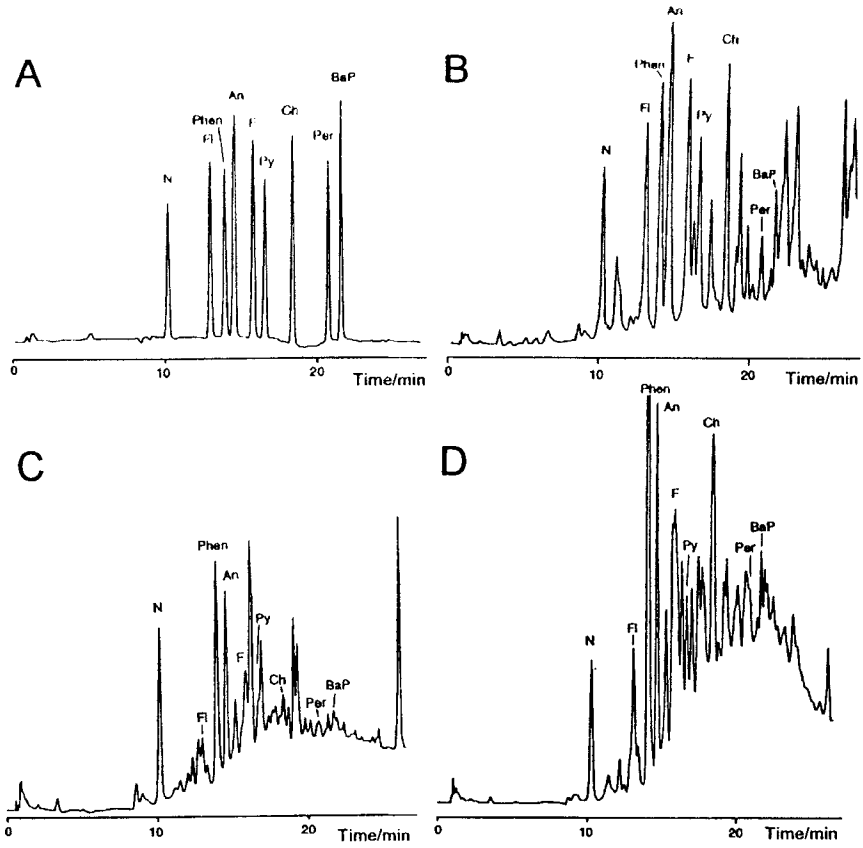

Figure 1. Separation of PAHs by HPLC and UV detection at 254 nm. A: mixture of PAH standards; B: fresh Baltic Herring sample spiked with standards; C: smoked Baltic Herring; D: broiled Baltic Herring. Abbreviations: see Materials section. HPLC-conditions: see Methods section

and pyrene, respectively. Fresh Baltic Herring samples were chosen for spiking matrix, because the amount of PAHs was below the UV-detection limit. Typically, concentrations of individual PAHs in Baltic Herring are less than 5 ng/g eatable part of the fish in dry weight basis.[23]

Fig. 1B. is a chromatogram of the spiked Baltic Herring sample, and Fig. 1C. and Fig. 1D. represent chromatograms of smoked and broiled fish samples, respectively.

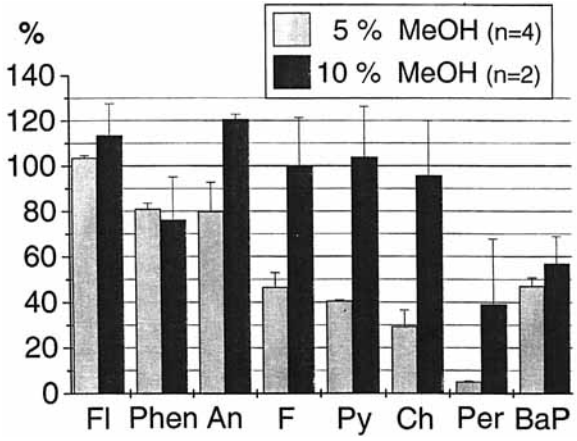

Figure 2. Effect of modifier addition to the recoveries of the individual PAHs spiked into fish tissue. Abbreviations: see Materials section.

As reported in the literature,[9-10] the extraction of spiked PAHs from inert quartz sand matrix was successful, e.g. at 250 atm and 55 °C recoveries varied from 80 to 100 % with R.S.D. of 3-11 %. The optimum conditions for extraction of PAHs from dried fish were studied by varying pressure, temperature and modifier concentration. SFE of fish material showed that the PAHs containing more than three rings were poorly isolated from tissue with neat CO_2. Our findings agreed with that of Nam et al.[15] They increased the solubility of PAHs by adding toluene directly into the sample in the extraction cell.

In our method, methanol, as a dynamic modifier, was added through another pump. All the PAHs studied were more soluble to the binary fluid than to the neat CO_2. Depending on the PAH-compound, the highest recovery was obtained with adding 5 or 10 % of methanol, as can be seen in Fig. 2. They reached recovery of 75-100 %, except perylene and bentzo(a)pyrene.

One explanation for this enhancement of extraction efficiency by the use of modifier may be the occupation of the active sites of fish matrix with modifier.[5,11] This may be the case in the study of Nam et al..[15] The other possible reason may be the co-elution of PAHs with lipids, which has been the problem in solvent extraction.[22] This might be true in our study, because the increasing of the polarity of the fluid by the modifier addition resulted in the extraction of reasonable amount of lipids.

The quantification of the PAHs in the extracts of herring products was sometimes difficult, because lipophilic compounds interfered the analysis of PAHs by HPLC-UV at 254 nm. Attempts were made to select such optimum extraction conditions (p and T), that the amount of lipids in the SFE-extracts decreased without affecting on the recovery of PAHs. The decreasing of the temperature or pressure lowered the solubility of fat to the fluid, but at the same time, recoveries of PAHs were reduced. A quite often used method for decreasing the co-extraction of lipids is to add aluminum oxide in the extraction cell.[17,24-26] In our study, co-extraction of lipids was only slightly reduced by mixing 1.0 g of aluminum oxide to 0.6 g sample prior to the extraction. Therefore, we ignored the use of alumina in the extraction cartridge, and instead, solid phase purification methods described in the methods section were used prior to the quantitative determination by HPLC-UV.

Samples of two distinct Baltic Herring (*Clupea harengus*) products showed great difference in PAH content and distribution of individual compounds as shown in Fig. 3. The amount of PAHs in freeze-dried, fresh samples was too low to be shown in the same scale. The values in the Fig. 3. are multiplied by response factors. The total PAH contents quantified in this work was about 180 ng/g and 1300 ng/g, in smoked and broiled fish samples, respectively. These concentrations are calculated to be corresponding with the edible part of the undried product. The amount of PAHs in smoked fish is in good agreement with published results,[2-3,27-28] although the correlation is difficult due to the differences in fish species,[27] isolation and chromatographic methods,[2-3,27-28] and difference between compounds quantified.[28] In both fish products studied the proportion of fluoranthene was the highest of the PAHs, i.e. about 220 ng/g (40 %) and 1.1 mg/g (30 %) on dry weight basis in smoked and broiled fish, respectively. Fortunately, the amounts of more harmful compounds, e.g. the concentrations of B(a)P were found to be smaller, i.e. about 40 ng/g in smoked and 400 ng/g broiled fish samples on dry weight basis. The PAH-content in edible parts of smoked fish depends obviously on the concentrations of these compounds in the wood smoke and the manufacturing method. The very high concentrations found in broiled fish sample has to be referred with the manufacturing method and consumption manners of this product in Finland. The daily intake of PAHs might increase by eating broiled fish, because according to national habit, the product is eaten as a whole, including the burned skin. In contrast, smoked fish is eaten without skin.

The sensitivity of the SFE-method with HPLC-separation and UV-detection is in ppb-range, which corresponds well with the concentration of PAHs in smoked Baltic Herring samples. More sensitivity is needed, if the

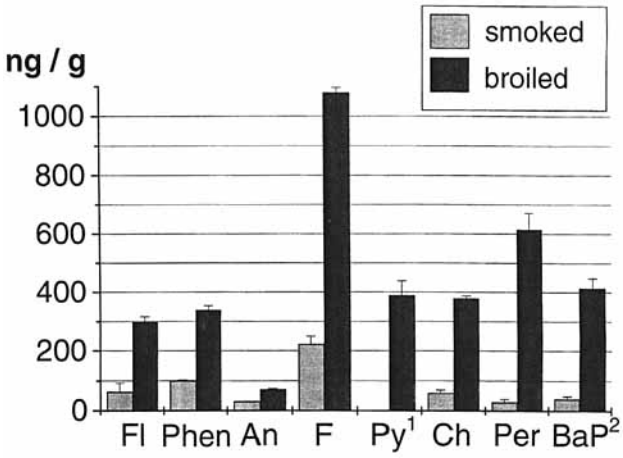

Figure 3. Concentrations of individual PAHs in smoked and broiled Baltic Herring samples (ng/g dry weight). Values are corrected with response factors. Abbreviations: see Materials section. [1] Not quantified from smoked fish.[2] n=2.

PAHs are determined in trace levels, like in fresh fish samples. It can be achieved by increasing the sample size, or increasing the sensitivity or selectivity of the chromatographic determination.

CONCLUSION

The extraction of one sample was completed in 20 minutes, while several hours were consumed in the conventional solvent extraction method. Despite the subsequent purification steps the savings in time and solvent consumption are remarkable. In the future, we consider the increasing of the sensitivity and specificity of the method by fluorescence or mass detection. At the same time the cleanup of the sample could be simplified, and the whole sample preparation procedure minimized.

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