Review paper
Pregledni znanstveni prispevek

SINGLE CELL OILS PRODUCTION AND APPLICATION

Nataša FIDLER[a)], Berthold KOLETZKO[b)] and Thorsten U. SAUERWALD[c)]

[a)] Univ. of Ljubljana, Biotechnical Fac., Zootechnical Dept., Groblje 3, SI-1230 Domžale, Slovenia, Ph.D., M.Sc.
[b)] Ludwig-Maximilians-University, Dr. von Haunersches Kinderspital, Kinderklinik and Kinderpoliklinik, Div. Metabolic Diseases and Nutrition, Lindwurmstr. 4, D-80337 Munich, Germany, Prof., Ph.D.
[c)] Same address

Received June 30, 1999, accepted October 05, 1999.
Delo je prispelo 1999-06-30, sprejeto 1999-10-05.

ABSTRACT

Microorganisms have been receiving increased attention as sources of novel lipids. Those that accumulate more than 20-25 % of their biomass as oil may be termed oleaginous and their oils single cell oils (SCOs), unicellular oils or microbial oils. For the lipid accumulation in yeasts, moulds and eukaryotic algae, but not in bacteria, the presence of enzyme ATP-cytrate lyase is of vital importance. This enzyme serves to produce acetyl-CoA, which is the substrate for fatty acid biosynthesis. Nitrogen limitation is the most frequently used condition to favour lipid accumulation. Oleaginous organisms differ from nonoleaginous ones in being able to convert carbon from the growth medium into the intracellular lipid, after the nitrogen has been depleted from the medium, provided that the supply of carbon stays plentiful. Biosynthetic pathways of n-6 and n-3 polyunsaturated fatty acids from the saturated and monounsaturated precursors with the chain elongations and desaturations are presented. The suitability of an microalgal triglyceride-SCO highly enriched in docosahexaenoic acid (DHASCO®) as a source for nutritional supplementation for formula milk is compared to fish oil. Some safety evaluation studies of SCOs are presented. For the safe use of SCOs in infant formulas even further safety studies should be performed. By growing microalgal strains in a medium containing D-[1-^{13}C]glucose, SCOs enriched with the stable isotope ^{13}C can be produced. Some examples of recent research and diagnostic applications of ^{13}C-labelled SCOs to study fatty acid metabolism are outlined. In conclusion, SCOs in combination with stable isotopes have become indispensable to study metabolic pathways.

Key words: microbiology / oleaginous microorganisms / single cell oil / docosahexaenoic acid / stable isotopes

PROIZVODNJA IN UPORABA ENOCELIČNIH OLJ

IZVLEČEK

Mikroorganizmi postajajo vse bolj zanimivi kot vir novih lipidov. Mikroorganizme, ki lahko nakopičijo več kot 20-25 % biomase v obliki olj, imenujemo oljni mikroorganizmi, njihova olja pa enocelična olja (single cell oils; SCOs) ali mikrobna olja. Pogoj za kopičenje lipidov pri kvasovkah, plesnih in evkariontskih algah, ne pa pri bakterijah, je vsebnost encima ATP-citrat liaza. Omenjeni encim sodeluje pri pretvorbi citrata v oksalacetat in acetyl-CoA, ki je substrat za sintezo maščobnih kislin. Najpogosteje spodbujamo kopičenje lipidov z omejevanjem dušika v gojišču. Oljni mikroorganizmi so namreč sposobni pretvarjati ogljik iz rastnega medija v intracelularne lipide tudi potem, ko v gojišču že zmanjka dušika. Predstavljamo potek biosinteze n-6 in n-3 večkrat nenasičenih maščobnih kislin iz njihovih nasičenih in enkrat nenasičenih predhodnikov s pomočjo podaljševanja in desaturacije. Podajamo primerjavo ustreznosti uporabe enoceličnega triacilglicerolnega olja alg, bogatega z dokozaheksaenojsko kislino (DHASCO®), z

ustreznostjo ribjega olja kot vir prehranskega dodatka za mlečni nadomestek. Predstavljamo tudi nekaj študij o varnosti uporabe enoceličnih olj v prehrani živali in ljudi. Za varno uporabo SCOs v mlečnih nadomestkih bi bile dobrodošle še dodatne podrobnejše raziskave. Z gojenjem alg v mediju, ki vsebuje D-[1-^{13}C]glukozo, je možno pridobivati enocelična olja, obogatena s stabilnim izotopom ^{13}C. Orisali smo nekaj novejših primerov uporabe ^{13}C-označenih enoceličnih olj v raziskovalne in diagnostične namene, ki omogočajo raziskave presnove maščobnih kislin. Zaključujemo, da so postala enocelična olja v kombinaciji s stabilnimi izotopi nenadomestljiva za študij presnovnih poti.

Ključne besede: mikrobiologija / oljni mikroorganizmi / enocelično olje / dokozaheksaenojska kislina / stabilni izotopi

SINGLE CELL OILS

Oils derived from microbial sources are named microbial oils, unicellular oils or single-cell oils (SCOs). The term SCO is used as a parallel to Single Cell Protein (SCP) to denote oils of microbial origin. It indicates a triacylglycerol type of oil, similar to that found in plant and animal edible oils and fats (Kyle *et al.*, 1992, Ratledge, 1993, Boswell *et al.*, 1996).

OLEAGINOUS MICROORGANISMS

Like all living cells, also microorganisms contain lipids. Few species of microorganisms can even produce abundant amounts of lipids. Those microorganisms may be termed, in parallel with the designation given to oil-bearing plant seeds, oleaginous. With few exceptions, oleaginous microorganisms are eukaryotes, including algae, yeasts and moulds (Hammond and Glatz, 1988).

Precise definition of oleaginous microorganisms poses some difficulty. A pragmatic definition would suggest those microorganisms that contain more than 20-25 % of their biomass as oil. For yeasts, moulds and eukaryotic algae, but not for bacteria, also a biochemical definition has been proposed. Boulton and Ratledge (1981) observed a correlation between the possession of the enzyme citrate ATP-citrate lyase and the ability of yeast to accumulate more than 20 % of its biomass as lipid. The significance of the enzyme is that it serves to produce acetyl-CoA (from citrate), which is the substrate for the fatty acid biosynthesis.

$$\text{citrate} + \text{ATP} + \text{CoA} \longrightarrow \textbf{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + P_i$$

Acetyl-CoA cannot be produced in the cytoplasm from pyruvate. Oleaginous microorganisms accumulate citrate in the mitochondria which is then transported into the cytoplasm and there cleaved by ATP-citrate lyase. Nonoleaginous organisms do not possess the citrate-cleaving enzyme and must rely on less effective means of producing acetyl-CoA in the cytoplasm. The possession of ATP-citrate lyase may be thus considered as a key to oleaginicity (Ratledge, 1986).

As prokaryotic microorganisms do not have the compartmentalisation of the mitochondrion to separate acetyl-CoA formation from acetyl-CoA utilisation for fatty acid biosynthesis, there is no need for ATP-citrate lyase. Absence of this enzyme in oleaginous bacteria thus has no biochemical significance (Ratledge and Boulton, 1985).

BIOCHEMISTRY OF LIPID ACCUMULATION

It has been known for a long time that many eukaryotic microorganisms will increase their lipid content if they become depleted for a nutrient, provided that the supply of carbon to the cell remains abundant (Boulton and Ratledge, 1985). Oleaginous organisms differ from nonoleaginous ones in being able to continue to convert carbon from the growth medium into

intracellular lipid, after nitrogen has been depleted from the medium (Hall and Ratledge, 1977, Hammond and Glatz, 1988, Kyle *et al.*, 1992). Due to nutrient deprivation, the organism is unable to synthesise essential cell materials such as proteins and nucleic acids etc., and thus the cell division stops. During nitrogen starvation both oleaginous and nonoleaginous microorganisms continue to take up carbon (glucose), but only oleaginous organisms metabolise it and increase their ATP/AMP ratio in the cell. The existing cells become large or fatter as their lipid droplets continue to grow (Hammond and Glatz, 1988). The pathway of lipid biosynthesis (conversion of carbohydrate to lipid) in oleaginous microorganisms has been reviewed in detail elsewhere (Ratledge, 1986).

Oleaginous microorganisms accumulate lipids mainly in the form of triacylglycerols with esterified straight chain fatty acids containing 0 to 3 double bonds. Unsaturated fatty acids are preferentially incorporated into the sn-2 position of glycerol (i.e., the central carbon atom). Both saturated and unsaturated fatty acids occur on the sn-1 and sn-3 positions (Ratledge, 1986).

Saturated fatty acids are synthesized from acetyl-CoA by the concerted action of two complex enzyme systems, acetyl-CoA carboxylase and fatty acid synthetase (Boulton and Ratledge, 1985).

Long chain polyunsaturated fatty acids (LCP) are synthesized from the saturated or monounsaturated precursors with the elongation and desaturation pathway (Fig. 1). Plants and unicellular phytoplankton algae (Unsaturated…, 1992) and some fungus (Kendrick in Ratledge, 1992a), but not animals and humans, possess the enzymes Δ^{12} and Δ^{15} desaturases, which in the presence of oxygen transform oleic acid (C18:1n-9) into linoleic acid (C18:2n-6) (Δ^{12} desaturase) and this last one further into α-linolenic acid (C18:3n-3) (Δ^{15} desaturase). Due to absence of these two enzymes in humans, linoleic and α-linolenic acid are essential fatty acids in human and animal nutrition (Unsaturated..., 1992, Kendrick in Ratledge, 1992b). In addition to enzymes showed in fig. 1, some moulds also contain Δ^{17} desaturase, that can convert arachidonic acid (20:4n-6, AA) into eicosapentaenoic acid (C20:5n-3, EPA) (Kendrick and Ratledge, 1992b, Ratledge, 1993).

THE IMPORTANCE OF DHA IN NUTRITION

DHA is an LCP with 22 carbon atoms and 6 double bonds, the first one located at the third carbon atom from the methyl terminus (n-3 LCP). DHA is an important structural component in tissue membranes of the human body. It is the predominant n-3 LCP found in the human brain and important structural component of neurological and retinal tissues (Crawford, 1993, Koletzko, 1990, Koletzko *et al.*, 1998a, Nettleton, 1993, Neuringer *et al.*, 1988).

DHA can be provided by dietary sources, by liberation from body stores or by endogenous synthesis from precursor fatty acids, like α-linolenic acid. Humans can elongate and desaturate the precursor α-linolenic acid (Fig. 1), but presumably very slowly (Makrides *et al.*, 1996, Martin *et al.,* 1993, Nettleton, 1993, Simopoulos, 1991).

An optimal supply with DHA is important during the whole life period (Unsaturated…, 1992), but especially during the early development. The most rapid period of brain growth occurs during the last trimester in utero and during the first months of postnatal life. During this periods, the fetus acquires DHA from the mother by placental transfer and the infant later from the breast milk. Insufficient early supply can affect later development of visual and neural functions (Carlson *et al.,* 1993, Gibson *et al.*, 1996, Koletzko *et al.*, 1998a).

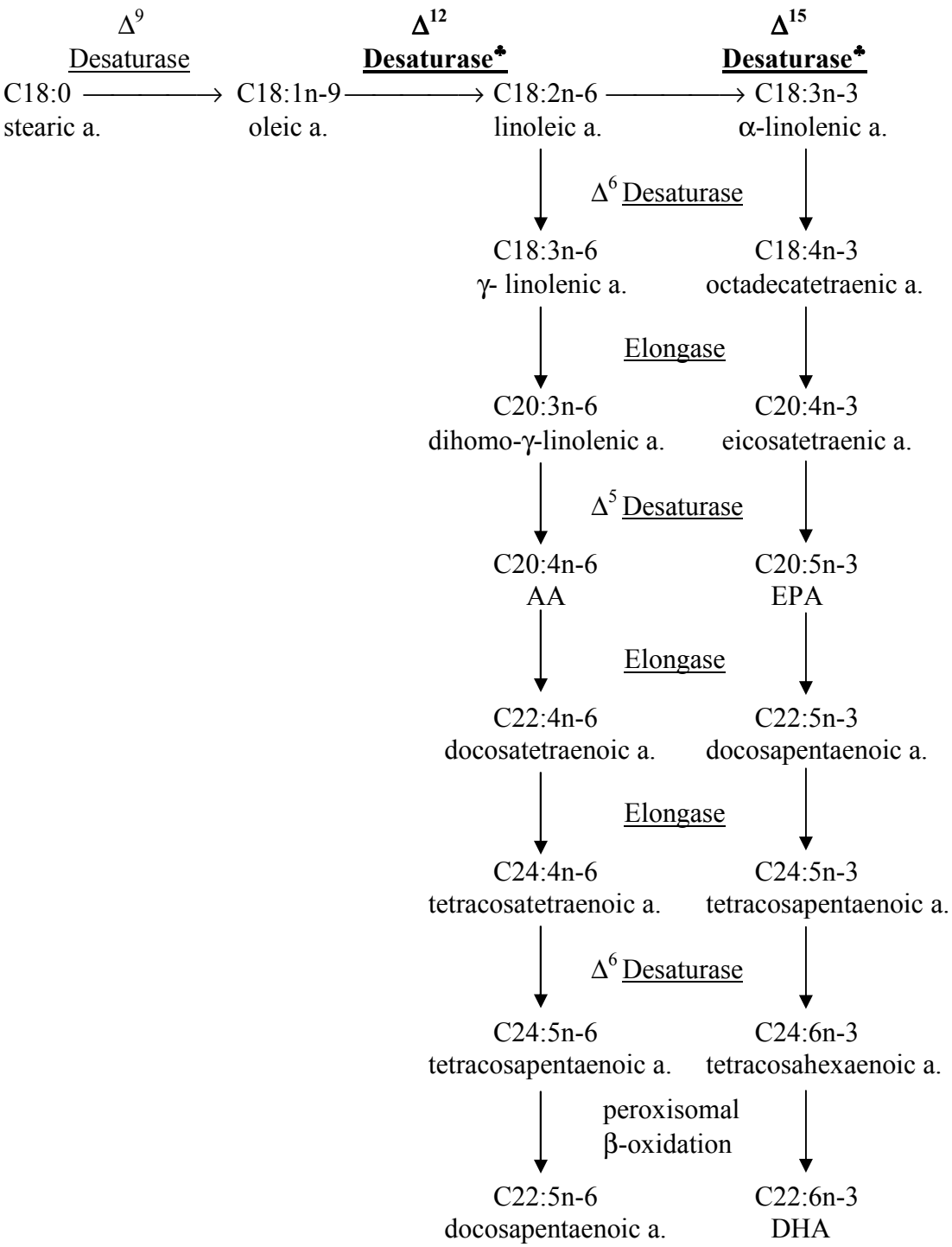

Figure 1. Biosynthetic pathways of n-6 and n-3 polyunsaturated fatty acids of mammalian physiological importance (modified after Moore *et. al.*, 1995 and Kendrick and Ratledge, 1992b).

♣Δ^{12} and Δ^{15} desaturases are not found in animals and humans

Slika 1. Biosinteza za sesalce fiziološko pomembnih večkratnenasičenih maščobnih kislin (povzeto po Moore s sod., 1995 ter Kendrick in Ratledge, 1992b).

♣živali in ljudje nimajo Δ^{12} and Δ^{15} desaturaz

COMPARISON OF FISH OIL AND SCO AS A SOURCE OF DHA

Sea fishes are among the richest sources of DHA in human nutrition, however they can not produce LCP by themselves. Fishes derive their LCP from uptake of the phytoplankton that they eat. This algal source can be used for the production of SCO rich in DHA. Besides DHA, fish oils contain also several other LCP, such as EPA and docosapentaenoic acid, many of which have other specific bioactivities. Due to their unsaturated nature, all of these LCP increase the susceptibility of fish oils to oxidation (Boswell *et al.*, 1996).

Some clinical studies using fish oil (containing DHA and EPA, Table 1) to provide DHA in infant formulas resulted in depression of the growth rate of the infants. This was likely due to the significant drop of AA levels caused by EPA in fish oil (Carlson *et al.*, 1992, Koletzko and Braun, 1991).

Unlike fish, can certain microalgae due to their enzymes, synthesise DHA *de novo*. Kyle *et al.* (1992) screened different phytoplankton species for their ability to produce DHA. The microalgal strain MK 8805 yields about 33 % of its biomass in an extractable triglyceride oil (DHASCO^{TM}), containing about 35 wt. % of DHA (Tab. 1). One of the most unique features of this algal strain is the simple fatty acid profile compared to that of fish oil. It contains no LCP in appreciable amounts other than DHA, which is presumed to be a direct consequence of *de novo* synthesis of DHA. Because of such a high DHA content, compared to fish oil for the equivalent amount of DHA, this algal oil is substantially less unsaturated and consequently less prone to oxidation (Kyle *et al.*, 1992).

Table 1. Weight percentage of EPA and DHA in various fish (Unsaturated…, 1992) and microalgae (Kyle *et al.*, 1992)

Preglednica 1. Utežni odstotek EPA in DHA v različnih vrstah rib (Unsaturated…, 1992) in mikroalg (Kyle s sod., 1992)

Organism	DHA (wt. %)	EPA (wt. %)
Microalgae		
MK8805[a]	35	0
MK8908	0	5
Fish		
Herring	3	3
Menhaden	10	14
Cod liver	27	6
Trout liver	7	16

[a] used for production of DHASCO^{TM}

An important role for the quality of fat absorption in infants plays also the positional distribution of fatty acids within the triacylglycerol molecule (Hansen *et al.*, 1997). The position of DHA within the triacylglycerol molecule in fish oil at sn-2 differs from that in human milk (Christenson *et al.*, 1993). Due to positional preferences of DHA for the sn-1 und sn-3 position in algal oils, they are more similar to mammal oil (human milk fat) than to fish oil (Kyle *et al.*, 1992).

SAFETY OF SCOs

As SCOs rich in LCP are novel food sources in human diet, their safety for use in infant feeding requires thorough evaluation. An expert committee in the United Kingdom provided precise guidelines for thorough evaluation of nutritional and clinical effects of new ingredients in infant formulas (Department of Health, 1996, reviewed elsewhere by Koletzko and Sinclair, 1999). There was a concern that some microorganisms used for SCO production may produce biotoxic substances and novel sterols (Kyle and Ratledge, 1992, Reiss, 1993, Withers *et al.*, 1978, reviewed by Koletzko and Sinclair, 1999). It has been proposed that SCOs used for infant formula production should be derived from non-pathogenic microorganisms and be free of contaminating substances (Koletzko and Sinclair, 1999).

Boswell *et al.* (1996) performed safety evaluation studies of ARASCO® (fungal oil, highly enriched in AA), DHASCO® (algal oil, highly enriched in DHA) and Formulaid® (a combination of ARASCO® and DHASCO®) in rats. They concluded that the examined SCOs have no demonstrable toxicity and that administration of these SCOs is no less safe than administration of sunflower oil. Innis and Hansen (1996) studied the acute effect of increasing intakes of the same ARASCO® and DHASCO® blended together in healthy adult males. Their results show that the blend of microalgal- and fungal-derived oils containing AA and DHA are efficient in increasing plasma lipid concentrations of AA and DHA. No apparent adverse effects were found during a 14-day supplementation period.

Although the results of mentioned studies confirm that AA and DHA in these SCOs are highly bioavailable and thus may offer a new source of these fatty acids for nutritional purposes, their use in infant formulas might still need some additional evaluations, for example all those proposed by an expert committee in the United Kingdom (Department of Health, 1996).

APPLICATIONS OF SCOs TO STUDY FATTY ACID METABOLISM

A large number of applications of SCOs as a single source of DHA and/or AA, have been used in recent research studies in experimental animals as well as in humans. For example Makrides *et al.* (1996) studied the effect of maternal DHA (DHASCO®) supplementation on breast milk composition. These studies enabled detailed research of metabolism and biological functions of these fatty acids.

An advanced step to study metabolism and function of fatty acids was achieved by growing the microalgae strain in a defined medium containing D-[1-^{13}C]glucose, which produces SCOs, enriched with the stable isotope ^{13}C. Stable isotope labelled fatty acids (for example [^{13}C]DHA) were used as tracers in metabolic research in experimental animals (Brossard *et al.*, 1994, Brossard *et al.*, 1996a, Brossard *et al.*, 1996b, Croset *et al.*, 1996) as well as in humans *in vivo* (Brossard *et al.*, 1996b, Fidler, 1999, Szitanyi *et al.*, 1999, reviewed by Koletzko *et al.*, 1998b). The potential adverse effects of stable isotope use have been evaluated in a large number of studies (Jones and Leatherdale, 1991, Klein and Klein, 1986, Koletzko *et al.*, 1997). The wider usage of stable isotopes is enhanced by increased consciousness of the potential risk of ionising radiation (for example ^{14}C) as well as limitations due to more restrictive legislations, remarkable improvements in analytical technologies, specially mass spectrometry, and after all the availability of numerous stable isotope labeled substances (Koletzko, *et al.*, 1997). Several other studies, using stable isotopes for research and diagnostics purposes, have been reported (Demmelmair *et al.*, 1995, Demmelmair *et al.*, 1998, Hachey *et al.*, 1987, Koletzko *et al.*, 1998b, Pacy *et al.*, 1989, Sauerwald *et al*., 1996). With these studies using stable isotopes, new insights into kinetics and mechanisms of fatty acid absorption, transport, conversion rate and oxidation have been obtained.

The presented examples demonstrate the possible opportunity for SCOs as a novel source of polyunsaturated fatty acids, such as DHA and AA for nutritional supplementation. For their use in infant formulas all necessary safety evaluation studies should still be performed. Recent developments of SCOs in combination with stable isotopes have become indispensable to study a range of metabolic enigmas, including some of those which were insoluble by the traditional techniques.

POVZETEK

Mikroorganizme, ki kopičijo več kot 20 do 25 % svoje mase v obliki maščob, imenujemo oljni mikroorganizmi, njihova olja pa enocelična olja ali mikrobna olja. Za oljne kvasovke, plesni in evkariontske alge je značilna prisotnost encima ATP-citrat liaza. Oljni mikroorganizmi so sposobni pretvarjati ogljik iz rastnega medija v intracelularne lipide tudi takrat, ko je v gojišču že zmanjkalo dušika in ni več možna sinteza bistvenih celičnih sestavin, kot so beljakovine in nukleinske kisline. Enocelične alge rastlinskega planktona (Unsaturated…, 1992) in nekatere glive (Kendrick in Ratledge, 1992a) vsebujejo encima Δ^{12} in Δ^{15} desaturazo, ki pretvorita oljno kislino (C18:1 n-9) v linolno (C18:2 n-6), le to pa v α-linolensko (C18:3 n-3). Zaradi odsotnosti omenjenih dveh encimov pri ljudeh in živalih sta linolna in α-linolenska kislina v prehrani ljudi in živali nenadomestljivi (Unsaturated..., 1992, Kendrick in Ratledge, 1992a). Poleg encimov, ki jih prikazuje slika 1, vsebujejo nekatere glive, zlasti plesni, tudi Δ^{17} desaturazo, ki omogoča pretvorbo arahidonske kisline (C20:4 n-6, AA) v eikozapentaenojsko kislino (C20:5 n-3, EPA) (Kendrick in Ratledge, 1992a, Ratledge, 1993). V nasprotju z ribami lahko nekatere mikroalge sintetizirajo dokozaheksaenojsko kislino (DHA) na novo. Rod mikroalg MK 8805 sintetizira trigliceridno olje s kar 35 ut. % DHA. Omenjeno olje ne vsebuje drugih dolgoverižnih večkrat nenasičenih maščobnih kislin, zaradi česar je bistveno manj podvrženo oksidaciji kot ribje olje. Poleg tega je zaradi porazdelitve DHA na sn-1 in sn-3 položaju v triacilglicerolni molekuli mnogo bolj podoben olju sesalcev kot ribje olje. Zaradi naštetih lastnostih je primerno kot prehranski dodatek ali dodatek za mlečni nadomestek (Kyle s sod., 1992). Ker so SCO nov vir hrane, so nekaj raziskav o varnosti njihove uporabe v prehrani ljudi in živali že izvedli. Za varno uporabo SCO v mlečnih nadomestkih bi bile, podobno kot za vse druge nove sestavine v mlečnih nadomestkih, zaželene še dodatne raziskave o varnosti njihove uporabe (Koletzko in Sinclair, 1999). Gojenje mikroorganizmov na gojišču z D-[1-^{13}C]glukozo omogoča sintezo enoceličnih olj, obogatenih s stabilnim izotopom ogljika (^{13}C). Uporaba enoceličnih olj v kombinaciji s stabilnimi izotopi ter velik napredek analitskih tehnik, zlasti masne spektroskopije, so odprli nove možnosti v odkrivanju še nepoznanih presnovnih poti maščobnih kislin *in vivo*.

Abbrevations:

AA	arachidonic acid
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
LCP	long chain polyunsaturated fatty acid
SCOs	single cell oils

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