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Enantiomeric separation of triacylglycerols containing very long chain fatty acids

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ABSTRACT

Enantiomers of triacylglycerols (TAGs) containing any combination of very long chain fatty acids (VLCFAs) and/or very long chain polyunsaturated fatty acids (VLCPUFAs) with diolein, dilinolein and didocosahexaenoin were synthesized. Gradient non-aqueous reversed-phase high-performance liquid chromatography/high resolution atmospheric pressure chemical ionization-tandem mass spectrometry (NARP-HPLC/HRMS^2-APCI) and chiral liquid chromatography were used for the separation and identification of molecular species of these TAGs. Further, NARP-LC and chiral LC were used to separate natural mixtures of TAGs obtained from four natural sources, i.e. ximenia oil (*Ximenia americana*), green alga (*Botryococcus braunii*), brewerís yeast (*Saccharomyces pastorianus*) and a dinoflagellate (*Amphidinium carterae*). The ratio of regioisomers and enantiomers in individual samples was determined and a hypothesis has been confirmed on the biosynthetic pathway of natural TAGs, which is based on the preferential representation of VLCFAs and VLCPUFAs in the *sn*-1 position of the glycerol backbone.

1. Introduction

Vegetable oils and animal fats consist predominantly of triacylglycerols (TAGs) which are esters of three fatty acids (FAs) with glycerol. Natural TAGs are present in living organisms as mixtures of different types of molecules, in many cases as stereoisomers, i.e. regioisomers or enantiomers. Natural TAG mixtures, whether of plant or animal origin, always contain tens, hundreds or even thousands of molecular species depending on the number of bound fatty acids (see Table S1). Milk fat [1] and oil from marine organisms [2] are considered to be the most complex mixtures of TAGs. The number of identified molecular species of TAGs reaches hundreds, e.g. 762 regioisomeric TAGs from krill oil [3] and 568 for human milk [1].

Since four enzymes are primarily involved in TAG biosynthesis, three of which are acyltransferases [4] which specifically esterify each of the glycerol hydroxyls, the resulting combinations are not random. So, for example, *sn*-16:0/18:1/16:0 and *sn*-16:0/16:0/18:1 (16:0 is palmitic and 18:1 oleic acid) are present in sunflower oil at a ratio of 100: 0, whereas in pork fat they occur in a ratio of 8:92 [5]. Physical properties of regioisomers such as *sn*-18:0/18:2/18:1 and *sn*-18:0/18:1/18:2 (where 18:0 is stearic and 18:2 is linoleic acid) vary considerably. Appropriate melting points of, for example, α crystalline forms are ~−4 °C and ~−13 °C [6] and the difference of 9 ° is due simply to a change in the positions of two FAs, i.e. is caused by changing the acids esterified to primary and secondary hydroxyl. Molecular structure of TAG is therefore of great importance in terms of nutritional, biochemical and technological aspects.

The number of carbon atoms in the acyl chains of natural TAGs is usually 16, 18, 20 or 22. This results from their biosynthesis, where the FAs in plants and animals are biosynthesized from acetyl-CoA (malonyl-CoA) and therefore contain even-numbered chains. Fatty acids can be classified, e.g., by carbon chain length and number of double bonds. In FAs called long-chain fatty acids (LCFAs), chain lengths range from 12 to 22 carbon atoms, of which LCFAs with 16 and 18 carbon atoms are the most common types of FAs found in nature. FAs that are longer than C22 [7] are called very long chain fatty acids (VLCFAs) and are less widespread than LCFAs. VLCFAs with chain lengths above 26 carbon atoms are often called ultra-long chain FA (ULCFA) [8] and are found from fungal spores to specialized mammalian tissues (the brain, retina, etc.). In the plant kingdom, they range from algae to higher (flowering) plants. The

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chain lengths are sometimes defined differently; see, for example, the definition by Poulos et al. [9] which defines VLCFAs as 24–28C fatty acids and ULCFAs as fatty acids having a chain of 30–38 carbon atoms. If they contain 2 or more double bonds, they are called polyunsaturated FAs, and in combination with differently long chains they are abbreviated as PUFAs, VLCPUFAs, etc. Unfortunately, with very few exceptions (sponges), their representation in organisms is very low, usually below 1% of all FAs.

One of the most common methods for separating natural TAGs is non-aqueous reverse phase liquid chromatography (NARP-LC). It was used to separate hundreds of TAGs [10–12], which were then identified by electrospray ionization mass spectrometry (ESI–MS) and/or atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). Stereospecific analysis of individual molecular species of TAGs is relatively difficult. Using APCI, the regioisomers of TAGs can be distinguished by the lower relative occurrence of the $[M+H\text{-}R_2COOH]^+$ fragment ion which results in a neutral loss of FA from the *sn-2* position [13–15]. If the TAG is asymmetric, i.e., if the two primary hydroxyl groups are esterified by different FAs, then the TAGs may be chiral, i.e. they may form two enantiomers, e.g., di-stearoolein (*sn*-18:0/18:1/18:1 and *sn*-18:1/18:1/18:0).

The separation of enantiomers of TAGs is most often carried out on a stationary phase based on cellulose tris(3,5-dimethylphenylcarbamate) or cellulose tris(3-chloro-4-methylphenylcarbamate) [16]. In the case of enantiomers, it is always necessary to obtain a standard of at least one of the two possible enantiomers for identification because the order of elution from the column is virtually impossible to predict. Only minor structural modifications of the stationary phase, e.g. substitution of methyl for Cl (cellulose tris(3,5-dimethylphenylcarbamate) versus cellulose tris(3-chloro-4-methylphenylcarbamate)), where methyl is a group of electron donating and the chlorine group is electron withdrawing and therefore the electron density on the phenyl group, has an effect on the elution of TAGs [17]. Examples of important chiral separations of TAGs have previously been published [18–22], and the type of columns, the mobile phase, the molecular weight range (in Da), including the origin of samples and references have been given, so here we mention only the most recent articles concerned with, e.g., the composition of regioisomers and enantiomers of TAGs in immature chicken egg yolk, mature chicken yolk, and chicken meat [23].

For the analysis of TAGs containing VLCFAs, the sources (organisms) already described were selected. In the case of higher, i.e. flowering plants, VLCFAs are present e.g. in the seeds of the genus *Tropaeolum* [24] or *Ximenia* [25–27]. *Ximenia americana* is a small sprawling tree of woodlands native to the tropics. The fruits are lemon-yellow or orange-red. In all cases, TAGs contain monoenoic FAs (erucic, i.e. 22:1ω-9, nervonic, i.e. 24:1ω-9, ximenic, i.e. 26:1ω-9 and 19-octacosenoic, i.e. 28:1ω-9 acids) as the major VLCFAs. For the next analysis, we used ximenia oil (*Ximenia americana*) [26].

Mitei et al. [25], who published the data on TAGs in ximenia oil obtained by direct inlet high resolution positive ESI, identified only 7 molecular species of TAGs. Since tandem MS was not used, the TAGs were characterized only by the carbon number: double bond(s) ratio, i.e. CN: DB, given as 64:3, 64:5, 62:2, etc. Based on their FAs analysis, however, it can be assumed that, for example, the 64:3 TAG contains two oleic (18:1ω-9) and one 19-octacosenoic (28:1ω-9) acids. Furthermore, the same authors, based on chemical shift (13C NMR) of ester carbon (about 170 ppm) found that saturated FAs were distributed only at the *sn*-1/3 position while *sn*-2 contains unsaturated FAs.

Further, yeasts, in particular the genus *Saccharomyces*, are the most widely used microorganisms in biotechnological production of beer and wine. Worldwide production of yeast biomass remaining after beer production is about half a million tons of dry matter. Already in 1973 Welch and Burlingame [28] described the presence of VLCFAs up to tetratriacontanoic acid in the yeast species *Saccharomyces cerevisiae*, but monounsaturated FA only up to 28:1 (octacosenoic acid) were identified. Other papers dealing with VLCFA in yeast and specifically *S. cerevisiae* have been published only sporadically [29,30]. After over 40 years, the yeast obtained from beer production by bottom fermentation was analyzed by reversed-phase LC–MS using positive APCI or ESI [31] and TAGs as well as phospholipids containing VLCFAs [32] were identified. In total, 22 TAGs containing at least one VLCFA [31] were identified.

The green alga of the genus *Botryococcus* is known to be used as a potential source of biofuel because, unlike other microorganisms, it produces long-chain hydrocarbons and, in addition, produces them extracellularly. It is understandable that if alkadienes and alkatrienes with odd carbon numbers – 23–31 – or botryococcenes containing 30–37 carbon atoms are produced, the alga must also produce VLCFAs, which are their biosynthetic precursors. Lipid analysis by LC–MS confirmed that this alga contain TAGs with saturated and monounsaturated VLCFAs [33].

The last microorganism to be analyzed was *Amphidinium carterae* from the phylum Dinoflagellata (Dinophyta or Dinozoa). These unicellular flagellates live in freshwater, brackish or marine environments. They are often mixotrophic organisms, and their cells are covered by a complex structure formed by membranes and flattened vesicles. A bloom of certain dinoflagellates can result in a visible coloration of the water colloquially known as red tide, which can cause shellfish poisoning. Dinoflagellates produce characteristic lipids, especially VLCPUFAs [34–38], but the contents and structures of TAGs and phospholipids have also been described [38,39].

One of the most serious problems in analyzing regioisomers and enantiomers is the very poor availability of commercially available TAGs. The first step is almost always the necessity to prepare the appropriate standards by organic synthesis. We describe here a method for separating and identifying regioisomers and enantiomers of TAGs that enable identification of molecular species containing VLCFAs and VLCPUFAs, respectively.

2. Experimental

2.1. Standards and instrumentation

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18:1/18:1-PC) and 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (18:2/18:2-PC), 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphocholine (22:6/22:6-PC), 6(*Z*),9(*Z*),12(*Z*),15(*Z*),18(*Z*),21(*Z*)-tetracosahexaenoic, 15(*Z*)-tetracosenoic, 17(*Z*)-hexacosenoic, tetracosanoic, and hexacosanoic acids were purchased from Larodan (Malmo, Sweden). Ximenia oil was purchased from Augustus Oils Ltd. (Hampshire, England). All other chemicals were purchased from Sigma-Aldrich (Prague, CR).

2.2. Cultivation of microorganisms

For analyses of TAGs from microorganisms, the following organisms were used: green alga (*Botryococcus braunii)* [40], brewerís yeast (*Saccharomyces pastorianus*) [31] and dinoflagellate (*Amphidinium carterae*) [33] which have been previously cultivated in the laboratory under controlled conditions.

2.3. Enzymatic synthesis of TAGs

1,2-diacyl-*sn*-glycero-3-phosphocholines (18:1/18:1-PC, 18:2/18:2-PC and/or 22:6/22:6-PC) were treated with phospholipase C (Type I from *Clostridium perfringens* (*C. welchii*)) as described by Christie [41]. In brief, phospholipase C (3 mg) in 0.5 M tris(hydroxymethyl)methylamine (tris) buffer (pH 7.5; 2 mL),

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