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Long-Chain Omega-3 Specialty Oils

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THE OILY PRESS
An imprint of PJ Barnes & Associates
Bridgwater, England

Published by **The Oily Press**
an imprint of PJ Barnes & Associates
PO Box 200, Bridgwater TA7 0YZ, England
Tel: +44-1823-698973, Fax: +44-1823-698971
E-mail: editor@pjbarnes.co.uk
Web site: http://www.pjbarnes.co.uk

ISBN 978-0-9552512-1-4

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This book is **Volume 21** in **The Oily Press Lipid Library**

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Typeset in 10½/12pt Times New Roman by
Ann Buchan (Typesetters), Middlesex, UK
Printed and bound in Great Britain by Athenaeum Press Ltd,
Gateshead, Tyne & Wear

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CHAPTER 1

Omega-3 fatty acids – Introduction

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A. What are omega-3 fatty acids?

The most important polyunsaturated fatty acids are straight-chain compounds with 18, 20, or 22 carbon atoms per molecule and two to six *cis* olefinic centres, each separated from the next by one methylene function. These have the general structure shown below:

$$CH_3(CH_2)_xCH{=}CH[CH_2CH{=}CH]_y(CH_2)_zCH_2COOH$$

and are divided into families depending on the value of x. The members of each family of polyunsaturated fatty acids thus share a common structural feature in the position of the double bond closest to the methyl end group of the molecule. For example, for the family of polyunsaturated fatty acids for which x=1, unsaturation starts on the third carbon atom from the methyl end group. Such acids are designated as omega-3 (also known as *n*-3) fatty acids.

Throughout this book, an abbreviated nomenclature is used to designate the structures of fatty acids. For example, docosahexaenoic acid (DHA), the common term for all-*cis*-4,7,10,13,16,19-docosahexaenoic acid, can be designated as Δ4,7,10,13,16,19-22:6 or simply as 22:6*n*-3, indicating a fatty acid with 22 carbon atoms and 6 double bonds, the first double bond being found on the third carbon atom from the methyl end group (see Figures 1 and 2). Trivial names associated with the omega-3 series include linolenic (18:3), moroctic and stearidonic (18:4), timnodonic (20:5), clupanodonic (22:5) and cervonic (22:6), though not all of these remain in use. Several of these names were used before the structures were correctly identified.

The polyene acids are biosynthesized through a combination of metabolic elongation and desaturation processes, starting with an unsaturated acid. This is palmitoleic acid (16:1) for the omega-7 (*n*-7) family, oleic acid (18:1) for the omega-9 (*n*-9) family, linoleic acid (18:2) for the omega-6 (*n*-6) family, and α-linolenic acid (18:3) for the omega-3 (*n*-3) family. These metabolic changes are particularly important in animal systems and lead to C_{20} and C_{22} polyunsaturated fatty acids of considerable nutritional significance. The most important

Omega-6 (*n*-6) family	**Omega-3 (*n*-3 family)**
18:2 (9,12) *linoleic*	18:3 (9,12,15) *α-linolenic (ALA)*
↓ **Δ6 desaturase**	↓ **Δ6 desaturase**
18:3 (6,9,12) *γ-linolenic*	18:4 (6,9,12,15) *stearidonic*
↓ **elongation**	↓ **elongation**
20:3 (8,11,14)	20:4 (8,11,14,17)
↓ **Δ5 desaturase**	↓ **Δ5 desaturase**
20:4 (5,8,11,14) *arachidonic*	20:5 (5,8,11,14,17) *eicosapentaenoic (EPA)*
	↓ **elongation**
	22:5 (7,10,13,16,19) *docosapentaenoic (DPA)*
	↓ **elongation**
	24:5 (9,12,15,18,21)
	↓ **Δ6 desaturase**
	24:6 (6,9,12,15,18,21)
	↓ **ß-oxidation**
	22:6 (4,7,10,13,16,19) *docosahexaenoic (DHA)*

Figure 1. The omega-6 and omega-3 families of polyunsaturated fatty acids. The final stages of docosahexaenoic acid (DHA) synthesis follow a slightly different pathway, proceeding through the C_{24} acids. The term long-chain polyunsaturated fatty acids (LC-PUFA) is reserved for acids with more than 18 carbon atoms.

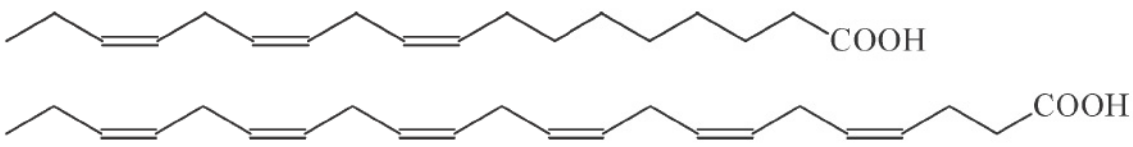


Figure 2. Schematic diagrams of: (above) α-linolenic acid (ALA; 18:3*n*-3) and (below) docosahexaenoic acid (DHA; 22:6*n*-3), showing the omega-3 double bond (the leftmost double bond on each diagram) in each of these acids.

are the omega-6 and omega-3 families, derived from linoleic and α-linolenic acids, respectively.

The changes occurring in mammalian systems are set out in Figure 1. As the same enzymes are required for each family, there is competition for access to these enzymes. The ratio of omega-6 to omega-3 fatty acids required in the diet for optimum health is a matter of current debate and is discussed later. This book is devoted to the omega-3 family of fatty acids, but these are so closely associated with the omega-6 family that some reference must also be made to the latter.

The final stages of DHA synthesis follow a slightly different pathway from that of the omega-6 fatty acids, proceeding through C_{24} acids. The term long-chain polyunsaturated fatty acids (LC-PUFA) is reserved for acids with more than 18 carbon atoms.

Table 1. Enzymes and substrates involved in the conversion of oleate to linoleate and of linoleate to linolenate

Substrate (acid)	Substrate (lipid)	Desaturase	Product
Oleate	Phosphatidylcholine	Δ12	Linoleate
Linoleate	Monogalactosyldiacylglycerol	Δ15	α-Linolenate

The LC-PUFA are further metabolized to a range of compounds with 20 or 22 carbon atoms (or more), including prostaglandins, leukotrienes, resolvins and endocannabinoids, which have been associated with one or another of several disease conditions such as atherosclerosis, thrombosis, angina, stroke, psoriasis, arthritis, asthma, depression, schizophrenia, obesity and others, including impaired cognitive and joint function. Good health may require an appropriate dietary ratio of C_{20} to C_{22} PUFA.

The special nature of docosahexaenoic acid in membranes has been discussed by Gawrisch *et al.* (2003) and by Stillwell & Wassall (2003).

B. How are omega-3 fatty acids formed in plants and animals?

In the cytoplasm of plants, desaturation converts oleate in the form of a phosphatidylcholine to linoleate, and converts linoleate as its monogalactosyldiacylglycerol derivative to linolenate. These changes take place through the action of a Δ12 and Δ15 desaturase, respectively (Table 1). The additional double bonds have the *cis* configuration and are in a methylene-interrupted relation to other double bonds. The 1,4-diene unit is characteristic of the common polyunsaturated fatty acids and is to be distinguished from the 1,3 (conjugated) systems found in carotenoids and some less-common fatty acids, and the 1,5 system found in polyisoprenoids.

A Δ6 desaturase is common in animal systems but less so in the plant world. However, it is active in the biosynthesis of γ-linolenic acid (6,9,12-18:3) from linoleate and of stearidonic acid (6,9,12,15-18:4) from α-linolenate.

The C_{20} and C_{22} polyenes that characterize animal systems, and particularly fish lipids, either do not exist in land plant systems or are exceedingly rare. However, the leaves, stem, and whole plant of purslane (*Portulaca oleracea*) has been reported to be a vegetable source of omega-3 acids, with low levels of the 20:5, 22:5 and 22:6 members. This surprising and unlikely result needs to be confirmed (Omara-Alwala *et al.*, 1991). These acids have not been identified in purslane seed oil, which contains only the conventional palmitic, stearic, oleic, linoleic and linolenic acids.

The production of important LC-PUFA such as arachidonic (Δ5,8,11,14-20:4), eicosapentaenoic (Δ5,8,11,14,17-20:5) and docosahexaenoic (Δ4,7,10,13,16,19-22:6) acids in plant systems is a challenge for plant geneticists. Research has got as far as proof of concept, but much remains to be done before an

economically viable system can be developed. The production of even modest levels of LC-PUFA in a plant that can be grown easily and widely will be a very significant development (see Section C.6).

It is important to recognize that animals cannot make linoleic acid (18:2*n*-6) or α-linolenic acid (ALA; 18:3*n*-3) for themselves, nor can they interconvert omega-6 and omega-3 acids. In animals, these two C_{18} PUFA must be derived from a dietary source of plant origin. LC-PUFA entering into the diet by eating meat or fish have also come originally from plant systems (including phytoplankton in the case of fish). Once linoleic acid and ALA have been ingested they suffer a number of fates (Section D), one of which is metabolism by chain-extension and desaturation between existing double bonds and the carboxyl group to produce important polyunsaturated C_{20} and C_{22} acids including, in particular, arachidonic acid (20:4*n*-6), eicosapentaenoic acid (EPA; 20:5*n*-3) and docosahexaenoic acid (DHA; 22:6*n*-3) (Figure 1). An important issue is whether it is possible for animals in general, and humans in particular, always to produce adequate supplies of EPA and DHA from a sufficient intake of ALA, or whether there are occasions when these two long-chain omega-3 essential fatty acids must themselves be part of a healthy diet.

Nutritional attitudes to PUFA have changed over the years. First, they were considered as a single group, distinct from saturated and monounsaturated acids, then they were subdivided into omega-6 and omega-3 PUFA and the ratio of these two was considered as a useful dietary index. Today this concept is considered to be flawed, and separate recommendations are increasingly being given for ALA and for EPA/DHA. ALA is almost entirely of plant origin, while EPA and DHA come from animal sources, particularly fish.

C. Dietary sources of omega-3 fatty acids

This discussion focuses on dietary sources of three omega-3 fatty acids (ALA, EPA and DHA), with a passing reference to stearidonic acid (18:4). What follows should be read in conjunction with the more detailed information given in the later chapters on fish oils and microbial oils.

1. ALA

Among the so-called commodity vegetable oils, only three contain ALA in significant amounts. These are: soybean oil, with 8% ALA and an annual production of 33.3 million tonnes in 2005; rapeseed/canola oil, with 10% ALA and an annual production of 16.0 million tonnes in 2005; and linseed (flaxseed) oil, with ~50% ALA and an annual production of 0.6 million tonnes in 2005. Linseed oil is used mainly for industrial purposes and only small amounts are used for human dietary purposes, either as the seed itself or as cold-pressed (flaxseed) oil. As a consequence, soybean and rapeseed oils are the major

dietary sources of ALA. Gunstone (2005) calculated that in 2004–2005 the agricultural industry produced 3.5 million tonnes of ALA, mainly from soybean oil (1.9 million tonnes) and rapeseed/canola oil (1.4 million tonnes), but the true amount available for dietary intake is less than this because of its modification during partial hydrogenation to 18:2 and 18:1 isomers or through high-temperature deodorization to acids with *trans* unsaturation. Also, varieties of both soybean and rapeseed oils with reduced levels of ALA are becoming more widely available (see for example Caiping Su *et al.*, 2003; Warner & Gupta, 2003). These are attractive to the food industry because they can be used for frying purposes without the need for prior brush hydrogenation to lower the level of ALA. One soybean plant, to be grown in commercial quantities in the USA from 2006 onwards, has less than 3% ALA.

Several minor oils are also useful sources of dietary ALA. In a recent survey, Gunstone (2007) listed almost 20 oils with ALA levels between 5% and 65%, of which the following are typical and have been selected on the basis of their ease of availability: camelina (30–40%), hemp (15–25%), kiwi (63%), perilla (57–64%), rosehip (32%), walnut (13–15%), and wheat germ (5%). Camelina oil, for example, is being used in the production of spreads to increase their content of omega-3 acids (Anon., 2005).

ALA is a significant component of the lipids present in green leaf tissue, though the amount of lipid derived from such sources is limited. Duo Li *et al.* (2003) cite the following values for the ALA content (mg/100 g) of fresh green vegetables: watercress (180), spinach (129), broccoli (110), Brussels sprouts (98), and Cos lettuce (94), indicating an average figure of around 0.1% of the fresh leaves.

2. *Stearidonic acid*

Stearidonic acid (Δ6,9,12,15-18:4) is the omega-3 equivalent of γ-linolenic acid and is available as a dietary supplement. Only a few seed oils contain stearidonic acid but *Echium plantagineum* is cultivated as a source of this acid (Table 2). With over 60% of the acids having three or four double bonds the oil is highly unsaturated. Another convenient source of stearidonic acid is the

Table 2. Component acids of oils containing γ-linolenic acid (18:3*n*-6) and stearidonic acid (18:4*n*-3)[a]

Oil	Typical acid concentration (%, w/w of oil)						
	16:0	**18:0**	**18:1**	**18:2*n*-6**	**18:3*n*-6**	**18:3*n*-3**	**18:4*n*-3**
Blackcurrant	7	2	11	47	17	13	3
Echium	6	3	14	13	12	33	17

[a]Data taken from Clough (2001a,b).

Table 3. Levels of fat, EPA and DHA in selected fish[a]

Fish	Concentration (g/100 g tissue)		
	Fat	EPA	DHA
Herring	17.8	2.04	0.68
Sprat	16.6	1.33	1.90
Tuna	15.5	1.08	2.29
Salmon	13.6	0.71	2.15
Sardines in oil	13.9	1.20	1.24

[a]Adapted from Trautwein (2001).

more readily available blackcurrant seed oil, even though it contains only 2.5–3.0% of this acid. Stearidonic acid is the first metabolite in the conversion of ALA to EPA and DHA (Figure 1), and arguments for the inclusion of γ-linolenic acid in dietary supplements can also be applied to stearidonic acid (Clough, 2001a,b; Kallio, 2003). Eckert *et al.* (2006) claimed that soybeans with Δ6 desaturase (from borage oil) and Δ15 desaturase produce an oil containing around 30% of both ALA and stearidonic acid.

3. *EPA and DHA from fish oils*

Long-chain PUFA in the human diet are mainly obtained by eating fatty fish, by taking supplements of fish oil sometimes enriched in EPA and/or DHA, or by consuming food to which fish oil has been added. Table 3 contains information on widely consumed oily fish that represent valuable sources of EPA and DHA. Table 4 contains similar information for a range of commercial fish oils. The sum of EPA, DPA (docosapentaenoic acid; 22:5*n*-3) and DHA ranges from 11% to 33%, with DPA present only as a minor component. The major LC-PUFA is sometimes EPA, sometimes DHA, and sometimes both are found at similar levels (Hjaltason & Haraldsson, 2006a,b; Haraldsson & Hjaltason, 2006). DPA (22:5) is present in fish oils at only low levels, but levels are somewhat higher (up to 5%) in the fats of marine mammals such as seals (Spurvey *et al.*, 2001; Durnford & Shahidi, 2002; Shahidi & Ying Zhong, 2005). Many fish oils contain small amounts of 21:5*n*-3, which may result from the α-oxidation of 22:5.

The fishing industry provides fish for human consumption, along with fishmeal as a valuable protein source (6.0 million tonnes in 2005) and fish oil (1.0 million tonnes in 2005) (Oil World Annual, 2006). Most industrial fish oil is now used in aquaculture as part of the feed for farmed fish (72% in 2003), a diminishing portion is hardened for use in spreads (15% in 2003), and a small but rapidly growing amount is used as high-quality fish oil in capsules or after incorporation into other foods (4% in 2003) (I.H. Pike, personal communication). The balance is used for industrial and other purposes.

Table 4. Levels of omega-3 acids in commercially available fish oils[a]

Fish	Concentration (% of total fat)			
	EPA (20:5)	DPA (22:5)	DHA (22:6)	Total
Anchovy	22	2	9	33
Jack mackerel	13	2	15	30
Menhaden	14	2	8	24
Sardine/pilchard	16	2	9	27
Capelin	8	–	6	14
Herring	6	1	6	13
Mackerel	7	1	8	16
Norway pout	9	1	14	24
Sand eel	11	1	11	23
Sprat	6	1	9	16
Tuna	6	2	22	30
Cod liver oil	8	1	11	20
Minke whale	4	2	5	11
Harp seal	8	3	10	21
Greenland shark	3	2	6	11
Farmed salmon	7	3	11	21
Blue whiting	9	1	10	20

[a]Adapted from Hjaltason & Haraldsson (2006a). EPA, eicosapentaenoic acid. DPA, docosapentaenoic acid. DHA, docosahexaenoic acid. –, Not reported.

4. *DHA from microbial oils*

Oils containing DHA are now commercially available from microbial sources (Ratledge, 2004; Ratledge & Hopkins, 2006; see also Chapter 3). Three DHA-rich oils are shown in Table 5. As yet there is no single cell source of EPA. The oils are used mainly in infant formulas, or the dried biomass is used to feed animals to produce elevated levels of DHA in eggs or meat or to feed fish larvae and shrimp. In his earlier paper Ratledge (2004) indicated a production level of 650 tonnes of single cell oils in 2003, and this is likely to have increased since that date.

Dried biomass is usually extracted with hexane, but this powerful solvent extracts more than triacylglycerols, in particular also extracting free acids and phospholipids. Consequently the product may require relatively harsh refining procedures with loss of product. Wang *et al.* (2004) have described a soft

Table 5. Major microbial sources of DHA

Product name	DHA (%)	Source
DHASCO™	40–45	*Crypthecodinium cohnii*
DHASCO-S™	25	*Schizochytrium* sp.
DHActive™	46	*Ulkenia* sp. (probably)

Table 6. Concentrations of selected fatty acids in beef from cattle given three different feeding regimes[a]

Feed	Fat/fatty acid concentration (mg/100 g lean tissue)							
	Total fat	Saturated fat	18:1 *trans*	ALA	EPA	DPA	DHA	Total omega-3
Grass	2138	801	47.0	42.1	32.0	53.9	7.1	135.1
STGF	1738	644	76.5	13.5	14.7	36.9	5.3	70.4
LTGF	3175	1172	111.6	15.1	12.3	36.2	4.2	67.8

[a]Adapted from Mann (2005). STGF, short-term grain feeding with feed-lot ration 80 days pre-slaughter. LTGF, long-term grain feeding with feed-lot ration 180–200 days pre-slaughter.

extraction procedure. Dried biomass is mixed with a carrier oil (sunflower or some other appropriate vegetable oil), milled, and then pressed. The recovered lipid contains less peroxide, free acid, and phospholipid, but the LC-PUFA oil is diluted with the vegetable oil and the DHA level may be reduced to 5%. However, this is sufficient for many purposes. Another advantage is that the extracted oil contains the antioxidants present in the vegetable oil and these protect the highly unsaturated microbial oil.

5. *LC-PUFA from animal fats*

Mann (2005) has pointed out that red meat is the second-best source of LC-PUFA after fish. The levels of these acids in meat are dependent on the diet of the animal. Pasture-fed animals living on forage rich in ALA produce higher levels of long-chain omega-3 acids (especially DPA) than animals fed on grain or seed meals rich in omega-6 acids (Table 6). These factors influence the intake of LC-PUFA by Australians according to their dietary habits. Australian intakes are 280 and 140 mg/day of omega-3 LC-PUFA for high and average meat eaters, respectively (Table 7).

Nuernberg *et al.* (2002) compared the levels of omega-3 fatty acids in the muscle of beef cattle (German Simmental and German Holstein bulls) fed on concentrate and on pasture. These sources provide diets rich in omega-6 and omega-3 acids, respectively. The pasture-fed animals had lower omega-6: omega-3 ratios and contained useful quantities of omega-3 LC-PUFA, though this was mainly in the form of DPA (Table 8).

Ailhaud *et al.* (2006) discussed changes in the fatty acid composition of animal products in the French diet between 1960 and 2000. Changes over this time period (Table 9) have resulted mainly from alterations in the composition of the animals' diets, and have had a marked effect on the omega-6:omega-3 ratio in the human diet.

By feeding hens diets enriched in omega-3 fatty acids through the inclusion of fish oil or of algal biomass, it is possible to produce eggs with enhanced

Table 7. Meat, total fat and LC-PUFA intakes of Australian subjects in four different dietary groups[a]

	Intake per dietary group			
Dietary component	**High meat**	**Moderate meat**	**Ovolactovegetarian**	**Vegan**
White meat (g/day)	110	54	0	0
Red meat (g/day)	270	109	0	0
Fish (g/day)	28	26	1	1
Total fat (% of energy intake)	38	33	33	28
Fatty acids (mg/day):				
20:4*n*-6	240	100	30	0
20:5*n*-3	70	40	0	0
22:5*n*-3	90	30	0	0
22:6*n*-3	120	70	10	0
Total omega-3 LC-PUFA (mg/day)	280	140	10	0

[a]Adapted from Mann (2005). High meat, average figures for 18 high-level meat eaters. Moderate meat, average figures for 54 moderate-level meat eaters. Ovolactovegetarian, average figures for 42 ovolactovegetarians. Vegan, average figures for 18 strict vegetarians.

Table 8. Fat content and fatty acid composition of *longissimus* muscle in animals fed different diets[a]

	German Simmental bulls fed:		German Holstein steers fed:	
Fat/fatty acid content of muscle	**Concentrate (4 bulls)**	**Pasture (6 bulls)**	**Concentrate (4 steers)**	**Pasture (5 steers)**
Fat (g/100 g wet wt)	1.70	1.98	4.54	3.94
Saturated fat (wt% of total fat)	40.3	45.2	43.4	45.2
Monounsaturated fat (wt% of total fat)	35.6	37.2	45.7	46.8
Omega-6 fatty acids (wt% of total fat)	18.9	7.5	5.9	2.5
Omega-3 fatty acids (wt% of total fat)	1.4	5.5	0.6	2.2
Omega-3 fatty acids (mg/100 g wet wt)	24.6	108.6	28.8	86.3
Omega-3 LC-PUFA (wt% of total fat)	1.1	3.1	0.37	1.3
Omega-6:omega-3 ratio	13.7	1.3	9.2	1.2

[a]Adapted from Nuernberg *et al.* (2002).

Table 9. Changes in the levels of dietary fatty acids derived from milk, eggs and meats in France between 1960 and 2000[a]

	Fatty acid content of animal products in the diet (% of total fatty acids)						
Year	**16:0**	**18:1**	**18:2*n*-6**	**18:3*n*-3**	**20:4*n*-6**	**Omega-3 LC-PUFA**	**Omega-6:omega-3 ratio**
1960	25	33	5.5	2.4	0.4	0.5	2
2000	29	31	6.2	0.6	0.7	0.2	9

[a]Adapted from Ailhaud *et al.* (2006).

levels of DHA – up to about 3%. These provide a satisfactory dietary means of enhancing circulating blood levels of DHA for mothers and infants during pregnancy (Smuts *et al.*, 2003). Grune *et al.* (2001) reported that the enrichment of eggs with omega-3 PUFA through dietary fish oil requires vitamin E supplementation to inhibit oxidation. In a review on omega-3 fatty acid enrichment of edible tissue in chickens and turkeys, Rymer & Givens (2005) conclude that tissue levels of ALA, EPA and DHA can be increased by adding each of these acids to the diet of the birds, though the response to enhanced DHA intake is weaker than for either ALA or EPA. There is, however, little evidence that ALA or EPA is metabolized to other LC-PUFA in these birds.

Givens & Gibbs (2006) report that the daily UK ingestion of EPA and DHA is around 244 mg/day, well below the recommended minimum level of 450 mg/day. The intake will be higher than average for the quarter of the population who eat fish, but lower for the larger number that do not. Givens & Gibbs consider that meats and dairy products enriched in these acids could provide, on average, an additional 230 mg/day. Poultry flesh would be a good target for enrichment because of the speed with which this commodity can be produced and its large and increasing consumption. Garg *et al.* (2006) reported means of delivering recommended levels of LC-PUFA in human diets through the use of a range of fortified foods, including dairy products, eggs, and a range of baked goods.

6. LC-PUFA from plant lipids

The need for omega-3 LC-PUFA and the problems of getting adequate supplies of these from fish or from algal resources has been reviewed by Heinz who, along with other research groups, is striving to develop plant sources of these acids (Heinz, 2006). This has already been achieved in several ways involving gene transfer from other sources. Many difficulties remain to be overcome, not least, for some communities, the fact that these results have only been achieved by genetic engineering (Abbadi *et al.*, 2004; Qi *et al.*, 2004; Napier *et al.*, 2005; Robert *et al.*, 2005; Wu *et al.*, 2005).

7. LC-PUFA in structured lipids

Natural sources of LC-PUFA are not always optimal in their content of these acids, in the nature of other acids also present, and in their bioavailability. There is now widespread interest in preparations rich in triacylglycerols of the type MLM, where M stands for medium-chain acids that are easily metabolized and L represents a long-chain acid such as a LC-PUFA. Procedures to achieve this have been reviewed by Wijesundera (2005).

D. Omega-3 fatty acids – physiological and medical effects

Omega-3 fatty acids have three important physiological functions. First, they serve as major components of biological membranes and are important in membrane structure and function (Gawrisch *et al.*, 2003). DHA is present in high concentrations in lipids of the retina, brain and sperm. Second, they can alter gene expression – down-regulating some enzymes and up-regulating others. Third, EPA (20:5*n*-3) has an important role in the regulation of eicosanoid production from arachidonic acid (20:4*n*-6) through competition for the metabolizing enzymes. The eicosanoids derived from EPA and arachidonic acid show different properties, and it is important that these be appropriately balanced. For the most part EPA is considered to have a functional role – operating through its metabolites – whereas DHA has a structural role.

The importance of omega-3 LC-PUFA for human health is apparent in several of its physiological effects. It is known that blood pressure is reduced by DHA (with EPA having a lesser effect), that plasma total and LDL (low-density lipoprotein) cholesterol levels and serum triacylglycerol levels are reduced, and that thrombosis risk is decreased. There is strong support for the use of omega-3 acids in the secondary prevention of acute coronary syndromes. The mechanism by which these acids protect against cardiac death is not known with certainty, but it may relate to their ability to prevent cellular damage during periods of ischemic stress. Animal studies suggest that omega-3 acids reduce or protect against tumour development, though the limited human studies have been inconclusive. Omega-3 acids (especially EPA and DHA) provide benefits for sufferers with arthritis or Crohn's disease. Patients with neuropsychiatric disorders such as depression and schizophrenia may also benefit from diets rich in omega-3 acids, and EPA is being examined intensively for this purpose. In short, omega-3 acids (or the lack of these) have been linked with a wide range of disease conditions including atherosclerosis, thrombosis, angina, stroke, psoriasis, arthritis, asthma, depression, schizophrenia and obesity (Trautwein, 2001; Lands, 2005; Brouwer *et al.*, 2006). However, Brouwer *et al.* (2006) argue that the influence of omega-3 acids on heart disease remains unproven. Horrobin (1998) considered that schizophrenia, which has

been linked to omega-3 acids, might be the illness that 100 000 years ago made us human.

Several interesting papers were published in the proceedings of a congress on 'Dietary Fats and Health' (Knapp *et al.*, 2003), including a section on DHA function. Other congress papers covered: the immune response and auto-immunity – a question of omega-6 essentiality and the balance between omega-6 and omega-3 (Harbige, 2003), omega-3 PUFA and inflammation (Calder, 2003), and the consumption of fish as a source of omega-3 fatty acids beneficially associated with levels of HDL (high-density lipoprotein) cholesterol and triacylglycerols (Dewailly *et al.*, 2003). Lands published the second edition of his book *Fish, Omega-3 and Human Health* in 2005. He has long been a exponent of the importance of dietary omega-3 fatty acids, and the book ends with a section on choosing diets. Other relevant aspects are reported by Calder & Burdge (2004) in a chapter covering the structure and biology of fatty acids.

E. Omega-3 fatty acids – does chain length matter?

Many food products are advertised as rich in omega-3 acids without any clear indication of how much is ALA and how much is LC-PUFA. Also, lists of foods recommended for their content of omega-3 acids frequently do not distinguish between those containing C_{18} and the $C_{20/22}$ acids. Does this matter? Is ALA an adequate precursor of the LC-PUFA, always or ever? Discussion of metabolic processes such as those set out in Figure 1 has sometimes given the impression that this is the most important metabolic fate of ALA. We now know that this is not true in mass terms, and we have to consider whether sufficient ALA is converted to EPA and DHA or whether these important acids need to be part of our dietary intake.

How effectively is ALA metabolized to DHA? In animal studies, dietary DHA is reported to be a more effective source of tissue DHA than ALA by a factor of 7–20. Feeding ALA leads to an increase in tissue ALA levels, to small increases in tissue EPA and DPA levels, and to an even smaller increase in tissue DHA levels. Results vary in different tissues, and plasma levels, though easy to measure, are not always a good indication of tissue levels. *In vivo* conversion of ALA to EPA and DHA appears to be a slow process and not as effective a source of LC-PUFA as direct consumption of these acids from fish lipids. Reducing the intake of linoleic acid assists the conversion of ALA to LC-PUFA. Δ6 desaturation is rate-limiting in both the omega-6 and omega-3 series.

In reviews of the role of ALA for mammals, Sinclair and coauthors (Sinclair & Duo Li, 2001; Sinclair *et al.*, 2002) describe four important metabolic processes:

- β-oxidation to provide energy. In the rat up to 60% of ALA is expired as CO_2 and this is greater than for other dietary acids. In humans, 16–20% is expired as CO_2 within 24 hours.
- Carbon recycling. This involves conversion, via acetate, to other lipids such as saturated and monounsaturated acids and cholesterol.
- Deposition of ALA, especially in skin and fur.
- Conversion to LC-PUFA. There is only a limited ability to convert ALA to DHA via the seven-step process that occurs in the endoplasmic reticulum. The ability to do this differs in different tissues; some, such as brain, retina and reproductive tissues, may be better able to meet their requirements than others.

Sinclair *et al.* (2002) conclude that β-oxidation is the major metabolic route for ALA, accompanied at lower levels by accumulation in specific sites such as the carcass, adipose tissue, skin, and fur and by conversion to EPA and DHA. It is generally considered that as little as 1–5% of dietary ALA is converted to DHA.

Cunnane (2001) reported that in suckling rats 80% of ingested ALA is released as carbon dioxide, with lower levels appearing as accumulation (<5%), excretion (~2%), conversion to LC-PUFA (<5%), and synthesis of cholesterol and fatty acids (5–10%). It is clear that in this experiment metabolic conversion to LC-PUFA occurred only to small extent. In a later publication, Cunnane *et al.* (2003) argued that the extensive β-oxidation of PUFA in neonates has two significant endpoints: use as an energy source, and use as a ketogenic substance for lipid synthesis.

Poumes-Ballihaut *et al.* (2001) discussed the comparative bioavailability of dietary ALA and DHA in the growing rat. Their results, summarized in Table 10, again show a high level of oxidation for both acids. Accumulation was somewhat higher than reported by Cunnane for suckling rats, and there was some conversion of ALA to LC-PUFA. Most of the DHA was deposited in the skin and carcass.

Table 10. Fate of dietary ALA and DHA in growing rats[a]

	Fate (% of intake)	
Dietary acid	**ALA**	**DHA**
Disappearance by β-oxidation	58.9	64.5
Faecal excretion	0.4	0.5
Total accumulation:	38.4	35.0
as ALA	21.2	0
as DHA	14.0	30.1
as EPA + DPA	3.2	4.9

[a]Adapted from Poumes-Ballihaut *et al.* (2001).

Zhong Fu & Sinclair (2000) found that high ALA intake by guinea pigs led to increased levels of ALA in all tissues except the intestines, brain, carcass and skin. Whole-body LC-PUFA levels were less than 5% of ALA intake, and the main LC-PUFA present was DPA. ALA is also excreted via skin or fur or is subject to β-oxidation rather than being converted to DHA.

From their study of suckling rat pups, Bowen & Clandinin (2000) concluded that an enhanced intake of linolenic acylglycerols significantly increased the level of this acid in the whole body, brain, skin, muscles, and epididymal fat pads but had virtually no influence on the levels of DHA.

F. Omega-3 fatty acids – dietary intakes and recommendations

This section on dietary and recommended intakes of omega-3 acids reflects the dual concerns that intakes are generally too low and that, because of the inefficient metabolism of ALA to EPA and DHA – particularly for men – separate recommendations are required for ALA and for LC-PUFA (EPA and DHA). Some reports suggest that it may be necessary to have individual recommendations for EPA and for DHA since these serve different purposes from each other, and also separate recommendations for males and females because of their differing ability to metabolize ALA.

Recent reviews by Meyer *et al.* (2003) and by Astorg *et al.* (2004) indicate that in Australia and in France dietary intake of ALA comes from both vegetable sources (oils, cereal foods made with oils, vegetable preparations) and animal sources (meats and dairy products). Many people in these countries are not ingesting adequate levels of this acid, and the uptake of ALA in developed countries is often below the recommended level. For example, Astorg *et al.* claimed that "95% of the [human subjects in the] sample consumed less than 0.5% of energy as ALA, which is well below the current French recommendation for adults of 0.8% of energy". Trautwein (2001) listed intakes for men and women in the USA and in 14 different European countries (summarized in Table 11; see also Table 12). The European intakes showed a wide range between different countries. The intake of linoleic acid, for example, was 6.7 and 5.3 g/day in Sweden for men and women, respectively, but 19.0 and 13.2 g/day in the Netherlands. For ALA, contrasting values were shown in France (0.6 and 0.5 g/day) and in Denmark (2.2 and 2.1 g/day) for men and women, respectively.

Table 13 contains part of an interesting analysis of the sources of omega-3 acids in the Australian diet (Meyer *et al.*, 2003). This table shows, for example, that for Australians meat and meat products provide approximately 21% of their EPA intake, 12% of their DHA intake, and almost half of their DPA. Compared with those in many other countries Australians generally consume high levels of meat and meat products. A similar set of data is available for consumption patterns in the UK (Table 14).

Table 11. Daily intake of linoleic acid and α-linolenic acid (ALA) for men and women in the USA and 14 European countries[a]

	Daily intake (g/day)			
	Linoleic acid		ALA	
Region	Men	Women	Men	Women
Europe	6.7–19.0	5.3–13.2	0.6–2.5	0.5–2.1
USA	16.0	11.0	2.0	1.0

[a]Adapted from Trautwein (2001).

Table 12. Daily dietary intakes of PUFA for men and women in France[a]

	Daily intake	
Fat/fatty acid	Men	Women
Total fat	94.1 g	73.5 g
LA	10.6 g	8.1 g
ALA	0.94 g	0.74 g
AA	204 mg	152 mg
EPA	150 mg	118 mg
DPA	75 mg	56 mg
DHA	273 mg	226 mg

[a]Adapted from Astorg *et al.* (2004). LA, linoleic acid (18:2*n*-6). ALA, α-linolenic acid (18:3*n*-3). AA, arachidonic acid (20:4*n*-6). EPA, eicosapentaenoic acid (20:5*n*-3). DPA, docosapentaenoic acid (22:5*n*-3). DHA, docosahexaenoic acid (22:6*n*-3).

Table 13. Food sources of omega-3 acids in the Australian diet[a]

	% Contribution to total dietary omega-3 intake[c]				
Food group[b]	ALA	EPA	DPA	DHA	EPA+DPA+DHA
Meat	15.3	20.6	49.2	11.8	19.6
Cereal	23.8	0.1	0.4	4.3	2.5
Fish	3.5	75.9	50.4	73.1	70.8
Egg	0.5	1.1	–	10.0	6.0

[a]Adapted from Meyer *et al.* (2003). [b]Foods in each category include dishes based on these commodities; for example, 'cereal' includes cooked foods based on cereals and fats, such as biscuits and pastries. [c]These figures indicate the main dietary sources of each of these acids. Thus, for example, dietary DPA is obtained almost entirely and equally from meat (49.2%) and fish (50.4%). When the three long-chain omega-3 PUFA are considered together, this group is seen to come mainly (70.8%) from fish. –, Not reported.

Table 14. Amounts and sources of fat consumed in the UK during 2000–2001[a]

	Dietary intake					Dietary source (% of total dietary intake)[c,d]				
	Men		Women		Rec'd[b,c]	Meats[e]	Cereals[f]	Milk[g]	Fats[h]	Snacks[i]
Energy	9.72 MJ (2313 kcal)		6.87 MJ (1632 kcal)							
Fat:	(g in diet)	(% of energy)	(g in diet)	(% of energy)	(% of energy)					
total	86.5	35.8	61.4	34.9	35	23	19	14	12	10
saturated	32.5	13.4	23.3	13.2	11	22	18	24	11	7
trans	2.91	1.2	2.04	1.2	2	21	26	16	18	6
cis-monoene	29.1	12.1	20.2	11.5	13	27	17	10	11	12
omega-3 PUFA	2.27	1.0	1.71	1.0	–	17	17	4	7	17
omega-6 PUFA	12.9	5.4	9.49	5.3	6.5	18	20	3	14	13

[a]Taken with permission from Gunstone (2004) and based on data in the websites www.nutrition.org.uk/ndnsadults and www.food.gov.uk/science/101717/ndnsdocuments/ndnsv2. These figures relate to the UK National Diet and Nutrition Survey conducted between July 2000 and June 2001 with 1724 adults aged between 19 and 64. They are based on consumption over 7 days, including food consumed outside the home. Women who were pregnant or breastfeeding were excluded from the survey. [b]Recommended levels. [c]The figures in the final columns apply to men and women together. [d]Also included in the full tables were data on eggs and egg dishes; fish and fish dishes; vegetables (excluding potatoes); fruit and nuts; sugar, preserves and confectionery; drinks; and miscellaneous. [e]Meat and meat products. [f]Cereals and cereal products. [g]Milk and milk products. [h]Fat spreads, salad oils, frying oils, etc. [i]Potatoes and savoury snacks. –, Not reported.

According to Burdge *et al.* (2003) and Burdge (2004), the UK intake of ALA at ~1.5 g/day is 10 times lower than the intake of linoleic acid, and ALA levels in blood and cellular lipids are typically below 0.5%. In contrast, EPA and DHA are usually present at levels of 1% and 5%, respectively, even though dietary intakes of these acids are 15–25 times lower than that of ALA.

New evidence suggests that men and women differ in their ability to convert ALA to EPA and DHA. Men convert 8% of ALA to EPA, 4–8% to DPA, and <0.05–4% to DHA. Burdge (2004) considers the average DHA conversion level to be at the lower end of this range, probably only ~0.1%. In women these conversion levels are much higher, with 21% of ALA being converted to EPA and 9% to DHA. These differences may be caused by modulation by estrogen and may be related to the greater need for DHA by women, especially during pregnancy and lactation. Burdge also reports that increased consumption of oily fish down-regulates the conversion of ALA to LC-PUFA. This observation may be counter-intuitive, but perhaps the levels of LC-PUFA need to be controlled because of the burden they place on antioxidant defences.

Dietary intakes of omega-6 PUFA are mainly as linoleic acid and only to a small extent as arachidonic acid. It has been suggested that dietary levels of the omega-6 acids should be around 6% of energy intake, with 3–10% representing a safe range, but there is a growing opinion that even these levels might be too high and arguments have been put forward for an intake of only 2% and a maximum of 3% (Gunstone, 2004). It has been calculated that, with about 20–40% of human body mass being fat and 15–20% of body fat being linoleic acid, adult Americans have more than 3 kg of linoleic acid in their tissues (US National Institutes of Health; ods.od.nih.gov/eicosanoids). This large reservoir of omega-6 acid may need three years to equilibrate with a change in dietary lipids. ISSFAL (the International Society for the Study of Fatty Acids and Lipids) recommend an ALA level of 1% of energy intake (2.22 g/day on a 2000 kcal/day diet) as long as the linoleic acid level does not exceed 2% of energy intake (www.issfal.org.uk/adequate-intakes.html).

Intakes of omega-3 LC-PUFA are generally below recommended levels (see next paragraph). For example, the average UK intake has been reported to be only 215 mg/day; the average consumption in Australia and the USA, at <100–200 mg/day, is also far short of the recommended levels (Givens & Gibbs, 2006). Givens & Gibbs (2006) recently reported a daily consumption of 244 mg/day of EPA and DHA together in the UK. Fish-eaters attain a higher level, but the large number of people who do not consume fish have even lower intakes.

For omega-3 acids separate recommendations are now usually given for ALA and for EPA/DHA. One authority suggests an intake of 1% of energy within a range of 0.5–2.5% for ALA, and 0.5% within a range of 0–2.0% for EPA and DHA. Another group has expressed this slightly differently, at 1.0% for ALA and 0.3% for the higher acids, with a minimum of 0.1% for each of

EPA and DHA (Gunstone, 2004). The American Heart Association now recommends that patients with known coronary heart disease consume ~1 g/d of EPA and DHA. For those without known coronary heart disease the recommendation is for at least two servings of fish (preferably oily fish) each week – equivalent to ~500 mg/day. Garg *et al.* (2006) summarize recent recommendations. These range from 200–650 mg/day for the general population, with higher levels of up to 1000 mg/day for those at risk of cardiovascular disease. A recent UK proposal is for 450–1800 mg/day.

Because DHA is such an important component of the brain, appropriate supplies of this acid are required by a developing fetus and will be supplied by the mother via the umbilical cord. This need continues after birth and should be met through human breast milk, which will contain adequate levels of DHA in a well-nourished mother. Problems arise when the child is not breast-fed and, more significantly, with pre-term infants, since brain development is particularly marked in the final trimester before birth. It is now recommended that during pregnancy and lactation a woman's dietary fat intake should be as for other adults except that the intake of DHA should be at a minimum level of 300 mg/day (Carlson *et al.*, 2001). Infant formulas for Western countries should contain linoleic acid (10.0%), ALA (1.50%), arachidonic acid (0.50%), DHA (0.35%) and EPA (<0.10%), expressed as a percentage of total fatty acids. Slightly different levels are used in Japan, with linoleic acid at 6–10% and DHA at 0.6%. Yuhas *et al.* (2006) have examined the composition of breast milk fat from nine countries (Australia, Canada, Chile, China, Japan, Mexico, the Philippines, the UK, and the USA). They found arachidonic acid levels, at 0.36–0.49% of total fatty acids, to be fairly similar across all countries, but DHA levels ranged from 0.17% to 0.99%, with the lowest values for Canada and the USA and the highest levels for Japan.

The content of EPA plus DHA as a percentage of the total lipids of red blood cells has been suggested as a useful biomarker of omega-3 intake and it is recommended that this so-called 'omega-3 index' (the content of EPA+DHA as a % of total lipids expressed as fatty acids) should be considered as a new risk marker for death from coronary heart disease. A value of 8% is preferred. In a study of 163 individuals from Kansas, values lay between 1.7% and 12.4% (mean 4.9±2.1%), with 91% of subjects having scores below 8% and 44% having scores below 4% (Sands *et al.*, 2005).

G. Stability

PUFA easily undergo oxidative deterioration and other undesirable changes, and it is important to be aware of this during the processing, handling and storage of these labile substances (Kamal-Eldin *et al.*, 2002).

When subjected to thermal stress, even in a vacuum, EPA and DHA or fish oils containing these acids undergo a number of changes. They form dimers or

higher oligomers, cyclize to form cyclic monomers, and undergo stereomutation – though this last change is generally confined to one or two olefinic centres. Fournier *et al.* (2006) examined these changes during deodorization of a fish oil containing EPA (5.8%) and DHA (19.5%) for 3 hours under reduced pressure (1.5 mbar) with 2%/hour direct steam injection at 180, 220, and 250°C. They fully detailed the changes that occurred during the process and concluded that the deodorization of fish oil should be conducted at a temperature not exceeding 180°C.

Natural PUFA are all-*cis* isomers and their conversion to molecules with one or more *trans* double bonds will interfere with their normal physiological activity.

The ready oxidation of PUFA is a major difficulty in their use in food products. The consequences of rancidity and reduced shelf-life require serious attention, and this topic is covered in detail in Chapter 8 (see also, for example, Let *et al.*, 2004). It is desirable to reduce the access of air by encapsulation and to incorporate effective antioxidant mixtures. Garg *et al.* (2006) have discussed the use of microencapsulated fish oil as a means of producing a range of omega-3 fortified foods.

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CHAPTER 9

Analysis of oils and concentrates

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A. Introduction

In writing this chapter my objective has been to provide the reader with an introduction to reliable and, where possible, official methods which are currently used in the analysis of refined commercial marine and algal oils containing long-chain omega-3 fatty acids. Particular attention has been given to describing official methods used to determine fatty acid profiles and to quantify omega-3 levels in oils. These are of the utmost importance for trade in commercial oils and concentrates and yet continue to be a source of much confusion and uncertainty. Established methods for the measurement of oxidation products in refined oils, either via non-specific chemical tests of 'oil quality' markers or via chromatographic methods, are discussed in order to give the reader some context for their importance and typical usage. Since fish and algal oils are typically found in their triacylglycerol form in nature, a section covering the positional analysis of triacylglycerols (which might be important for oil authentication, for instance) and determination of the triacylglycerol content of oils is included. Finally, a brief overview is given of the types of analyses commonly performed to quantify trace contaminants in oils as necessitated by safety and regulatory requirements, as well as for reasons of oil stability.

Most commercial omega-3 oils are highly complex mixtures resulting from a combination of their natural composition along with components due to the effects of extraction, processing, enrichment, oxidation and pollution. It is therefore not surprising that a large number of analytical methods have been devised in order to define the composition of fish oils in ever-increasing detail. The methods in use today are a combination of the still-useful wet-chemistry approaches along with modern chromatography and spectroscopy or mass spectrometry. The unstable nature of polyunsaturated fatty acids (PUFA) also contributes greatly to this complicated picture. In order to avoid becoming overwhelmed by the available data, it is essential to approach edible oil

analysis with a broad understanding of the relevance of the various measurements in order to maintain perspective in the continuing quest for greater purity and enrichment.

Prior to the invention of gas–liquid chromatography (GLC or GC) by James & Martin (1952), the identification of PUFA was a complicated affair. A procedure in use in the early 1950s would likely have started with extensive fractionation of the oil. A sophisticated example of this would be use of the 200-cell automated all-glass counter-current chromatography system described by Craig *et al.* (1951) and employing a complex solvent system (Ahrens *et al.*, 1952). Individual fractions would then have been recovered and analysed using several different methods in order to arrive at an overall compositional analysis. These methods might have included determination of the iodine value using the Wijs method (AOAC, 1955), which gives an indication of the amount of unsaturation by reaction with iodine, or similarly by quantitative measurement of hydrogen uptake (Pack *et al.*, 1952). Another important method used to establish the number of ethylenic bonds involved isomerization of these PUFA in alcoholic alkali to give conjugated systems which could be readily measured by an ultraviolet (UV) spectrophotometer at characteristic wavelengths (Herb & Riemenschneider, 1953). Further separation of unsaturated fatty acids might also have been achieved using paper chromatography (Mangold *et al.*, 1955) with iodine staining. Thus, results from a number of experiments were required in order to infer fatty acid compositions. In contrast, a typical procedure today involves quantitative conversion of a triacylglycerol oil to the fatty acid methyl ester (FAME) form using one of a number of well-defined methods. The resulting FAME mixture is analysed by high-resolution GC with peak identification by retention times matched against readily available FAME standard mixtures. Quantitative determination of the entire fatty acid profile of the oil can be achieved in a matter of hours. Other tests are only necessary to investigate the *quality* of the oil, as defined by the presence of oxidation products or trace contaminants remaining after processing, or just occasionally to investigate or confirm the occurrence of novel or unusual fatty acids.

B. Principles used in the analysis of the fatty acid content of omega-3 oils as fatty acid methyl esters (FAME) using gas chromatography (GC)

Although triacylglycerol oils can be injected onto GC columns stable to high temperature, this type of analysis is largely limited to separation by decreasing volatility or increasing total acyl carbon chain length, referred to as acyl carbon number (ACN) (Ruiz-Gutiérrez & Barron, 1995; Mareš, 1988). Since marine oils may contain 50 or more fatty acyl groups, each of which may be present on one or more of the three positions of the glycerol backbone, this gives rise to such a large number of possible triacylglycerols that separation of all species by

GC alone cannot be achieved. However, the foundation for overcoming this problem was laid with the use of methyl ester derivatives (James & Martin, 1956). These have the advantage of simplifying the analytical problem by considering only the fatty acyl composition and ignoring the more complicated question of the positional distribution within the acylglycerol molecule, a problem which is still challenging today even with modern liquid chromatography/mass spectrometry (LC/MS) methods (Mottram, 2005). Since methyl esters are relatively volatile, non-polar derivatives, they are well-suited to analysis by GC (Christie, 1989) and are by far the most widely used derivatives in both qualitative and quantitative work (Medina *et al.*, 1992). However, it is worth noting that many other derivatives are used for specific applications, such as the use of picolinyl (3-hydroxymethylpyridinyl) ester or 4,4-dimethyloxazoline (DMOX) derivatives to give fragmentation patterns in electron impact mass spectra, which can be used to locate the positions of double bonds (Christie, 2003). Ethyl ester derivatives are of particular importance here, both as intermediates in the concentration of omega-3 oils (see Chapter 5) and as a finished product in dietary supplements. Since fatty acid ethyl esters (FAEE) have similar physical properties to FAME, they are amenable to direct analysis by GC. This makes it possible for some official methods, for example Ph.Eur. 2005:2063 (*European Pharmacopoeia*, 2005a) to dictate that ethyl esters should be analysed without first converting them to the methyl form. Direct GC analysis of FAEE does have the advantage of convenience but, while it could be argued that one should confine the analysis of FAEE to those components truly in the ethyl ester form, this method has the disadvantage that any residual triacylglycerols, partial acylglycerols or free fatty acids present do not contribute to the FAEE peak areas used to determine omega-3 levels. These non-FAEE components can be up to 7% of the oil as defined in the European Pharmacopoeia method (determined using uncorrected peak areas from size-exclusion chromatography with refractive index detection), and may actually contribute small additional amounts of nutritionally available omega-3 fatty acids. Thus, when quantifying omega-3 fatty acids of ethyl esters, slightly higher results may be observed following conversion to FAME than by direct analysis of the ethyl esters.

1. Methods for forming FAME from oils

A typical procedure used to prepare FAME for quantitative GC analysis is outlined in Figure 1.

There are many known methods available for the conversion of oils into FAME – a process often referred to as 'transesterification' (Christie, 1990a,b, 1993). The methods broadly fall into three categories: acid-catalysed methylation, base-catalysed methylation, and a two-step process involving treatment with dilute NaOH in methanol followed by acid-catalysed methylation using

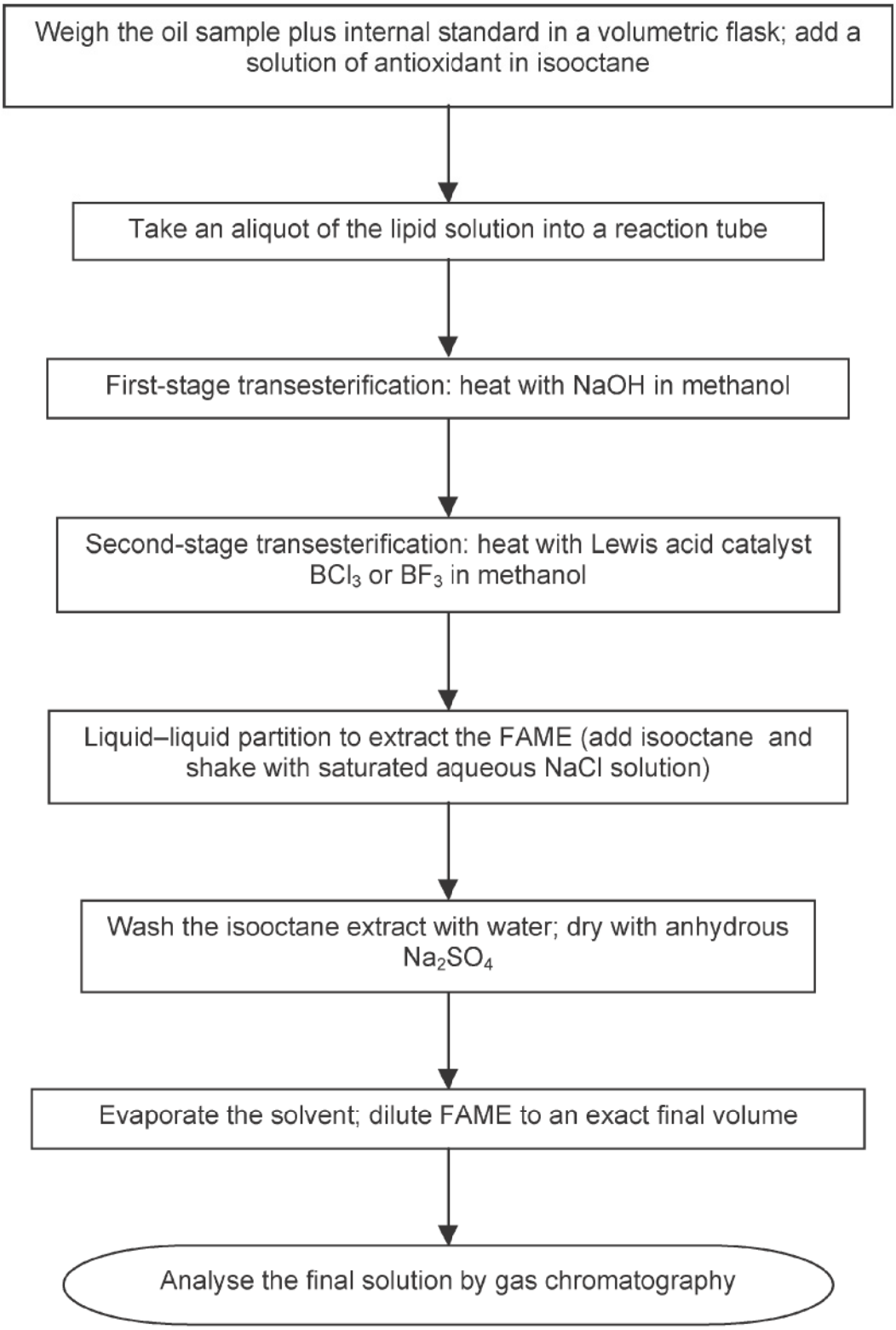


Figure 1. Flow chart showing a typical FAME (fatty acid methyl ester) analysis procedure for oils.

BF_3 (or BCl_3) in methanol. It is the two-step process which has been adopted by official methods (Figure 1), despite suggestions that it is unnecessary or even detrimental compared to direct acid- or base-catalysed processes (Christie, 1994; Ackman, 1998). A detailed discussion of the use of the two-step process (Ackman, 1998) points out that the first step (brief heating with alkali), which is often described as 'saponification', is in fact largely transesterification leading to direct formation of FAME. Any residual free fatty acids are converted to FAME in the second step (heating with BF_3–methanol). At this point it is an academic debate as to whether or not the two-step process is necessary, as it is required by official methods (see Table 1) and has been successfully used for many years.

2. *Official methods for FAME analysis*

A comparison of two of the most widely used official methods (in the European Union and North America) is given in Table 1. The American Oil Chemists' Society (AOCS) method, later adopted by AOAC International (Joseph & Ackman, 1992) was derived from the considerable contributions of Ackman and collaborators. The European Pharmacopoeia method arose from later refinements, with emphasis on accurate quantification for fish oil concentrates (Tande *et al.*, 1992). There are notable differences between the two methods, which merit some explanation, since they can lead to significant discrepancies in omega-3 measurements between laboratories. (Einig & Ackman, 1987).

The most fundamental difference between the two methods is the use of theoretical response factors for FAME in the AOCS method, versus the use of experimentally determined ('empirical') response factors in the European Pharmacopoeia method. This is discussed in detail in Section B.3 below.

The European Pharmacopoeia method requires considerably more oil to test than that of the AOCS – though this is rarely a problem when considering commercial oils which are available in bulk. For good quantification, care must be taken to ensure that the peak areas of internal standard and analytes are similar, and this is treated differently by the two methods. In the AOCS method, the fixed sample amount results in a ratio of sample to internal standard that may not be optimal for analysing a concentrate – for example, an oil that contains 50% DHA (docosahexaenoic acid; 22:6*n*-3) would have a ratio of DHA to internal standard of about 5:1. However, the AOCS method indicates in its numbered notes that if the peak height of the internal standard is less than half that for EPA (eicosapentaenoic acid; 20:5*n*-3) or DHA, the analysis should be repeated with twice the amount of internal standard. This would result in a DHA:internal standard ratio of 2.5:1 in the above example, compared with about 2:1 for the European Pharmacopoeia method.

The European Pharmacopoeia method makes use of the antioxidant butylated hydroxytoluene (BHT), added to the lipid prior to derivatization to minimize

Table 1. Comparison of the European Pharmacopoeia (Ph.Eur.)and American Oil Chemists' Society (AOCS) methods for FAME analysis of omega-3 oils

Method	AOCS Ce 1b 89a	Ph.Eur. 4.3:2.4.29
Similar methods	AOAC 991.39	
Sample amount	25 mg + 2.5 mg internal standard in 25 ml isooctane	400–500 mg if contains 30–50% EPA+DHA 300 mg if contains 50–70% EPA+DHA 250 mg if contains 70–80% EPA+DHA + 70 mg internal standard in 10 ml isooctane with BHT antioxidant
Amount derivatized	25 mg + 2.5 mg internal standard	2 ml = 50–100 mg + 14 mg internal standard
First-step esterification	1.5 ml 0.5 M NaOH–MeOH; 5 min	1.5 ml 0.5 M NaOH–MeOH; 7 min
Second-step esterification	2 ml 12% BF_3–MeOH; 30 min	2 ml 12% BCl_3–MeOH; 30 min
Extraction	Isooctane–saturated aqueous NaCl	Isooctane–saturated aqueous NaCl
Internal standard	23:0 ME for FAME	23:0 ME for FAME
	23:0 EE for FAEE	23:0 EE for FAEE
Response factor solution	None – uses theoretical factors	Reference A solution (EPA–EE + DHA–EE + internal standard), derivatized as sample[a]
GC conditions	30 m × 0.25 mm (or 0.32 mm) internal diameter	At least 30 m × 0.25 mm, 0.25 µm film and 1:200 split
Analysis of ethyl esters	Analyse directly as FAEE	Analyse directly as FAEE: if >50% (EPA+DHA), see Ph.Eur. 2005:2063; if >80% (EPA+DHA), see Ph.Eur. 2006:1250
Determination of total omega-3 content	Not specified. Could use theoretical factors for all FAME	Use the average EPA+DHA experimental response factor for omega-3 FAME other than EPA+DHA (i.e. for 18:3, 18:4, 20:4, 21:5, 22:5)

[a]A mixture of pure EPA ethyl ester, DHA ethyl ester, and internal standard (referred to as 'reference A solution') is derivatized alongside the sample. EPA, eicosapentaenoic acid (20:5*n*-3). DHA, docosahexaenoic acid (22:6*n*-3). BHT, butylated hydroxytoluene. EE, ethyl ester. ME, methyl ester.

oxidation of PUFA. However, it has been shown (Moffat *et al.*, 1991; Christie, 1994) that BHT can form a number of compounds during reaction with NaOH–methanol and BF_3–methanol (and presumably BCl_3–methanol), which appear between 15:0 and 16:0 on the polar columns used in these methods. The choice of BCl_3 instead of BF_3 as an acid catalyst was suggested to yield higher EPA and DHA peak areas relative to the internal standard (Moffat *et al.*, 1991) and may lead to fewer artefacts (Christie, 1990a), although the reaction rate was shown in early studies to be slower for BCl_3 (Morrison & Smith, 1964). BCl_3 is also less volatile and more stable at room temperature than BF_3.

Both methods are similar in column specification – the use of various versions of bonded polyethylene glycol phases ('macrogol' or 'carbowax'), at least 30 m long and with an internal diameter of 0.32 mm or 0.25 mm. There are numerous commercial suppliers of these columns and a key test of separation performance is to ensure that DHA is resolved from 24:0 and 24:1, as illustrated in Figure 2. In our laboratory, we routinely achieve good separation of all of the seven measured omega-3 fatty acids in under 10 minutes (Figure 2b; note that 21:5*n*-3 elutes just before 23:0, but is not labelled on this figure since it is only present at trace amount) – much more rapidly than the official methods, which indicate 30–55 minutes. This is achieved on the same columns through the use of hydrogen carrier gas and with adjustment of the flow and temperature programs. However, it should be noted that there is inevitably a compromise between speed and performance for the more volatile short-chain FAME, as can be seen for the loss of resolution around 18:1 isomers in Figure 2b. Fast methods using shorter microbore columns have been reported (Mondello *et al.*, 2006; Masood *et al.*, 2005) but any such non-standard method for in-house use should be carefully validated against standard methods for quantitative studies.

There is a clear deviation between the two methods concerning instrument performance qualification – the AOCS method gives example chromatograms to indicate expected chromatographic performance, whereas the European Pharmacopoeia specifies stringent additional tests. These tests demonstrate injector discrimination, the accurate recovery of EPA and DHA ethyl ester spikes added to the sample oil prior to derivatization, and the measurement of any peak coeluting with the internal standard. The importance of GC and method performance for accurate FAME quantification has been demonstrated (Tande *et al.*, 1992), but the required tests do add a considerable overhead to the analysis.

3. *Quantification in FAME analysis*

The flame ionization detector (FID) is by far the most universally used GC detector for organic compounds (Ettre, 2002). It has an enormous linear dynamic range (~10^7) and high sensitivity and is robust to normal GC operating conditions, giving stable baselines (Henrich, 1995). The principle of operation

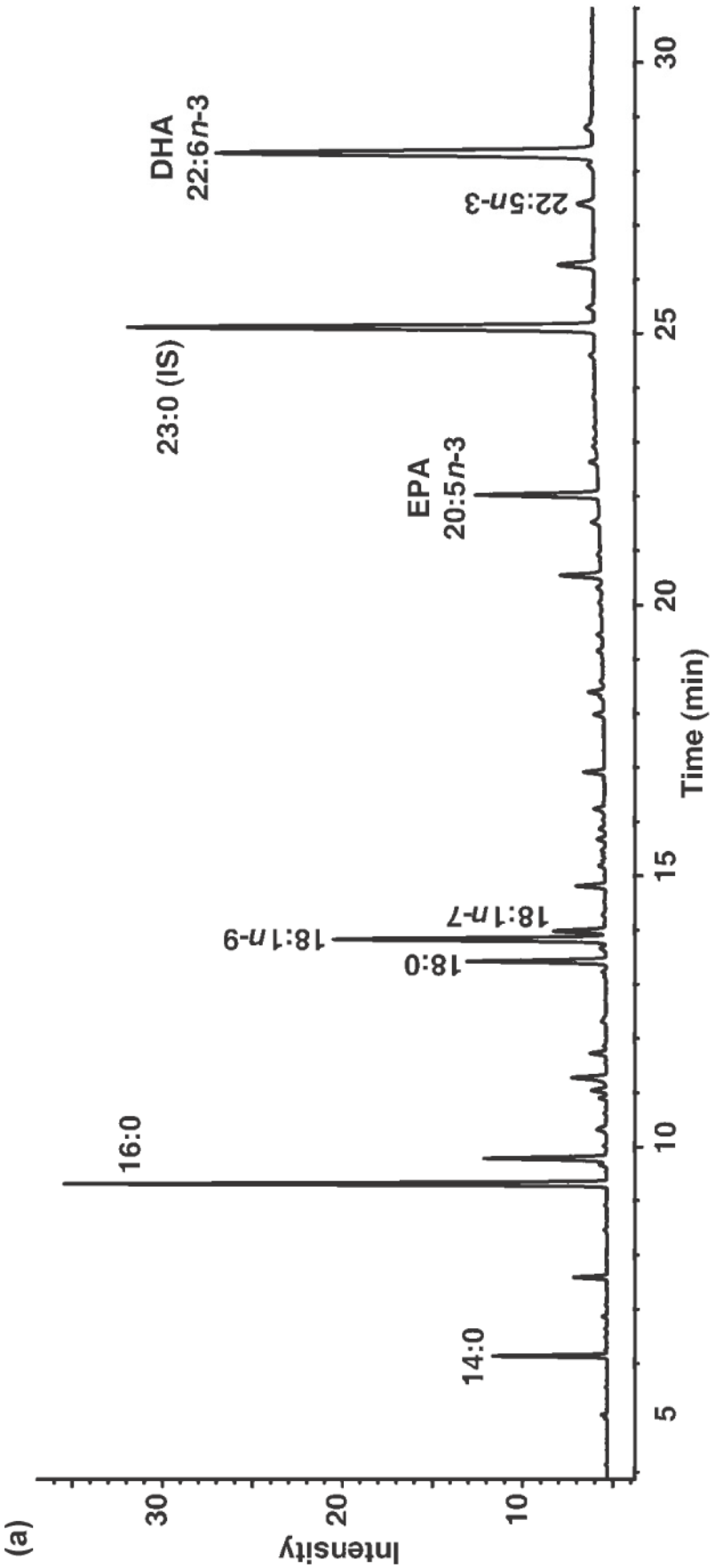


Figure 2. Continued on page below

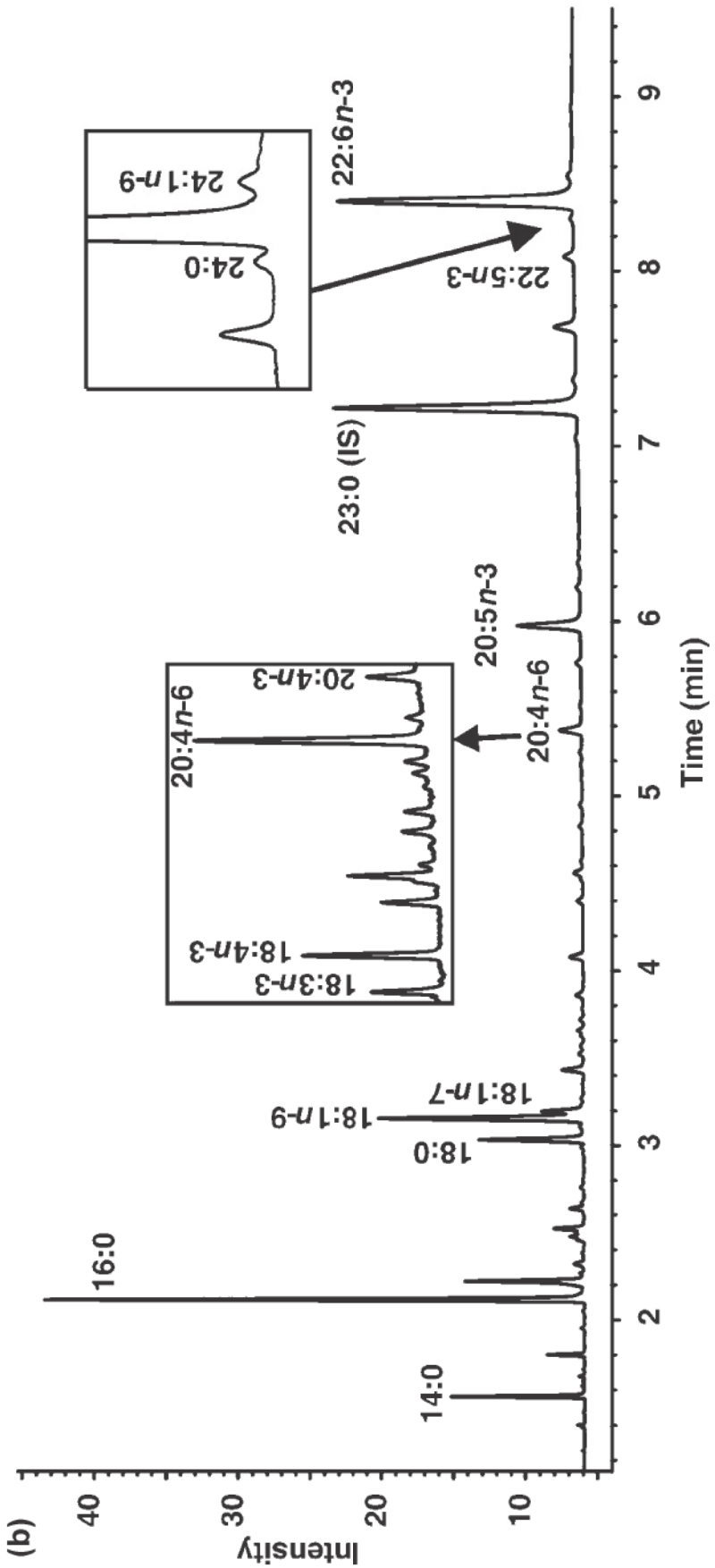


Figure 2. GC FAME analysis of tuna oil: (a) 30 minute analysis using the method described in Ph.Eur. 2.4.29; (b) using an optimized fast program (9 minutes) on the same 30 m Restek Famewax capillary column. IS, internal standard.

is based on ions being produced in a small hydrogen–air flame and striking an electrode where they are detected via an amplified current. The GC effluent is introduced into the flame gas; when an organic compound is eluted, flame reactions result in a rise in the measured current that is approximately proportional to the amount of carbon in the sample (Henrich, 1995). Typical response factors for organic carbon-containing compounds are ~0.015 coulombs per gram of carbon, although the actual response is affected by the presence of heteroatoms and unsaturation. It has been shown (Ackman, 1964) that rules can be developed for predicting the response factor of an organic compound based on its known structure, using the concept of an 'effective carbon response' or 'effective carbon number' (ECN) contribution by the constituent groups of the compound. For instance, each aliphatic carbon contributes an ECN of 1.0, whereas each carbonyl group contributes an ECN of 0.0 (Ackman & Sipos, 1964a,b). These rules form the basis for the development of the so-called 'theoretical response factors' for FAME that are in use today. The concept, as used in the current AOCS method, is that under ideal chromatographic conditions, the response of a given FAME relative to that of the internal standard can be predicted using calculated response factors (Craske & Bannon, 1987, 1988). Thus, the GC-FID response of an accurately known mass of pure methyl tricosanoate (the internal standard) is the fundamental measurement from which the amount of omega-3 fatty acids in an oil sample is determined, with measured GC peak responses corrected using theoretical factors. It is implicitly assumed that any losses during derivatization and analysis are the same for both omega-3 PUFA and the saturated internal standard.

An alternative approach (Einig & Ackman, 1987) is to base quantification on the GC-FID response to accurately determined weights of pure EPA and pure DHA in the ethyl ester form, which are currently commercially available at >99% purity. By preparing a mixture of known amounts of EPA and DHA as ethyl esters, plus methyl tricosanoate as an internal standard, the response factors for EPA and DHA following methylation can be experimentally determined. These experimental response factors are applied to sample responses in exactly the same way as the theoretically derived response factors described above; the internal standard response in this case essentially corrects only for volumetric losses. This is the approach used in the European Pharmacopoeia method. The advantage of this method is that experimental conditions should be properly accounted for since there are no assumptions of FID response, nor is it assumed that omega-3 fatty acids behave in the same way as the saturated internal standard during derivatization. A drawback of this method is that it relies upon the accurate preparation of pure standards of EPA and DHA ethyl esters, which have a limited shelf-life, and that it requires additional samples for derivatization and analysis for every batch of samples analysed. The European Pharmacopoeia method specifies that the total omega-3 content of a marine oil is made up of EPA (20:5*n*-3), DHA (22:6*n*-3), α-linolenic acid

(18:3*n*-3), 18:4*n*-3, 20:4*n*-3, 21:5*n*-3 and 22:5*n*-3. Since this method only determines response factors for EPA and DHA, the weight percentages of the other five omega-3 fatty acids are estimated using the average of the EPA and DHA response factors.

4. Other approaches to the quantification of fatty acids

An interesting alternative approach, currently being investigated by the AOCS (draft method Ce 1i–07), uses the triacylglycerol form of the 23:0 internal standard, which undergoes methylation in the sample oil (unlike when the methyl ester form is used), along with experimental GC response factors obtained using an accurately prepared mixture of a range of pure FAME standards. At the time of writing this chapter, the performance of this method has not been proven, but it seems likely that future revisions of official methods will encompass such variations of internal and external standards, perhaps coupled to faster chromatography as technology permits. Another approach recently reported uses GC/MS to quantify FAME either with ethyl ester or deuterium-labelled FAME internal standards for quantification (Thurnhofer & Vetter, 2006). The additional dimension of information available in GC/MS compared to GC/FID make this a more definitive technique, useful in confirming GC peak identities, and already widely used in the structural characterization of novel fatty acids (Christie, 2003). Unfortunately there are wide differences in response factors for FAME in electron impact (EI) mass spectra, so a challenge in using GC/MS is the requirement for additional standards in order to quantify multiple FAME. For some applications, this disadvantage may be offset by the greater sensitivity of mass spectrometry in the selected ion recording (SIR) mode (Thurnhofer & Vetter, 2006).

C. Fish oil quantification, authenticity and triacylglycerol positional analysis by nuclear magnetic resonance (NMR) spectroscopy

1. Quantification of DHA by high-resolution ^{1}H NMR

Two of the major limitations of analysing fish oils by GC after conversion to FAME are the overhead required for the preparation of FAME and the lack of regiospecific information obtained concerning the positional distribution of the fatty acids on the glycerol backbone of the triacylglycerols. In contrast, NMR analysis requires little sample preparation other than to dissolve the oil in a suitable solvent (usually deuterochloroform), and ^{13}C NMR has been used to determine the distribution of DHA and EPA between the 1(3)- and 2-positions on the glycerol backbone (Gunstone & Seth, 1994). Furthermore, although quantification of all fatty acids present in fish oil is not possible by NMR as it is by GC, *non-destructive* quantification of DHA has been achieved by high-

resolution ^{1}H NMR (Aursand *et al.*, 1993; Igarashi *et al.*, 2000, 2002). This is possible because of the unique environment of the C2 and C3 methylene groups in DHA, located between the electron-withdrawing carboxyl group at C1 and the double bond at C4. Thus, the signals from DHA C2 and C3 methylene protons are shifted downfield away from the signals of other fatty acid C2 methylenes. Comparison of peak integrations for the DHA C2,3 methylene protons with those for other C2 methylene protons, with reference to the known concentration of an internal standard, allows determination of the DHA content both as a percentage of total fatty acids and as an absolute weight percentage in the oil. In a similar way, the total omega-3 fatty acid content can be obtained from the ^{1}H NMR spectrum by considering the terminal methyl protons for omega-3 fatty acids, which resonate downfield compared to other fatty acids due to the proximity of the double bond. An IUPAC (International Union of Pure and Applied Chemistry) international interlaboratory study on these methods for six marine oils achieved reproducibility relative standard deviations (RSD_R) of 1.73–4.27% for DHA concentration (mg/g). Thus, NMR provides an alternative approach to GC for the determination of DHA and total omega-3 content in fish oils. However, at present it likely has limited use due to the high cost of high-resolution NMR spectrometers, and the currently restricted scope of the NMR method compared with traditional FAME analysis.

2. *Positional distribution by high-resolution ^{13}C NMR*

For a given fatty acid in a triacylglycerol molecule, it is known that the chemical shift in the ^{13}C NMR spectrum for the C1 carbon is 0.4 ppm higher in the case of *sn*-1(3) acylglycerol substitution than for *sn*-2 substitution (Gunstone, 1990). Fish oils typically show eight strong resonances in the 172–173 ppm region (Gunstone & Seth, 1994; Siddiqui *et al.*, 2003), which correspond to four pairs (i.e. *sn*-1(3) and *sn*-2) of resonances for fatty acyl groups with a double bond at C4 (DHA), C5 (EPA), C6 (stearidonic acid, 18:4*n*-3) and a fourth pair of resonances comprising most other fatty acyl groups. The practical outcome of all this is that the distribution of EPA and DHA between *sn*-1(3) and *sn*-2 positions can be obtained from the peak areas of the two resonances for each. If a fatty acid were randomly distributed between *sn*-1, *sn*-2 and *sn*-3 substitution, then the ratio would be 2:1, since the method does not distinguish between *sn*-1 and *sn*-3 positions. In this way, it has been demonstrated that in many types of fish oils, DHA is preferentially found at the *sn*-2 position; for example, for menhaden oil, 4.8 mol% of DHA is found on the *sn*-1(3) positions versus 7.6 mol% on the *sn*-2 position (Gunstone & Seth, 1994).

The positional distribution of EPA and DHA in natural fish oils is therefore not random, and ^{13}C NMR provides a reliable means for its determination. In contrast, omega-3 concentrates may be formed via conversion to ethyl esters and subsequent reformation of the triacylglycerol structure. Therefore, it is

Table 2. Authentication of farmed salmon oil by ^{13}C NMR[a]

Fatty acyl group	Signal	Chemical shift (ppm)	Mol% *sn*-2 (β)
DHA	*sn*-2 (β)	172.05–172.09	60–70
	sn-1,3 (α)	172.43–172.47	
EPA	*sn*-2 (β)	172.52–172.56	25–35
	sn-1,3 (α)	172.90–172.94	
18:4*n*-3	*sn*-2 (β)	172.56–172.60	40–55
	sn-1,3 (α)	172.95–172.99	

[a]Using the method described in Ph.Eur. 01/2005:1910 (European Pharmacopoeia, 2005b).

unlikely, whether chemical or enzymatic transesterification of the ethyl ester concentrates are used, that the final triacylglycerols will have the same positional distribution of EPA and DHA (or other fatty acids) as in the starting oil. Hence, ^{13}C NMR can be used to determine if an oil has a positional distribution of EPA and DHA close to its natural state, or whether it has been altered by the addition of a triacylglycerol concentrate, ethyl ester or other type of fish oil that has changed the EPA and DHA positional distribution. This idea has been exploited in a European Pharmacopoeia monograph for farmed salmon oil (European Pharmacopoeia, 2005b) to demonstrate authenticity. Table 2 lists the defined signal ranges and acceptable percentages of EPA, DHA and stearidonic acid on the *sn-2* position. Hence, in salmon oil, 60–70% of the total DHA is on the *sn-2* position, compared with only 25–35% of the total EPA; a random distribution would be 33%. It is expected that this method will be applied to other types of fish oils, and an additional monograph for farmed cod liver oil has recently been published (European Pharmacopoeia, 2006a).

D. Measurement of oxidation products in refined fish oils

A fundamental limitation to the more widespread acceptance of fish oils – either delivered encapsulated as a supplement, or incorporated into a functional food – is the ubiquitous presence of oxidation products in the oils. These give rise to a whole range of undesirable attributes: fishy and rancid flavours and odours, unattractive colouration, and poor stability of the product. Their presence may be greatly exacerbated in omega-3 concentrates since alteration of the natural fish oil triacylglycerol structure, loss of natural antioxidants, and elevated concentrations of the least oxidatively stable components of the oil all contribute to enhanced potential for the formation of undesirable oxidation products. Measurement of the presence of primary and secondary oxidation products – markers of oil quality – is therefore an essential part of fish oil production. There are essentially two levels on which this can be done. The first is by use of the chemical tests described below, which are non-specific tests

developed to determine the presence of general compound classes. However, as pointed out by Macfarlane *et al.* (2001), such tests do not reliably correlate with taste and odour measurements by human sensory panels. Therefore, the next level of sophistication for oil quality measurements is identification of the actual compounds responsible for undesirable flavours and odours. Much progress has been made in this area by the use of sophisticated headspace-GC/MS methods and 'electronic nose' technologies. It should also be mentioned that both primary and secondary lipid oxidation products can be identified by using LC/MS techniques, as recently reviewed (Kuksis, 2005; Byrdwell, 2005). Unlike the GC methods for FAME derivatives described above, LC/MS techniques may allow structural elucidation of oxidation products, such as oxidized forms of triacylglycerols, without prior derivatization. This will perhaps open the way for more specific markers of oil oxidation than those in common use today.

1. Peroxide value

A summary of many of the commonly used measurements of fish oil quality is given in Table 3. This is by no means a complete list of all known methods, but rather a summary of those most commonly used – others can be found by consulting, for instance, the latest edition of the *Official Methods and Recommended Practices of the American Oil Chemists' Society*.

An in-depth discussion of lipid oxidation has been given by Frankel (2005). In outline, it is believed that the oxidation of lipids follows a free radical chain mechanism. The first stage is initiation, whereby the lipid radicals are initially formed by various mechanisms including dissociation of preformed hydroperoxides, the presence of trace redox metal ions, UV light and temperature (Kamal-Eldin *et al.*, 2003). Once lipid radicals are formed, propagation occurs, whereby a hydroperoxide radical ($RCOO^{\bullet}$) abstracts a hydrogen atom most readily from unsaturated lipids to form a hydroperoxide (RCOOH) plus a further lipid radical. As oxidation proceeds, hydroperoxides accumulate and so measurement of their concentration gives a useful measure of active lipid oxidation. Hydroperoxides can be identified by LC and LC/MS methods (Bauer-Plank & Steenhorst-Slikkerveer, 2000; Kuksis, 2005), but the most commonly used measurement for bulk oils is the peroxide value (PV). In the PV test, an oil sample is dissolved in isooctane or chloroform containing acetic acid, and the presence of hydroperoxides causes oxidation of a potassium iodide solution to liberate iodine. The amount of iodine formed is quantified by titration with sodium thiosulfate to a starch endpoint, although potentiometric endpoint determination is also used (e.g. see draft ISO NP27107). A relatively large amount of oil is used in the test (5 g in AOCS procedure Cd 8 53), and it has been pointed out (Crowe & White, 2001) that accurate PV determination can be obtained with sample sizes as low as 0.5 g for vegetable oils. Our

Table 3. Typical methods used in the measurement of oil quality, oxidation products and other lipid components

Measurement	Principle	Official methods
Peroxide value (PV)	Oxidation of potassium iodide by hydroperoxides in oils	AOCS Cd 8-53 AOCS Cd 8b-90 ISO 3960:2001 IUPAC 2.501
Conjugated dienes (CD) and trienes	Measurement of diene hydroperoxides by UV absorbance at ~233 nm; trienes at ~268 nm	AOCS Cd 7-58 ISO 3656:2002 IUPAC 2.206
p-Anisidine value (AV)	Reaction of secondary oxidation productions (aldehydes) in oils with *para*-anisidine reagent	AOCS Cd 18-90 ISO 6885:2006 IUPAC 2.501 Ph.Eur. 2.5.36
Polymer (oligomer) content	Size exclusion (gel permeation) chromatography	AOCS Cd 22-91 Ph.Eur. 2005:1352 Ph.Eur. 2005:2063 Ph.Eur. 2006:1250 ISO 16931:2001 IUPAC 2.508
Acid value	Measurement of free fatty acid content of oils by titration with KOH	AOCS Cd 3d-63 ISO 660:1996 Ph.Eur. 2.5.1 IUPAC 2.201
Trans isomers	FTIR absorbance at 966 cm^{-1} for a *trans* double bond	AOCS Cd 14d-96 AOCS Cd 14d-99
Unsaponifiable matter	Gravimetric determination of non-saponifiable components (e.g. sterols)	AOCS Ca 6b-53 Ph.Eur. 2.5.7 ISO 18609:2000
Total cholesterol	GC–FID measurement on the non-saponifiable oil fraction	AOCS Ch 6-91 Ph.Eur. 2.4.32
Tocopherol (antioxidant) content	LC with fluorescence detection	AOCS Ce 8-89 ISO 9936:2006

FTIR, Fourier transform infrared. GC-FID, gas chromatography flame ionization detection. LC, liquid chromatography. AOCS, American Oil Chemists' Society. ISO, International Standards Organization. IUPAC, International Union of Pure and Applied Chemistry. Ph.Eur., European Pharmacopoeia.

experience with deodorized fish oils has been that 1–2 g is required for good agreement, consistent with the recommendations given in ISO method 3960.

2. *Conjugated diene test*

An alternative test for measuring the early stages of oxidation of highly unsaturated oils is the conjugated diene (CD) test. It has been known for a long time (e.g. see Bolland & Koch, 1945) that hydroperoxides containing a conjugated diene structure are major products of PUFA oxidation. Since these compounds have a strong UV absorption maximum at ~234 nm, they can be detected by a simple test using a UV spectrophotometer. Measurement of the conjugated diene content can be useful in the context of a stability study, but is much less frequently reported than PV. For PUFA oxidation, conjugated trienes are also observed, but at wavelengths of ~270 nm.

3. *Anisidine value (AV)*

Termination of the free radical oxidation mechanism occurs once high levels of peroxyl radicals accumulate; these may react with each other to form non-radical products. Alternatively they may result in the formation of reactive alkoxyl radicals which can initiate branching reactions or terminate in other non-radical species. A more desirable reaction is termination via interaction with the antioxidant. The key point is that the decomposition of hydroperoxides leads to a variety of volatile, non-volatile and polymeric 'secondary' oxidation products (Kamal-Eldin *et al.*, 2003). For fish oils, these include unsaturated short-chain aldehydes which give rise to the characteristic rancid fishy odours and taste. A common method used to determine the presence of aldehydes is the *p*-anisidine value (AV), in which oil in solvent with acetic acid reacts with *p*-anisidine reagent; the absorbance of the resulting solution at 350 nm is measured. Sometimes, a total oxidation value (TOTOX), defined as [2 × (PV+AV)] is reported for fish oils (e.g. Council for Responsible Nutrition, 2006) in order to define a maximum of total primary and secondary oxidation products in an oil. In stability studies of oils it is often observed that PV rises initially as oxidation of the oil commences, then later decreases once secondary oxidation products are formed. The latter is accompanied by a rise in AV as aldehydic compounds accumulate, but with a lag in maximum relative to PV. As discussed above, it is important to remember that there is no direct correlation between AV (or PV) and the taste of fish oils. This is because the odour and taste thresholds of certain specific oxidation products of fish oils (e.g. *t,c*-2,6-nonadienal) are orders of magnitude lower than others (Kulås *et al.*, 2003), so that extremely low concentrations of these particular compounds have a major impact on oil sensory quality. In contrast, AV is a non-specific test giving a single result for an unknown combination of saturated and unsaturated aldehydes.

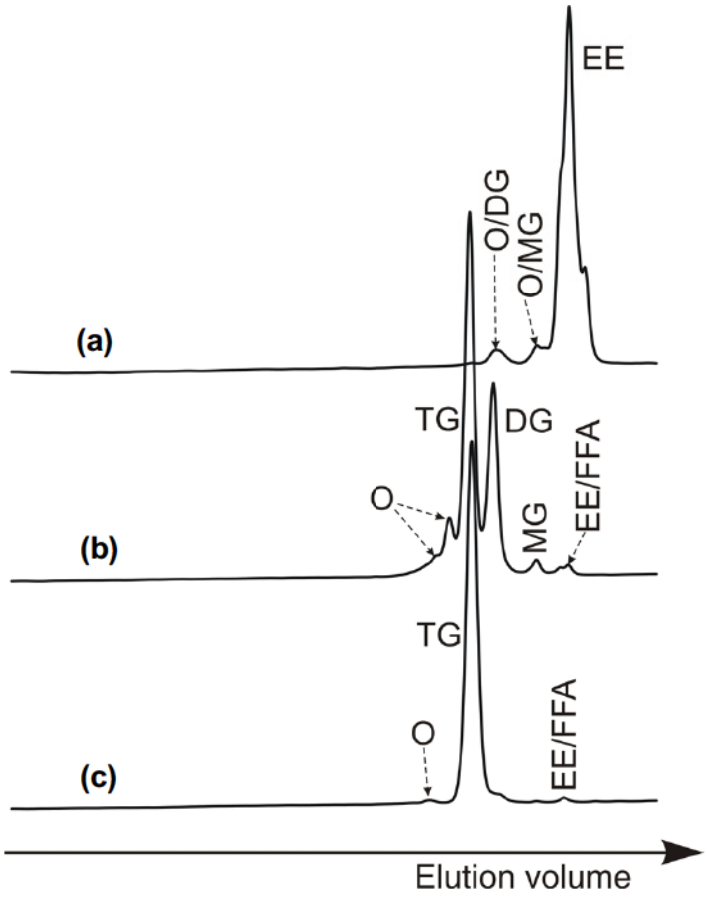


Figure 3. Determination of oligomers in oils using size exclusion chromatography (SEC). (a) An ethyl ester (EE) oil, showing two peaks at lower elution volumes – these could be oligomers (O), monoacylglycerols (MG) or diacylglycerols (DG), as indicated. (b) A partially re-esterified omega-3 concentrate predominantly comprised of triacylglycerols (TG) and diacylglycerols (DG). A relatively high level of oligomers is present (O). (c) A natural 18/12 triacylglycerol oil, showing low levels of oligomers (O).

4. Measurement of lipid oligomers by size exclusion chromatography

Lipid dimers or oligomers linked through peroxyl, ether or carbon–carbon bonds can form as a result of the condensation of peroxyl, alkoxyl and alkyl radicals (Frankel, 2005). These oxidation products are formed readily when fish oils are heated in the presence of oxygen, and are well known in other oils subjected to extreme thermal oxidation, such as frying oils. Since they are large relative to triacylglycerol or ester monomers, they can be separated using size exclusion (gel permeation) chromatography (SEC), usually using several (2 or 3) styrene-divinylbenzene copolymer columns in series, a mobile phase of tetrahydrofuran, and refractive index detection. In SEC, the compounds with the largest hydrodynamic radius elute first, so that oligomers elute at shorter times (or lower elution volumes) than triacylglycerols, which in turn elute before ethyl esters (Figure 3). In the case of triacylglycerols, oligomers

(especially dimers) may be poorly separated from monomeric triacylglycerols by SEC. Since oligomers are present at much lower abundance than the monomers, obtaining accurate oligomer peak areas can be difficult and arbitrary. In the case of ethyl esters, the situation is complicated by the fact that oligomers resulting from the oxidation of ethyl esters, traces of triacylglycerols or partial acylglycerols may also be present and cannot be separately identified (Figure 3c). This situation is acknowledged in the European Pharmacopoeia method for ethyl esters 60 (European Pharmacopoeia, 2005a), where the allowed limit is 7.0% defined as a sum of partial acylglycerols and oligomers. However, the interference from mono-, di- and triacylglycerols can be removed by following the analytical procedure for methylation (or ethylation) using $NaOH/BF_3$ (see Section B above) before identifying the oligomers by SEC; this is the approach adopted in the European Pharmacopoeia method for ethyl esters 90 (European Pharmacopoeia, 2006b).

5. *Trans isomers*

Natural fish oils typically contain almost all carbon–carbon double bonds with a *cis* configuration. Oxidation or thermal isomerization during processing steps such as deodorization can lead to the formation of various *trans* isomers of fatty acids or other compounds (Fournier *et al.*, 2006a). There is currently much interest in levels of *trans* isomers, largely brought about by the negative health effects of consuming hydrogenated oils that are rich in *trans* fatty acids (Ratnayake, 1995). This has led to legislation requiring the labelling of the *trans* content of foods in some countries. There are two methods commonly used for the measurement of *trans* fatty acids (Mossoba *et al.*, 2003). With suitable choice of columns and conditions, gas chromatography can resolve many *trans* FAME isomers from *cis* isomers for quantification; such methods include AOCS method Ce 1f 96, AOAC method 996.06 (House, 1997), and most recently AOCS method Ce 1h 05. These methods were developed for refined and partially hydrogenated vegetable oils in which the predominant *trans* fatty acids contain only one or two degrees of unsaturation. However, since the number of possible *trans* isomers rises geometrically with the number of double bonds, it becomes effectively impractical to resolve all of the *trans* isomers of EPA and DHA, with five and six double bonds respectively, using GC (Fournier *et al.*, 2006b). The other widely used method is based on the absorption band at 966 cm^{-1} in the infrared spectrum of an oil containing isolated *trans* double bonds, as implemented for example in AOCS methods Cd 14 95 (transmission measurement) and Cd 14d 96 or Cd 14d 99 (single bounce attenuated total reflection measurement). However, as stated in AOCS method Cd 14d-96, the limit of quantification (0.8%) is likely higher for complex systems, the method is unsuitable for systems containing more than 0.5% of conjugated unsaturation, and in general unsuitable for any materials

with absorption bands around 966 cm^{-1}. Thus, this method is not reliable for analysing omega-3 concentrates with high degrees of unsaturation and low percentages of *trans* isomers, and underlying absorption bands that would interfere with the measurement. Very high levels of *trans* isomers could be measured in this way, but as pointed out by Fournier *et al.* (2006a), a suitable choice of processing conditions such as deodorization temperature can minimize *trans* isomer formation so that levels of *trans* isomers above 5–10% should not be present in finished omega-3 oils. In summary, the measurement of low levels of *trans* isomers in omega-3 oils is an area where methods are not yet highly developed.

6. *Free fatty acids and unsaponifiable matter*

Other lipid components commonly measured in commercial omega-3 oils include free fatty acids and unsaponifiable matter (Table 3).

Free fatty acids may be formed by the hydrolysis of oils, and levels are minimized during processing. Their measurement is based on a simple acid–base titration using potassium hydroxide solution. The result may be expressed as the 'acid value' (the number of milligrams of KOH required to neutralize the free fatty acids in 1 g of oil) or more often converted to the equivalent percentage of oleic acid, since the average molar mass of free fatty acids present may not be known.

Unsaponifiable matter refers to oil components which are not converted to soaps following caustic treatment, but which are soluble in typical oil solvents. This includes natural components such as cholesterol and pigments, as well as other components that may result from contamination or degradation, such as hydrocarbons or alcohols. Thus, total cholesterol levels in omega-3 oils can be measured by GC-FID, without interference from the much more abundant fatty acids, by quantitatively recovering the unsaponifiable matter (Table 3). The internal standard 5α-cholestane is usually added prior to the saponification step. A high level of unsaponifiable matter can lead to a greater than expected difference between weight percentage and peak area percentage measurements of FAME by GC, since unsaponifiable matter is a component of the oil weight but is not converted to FAME. Tocopherols, which are often added to omega-3 oils as antioxidants, could also appear as unsaponifiable matter. The standard method used to determine tocopherol content is LC, with UV or preferably fluorescence detection, which allows the quantification of all four tocopherol isomers as well as a number of tocotrienols.

E. Measurement of contaminants and impurities in refined fish oils

One of the key advantages to the consumer of consuming refined fish oil supplements rather than fresh fish is the guarantee given by reputable suppliers

that contaminants such as heavy metals, or organic pollutants known to accumulate in animal fat such as polychlorinated biphenyls (PCBs) or polychlorinated dibenzo-dioxins or -furans (PCDDs or PCDFs), are reduced to the lowest possible levels. Many regulations on allowable limits of trace contaminants have been driven partly by toxicological evidence and partly by advances in analytical chemistry that have allowed lower and lower concentrations to be measured with increased specificity. Often, measurements of trace contaminants require specialized analytical facilities not readily available in lipid laboratories, and hence are performed by expert contract laboratories. For some ultra-trace contaminants, measurements may still be required at, or close to, the current limits of detection (LOD) for the analytical methods, such as for PCDDs, PCDFs and coplanar PCBs. In these cases, the measured limits of detection achieved by the chosen laboratory – a function of the equipment, method and levels achieved for method blanks – have a very significant bearing on the final result, since the conservative estimates required by regulations may specify that a 'non-detect' for a particular congener or component is counted in the totals as either the LOD or half the LOD. Thus, it is imperative to choose skilled laboratories that regularly achieve low LODs in order to avoid overestimates of total contaminant levels.

There are few official methods specifically for the quantification of persistent organic pollutants and trace metals in omega-3 or other edible oils. Furthermore, the list of compounds to be monitored continues to grow – for example the polybromo-diphenyl ethers (PBDEs) used as flame retardants were added to the list of compounds to be monitored over the last few years. A wide range of polycyclic aromatic compounds (PAC or PAH) and pesticides are routinely monitored. Many testing laboratories will use a combination of prescribed procedures, such as US EPA Method 1613 ("Analysis of dioxins and furans in wastewater for PCDD and PCDF"), and in-house adaptations for the matrix of interest. Similarly, commercial laboratory procedures for trace heavy metals in oils might use a method for water such as US EPA 200.7 (ICP-OES) or EPA 200.8 (ICP-MS), but this would be combined with suitable pre-treatment (e.g. EPA 305.1; "Digestion in nitric acid"). In addition to posing health concerns, some trace metals are known to promote oxidation in oils (Frankel, 2005), most notably Fe and Cu. Fortunately, official methods do exist to measure these metals at ppb levels in oils using graphite furnace atomic absorption spectroscopy (GF-AAS; see AOCS method Ca 18b 9) and inductively coupled plasma optical emission spectroscopy (ICP-OES; see AOCS method Ca 17 01).

Analysts should be aware that more sophisticated methods, such as US EPA 1668 ("PCBs in water, soil – isotope dilution HRGC/HRMS") will likely give lower detection limits and fewer interferences than less sophisticated methods, such as using GC with an electron capture detector alone – albeit at a greater cost. Overall, there is a real need in this area to employ test samples and

certified reference materials in representative matrices in order to demonstrate acceptable accuracy when measuring trace-level contaminants.

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CHAPTER 3

Microbial oils: production, processing and markets for specialty long-chain omega-3 polyunsaturated fatty acids

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A. Introduction

The omega-3 (*n*-3) rich microbial lipids that are currently produced, and are the topic of this chapter, are normally referred to as single cell oils (SCOs). More generally, SCOs are defined as oils derived from microbial cells and which are destined for human consumption. The name was coined by one of us (in the early 1980s) to draw the parallel with single cell protein (SCP), which was being developed using bacteria and yeasts grown on various feedstocks, including alkanes, with the whole biomass, without fractionation, being used as an animal feed material. This term SCP had already obtained some public acceptance at the time and had been created to avoid the term 'microbial proteins', which was considered to have a potentially negative connotation to consumers. In much the same way, SCO was coined to avoid having to use the term 'microbial oils' or, possibly even worse, 'fungal or mould oils'.

That microbial cells produce cellular lipids similar to those found in edible plants has been recognized for over 100 years, and the prospect of harnessing this ability was attempted as early as World War I (1914–1918). Henkel, the major German oil and fats company, had spearheaded this work in trying to develop a yeast or fungal source of lipid to supplement plant-derived oils that were limited in supply due to the global conflict. Although the process was developed further during World War II (1939–1945), production using *Geotrichum candidum*, *Candida utilis* (which is not a fat-accumulating species)

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This review reflects the opinions of the authors and does not necessarily reflect the opinion of Martek Biosciences Corp., the employer of JPW.

and some species of *Fusarium* was only carried out on a relatively small scale due to difficulties in scaling up the process. The sole recipients of the microbially derived lipid produced were army horses, which were fed the complete yeast or fungal biomass containing the lipid directly rather than an extracted oil. Although this may seem a rather trivial application of the lipid-rich biomass, it should be pointed out that during this war Germany used over one million horses, which clearly needed regular feeding and whose nutritional requirements could not be ignored. [A short account of this early work on SCOs has been given by Ratledge (1992); more detailed discussion of the historical aspects of the subject can be found in the early review of Woodbine (1959) and the short account provided by Bunker (1946).] A microbial oil, as such, was not produced at this time for direct human consumption.

In the period directly post-World War II, major developments were made to intensify agriculture in most countries, certainly in all western countries, in order to feed populations as cheaply as possible. The result of this agricultural progress was a decrease in the cost of plant-derived oils to a level that made microbial oils economically unattractive (a situation that persists to this day if a simple bulk cost–price comparison is made).

Consequently, microbial oils as a direct substitute for plant equivalents were not pursued. It was realized, however, that although microbial oils resembled plant oils in their general characteristics – the profile of fatty acids, the presence of these fatty acids as triacylglycerols, and also the absence of unusual fatty acids or lipid types – some microbial oils were distinct from those oils that could be obtained from plant sources (see below).

Interest in microbial oils resurfaced in the mid 1960s in the UK due to a curious misapprehension that arachidonic acid (ARA; 20:4*n*-6) was a chicken flavour precursor. With this as a basis for their research, a small group was set up at Unilever Research Laboratory in the UK (under the leadership of Dr Robert Shaw) to investigate potential sources of ARA as it was not present in any known plant oil and could only be produced using animal organs or hen's eggs. (The association of ARA with eggs was possibly significant in suggesting that it might have a role in the eventual flavour of chickens.) This work led to the identification of a number of eukaryotic microorganisms that synthesized ARA (Shaw, 1966) and, although this work was not supported through to fruition, as it was belatedly realized that ARA was not a chicken flavour compound, it set the stage for future developments in the field of SCOs in general.

The first SCO to become a commercial reality was another omega-6 PUFA-rich oil: γ-linolenic acid (GLA; 18:3*n*-6). This fatty acid has been highly prized for hundreds of years as the active ingredient in evening primrose oil. The oil itself has been known as the 'King's Cure-All' in recognition of its supposed therapeutic properties and was, and still is, sold in the UK and elsewhere for the alleviation of numerous complaints but principally premenstrual tension. In the

late 1970s, when work on GLA production in fungi first began, evening primrose oil itself was expensive at over £50/kg. (This might be compared to the costs of normal commodity plant oils at that time of about £0.3/kg.) The previous work of Shaw (1966) had revealed that this fatty acid was present in many lower fungi, some of which produced relatively high amounts of it. A subsequent survey of likely sources of GLA in fungi (see Ratledge, 2006), revealed that one fungus, the dimorphic zygomycete *Mucor circinelloides*, was a good lipid producer (25% extractable oil content of the cells) that was also relatively rich (~20%) in GLA. Despite competing directly with a plant source containing the same desirable PUFA as the active ingredient, this SCO, named somewhat romantically 'Oil of Javanicus' (as a reference to the earlier taxonomic designation of the production organism as *Mucor javanicus*), enjoyed an albeit modest and somewhat short-lived commercial success due to its distinctive fatty acid profile that contained over twice amount the GLA as evening primrose oil.

The process was developed at the University of Hull in the UK and was commercialized by J&E Sturge Ltd using a large-scale fermentation plant (220 m^3) in Selby, Yorkshire, UK, usually used for the production of citric acid. This process ran from 1985 to 1990. Its demise was occasioned by the launch of borage oil, a plant oil also containing GLA but at levels slightly higher (~22%) than that produced by the fungus and, significantly, at a cost below that of the SCO. [A detailed account of the microbial production of GLA and of the work that led up to its commercialization and eventual demise has been given by Ratledge (2006).]

During the late 1980s and 1990s attempts were made to produce a SCO to compete against another plant-derived lipid. In this case the target was a microbial facsimile of cocoa butter. The impetus for this research was the record high price of cocoa butter during the late 1980s. In the case of cocoa butter, the key characteristic is not so much the specific nature of the fatty acids contained in the lipid [the predominant ones being stearate (18:0), oleate (18:1) and palmitate (16:0), which are widespread in plant oils as well as in microbial oils], but the ratio of these fatty acids (~1:1:1) on the glycerol backbone and the need for the *sn*-2 position to be occupied by the oleate moiety. It is this structural configuration that gives cocoa butter (and therefore chocolate) its phase transition properties and its 'mouthfeel'. Several different approaches to developing a microbial cocoa butter equivalent were taken by research groups in the Netherlands, New Zealand, Canada and the UK, including the use of desaturase inhibitors and desaturase-deficient mutants to prevent the conversion of stearic acid to oleic acid and thus lead to a build-up of the former fatty acid at the expense of the latter (Moreton, 1988; Smit *et al.*, 1992; Davies, 1988, 1992). Although some impressive science was conducted in this endeavour, and a microbial lipid with the correct fatty acid profile and properties was achieved, this application for SCO never achieved commercial success as, by

the early 1990s, the price of cocoa butter had more than halved and competition from a microbial source was no longer economically feasible (see Davies, 1992).

More recent (and successful) attempts to commercialize SCOs have targeted oils rich in fatty acids that are not found in oils from 'traditional' sources, i.e. plants or animals (including fish). Plants, in general, lack an elongase enzyme system (see Figure 1) that is capable of acting on C_{18} fatty acids and, as such, they do not normally synthesize fatty acids with a chain length of >18. A few plants do, however, produce longer-chain fatty acids, but these almost invariably have only one double bond in the molecule: for example, the occurrence of erucic acid (22:1*n*-9) in European rapeseed oil. Animal oils, in contrast, are replete with a full range of fatty acids including those with multiple (methylene-interrupted) double bonds and chain lengths of >18, the so called long-chain polyunsaturated fatty acids (LC-PUFA). Depending on the source, animal-derived oils possess variable and complex fatty acid profiles arising from the significant contribution that the animals' own diet makes to the lipids found within the various cells and tissues. As a result, animal oils that are very rich in a single LC-PUFA, rather than a mixture of several LC-PUFA, are difficult to source.

The current 'crop' of SCOs, which have already surpassed their predecessors in terms of public acceptance and commercial success, are oils that are very rich in a single LC-PUFA which is not readily available from traditional sources, and contain fatty acids that are generally accepted as beneficial to human health. Although SCOs rich in both omega-6 fatty acids (e.g. the ARA-rich oil) and omega-3 fatty acids [e.g. docosahexaenoic acid (DHA; 22:6*n*-3)] are currently commercially available, due to the remit of this chapter, only the DHA-rich SCOs will be discussed in any detail here. Some information on the production of the ARA-rich oil using the fungus *Mortierella alpina* has been given by us elsewhere (Wynn & Ratledge, 2005, 2006). Prospects for the microbial production of eicosapentaenoic acid (EPA; 20:5*n*-3) are given in Section H.2.

B. Biochemistry of fatty acid biosynthesis and lipid accumulation in oleaginous microorganisms

Essentially, all living cells are capable of *de novo* synthesis of fatty acids and the derived fatty acyl-containing lipids (the exceptions being obligate parasites that rely on the lipid-synthesizing capabilities of their hosts, and also a primitive group of bacteria, known as the Archaebacteria, that produce highly branched-chain fatty acids derived from isoprene units and do not biosynthesize straight-chain fatty acids). The range of fatty acids that an organism is capable of producing *de novo* (from acetyl subunits) varies enormously, however. As stated above, terrestrial plants have limited capabilities for the synthesis of fatty acids with chain lengths of >18 carbons and none contain LC-PUFA (that

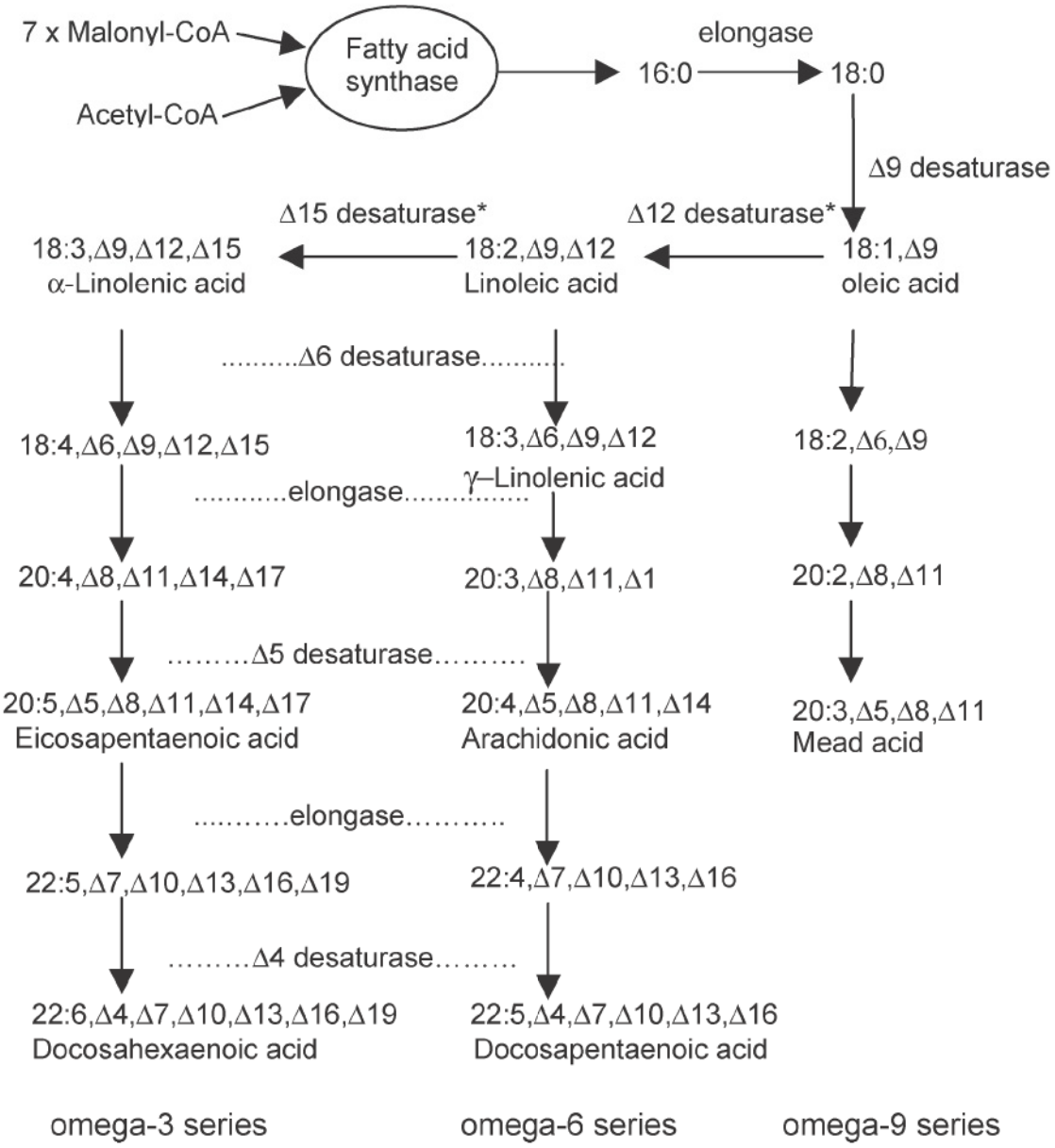


Figure 1. Biochemical conversions of fatty acids by fatty acid desaturases and elongases leading to long-chain polyunsaturated fatty acids (LC-PUFA) of the omega-3, omega-6 and omega-9 series. These reactions may be found, in whole or in part, in microorganisms and animals, though in animals the Δ15 and Δ12 desaturases (indicated by *) do not occur so that linoleic acid (18:2*n*-6) and α-linolenic acid (18:3*n*-3) must be obtained from the diet. In plants, only reactions up to the C_{18} series [including stearidonic acid (18:4*n*-3)] occur. Certain marine microorganisms (including a few bacteria and marine microalgae – dinoflagellates and thraustochytrids which are used commercially to produce DHA) have a completely separate biosynthetic mechanism for the synthesis of EPA, DHA and docosapentaenoic acid (DPA; 22:5*n*-3); see Figure 3.

is, fatty acids with 20 or more carbons and 3 or more double bonds – although plants of the genus *Echium* do produce stearidonic acid (18:4*n*-3), but this is usually regarded as the longest and most unsaturated plant fatty acid). Therefore, as the cellular lipid content of plants reflects solely their innate ability to

synthesize fatty acid precursors, plant oils do not generally contain any appreciable amount of fatty acids longer than 18 carbons and have relatively simple fatty acid profiles. Animal lipids, in comparison, are generally far more complex and varied (even between animals of the same species), as the fatty acid profile is determined more by the dietary fat intake than by the animal's innate ability to synthesize fatty acids. Although animals have the ability to synthesize saturated fatty acids, and also possess a whole array of fatty acid desaturases and elongases, they do not possess a complete complement. Specifically, all higher animals lack distal desaturases (those desaturases capable of inserting a double bond between the Δ9 position and the methyl end of the fatty acid chain), specifically the Δ12 and Δ15 desaturases, and so animals have a strict dietary requirement for fatty acids containing these two specific double bonds. Consequently, two fatty acids – linoleic acid (18:2*n*-6), the precursor of the omega-6 fatty acids, and α-linolenic acid (18:3*n*-6), the precursor of the omega-3 fatty acids (see Figure 1) – are dietary requirements for all higher animals. These two fatty acids are therefore designated 'essential fatty acids', but dietary insufficiency of them is unknown due to the widespread and abundant provision of these fatty acids in plant oils and also in animal fats, where these fatty acids have been derived from the animal's own diet. A diet would need to be so poor in order to be deficient in these fatty acids that the individual would inevitably perish of another deficiency before the fatty acid deficiency would become apparent.

Microbial systems are differentiated from those of both animals and terrestrial plants in that species are known that not only are capable of synthesizing PUFA with >18 carbons (unlike plants) but also possess all the desaturases to synthesize fatty acids of the omega-3 and omega-6 pathways *de novo*. As a consequence, microbial oils possess the dual benefit of having simple fatty acid profiles (which can be very rich in a specific fatty acid) – like plant oils – while also being potentially able to contain LC-PUFA of the omega-3 pathway (e.g. EPA and/or DHA) – like animal oils. Indeed, it is a little-appreciated fact that the omega-3 LC-PUFA (EPA and DHA), for which marine fish oils are so highly prized and on which their health-benefit claims are based, are not synthesized by the fish (see Tocher *et al.*, 2006) but are produced by marine microorganisms on which the fish either feed or have residing within their intestines. It is select members of these marine microbial populations that are the production organisms for the omega-3 LC-PUFA microbial lipids that are currently produced commercially.

As microbial systems are far more amenable to study than the more complex systems of both animals and plants, the biochemistry of lipid synthesis and accumulation is well documented for microbial systems. In general, fatty acids are synthesized throughout the growth of a microorganism, being key structural components of all biological cell membranes (in the form of phospholipids) and without which cells could not be cells. It is, however, only during the so-

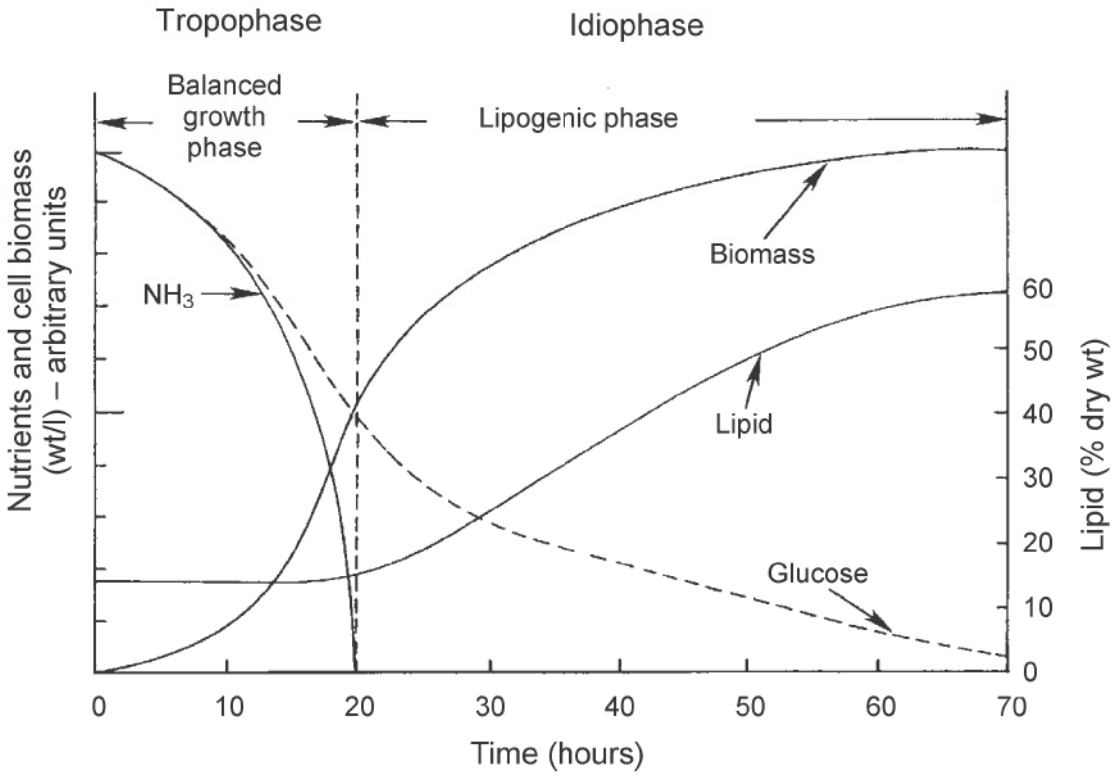


Figure 2. Idealized scheme showing the accumulation of lipid in oleaginous microorganisms. The organism of choice is grown in a culture medium whose composition is so arranged that the nitrogen source (usually NH_3) becomes quickly exhausted, after which the excess carbon substrate (usually glucose or sucrose) continues to be assimilated into the cells and is converted into storage lipid in the form of triacylglycerols. In large-scale fermentations, NH_3 does not become exhausted until the cells have reached a high density; the substrate is also usually added continuously into the fermentor to avoid any inhibition of growth at high concentrations. In this way, cell yields of 170–210 g/l with oil contents from 50% to 70% have been obtained (see Barclay *et al.*, 2005). From Wynn & Ratledge (2005).

called 'idiophase', when growth has become limited by a depletion of some required nutrient (usually nitrogen, both in the environment and in laboratory/production fermentation, but in the presence of excess carbon), that significant amounts of fatty acyl cell lipid (in the form of triacylglycerols, TAG) are accumulated (see Figure 2). Just as microbial systems vary widely in the fatty acids they produce, as a consequence of their genetic complement of fatty acid desaturases and elongases, they also vary widely as to the extent to which they accumulate TAG as a storage material.

Prokaryotic microorganisms (that is, ones without a defined nucleus – i.e. the bacteria, including the cyanobacteria, formerly known as the blue–green algae) do not accumulate significant amounts of TAG, but instead tend to store carbon/energy in the form of polyhydroxyalkanoates (see Wynn & Anderson, 2006). Eukaryotes (microorganisms having a defined nucleus and thus encompassing all the yeasts, fungi and microalgae, except for the cyanobacteria), however, often do accumulate significant amounts of TAG, and species that accumulate up to 60–80% of their dry weight as TAG under optimal conditions

have been reported (see Wynn & Ratledge, 2005, 2006). Those species that do accumulate high levels of intracellular TAG stores (above an arbitrarily designated 20%, w/w dry weight) are described as 'oleaginous'. Oleaginous species are clearly the species of most interest when sourcing a production organism for SCO manufacture.

The basis of microbial oleaginicity is relatively well documented and appears to be linked to the possession of two specific enzymes (ATP:citrate lyase and malic enzyme) that are involved in the provision of the primary substrates (acetyl-CoA and NADPH, respectively) for the fatty acid biosynthetic machinery. Neither of these two enzymes occurs in non-oleaginous species (malic enzyme does occur in the mitochondria of non-oleaginous yeasts, but is in the wrong compartment of the cell to be effective in fatty acid biosynthesis) and thus their presence in the oleaginous species explains the propensity of these organisms to accumulate lipid when placed in the appropriate conditions (see Figure 2). A full account of the biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms has been given by Ratledge & Wynn (2002) and a shorter one by Ratledge (2004); interested readers are referred to these for further information if this should be required.

Although the biochemical pathway for fatty acid biosynthesis (including LC-PUFA) is well documented and all of the biosynthetic genes encoding desaturases and elongases have been cloned (and in many cases patented), the microbial world retains the ability to spring surprises on the complacent scientist. This has certainly been the case in terms of LC-PUFA biosynthesis as, within the past ten years, a completely novel and unexpected mechanism for the production of LC-PUFA has been identified in certain marine microorganisms.

The initial observation, made by Yazawa *et al.* (1988), that led to the discovery of this new pathway was that certain marine bacteria (species of *Vibrio* and some new genera known as *Shewanella* and *Moritella*) had the ability to synthesize significant amounts of LC-PUFA (either DHA or EPA). Previously, the ability to synthesize LC-PUFA was not associated with prokaryotic systems, with the exception of the cyanobacteria (previously known as the blue–green algae). Furthermore, these prokaryotic LC-PUFA producers were often isolated from environments that were anoxic (without oxygen) and were able to produce LC-PUFA even under these conditions (Bowman *et al.*, 1997). Although the production of LC-PUFA under anaerobic conditions was apparently at odds with the involvement of oxygen-requiring desaturases, the large cluster of genes that was isolated from one of the LC-PUFA synthesizing bacteria was still assumed to encode conventional fatty acid elongases and desaturases with activities equivalent to those in other systems. These bacterial genes could be isolated and shown to confer the ability to synthesize LC-PUFA when transferred into a host bacterium (such as *Escherichia coli*) not normally able to synthesize such fatty acids [see for example Orikasa *et al.* (2004, 2006a,b) and Okuyama *et al.* (2007) as later examples of this type of cloning

work]. This then opened up the way for a detailed analysis of the genes and of the proteins that were being produced from them. However, the significant breakthrough came with the analysis of the gene set from a eukaryotic microalga (*Schizochytrium* sp.) and not from the bacteria originally studied by Yazawa *et al.* (1988). This *Schizochytrium* sp., which is used for the commercial production of DHA (see below) and thus can synthesize this LC-PUFA in some abundance, was genetically analysed by Metz *et al.* (2001), who recognized that the genes responsible for LC-PUFA biosynthesis in these systems were more similar to those encoding polyketide synthases (PKS) than to those encoding fatty acid desaturases and elongases.

PKS systems have been known for many years in both bacteria and eukaryotic microorganisms as carrying out a wide variety of biosyntheses, with products ranging from relatively simple molecules, such as 6-methylsalicylic acid, to much more complex structures as may be found in the tetracycline and macrolide range of antibiotics. Many PKS systems are, however, like fatty acid synthases (FAS) in that they use acetyl-CoA as the basic building unit, which is initially converted to malonyl-CoA followed by a condensation of these two molecules (see Figure 3). For the biosynthesis of the LC-PUFA, and unlike 'conventional' fatty acid biosynthesis using the FAS system, the product of the condensation reaction between the growing acyl chain and malonyl-ACP in the PKS-catalysed system does not always undergo complete reduction (which occurs in the FAS system to produce a saturated fatty acyl chain – see Figure 3 for guidance), but may selectively retain some of the double bonds formed *in situ*. Some rearrangement of the configuration and position of these double bonds may occur in order to create the final methylene-interrupted *cis*-double-bonded fatty acid (see Figure 3). [The molecular size of the PKS system (about 500–600 kDa) is, however, much less than that of the FAS (~2000 kDa), which might suggest that, from an evolutionary point of view, the PKS may have been derived from genes of the FAS system undergoing some losses as well as additional modifications.]

That LC-PUFA were being synthesized, not via a fatty acid synthase (therefore not through a saturated fatty acid intermediate), but directly via a PUFA-PKS system was a paradigm-changing discovery. It explained how these systems were able to synthesize high levels of LC-PUFA (i) under conditions of low O_2 concentrations and (ii) without the apparent accumulation of any intermediate fatty acids (see Metz *et al.*, 2001). A suggested sequence of reactions in given in Figure 3. The reactions are carried out by a multi-subunit complex coded for by a genetic sequence that has now been identified not only in *Schizochytrium* sp., but also in the various marine bacteria that produce EPA and DHA (Metz *et al.*, 2001; Allen & Bartlett, 2002; Gentile *et al.*, 2003; Orikasa *et al.*, 2004, 2006a,b; Okuyama *et al.*, 2007).

The *Schizochytrium* sp. that Metz *et al.* (2001) had genetically analysed for its PUFA-PKS system also produces docosapentaenoic acid (DPA; 22:5*n*-6) of

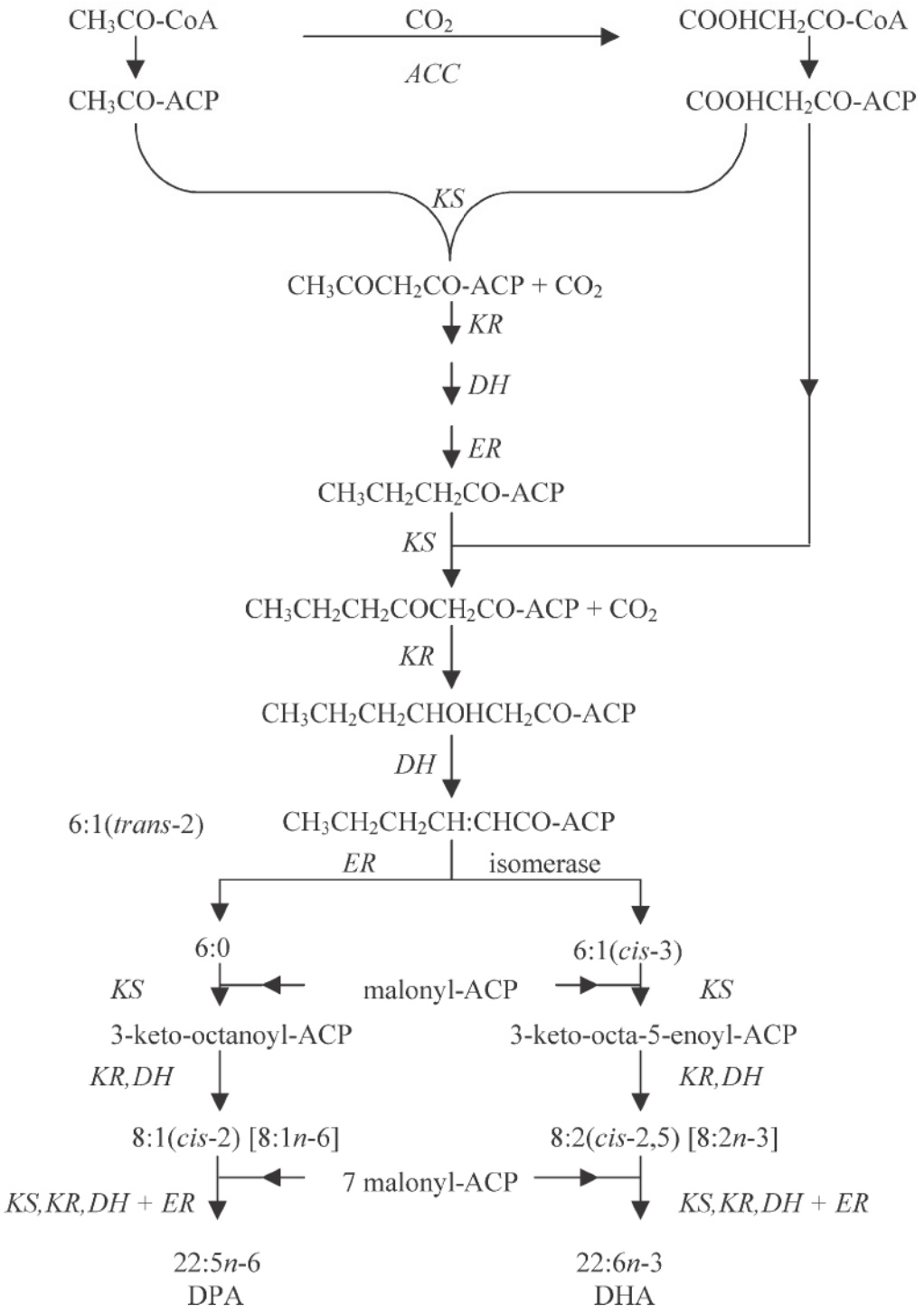


Figure 3. A suggested biosynthetic scheme to account for the formation of long-chain polyunsaturated fatty acids in marine microorganisms, and particularly in *Schizochytrium* sp. and other thraustochytrids, using a polyketide synthase (PKS) system (see Metz *et al.*, 2001). The pathway begins with the condensation of acetate, attached to an acyl carrier protein (ACP), and malonyl-ACP [derived from acetyl-CoA using acetyl-CoA carboxylase (ACC)] to give acetoacetyl-ACP. This reaction is carried out by ketosynthetase (KS), sometimes known as 'condensing enzyme'. The acetoacetyl-ACP is then reduced by a ketoreductase (KR) to 3-hydroxybutyryl-ACP, which loses water by a dehydratase (DH) reaction to give crotonyl-ACP, which is finally reduced by an enol reductase (ER) to give butyryl-ACP. Then follows a further addition of malonyl-ACP and the same sequence of reactions, involving KS, KR

the omega-6 series of fatty acids along with DHA of the omega-3 series (see below). It now has been shown (Hauvermale *et al.*, 2006) that when the genes coding for the PUFA-PKS synthesizing system are cloned from the *Schizochytrium* sp. into *E. coli*, not only is DHA produced but so is DPA, in the same proportions that occurred in the original alga. This therefore indicates that the PUFA-PKS system is so organized that there must be two routes of synthesizing both the omega-3 series of fatty acids and, simultaneously, the omega-6 series. A suggested model as to how these twin pathways may diverge from a common pathway is depicted in Figure 3, but it must be stressed that this scheme is highly conjectural and remains to be established by further genetic analysis.

Interestingly, *Schizochytrium* sp. still synthesizes 'conventional' short-chain fatty acids (14:0, 16:0, 18:0 and 18:1) along with the LC-PUFAs. These fatty acids are now known to be synthesized by an entirely separate fatty acid synthase (FAS) which functions alongside the PKS-PUFA system (Hauvermale *et al.*, 2006). The molecular organization of this FAS system resembles FAS systems found in fungi and yeast. It is under separate genetic control and can be inhibited by cerulenin, a well-known inhibitor of short-chain fatty acid biosynthesis, without affecting DHA/DPA biosynthesis (Hauvermale *et al.*, 2006).

The distribution of the PUFA-PKS system among eukaryotic marine microorganisms is still a matter of some debate and probably needs to be ascertained on a case-by-case basis. Such a system, or at least a related one, possibly also occurs in *Crypthecodinium cohnii* (see Ratledge, 2004), which is the other marine alga used for the commercial production of DHA – see below. If this is so, then the *Crypthecodinium* PUFA-PKS system will be different from the *Schizochytrium* system in that it does not produce DPA. Thus a branched pathway, as depicted in Figure 3, would not apply to the DHA biosynthetic pathway in *Crypthecodinium* and just the single route leading to DHA would then be functional. There has also been a report of a similar system in an organism related to *Schizochytrium*, *Ulkenia* sp. (Kiy *et al.*, 2005), which has been considered as a possible production organism to rival *Schizochytrium*.

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and DH, up to the formation of 6:1(*trans*-2)-ACP. In conventional fatty acid biosynthesis, this would be reduced (by ER) to 6:0 and the sequence would be repeated a further five times to give 16:0. For the PKS sequence leading to the formation of DHA, the 6:1(*trans*-2) intermediate might be isomerized to 6:1(*cis*-3), which undergoes condensation with malonyl-ACP leading to the formation of 8:2(Δ2,Δ5); then follows seven further cycles of condensation with retention of four of the seven double bonds to give DHA as the final product. As *Schizochytrium* sp. and related organisms (but not *Crypthecodinium cohnii*) also synthesize docosapentaenoic acid (DPA) of the omega-6 series, a branched pathway is postulated to occur as the ratio of DHA to DPA remains virtually constant under all conditions (Hauvermale *et al.*, 2006). A possible biosynthetic route may be by the reduction of 6:1(*trans*-2) to 6:0, using ER, followed by a similar cycle of reactions but in which the omega-3 bond no longer occurs and the most distal bond is then at the omega-6 position. (It must be emphasized that these pathways are entirely speculative, as none of the intermediates or individual reactions have yet been identified.)

The *Ulkenia* sp. also produces DPA along with DHA and so its biosynthetic machinery probably resembles that of *Schizochytrium*.

C. Advantages/disadvantages of SCOs versus 'traditional' omega-3 oils

As noted above, due to the fermentation costs associated with SCO production, it is not possible for SCOs with just 'conventional' fatty acyl groups to compete against the commodity oils from plants and animals on the basis of price alone. As a result, fish oils (as well as certain plant oils rich in α-linolenic acid, 18:3*n*-3) currently dominate the omega-3 PUFA supplement market for the adult population. Recent improvements in the processing of one SCO [produced by Martek Biosciences using *Schizochytrium* sp.; see Barclay *et al.* (2005)], however, have lowered the price to a level where the prices of this oil and certain fish oils are essentially equivalent on a weight of DHA basis. However, there are certain properties of SCOs, as a result of their production in fermentation vessels, that present distinct advantages over conventional oils and can be used to justify the premium price of these oils (Table 1).

At a time when the dietary importance of omega-3 LC PUFA is gaining more attention, both in the scientific and the popular press, there is a realization that fish oil, although currently the primary and cheapest source of DHA and EPA, may not be the best long-term solution to the increasing demand for these desirable fatty acids. Environmental issues, in terms of contamination of the marine ecosystems (and therefore of fish at the top of the marine food chain) and the spectre of overfishing with the potential collapse of fish stocks, have led both consumers and oil producers to look elsewhere for alternative sources of omega-3 fatty acids. The health threat of pollutants, such as methyl mercury and polychlorinated biphenyls (PCBs), in fish has become sufficiently severe that regulatory bodies, such as the FDA (Food and Drug Administration) in the USA, have published warnings regarding the consumption of certain fish by pregnant women and young children. The use of flaxseed oil as a substitute

Table 1. Advantages and disadvantages of single cells oils in comparison with traditionally derived oils

Advantages of single cell oils	Disadvantages of single cell oils
• Simple fatty acid profile • Not affected by climatic, geographical or environmental factors • Quality and supply can be guaranteed • Very rich in fatty acid of interest • Kosher, halal and vegetarian source • Superior sensory and stability profile	• High cost • Limited production capacity • Potentially adverse public perception

seems attractive; however, as humans possess a limited ability to convert the omega-3 fatty acid in flaxseed oil (18:3*n*-3) into EPA and, particularly, DHA (see Figure 1), the utility of this dietary source of omega-3 PUFA is limited in comparison to that of preformed DHA.

The realization that the Western diet has a suboptimal omega-3:omega-6 ratio, coincident with an unsustainable marine fish industry, means that alternative sources of omega-3 LC-PUFA are desperately required. Unfortunately, the aquaculture (fish farming) industry cannot be seen as a satisfactory solution as the farm rearing of marine species currently requires the input of marine fish oil/fishmeal to provide the omega-3 LC-PUFA required by these fish. As a result, the omega-3 LC-PUFA contained in farmed sea fish is in reality sourced from 'wild caught' sea fish and does not constitute a net increase in supply. Therefore, the only currently feasible way to increase the supply of omega-3 LC-PUFA for the human diet (without the risk of continued overfishing) is the production of omega-3 LC-PUFA rich SCOs.

As SCOs are sourced from microbial biomass grown under very closely controlled conditions, their production can permit very close regulatory scrutiny from a range of regulatory bodies. As a result, SCOs can be certified as vegetarian or even vegan, as well as being potentially kosher and halal. SCOs produced by Martek Biosciences are currently certified kosher by the Jewish Orthodox Union and halal by the Islamic Food and Nutritional Council of America. As a result, these oils can be incorporated into a wide range of food products without any risk of making the products unsuitable for any consumer group as a result of dietary restrictions. The use of fish oils is not so unrestricted.

Furthermore, as a consequence of the controlled production of SCO in large-scale fermentation vessels, both the quality and quantity of production can be closely controlled. This means that the fatty acid profile of an SCO can be controlled with a high degree of precision and the output of production plants can be rapidly increased (assuming sufficient plant capacity exists) or decreased to match any fluctuations in market demand. It also means that the specification of the oil remains constant irrespective of the vagaries of the weather, climate or season of the year. The supply is also impervious to political considerations. Neither the quality nor the production of fish oils can be so closely controlled due to variations caused by seasonal, geographical and dietary influences on the fish. Fishing quotas in various parts of the world's seas and oceans also has a considerable effect on supply. The dietary intake of fish also impacts on the composition of fish oils and leads these oils to have complex fatty acid profiles. As a result, it is impossible to achieve, except by prohibitively expensive fractionation, fish oils that have a high content of just one omega-3 LC-PUFA – especially a DHA-rich oil devoid of EPA, which is desirable for infant formula applications.

Historically, omega-3 rich oils have been noted for having a poor sensory

profile (a distinct 'fishy' taste/smell as well as an aftertaste or 'burp') that can limit their incorporation into foodstuffs. SCOs have a lower impact and a lessened sensory profile, and lack the distinctive fishy notes unless they are abused. In addition to having superior sensory characteristics, SCOs also benefit from having a better stability than fish oils, partly as a consequence of their simpler fatty acid profile and also because of the presence of lipid-soluble antioxidants produced along with the oil by the production microorganisms. Although the chemical nature of these antioxidants is unknown, they are responsible for the high stability of many SCO products at room temperature, in the air, and in daylight. Techniques to overcome the poor sensory attributes of fish oils are available (in terms of microencapsulation, etc.), but these have also been developed for SCOs to allow ease of incorporation into certain applications. These microencapsulation techniques do, however, add significantly to the cost of the oils.

D. Choice of production organism

As has been mentioned above, the fact that microbial systems synthesize oils containing LC-PUFA has been appreciated for a considerable length of time. The main factors that determine which of these microorganisms are most suitable as production organisms for the commercial production of SCO are: (i) amount and rate of LC-PUFA biosynthesis, and (ii) the ease of large-scale cultivation of the chosen organism. Both factors are important and a potentially useful microorganism must satisfy both criteria. As LC-PUFA rich oils are required in bulk (in many tens if not hundreds of tons), the capacity to generate, harvest and process very large quantities of microbial biomass is required to supply the demand. This limits the range of potential production organisms to those species that are oleaginous, i.e. those that are recognized to accumulate large intracellular stores of lipid in the form of triacylglycerols. This precludes the use of prokaryotic (bacterial) sources, as prokaryotes do not accumulate significant amounts of fatty acyl lipid. Although many oleaginous species are recognized among the eukaryotic microbes, the limitation here is to identify species that grow well and accumulate significant quantities of LC-PUFA of the omega-3 series. The biosynthesis of omega-3 LC-PUFA is normally associated with microorganisms from cold marine environments, although the synthesis of these fatty acids is not a prerequisite for survival under these conditions. Until recently, it was accepted that while the marine species that accumulated the highest percentages of omega-3 LC-PUFA in their cell lipid were isolated from the colder marine habitats, these species also accumulated less storage lipid and grew substantially slower than their close relatives from warmer climes. It has recently been shown, however, that after culture optimization, little difference is evident between the LC-PUFA yields from species isolated from either cold or warm waters.

Many of the microbial species that produce omega-3 LC-PUFA are photosynthetic, and this has led some investigators to attempt the phototrophic production of LC-PUFA. Although this approach looks economically attractive due to the availability of free energy (sunlight) and a free carbon source (atmospheric CO_2), in fact the phototrophic production of LC-PUFA is not practical. Only very low cell densities are attainable in open-air pond systems (where operating costs are at a minimum), which means that not only are very large ponds required, together with a climate that supplies the year-round sunlight required to 'power' the production, but also that vast quantities of culture have to be harvested in order to obtain the biomass prior to oil extraction. This, combined with the fact that open-air ponds or lagoons are difficult to operate as single-species systems, and the potential for both environmental pollution and seasonal (or year to year) climate variation, means that oil sourced using pond cultivation systems is no more controllable in terms of quality and quantity than fish-derived oils. Attempts to produce LC-PUFA rich oils phototrophically in closed systems (using 'photobioreactors') add significantly to the cost of the oil, as not only does energy now need to be inputted to maintain movement in the culture (to avoid self-shading issues similar to those encountered in open-pond systems) but also gas exchange issues have to be addressed to avoid O_2 build-up and CO_2 exhaustion from the system due to the photosynthetic process. This applies even to very simple photobioreactor cultivation systems using arrays of polythene tubing held in large racks outdoors and facing into the sun. Analysis has suggested that due to the complications associated with the intensive cultivation of phototrophs, or the requirement to harvest vast culture volumes, the cost of phototrophic production of LC-PUFA SCO is, in fact, many times higher than the heterotrophic production of similar oils. Despite the drawbacks of phototrophic production, some relatively small quantities of omega-3 LC-PUFA (specifically EPA-containing oils) are produced using photosynthetic microorganisms – such as 'V-pure' (a mix of DHA and EPA) produced by Water4Life and Enerex's 'Super Phytoplankton'. However, these are extremely low-volume products that are sold exclusively into the dietary supplement market and for which cost is not of paramount importance.

The choice of heterotrophic microorganisms that synthesize significant amounts of omega-3 LC-PUFA is somewhat limited, being restricted to certain marine microalgae. Initially, these species of microorganisms looked unattractive as sources of these LC-PUFA as they were difficult to cultivate to high cell densities in agitated tank fermentors (see for example Kendrick & Ratledge, 1992; Bajpai *et al.*, 1991). Despite this apparent barrier, two US-based firms, Martek Biosciences and OmegaTech, persevered during the 1980s and 1990s and were able to develop successful but competing processes for the production of a DHA-rich SCO from two different marine microalgae (see Kyle, 1996; Kyle *et al.*, 1992; Barclay, 1991; Barclay *et al.*, 1994). While Martek Biosciences

Table 2. Principal fatty acids of two commercial DHA-rich oils produced using microorganisms[a]

Fatty acid	Content (%, w/w of total fatty acids)	
	DHASCO[b]	Schizo-SCO[c]
14:0	20	8
16:0	18	22
16:1	2	12
18:0	<1	1
18:1	15	1
22:5*n*-6 (DPA)	ND	17
22:6*n*-3 (DHA)	39	41

[a]In each case, small amounts (<1%) of other fatty acids were also noted. [b]Produced using *Crypthecodinium cohnii* by Martek Biosciences Corp; 'DHASCO' is a registered trademark of this oil. Data from Arterburn *et al*. (2000c). [c]Produced using *Schizochytrium* sp. by Martek Biosciences Corp. Data from Barclay *et al.* (2005). ND, not detected.

used a dinoflagellate, *Crypthecodinium cohnii*, OmegaTech selected a stramenopile, *Schizochytrium* sp., as their production organism. In both cases, although the chosen strains accumulated significant amounts of DHA in the form of a triacylglycerol oil (see Table 2), cultivating these organisms to high cell densities at the very large scale required for commercial viability was a considerable technical and scientific achievement. In 2002 these two companies joined forces (under the umbrella Martek Biosciences Corp) to combine their expertise, and the joint company is now, by far, the most significant producer of microbial SCOs rich in LC-PUFA, being responsible for in excess of 95% of the global supply of microbially sourced omega-3 LC-PUFAs.

E. Current production

As stated above, the vast majority of microbially sourced omega-3 LC-PUFA currently available are the DHA-rich oils from either *Crypthecodinium cohnii* and *Schizochytrium* sp. produced by the US-based company Martek Biosciences Corp. Some competing processes are being operated outside the USA, specifically by Nutrinova (a subsidiary of Lonza) in the European Union (EU), and by Nagase & Co Ltd in Japan and the Far East.

The starting point for all industrial fermentations (particularly those involved in the production of a foodstuff or ingredient) is a well-defined working cell bank. This comprises a supply of culture samples ('cryostocks', which are stored at low temperatures) that are homogeneous and act as the starting point for each production fermentation run. A standardized starting point decreases variability between fermentation runs and helps maintain both reproducible quality and reproducible yield of the product. Vials from the cryostock are used to inoculate a succession of inoculum stages of increasing volume, starting

with small shake-flask cultures (~100–250 ml) and ending in a seed fermentation culture comprising normally 1–10% of the final production fermentor volume. The size of the final production tanks can vary but, in all commercial productions, the tanks used will be tens of thousands of litres in capacity and may exceed 100 000 litres. [When production of the first SCO was carried out, which was for an oil rich in γ-linolenic acid (see Introduction), the fermentors that were used had a capacity of 220 000 litres.] A typical fermentation flow diagram, from culture vial to final fermentor, is shown in Figure 4.

The size of the large production tanks makes control of the parameters required for optimum growth and lipid accumulation a significant engineering challenge. As lipid accumulation only really progresses after the active growth (i.e. cell division) has halted, due to the depletion of a culture component other than carbon (see Figure 2), the DHA fermentation can be considered to be a two-phase process. The first phase is the generation of the maximum amount of biomass the fermentor can sustain. This is limited by the amount of air that can be sparged through the fermentor (as the production organisms are all aerobic and so require plentiful dissolved O_2 for maximal growth) and the amount of power than can be put into the tank (in terms of agitation) to keep the culture well mixed and avoid the development of 'stagnant zones' in which both O_2 and nutrients can become locally depleted. Factors such as heating and/or cooling to maintain a suitable growth temperature can also affect the maximum amount of biomass a fermentor can sustain. All of the preceding factors are, in turn, greatly influenced by the fermentor size and design.

Once active growth has ceased due to the exhaustion of a key nutrient (usually the nitrogen source), the carbon source will continue to be taken up into the cells and used to synthesize storage compounds which, in the case SCO-production organisms, are intracellular triacylglycerols. The intracellular nature of the accumulated lipid is a key feature differentiating the omega-3 LC-PUFA fermentations from the majority of other large-scale industrial fermentations, in which the desired product is secreted. When a product is secreted, the production is not strictly limited by the culture density but rather by the organisms' ability to convert the medium components into the desired product and to secrete it. Therefore it is the conversion rate rather than the amount of biomass *per se* that determines the productivity and yield of these other processes; the yield can be essentially limitless as it is independent of the amount of biomass (many grams of a secreted product can theoretically be produced per gram of biomass) and more dependent upon factors such as inhibitory effects of the product. However when the product (such as a lipid) is intracellular then it is clear that the yield/productivity is closely tied to biomass generation, and yield can never exceed the amount of biomass in the fermentation vessel. It is, however, the productivity of the process, rather than the actual yield (that is the grams of product per litre), which is of key concern: the productivity of a process is expressed as the amount of product produced per

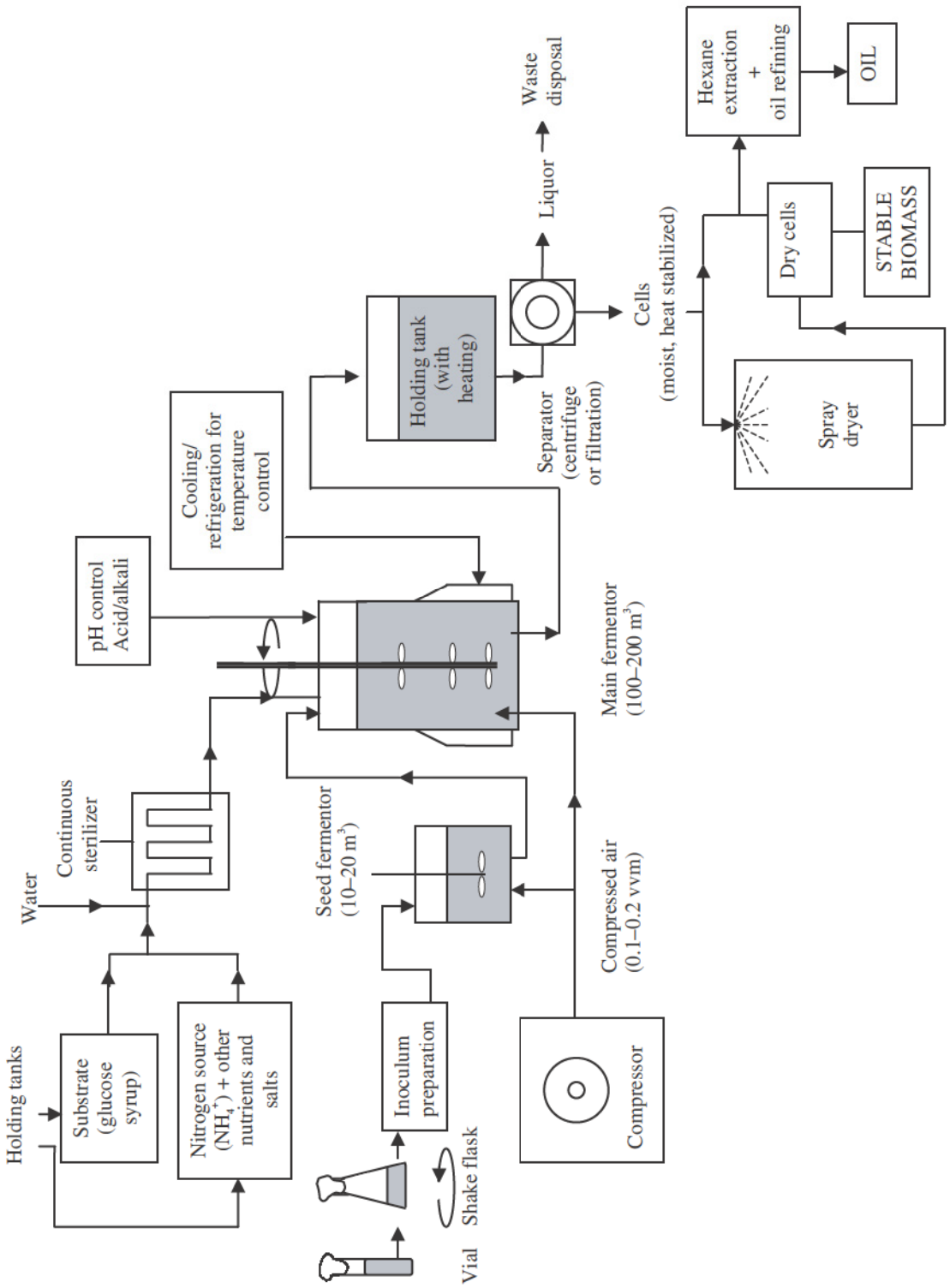


Figure 4. Diagrammatic representation of the fermentation process used for the production of microbial oils; see text for details (from Wynn & Ratledge, 2006). vvm, volume of air per volume of fermentor per minute.

litre of fermentor volume per unit time – in other words, how much product can be produced in a fermentor of given volume in the shortest possible time. For example, a yield of 100 g oil per litre produced over, say, 10 days of growth is not as good as 60 g oil produced in 3 or even 4 days.

The necessity to generate very high cell densities in the shortest possible time, pushing the limits of the mass transfer capacity of the fermentation vessels, has necessitated the development of feeding regimes. Here, the total amount of sugar needed to achieve a high cell density (together with a high oil content of the cells), if added at the beginning of the fermentation process, might be so high that it would inhibit the growth of the cells. If the objective is to produce a cell density of, say, 200 g/l with an oil content of 40%, this will require 500–600 g glucose/l, which would be clearly unattainable if added all at once at the beginning of the process. Consequently, glucose, or whatever substrate or feedstock is being used, must be added in a programmed manner to ensure that the concentration of sugar in the fermentor never exceeds a limit beyond which inhibition of the growth performance would result. Of course, it is also important that the concentration of sugar in the fermentor is never allowed to become exhausted, even at the end of lipid production. Under these circumstances, the cells would be placed in a starvation condition and would then begin to consume the very triacylglycerols that they had accumulated as a reserve carbon storage material to be mobilized if the external carbon source is exhausted. Thus there must be continuous monitoring of the concentration of the substrate within a fermentor to ensure that it is always between acceptable upper and lower limits.

Another key feature of LC-PUFA fermentations that is distinct from other fermentations is the use of marine microbes as the production organisms. Due to the marine origin of these species, they have a requirement for saline conditions for growth. All initial laboratory work with these species is usually carried out in a medium containing concentrations of NaCl similar to that of seawater (~19000 ppm Cl^-). However, such high levels of salt pose real issues when scaling up a process using these organisms, as stainless steel tanks are incompatible with high chloride concentrations and will quickly corrode.

Most industrial fermentors (and the associated piping) are constructed out of 304-grade stainless steel (the same grade commonly used for household utensils, such as knives and forks). Although this grade is suitable for intermittent exposure to highly saline conditions, its resistance is dependent upon short exposure times and thorough cleaning between exposures. Furthermore, during the preparation of an industrial fermentation medium, at some stage liquid has to be sterilized, often by heating to >120°C for an appreciable amount of time (>20 min). Chloride at high concentrations and at high temperatures has a highly corrosive effect over anything other than the shortest period of time. To overcome these corrosion problems two approaches are used in parallel. Firstly, the facilities used for these processes are constructed from higher

grades of steel (317L, 2205 or AL6XN), which then adds to the cost of building the fermentors. Secondly, the medium and strains have been developed, by appropriate screening and selection processes, to allow cultivation of the LC-PUFA omega-3 synthesizing production organisms at only a fraction of the Cl^- concentrations they experience in their natural habitat.

Once the fermentation has achieved a suitable yield, the biomass is removed from the tank and harvested; this involves a continuous centrifugation step capable of harvesting the large volumes of culture broth. Unused medium components are removed by washing and the biomass is spray dried prior to extracting the intracellular lipids with hexane, in a process similar to that used for extracting oils from plant seeds. After extraction, the processing of the LC-PUFA rich SCO continues to be similar to vegetable oil processing, with refining, bleaching and deodorizing steps yielding a brilliant yellow or golden coloured oil (depending on the origin) that has a distinctive but by no means unpleasant odour/taste profile that is predominated by a 'green' or 'herbal' note. The oils do not have the fishy tastes associated with omega-3 LC-PUFA rich oils from more traditional sources (i.e. fish).

The fatty acid profiles of the current commercially produced omega-3 SCOs are given in Table 2.

One aspect of the extraction/processing that separates these processes from vegetable oil manufacture is the need to limit exposure of the oil to both heat and atmospheric O_2. Due to its highly unsaturated nature (having six double bonds rather than the maximum of three found in plant-derived fatty acids), DHA is more prone to chemical oxidation than plant oils. To avoid problems associated with oxidation (including the development of 'fishy' odours and tastes if excessive), processing is accomplished as rapidly as is feasible, storage times are minimized (and, when unavoidable, storage is at low temperatures), and natural antioxidants (vitamin E, ascorbyl palmitate and rosemary extract) are added at various stages of processing to within closely defined limits.

The final oils can be blended with other oils to produce products with 'designer' fatty acid profiles (such as the 2:1 ARA:DHA ratio in the infant formula supplement 'Formulaid', produced by Martek Biosciences Corp), and are also now available in a variety of product forms such as powders and free-flowing oils and shortenings, to facilitate the incorporation of the oils into a variety of food/nutritional products with satisfactory stability and organoleptic properties. Some of the infant formula preparations that currently include DHA-rich SCOs (principally that from *C. cohnii*) are given in Table 3.

F. Safety

The first commercially produced SCO ('Oil of Javanicus', a rich source of the omega-6 PUFA γ-linolenic acid; 18:3*n*-6) was produced using a filamentous fungus, *Mucor circinelloides*, that had a long-established association with the

Table 3. Infant formulas containing DHA-rich oils from microbial sources (as of October 2006)

Product	Manufacturer/distributor
'Enfamil LIPIL'	Mead Johnson
'Similac ADVANCE'	Ross Abbott
'Good Start'	Nestlé
'Bright Beginnings'	PBM Products
'Parent's Choice'	Walmart
'SMA Gold' (launched internationally in 1998)	Wyeth

human food chain – being the organism used for the fermentative production of the oriental food materials known as tempeh and tapé (see Ratledge, 2006). As a result the safety of this pioneering SCO was not under any real doubt. The same could not be said for oils derived from any of the marine microbes used to source omega-3 LC-PUFA rich SCO. Before such oils could be added to the human food chain extensive safety data were required. As a result, extensive safety testing has been carried out on these oils; the scrutiny applied to these oils was heightened as one of the primary applications of the DHA-rich SCO was as a nutritional supplement for infant formula.

As oils containing DHA have long been associated with the human diet (in the form of fish oil), the inherent safety of the active ingredient (i.e. DHA) in the SCO was not a safety concern. Likewise, all the fatty acids present in the SCO were also routinely found in the human diet. Therefore the basis of the safety concerns for oils from microbial sources was due specifically to their origin.

Neither of the 'first generation' of omega-3 LC-PUFA containing oils, the *Crypthecodinium cohnii* and *Schizochytrium* derived oils, were produced from microorganisms that had shown any ability to produce toxins. Despite this, both oils were extensively studied by the manufacturers and the prospective customers to establish their safety, prior to being offered for sale to the general public. The safety testing of the *C. cohnii* oil is outlined in Tables 4 and 5. This safety evaluation has included *in vitro* assays, which failed to demonstrate any mutagenic or clastogenic (chromosome damaging) effects of these oils, pre-clinical studies, and finally clinical trials. In animal studies both acute and chronic administration failed to provoke any adverse effects on the animals studied (or their offspring). The doses used in both acute and chronic doses far exceeded the expected human doses, and the LD_{50} value (i.e. the concentration at which 50% of the experimental animals would have received a lethal dose) was far above the maximum dose it was possible to administer (>20 g/kg body weight). Indeed, no 'lethal dose' value could be derived from any of the studies, as no toxicity could be discerned: it may be imagined that it is very difficult to ask human volunteers to accept over 1000 g of oil per day (even if ethically

Table 4. *In vitro* and animal (*in vivo*) studies on the safety of DHASCO™ produced by Martek Biosciences Corp[a]

Study	Maximum DHASCO™ dose used	Model system employed	Conclusion	Reference
Ames mutagenicity test	5 mg/plate	*Salmonella typhimurium*	Not mutagenic	Arterburn *et al.* (2000c)
Forward mutation test	5 mg/plate	Mouse lymphoma cells	Not mutagenic	Arterburn *et al.* (2000c)
Chromosomal aberration test	5 mg/plate	Chinese hamster ovary cells	Not clastogenic	Arterburn *et al.* (2000c)
Acute toxicity	20 g/kg body wt	Rat	No deaths	Boswell *et al.* (1996)
Subchronic toxicity:				
28 day	1.25 g/kg body wt/day	Rat	Not toxic	Boswell *et al.* (1996); Arterburn *et al.* (2000b)
90 day	1.25 g/kg body wt/day	Rat	Not toxic	Arterburn *et al.* (2000a)
90 day, *in utero* exposure	3 g/kg body wt/day	Rat	Not toxic	Arterburn *et al.* (2000b); Burns *et al.* (1999)
Developmental toxicity test	1.25 g/kg body wt/day	Rat	Not toxic	Arterburn *et al.* (2000c)

[a]Data summarized in Wynn & Ratledge (2006).

Table 5. Human studies on the safety of DHASCO™ produced by Martek Biosciences Corp[a]

Dose (g/day)	Duration (weeks)	Number of subjects/group (experimental and control)	Conclusion	Reference
4	15	14	No effect on platelet aggregation/homeostatic factors	Agren *et al*. (1997)
4.5	6	12 vegetarians	No effect on thrombogenic factors	Conquer & Holub (1996)
15	13	6	No change in blood clotting or immune parameters	Nelson *et al*. (1997); Kelley *et al*. (1998)
1.5–7.3	2	8	No significant adverse effects	Innis & Hansen (1996)
0.75–1.5	4	12–15	No serious adverse effects	Otto *et al.* (2000)

[a]Data summarized in Wynn & Ratledge (2006).

allowable), and even experimental animals will not accept such loadings in their diets.

Human studies similarly failed to show any potential adverse effects, even at doses far in excess of those that would be routinely advocated. The safety of the *C. cohnii* oil in particular has now been confirmed by its use for over 5 years as a supplement for infant formula with no adverse effects being reported. It can be stated that of all oils and fats produced from any source, the safety of the microbial oils has been the most stringently evaluated. No adverse effects on infants, young people or adults in any country have ever been reported.

In light of the extensive safety data accumulated by Martek Biosciences, the omega-3 LC-PUFA oils have been given GRAS ('generally recognized as safe') status by the Food and Drug Administration in the USA and also by the equivalent body in the EU. The *Schizochytrium*-derived oil has been given 'Novel foods' status in the EU and is thus deemed to be acceptable for inclusion in food materials and as a dietary supplement.

Now that microbially derived oils have been demonstrated to be just as intrinsically safe as plant and animal oils, it is expected that the next generation of these oils will have a simpler task in gaining regulatory approval. In essence, newer oils will only have to confirm (by chemical testing) that they are compositionally 'essentially equivalent' to the existing SCOs, and it will be necessary for them to undergo only limited animal studies before they could reasonably expect regulatory approval.

G. Current applications for omega-3 LC-PUFA SCO

The most important application of a currently produced LC-PUFA omega-3 rich oil (a DHA-rich oil) is as a nutritional supplement for infant formula. The developmental benefits of this LC-PUFA are such that its inclusion in infant formula has been recommended by many international bodies, including the World Health Organization (WHO), the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and the International Society for the Study of Fatty Acids and Lipids (ISSFAL). Although DHA from fish oil is used in some countries in the EU and Asia, the contraindications concerning the co-supplementation of DHA together with EPA (the other major omega-3 LC-PUFA in fish oil) was sufficient that the US Food and Drug Administration initially only permitted the use of the DHA-rich SCO (devoid of EPA) as a DHA supplement for infant formula. Since the introduction of the SCO-derived DHA-rich oil (co-supplemented with an arachidonic acid rich SCO) in February 2002, the adoption by both manufacturers and consumers (or at least their parents) of infant formulas supplemented with microbially derived LC-PUFA is such that (at the time of writing, December 2006) in excess of 85% of all the infant formula sold in the USA (and some 25% of infant formula sold globally) contains these products (see Figure 5).

The realization of the critical importance of DHA in infant development (both *in utero* and post-partum) has prompted the inclusion of DHA-rich SCO in food supplements ('ExpectaLipil' from Mead Johnson and 'Optinate' from First Horizons Pharmaceuticals) and nutritional bars ('Oh Mama' bars from Vincent Foods) targeted at pregnant women, those who are hoping to become pregnant, and those who are nursing infants. Both applications aim to increase the DHA supply to the developing child (either via the placenta or via the mother's milk) and to help maintain the DHA status of the mother. Depletion of maternal DHA stores, as a direct result of donation to the foetus/infant, is a well characterized phenomenon during pregnancy/lactation, and the Western diet does not supply sufficient preformed DHA to restore this status between births in most cases. A decline in maternal DHA levels is one factor being considered as a cause of post-partum depression, so supplementation of women during the pregnancy/lactation period is of potential benefit to not only the developing neonate but also the mother (and subsequent siblings).

Infant formula supplementation and the pregnancy/lactation market currently constitutes over 90% of the global demand for omega-3 LC-PUFA rich SCOs. The remaining 10% is used for nutritional supplement capsules (as a vegetarian alternative to fish oil) and as a nutritional supplement for foodstuffs (see below). The latter is perceived to be an advantage where fish oils are considered to be a poor choice of DHA, either due to taste/smell problems or to their unacceptability because of dietary restrictions or fears (perhaps unfounded) of contamination. Within the past 12 months, a tuna fish oil with a low

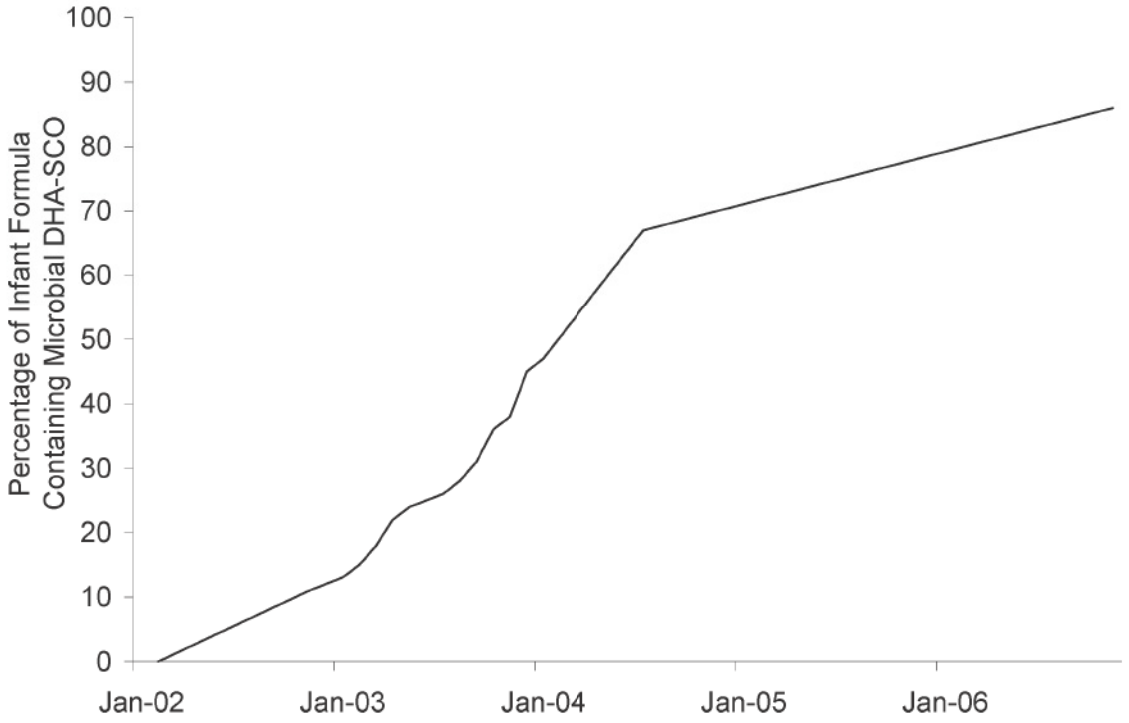


Figure 5. Inclusion of the DHA-rich oil derived from *Crypthecodinium cohnii* in US infant formulas between 2002 and 2006 (figures supplied by Martek Biosciences Corp).

content of EPA has obtained GRAS status for use in infant formula, but there is such concern over the introduction of even small amounts of EPA into the diet of neonates that the permitted levels at which this oil can be used delivers less than the recommended levels of DHA for infant formula supplementation. There is evidence that suggests that EPA may suppress the levels of arachidonic acid in the infant; thus, to maximize the benefit of DHA to infant nutrition, arachidonic acid is always included along with the DHA oil from *Crypthecodinium cohnii* in infant formulas (see Wynn & Ratledge, 2005, 2006).

H. Future applications of microbial omega-3 LC-PUFA

1. DHA-rich SCOs

The current level of interest in omega-3 LC-PUFA is identifying a number of nutritional and medical benefits for these oils. Indeed, it is now thought possible that many of the chronic diseases that are becoming ever more common, including heart disease, asthma, arthritis and even dementia/Alzheimer's disease, are not only linked to the ever-increasing age of the population but also to changes in the human diet. The evolution of modern humans appears to be associated with populations clustered around large bodies of water, either seas/oceans or large inland lakes. These bodies of water provided readily

available access by early humans to food in the form of fish and shellfish. It is reported that the diet of early humans was far richer in omega-3 LC-PUFA than the modern diet (an omega-6:omega-3 ratio of approximately 1:1, as compared with a ratio of >15:1 in the modern Western diet (Simopoulos, 2006). The suggestion has even been made that the high dietary intake of omega-3 LC-PUFA (specifically DHA) associated with a diet rich in fish played a pivotal role in the evolution of the large human brain (Crawford, *et al.*, 1999; Crawford, 2002; Broadhurst *et al.*, 2002).

As a result of the realization of the critical role of preformed DHA in the human diet, inclusion of this LC-PUFA in foodstuffs is beginning to attract the attention of food manufacturers, who are always keen to gain market share from increasingly health-conscious consumers. Although the most obvious way to increase the dietary intake of DHA would be to eat more of those foods that contain this fatty acid naturally (i.e. fatty sea fish) this is not as straightforward as it seems. While fish and fish product manufacturers promote the health benefits of DHA-rich sea fish, there are several reasons why increasing fish intake is not a desirable option for certain sections of the population. Obviously, for vegetarians the consumption of fish is untenable. But even within the non-vegetarian population, fish consumption has declined over the past few decades primarily because of disfavour with consumers. While many adults are probably willing to reincorporate sea fish into their diets, convincing children to do the same is more of a challenge. Moreover, more recently concerns over the safety of wild-caught sea fish have been raised by several governmental departments, based on the detection of high levels of organic mercury and PCB residues in certain species at the top of the marine food chain. As a result of these findings, recommendations on restricting the intake of sea fish for the most vulnerable groups (young children and pregnant women) have been made by the US FDA and other regulatory bodies. Safety concerns, of course, have an impact beyond those groups specifically targeted, and fears of environmental contamination of sea fish make the decision to reintroduce this foodstuff into the modern diet even more problematic. However, the health benefits of eating fatty fish, because of their omega-3 contents, far outweigh any possible slight detrimental effects. Sea fish should, in principle, be a sustainable resource, but years of overfishing have led to a decline in fish stocks that has resulted in quotas being imposed. As a result, the availability of sea fish is limited (this limitation being reflected in the increasing prices) and, despite the quotas, it is still considered by many that current fish stocks are unsustainable, and the eradication of certain species over the next 50–100 years is forecast.

Therefore, for a number of reasons there is a need for a non-fish alternative to obtaining an increased DHA consumption. The most obvious route is DHA-enriched foodstuffs. Attempts to fortify meat and eggs with LC-PUFA via feeding fish oil/fishmeal have been one approach, although in several instances the problems of fishy off-flavours have been a significant problem. Even when

these off-flavour issues can be addressed the problems associated with the environmental contamination of fish products and the lack of sustainability of the fish-derived DHA sources are leading more food manufacturers to investigate SCO-derived DHA. To date, foodstuffs supplemented with microbial DHA are available in both the USA and the EU in the form of soy milk, yoghurt and liquid eggs. It seems likely, therefore, that in the coming years microbial DHA will become a more commonplace food additive and will play a key role in restoring a more natural and healthy omega-3:omega-6 ratio to the human diet.

2. *Prospects for an EPA-rich SCO?*

While it has seemed relatively easy to identify useful microbial sources for DHA-rich oils, as well as for an arachidonic acid-rich oil from *Mortierella alpina* and, earlier, the GLA-rich oil from *Mucor circinelloides*, it has proved much more elusive to identify a microorganism that produces eicosapentaenoic acid (EPA, 20:5*n*-3) as a single PUFA. EPA is indeed produced by a variety of microorganisms, both of marine and soil origins. However, it is almost always produced in conjunction with either DHA itself or with arachidonic acid, or, in some cases, with both these LC-PUFA. An exception may be some marine bacteria (species of *Shewanella*, *Altermonas*, *Flexibacter*, *Psychroflexus* and *Vibrio*) that may produce EPA as the sole LC-PUFA (see Yazawa *et al.*, 1992 and Wen & Chen, 2003, 2005 for useful reviews of this topic). However, as these bacteria do not contain more than about 5% lipid (and many contain less than this) and, moreover, must also be grown at very low temperatures and, in some cases, under high pressure (see Wen & Chen, 2005), it is very difficult to obtain useful amounts of an EPA-rich oil from them. These bacteria would, however, be extremely useful as a source of the genes coding for EPA biosynthesis, which might then be introduced into plants (or other microorganisms) to achieve EPA production in a more amenable system (see also Section I below). It is not considered feasible to use these bacteria themselves for the commercial production of an EPA-rich oil.

Among the eukaryotic microorganisms that might provide sources of EPA, microalgae are probably the most abundant producers, although EPA also occurs in several genera of filamentous fungi, of which the most prominent are *Mortierella*, *Pythium* and *Saprolegnia*. However, all these fungi only produce EPA along with an approximately equal amount of arachidonic acid (ARA) (see Wen & Chen, 2005). Even *Mortierella alpina*, which is used commercially for producing ARA, does not seem able to produce EPA as the sole PUFA, irrespective of how the cultivation is progressed (Shimizu *et al.*, 1988, 1989). However, if there were ever a need for an ARA/EPA combination oil, then one of the above three fungi would clearly be of potential interest.

With respect to the microalgae, the best prospects for the production of a

nearly EPA-only oil would seem to be species of *Nannochloropsis*/*Nannochloris* (Yongmanitchai & Ward, 1989; Sukenik, 1991; Seto *et al.*, 1992; Zittelli *et al.*, 2000), *Navicula* (Tan & Johns, 1996) and *Nitzschia* (Wen & Chen, 2005). *Monodus subterraneus* and *Isochrysis galbana* have also been considered (Qiang *et al.*, 1997; Molina Grima *et al.*, 1992; see also Wen & Chen, 2003, 2005). However, in most cases, the microalgae have to be grown photosynthetically in photobioreactors which is a highly expensive process and is not conducive to producing an oil at a competitive price (see Section D). In the few cases where the algae have been grown heterotrophically, the yields have been low and considerably less than would be needed for commercial exploitation.

Thus, until recently it has seemed that there is no useful microbial source of EPA as a single LC-PUFA oil that might be produced in the same way as the DHA-rich oil from *C. cohnii*. However, it has recently been revealed that researchers at DuPont Central Research and Development (Wilmington, Delaware, USA) have genetically engineered the yeast *Yarrowia lipolytica* so that it is now able to produce EPA as its sole LC-PUFA. Normally, this yeast only produces fatty acids up to 18:2 but, by the introduction of 19 copies of 10 heterologous genes, a recombinant strain was produced which contained over 30% of its dry weight as an extractable triacylglycerol oil with almost 40% of the total fatty acids as EPA (Damude *et al.*, 2006; Zhu *et al.*, submitted). The genes introduced into this yeast included several desaturases (Δ5, Δ6 and Δ17) taken from two fungi, *Mortierella alpina* and *Saprolegnia declina*, and a Δ6 elongase to convert γ-linolenic acid (formed by the Δ6 desaturase from 18:2) into dihomo-γ-linolenic acid (20:3*n*-6). Additional genes also had to be included to ensure strong expression of the new elongase and desaturase genes, as well as genes for 'pushing' the flow (or flux) of carbon into lipid biosynthesis. This was done by replacing key genes in the fatty acid biosynthetic pathway with other genes that produced more active enzymes. It was also necessary to ensure rapid transfer of the newly synthesized fatty acyl chains into triacylglycerol formation, as the existing acyl transfer system was not efficient enough. The profile of the fatty acids of the engineered strain is given in Table 6, together with the profile of the original yeast.

This research programme demonstrates the strength of modern molecular techniques for designing organisms for the production of key fatty acids. Interestingly (perhaps very interestingly), the DuPont team has also indicated that by the addition of two more genes [a Δ5 elongase to convert EPA into docosapentaenoic acid (22:5*n*-3), and a final Δ4 desaturase] the recombinant yeast can be used to produce DHA, although not yet at commercially attractive levels (Damude *et al.*, 2006; Zhu *et al.*, submitted).

This genetically modified yeast appears to be sufficiently robust to be considered for commercial development; cell yields have already been reported to reach 100 g/l (Damude *et al.*, 2006; Zhu *et al.*, submitted), although details are not yet available concerning the scale of production. If the yeast does

Table 6. Fatty acyl profile of *Yarrowia lipolytica* containing an engineered omega-3 biosynthetic pathway for the production of EPA

	Content (%, w/w of total fatty acids)	
Fatty acid	**Wild-type yeast**	**Recombinant yeast[a]**
16:0	10	9
16:1	12	1
18:0	2	3
18:1	39	3
18:2	30	12
18:3*n*-6	ND	19
20:3*n*-6	ND	4
20:4*n*-6	ND	1
20:4*n*-3	ND	4
20:5*n*-3 (EPA)	ND	39

[a]The recombinant yeast was created by the introduction of 19 copies of 10 different genes taken from a variety of other microorganisms (see text and also Damude *et al.*, 2006; Zhu *et al.*, submitted). ND, not detected.

indeed prove to be genetically stable over a number of generations (a necessary prerequisite to growing it up to large-scale levels; see Figure 4), then it is possible that we shall see the launch of the next generation of SCO in the very near future. If improvements in the genetic constitution of the yeast continue to be made, then it is possible that *Y. lipolytica* could eventually be engineered to produce a DHA-rich oil to rival current commercial products. The acceptability of these LC-PUFA would, of course, depend on the usual evaluations by the various regulatory authorities, but the fact that the oils would be produced by a genetically engineered microorganism may not be a factor as the oil itself would be under scrutiny, not the yeast. In any case, the parent yeast was originally chosen because of its GRAS status and, although this status would not automatically be given to the genetically modified strain, it would nevertheless be a strong positive indicator in its favour.

I. Future of omega-3 LC-PUFA SCOs

It is a much-quoted and widely accepted truism within the SCO industry that it is impossible to compete against plant-derived oils. As plants cannot naturally synthesize PUFA with chain lengths greater than 18, the market for SCO LC-PUFA has been restricted to LC-PUFA with chain lengths of ≥20 (ARA and DHA). With the advent of molecular engineering, the potential for plants to accumulate significant levels of LC-PUFA is now also a real possibility. All the genes required for the synthesis of DHA have now been isolated from microbial sources (see Section B), and during the past two years significant advances have been achieved in overcoming the major bottlenecks to LC-PUFA

production in plants (the elongase reactions). Indeed, genetically modified plants capable of synthesizing omega-3 LC-PUFA have been produced at experimental scale. Adopting the elongase/desaturase route will inevitably yield an oil that resembles fish oil (i.e. containing both EPA and DHA), which will be of limited use in terms of infant formula applications. In contrast, expression of the PUFA-PKS system (see Section B above) in plants would generate a plant oil with a high DHA content and devoid of EPA, which could be an alternative to SCO DHA for infant supplementation.

Although there remain both technical and regulatory barriers for seed oils containing significant amounts of omega-3 LC-PUFA derived from genetically modified organisms (GMOs), it seems inevitable that these will be overcome within the next 10 years. When this happens, and assuming consumer acceptance – which in the USA is not a significant issue – the market for SCO-derived omega-3 LC-PUFA will become restricted to markets where its non-GMO status can be used to justify its premium price. It is very likely that the current anti-GMO views, widely held within various countries of the EU, will be severely challenged by the arrival of GMO oils. The dilemma will then have to be addressed, particularly among vegetarians, who are also often vociferous in their stand against GM products, as to whether it is better to carry on killing fish to obtain the desirable LC-PUFA or to obtain them from an apparently abhorrent GM plant source! Even if the advent of inexpensive plant oils eventually means the end for microbially derived SCOs, the legacy of these oils will remain in that the genes used to produce these oils in plants will have a microbial origin.

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