Determination of Particulate Fatty Acids as P-Bromophenacyl- or phenylphenacylesters using HPLC

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Key Words
Column liquid chromatography
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Summary
A method for the determination of fatty acids of the molecular weight range of propionic to erucic acid is reported. The acids are converted to either p-bromophenacyl- or p-phenylphenacylesters and the esters are separated by HPLC and detected by UV-absorption. Twentytwo esters, including saturated, monoolefinic and polysaturated ones were separated within one HPLC-run. The detection limit is about 5pmol per fatty acid. The application of the method to particulate material is described.

Introduction
Planktonic algal lipids contain varying amounts of fatty acids, as it is well documented in the review by Wood [1]. All these naturally occurring fatty acids are straight chain and even numbered, and the unsaturated ones have cis-configuration with very few exceptions. Saturated and mono-olefinic fatty acids are the main components of most of the algae species, but some species contain appreciable amounts of highly unsaturated acids. The composition of fatty acids found in marine particulate matter resembles the distribution pattern of planktonic fatty acids, especially when phytoplankton is a great part of particulate matter [2–5].

Most of the determinations of fatty acids were performed by GC-technique after esterification. But the improvement of HPLC-technique offers the possibility to determine fatty acids under very mild conditions and good resolution and, with the appropriate derivatization, also very sensitive. p-Bromophenacylesters of fatty acids exhibit molar absorptivity values in excess of $1.7 \times 10^4 \text{dm}^3 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ and thus makes it possible to detect fatty acids in very low concentrations [6]. The derivatization procedure of fatty acids with p-bromophenacylbromid is based on the work of Durst et al. [7] utilizing the catalytic properties of crown ethers.

The determination of low molecular weight, saturated fatty acids as their p-bromophenacylesters in an aqueous matrix had already be performed by some authors using either gas-chromatography with packed columns [8, 9] as well as glass-capillary columns [10] or HPLC [6, 11] for their separation.

In this work we derivatized saturated and unsaturated fatty acids with p-bromophenacylbromid and p-phenylphenacylbromid covering the range of C2-acids to C22-acids, which is the normal range of phytoplanktonic fatty acids. The separation was performed by HPLC and the procedure was applied to determine the composition of the fatty acids in particulate material from the Baltic Sea.

Experimental

Chemicals
Acetonitril and methanol were redistilled in a 150cm vacuum-jacket, silver mirrored all-glass distillation apparatus. Each batch was checked by UV-spectrometry for impurities. Clean water was gained from a four-step Millipore Super-Q system for ultra-clean water.

Methanolic potassium hydroxide solution (0.5molar, p.a. Merck) was diluted with methanol to give a 50molar solution. p-Bromophenacylbromid (Merck, 97%), recrystallized from methanol, and p-phenylphenacylbromid (p.a. Fluka) were dissolved in acetonitril to give a 20mmolar solution. Kryptofix 222 (p.a. Merck; 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo-(8,8,8)-hexacosan) was dissolved in acetonitril to give a 2mmolar solution.

The fatty acids used as standards (p.a. Serva) consisted of ten saturated acids (n-propionic acid = C3, n-valerianic acid = C5, caproic acid = C6, caprylic acid = C8, capric acid = C10, lauric acid = C12, myristic acid = C14, palmitic acid = C16; stearic acid = C18 and arachidic acid = C20),
seven mono-olefinic acids (trans-2-octenoic acid = C8:1\(\Delta 2\),
myristoleinic acid = cis-C14:1\(\Delta 9\), palmitoleinic acid = cis-
C16:1\(\Delta 9\), palmitolenic acid = trans-C16:1\(\Delta 9\), oleic acid =
cis-C18:1\(\Delta 9\), petroselinic acid = cis-C18:1\(\Delta 9\), erucic
acid = C22:1\(\Delta 13\)) and five polyolefinic acids (linoleic
acid = cis,cis-C18:2\(\Delta 9,12\), eicosadienoic acid = cis,cis-C18:
2\(\Delta 9,11,14\), linolenic acid = all cis-C18:3\(\Delta 9,12,15\), arachidonic
acid = all cis-C20:4\(\Delta 5,8,11,14\), and docosahexaenoic
acid = C22:6\(\Delta 4,7,10,13,16,19\)). They were dissolved
in methanol rendering 10 mmolar stock solutions, which
were stable for several weeks when stored in darkbrown
bottles in a refrigerator. From this stock solutions the p-
bromophenacyl- or p-phenylphenacyl esters were freshly
prepared. The esters were also stable for several weeks
when stored in the same manner.

**Apparatus**

The HPLC-system consisted of two constant flow reciprocating piston pumps (Constametric I and III, Milton Roy), an injection valve (Rheodyne), and a variable wavelength UV-detector (Schoeffel SFA 339) with a 12 \(\mu l\) flow cell. The two pumps were operated by a Gradient Master (Milton Roy) allowing one linear, five convex and five concave gradients for a binary solvent system. Details of the HPLC-columns and gradients are given in the figure captions of the appropriate chromatograms. Integration of the chromatograms were performed by a Hewlett-Packard 3390A Integrator. All derivatization procedures were carried out in 10 ml graduated test tubes with a ground joint glassstopper in a thermostated aluminium block.

**Derivatization of the Standards**

200 \(\mu l\) (2 \(\mu mol\)) of a fatty acid standard stock solution are neutralized to a phenolphthalein end point (pH 8.4) by about 40 \(\mu l\) of a methanolic potassium hydroxide solution (0.05 molar) by means of a microtitrator (smallest volume increment is 1 \(\mu l\)); 100 \(\mu l\) (0.2 \(\mu mol\)) of a Kryptofix 222 solution is added and the solution is heated gently for several minutes. Then 110 \(\mu l\) (2.2 \(\mu mol\)) of either a p-bromophenacylbromid or p-phenylphenacylbromid solution is added and the solution is heated for 15 min at 80°C in the aluminium block. The solution is then diluted with acetonitril to give a volume of 10 ml, resulting into a 200 \(\mu mol\) ester solution. 20 \(\mu l\) of this solution, corresponding to 4 nmol of one ester is directly injected into the HPLC-apparatus. If more then one acid is derivatized in one batch, the amounts of the reagents are calculated correspondingly. When lower concentrations of fatty acids are derivatized, the stock solutions of the fatty acids and the reagent solutions are diluted and then the same procedure as described is performed. We made series the highest concentration being 4 nmol per ester and per injection from a 200 \(\mu mol\) solution and the lowest concentration being 4 pmol per ester and per injection from a 200 nmol solution.

Alternatively: 200 \(\mu l\) (2 \(\mu mol\)) of a fatty acid standard stock solution are neutralized to a phenolphthalein end point (pH 8.4) by about 40 \(\mu l\) of a methanolic potassium hydroxide solution (0.05 molar) and the solution is then evaporated to dryness at reduced pressure. The residue is redissolved in about 1 ml acetonitril and a mixture of 100 \(\mu l\) (0.2 \(\mu mol\)) by Kryptofix 222 solution and 110 \(\mu l\) (2.2 \(\mu mol\)) of a p-bromophenacylbromid or p-phenylphenacylbromid solution is added. The solution is heated for 15 min at 80°C and is then directly used for injection.

**Derivatization of Samples**

The procedure is the same as described for standards. The unknown concentration of fatty acids in the sample is evaluated through the consumption of the 0.05 molar potassium hydroxid solution and the amount of the reagents are then calculated accordingly.

**Results and Discussion**

Crown ether catalysts aid by solvation of the cation the dissolution of salts in non-polar aprotic solvents. Anions of these salts in solutions have been shown to be unusually reactive [12, 13]. We therefore added the crown ether solution separately to the alkylating reagent to the alkali salts of the fatty acids to let the solution enough time for building of the cation-crown-ether complex. The chromatograms showed in that case reduced concentrations of by-products (Figs. 1 and 2) at the beginning and thus the interpretation of the chromatograms for the lower weight fatty acids is improved.

The derivatization procedure, using the evaporation step of the alkali salt solution, was found to give non-reproducible results in the following esterification of the fatty acids at the low pmol level. Also the background of by-products, most probably breakdown products of either the reagents or the fatty acids, was higher. This step therefore was abandoned. It was found, that the methanol from the KOH solution and the standard fatty acid stock solution did not interfere, when not evaporated before and when the esterification was performed as described in the derivatization procedure.

HPLC-chromatograms of 22 fatty acid esters are shown in Figs. 1 and 2. Both the p-bromophenacyl esters (Fig. 1) as well as the p-phenylphenacyl esters (Fig. 2) of all 22 acids could be resolved in one run. This includes the separation of steric isomers, as is demonstrated by the resolution of palmitoleic and palmitoleic acid and the separation of position isomers, as is shown by the resolution of oleic and petrosilinic acid. The range of the 22 fatty acids encloses most of the phytoplanktonic acids, so that screening of these acids by comparison with standard substances can easily be done.

In order to determine the reproducibility of the derivatization procedure and the chromatographic performance amounts of 2 \(\mu mol\), 200 \(\mu nmol\), 20 nmol and 2 nmol each of the 22 fatty acids were derivatized and run in triplicate, the corresponding amount of injected ester being 4 nmol, 400 pmol, 40 pmol and 4 pmol. The average standard deviation of peak areas of the triplicate runs lie between 0.14 and 0.96 for the different concentration ranges (Table 1). As expected the higher deviations are found in the lower pmol range. The greatest variations are at the

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