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INVESTIGATION OF FATTY ACIDS COMPOSITION IN FOOD ADDITIVES USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

Quantitative determination of fatty acids in commercial food additives, containing fish oil extracts, was done. A new simple methodology using preliminary separation of the mixture components with preparative thin layer chromatography, followed by derivatization procedure and, finally, gas chromatography-mass spectrometry analysis, was demonstrated to be useful. Five samples of pills available in the European market as food additives were monitored. It was observed that four of the samples have similar main components – triglycerides and free fatty acids. The quantitative determination of secondary compounds showed that samples 1, 2 and 5 have only one secondary component zone while samples 3 and 4 have four zones. The fatty acids composition of all tested samples showed significant similarities in both types of acids and in their relative strength.

Keywords: fatty acids, gas chromatography, mass spectrometry.

INTRODUCTION

Lipids are a main component of food. They ensure around 40 % of the calories in organism and are a very important component determining the nutrient content, consistence and taste of food. The fatty acids are the basic building blocks of all lipids. Fatty acids can be both odd- and even-numbered, with from two to nearby one hundred carbon atoms. Double bonds can have either *cis* or *trans* geometry, and acetylenic and allenic bonds occur. The latter can be part of a conjugated system of unsaturation or there can be several methylene groups between them. Also, there can be a host of further structural features, including branch points, alicyclic or heterocyclic rings, oxygenated functions, and many more. The exact number of different fatty acids of natural origin has never been tabulated, but it must be well over a thousand, and innumerable manmade ones can be added to the list [1].

Food treatment always leads to changes in the structure of lipids resulting into a complex mixture of fatty acids with unchanged, isomerized and/or oxidized residues. An important part of lipids are the polyunsaturated fatty acids, from which the ω-3 acids cannot

be produced in a human organism. They can be supplied only by food and especially seafood, which is very rich in them. They play an important role in the human body by reducing the levels of harmful cholesterol and triglycerides in the blood that are responsible for the emergence of heart disease, heart attacks, strokes and atherosclerosis. They also help to prevent many diseases and reduce the risk of blood thrombus formation. Fish oils as a food which are very rich in ω-3 fatty acids are a promising alternative for prevention and treatment of all the disorders mentioned above.

In the last decade the scientific interest in this group of compounds has strongly increased [2-5]. Due to increasing interest and use of fish oils as dietary supplements it is necessary to control their quality and safety.

In the present paper we describe our study for the creation of a new, easily reproducible method for quantitative determination of ω-3 fatty acids using gas chromatography-mass spectrometry (GC-MS).

EXPERIMENTAL

Five trade-marked fish oils, obtained from markets in France, Belgium and Germany were studied. The choice was made according to the geographical location of the countries [6]. Sample 1 – ''OMEGA 3 fortex''-1000 mg pills, made by''CAPSUGEL'', Ploermel, France; (available on the Bulgarian market); Sample 2 – ''DAS GESUNDE PLUS''-1000 mg pills, made by ''DM-DROGERIE MARKT'', Germany; Sample 3 – ''VISOLIE / HUILE DE POISSON''-500 mg pills, made in France; Sample 4 – ''EPA JUNIOR''-500 mg pills, made by ''BIOVER'', Belgium; Sample 5 – ''ELUSANES''-250 mg pills, made by ''PLANTES AND MEDECINES'', Castres, France. The content of ω-3 fatty acids 5,8,11,14,17-eicosapentaenoic acid (EPA) and 4,7,10,13,16,19-docosahexaenoic acid (DHA) for samples 1 and 2 only are shown on the package of the stock products. For the remaining samples no information on the fatty acids composition and content of minor components such as hydrocarbons, vitamins, etc., is given.

For thin layer chromatography (TLC) a standard mixture containing monoolein, diglycerides (1,2-diolein and 1,3-diolein), triglycerides (triolein) purchased from Sigma-Aldrich (UK) was used. All other reagents and solvents used in the current investigation were also purchased from Sigma-Aldrich (Poole, UK). Detection of the components under study during the thin layer chromatography (TLC) analyses was made by UV detection (366 nm) after treatment with 2,7-dichlorofluoresceine or 50 % H_2SO_4/MeOH. Alugram SIL G, 20x20 cm, 0.2 silica gel 60 (MACHAREY – NAGEL) plates were used for analytical TLC analyses. For preparative TLC analyses UNIPLATE SILICAGEL GF, 20x20 cm, 1000 microns (ANALTECH) plates were used.

General methodology for analytical TLC

100 mg of samples 1-5 were dissolved in 1 ml n-hexane. 5 μl sample and 2 μl standard mixture containing monoglyceride-monoolein, diglycerides-1,2-diolein and 1,3-triolein, triglyceride-triolein in equimolar quantities were laid on a TLC plate. Mobile phase of petroleum ether: acetone (100:6) was used for compound elution. The height of the TLC's elution was 18 cm continuously eluted without cover.

General methodology for preparative TLC

1 ml 10 % solution in hexane of every sample was laid on the TLC plate many times until a well defined layer is obtained. Mobile phase petroleum ether: acetone (100:5) was used for compound elution. The height of TLC chromatograms was 18 cm continuously eluted without cover. Different zones were identified according to the procedure mentioned above. The silica gel was scrapped off the plate and all components were extracted using petroleum ether. Further it was replaced by evaporation under vacuum. The obtained samples were analyzed by GC-MS.

General procedure for 2-alkenyl-4,4-dimethyloxazoline (DMOX) derivatives synthesis by means of so-called "hot" method

2-amino-2-methylpropanol was added to a sample of 2 mg fatty acids. The reaction mixture was stored under nitrogen atmosphere for 12 h at 180°C. At the end of the reaction time a TLC monitoring in the system n-hexane:MeOH (100:3) was made. After cooling the reaction mixture 5 ml mixture of n-hexane: ethyl ether (1:1) and 5 ml water was added. The obtained DMOX deriva-

tives are in the organic phase. The extraction procedure was repeated 3 times. The organic phases were dried over Na_2SO_4 and the solvent was evaporated under vacuum. The obtained DMOX derivatives of fatty acids were analyzed using a Gas Chromatograph model HP 5890 with electron trap MS 5972 model detector (70 eV). The temperature of the transfer line was 280°C.

The separation was done with a capillary column DB – 5 MS (AGILENT), 30 m x 0.25 mm x 0.25 μm. A temperature gradient from 120°C to 240°C at a rate of 40°C/min and final 15 minutes to 240°C was used. Argon was used as an eluent gas at a rate of 1ml/min.

Our analysis and conclusions regarding MS spectra were based on the literature data, especially those reported by W. W. Christie [7].

RESULTS AND DISCUSION

Initially, the active component was separated from the gelatin pill using a syringe. Further an analytical and preparative TLC separation of lipid components was made according to the procedure outlined in Experimental.

The components of the mixture were separated with the silica gel preparative TLC. Identification of the target zones in comparison with a standard mixture was made.

Our preliminary studies on different samples have shown that some of the oils might contain alkyldiacylglycerols, which are less polar than triglycerides. Their t_R is greater than that of triacylglicerols and appear on TLC immediately after the triacylglicerols. As a standard we have used lipid which does not contain alkyldiacylglycerols. The presence or absence of alkyldiacylglycerols can not be uniquely determined in the investigated samples.

The results obtained by thin-layer chromatographic separation showed that the main components of samples 1, 2, 4 and 5 are free fatty acids and triglycerides, with Rf 0.17 and 0.60 respectively. In samples 1,2 and 5 a zone of minor components with Rf 0,68 is outlined. Samples 3 and 4 contain four well-defined areas of minor components with Rf 0.70, 0.78, 0.86 and 0.95, respectively. In sample 3 is observed the presence of monoglycerols, triglycerols, methyl esters and sterol esters of fatty acids, respectively, with Rf 0.06, 0.60, 0.74, 0.82. Areas of all samples corresponding of triglycerols occur with tails, which from our previous studies we have reason to believe is due to the presence of alkyldiacylglycerols. In the preparative TLC the areas of triglycerols and their tails were isolated as one fraction. Fractions thus obtained, were used to determine the fatty acid composition of triglycerols. Additional analysis to determine the presence or absence of alkyldiacylglycerols are in progress.

All isolated by preparative TLC triglycerol fractions were subjected to the procedure for obtaining DMOX derivatives using the described in experimental section methodology.

Currently several methods for ω–3 acids determination are described in the scientific literature. Silver-based HPLC combined with mass spectrometry is used. However, this approach needs a complicated apparatus, very expensive reagent and materials as well as a long, complex derivatization procedure.

The classical method for different acyl groups' ratio determination includes several steps: acylglycerols hydrolysis, obtaining the methyl ester and further separation, identification and quantitative determination of the resulting methyl esters with GC. Unfortunately, the mass spectra of such derivatives rarely contain ions indicative of structural features; the positions of double bonds in the aliphatic chain, for example, cannot be determined. The reason is the strong capability of this type of bonds to isomerize to the length of an alkyl chain at the time of ionization. To overcome this shortcoming, most fatty acids are converted into more volatile compounds by their interaction with reagents containing a nitrogen atom. The reason for this is the preferred ionization of the nitrogen atom with formation

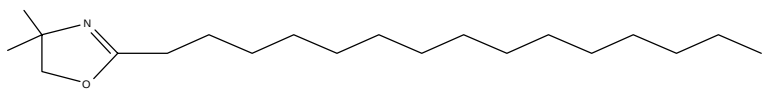

Fig. 1. General structure of DMOX derivatives of fatty acids.

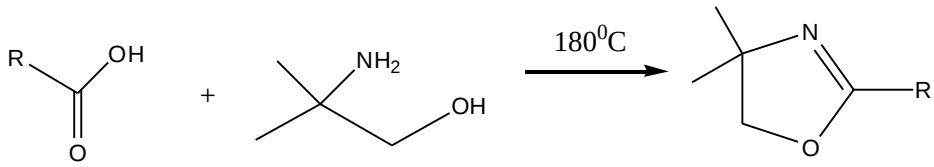

Fig. 2. General procedure for obtaining DMOX derivatives.

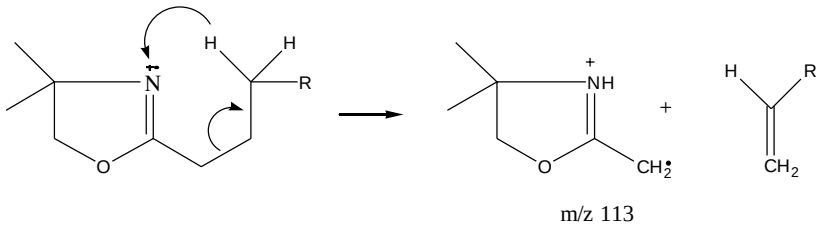

Fig. 3. m/z = 113 forming by radical fragmentation.

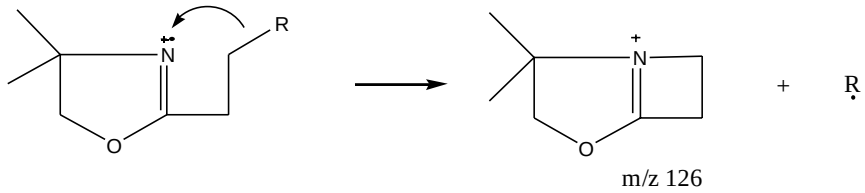

Fig. 4. m/z = 126 forming by radical fragmentation.

of cation - radicals, which can take hydrogen from a carbon atom in the allyl position to the double bond. The newly formed radical center undergoes further fragmentation, which makes it possible to determine the exact structure.

In our case a simplified procedure for preparative TLC was made, and then a GC-MS analysis was applied for the DMOX derivatives of fatty acids. This technique allows full determination of the position and number of the double bonds in the fatty acids chains as well as their steriochemistry. The fatty acids are not suitable for direct GC analysis mainly because of their interaction with the stationary phase during the analysis. There are many reported methods for obtaining various derivatives of fatty acids that improve the information from EI - mass spectra of branched chain and unsaturated acids, allowing for the establishment of the branches and the positions of double bonds. Several studies have shown that the most popular and preferred for chromatography DMOX are derived (Fig. 1).

The main advantages of DMOX derivatives are that they have a high volatility and compatibility with most of the stationary phases used in GC. The temperature of their elution is around 10°C higher than for the methyl esters of fatty acids [8], but the needed time for their separation is significantly shorter. In addition, GC-MS analyses of DMOX derivatives of fatty acids with good separation are described in the literature and can be used for comparative analysis. Unfortunately, there is a disadvantage in these compounds, namely their chemical instability. A little trace of water is able to open their ring very fast, but this can be easily prevented by storing them over dry agents.

Another advantage of the DMOX derivatives is that they can be obtained quickly and with high yield by a condensation reaction between the fatty acid (or its

esters, and in some cases even lipids)and 2-amino-2-methylpropan-1-ol (AMP) (Fig. 2).

Both ions at m/z =113 and m/z =126 in our GC-MS spectra showed that DMOX derivatives were successfully obtained. These ions were followed by a series of even-numbered m/z 126 +14n picks, resulting from the cleavage of each bond of the fatty acid chain. The mechanism of formation of these ion series can be explained by the migration of a proton to the charged part of the molecule which further forms a radical stabilized by subsequent fragmentation (Fig. 4):

For the derivatives of mono-and dimethyl branched fatty acids the spectrum shows characteristic variations in the intensity of m / z 126 +14 n series compared with that of fatty acids with normal chain [10]. It also shows local minima of intensities which display the location of the branching. MS of olefinic derivatives of fatty acids, with double bond format at between C7 to C15, are structurally specific. They show a specific molecular ion and fragment peaks used to determine the location of the double bond. In these spectra we observed an interval of 12 units between the allyl methylene group and the first carbon atom of the double bond. This range is accompanied by two more intense peaks due to allyl cleavage.

A series of DMOX derivatives of unsaturated fatty acids were separated and tested and their mass spectra allow the identification of these compounds in fish oils [11,12].

The obtained DMOX derivatives were subjected to GC/MS spectral analysis. The results of these tests are as follows:

• The saturated acids found are mainly myristic C14:0, palmitic C16:0 and stearic C18:0 acids.

• The monounsaturated acids found are mainly palmitoleic C16:1 (n-7), oleic C18:1 (n-9), vaccenic C18:1 (n-11) acids.

• The polyunsaturated acids found are mainly linoleic C18:2 (n-6), linolenic C18:3, eicosapentaenoic C20:5, docosahexaenoic C22:6 acids.

These acids are found in approximately equal amounts in all tested samples. From the obtained areas of peaks in the GC / MS chromatograms can be concluded that the unsaturated acids are found in greater amounts than the saturated. Most are monounsaturated fatty acids that are present as positional isomers. In moderation contain polyunsaturated fatty acids. In different samples in lower amounts were detected also other fatty acids, such as C15:0, C17:0, C20:0, C20:1.

CONCLUSIONS

During this study the following results were obtained:

• The basic lipid classes in food additives on the market, containing fish oils extracts, were determined with preparative TLC and GC-MS.

• The samples did not differ according to their main components

• The relative content of individual fatty acids in the investigated samples was similar.

• The fatty acids are mostly monounsaturated fatty acids in the form of positional isomers.

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