Trends in Chemical Engineering
Vol. 18, 2020

Review

Fractionation of fatty acid alkyl ester mixtures and opportunities for large-scale separation

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ABSTRACT

As derivatives of extracted triglycerides from natural resources, fatty acid alkyl esters (FAAE) are interesting base oleochemicals for the development of food additives, pharmaceutics and fine chemicals. In 2016, the global oleochemical market size was estimated at 20.2 billion USD with expected compound annual growth rate (CAGR) growth of 5.2% in the period of 2018 to 2025. Although there are significant market opportunities, prominent challenges in their large-scale separation into individual compounds of high purity hamper their current use. The major challenge is to isolate the FAAE without the risk of initiating degradation reactions. Despite the enormous potential of FAAE, a clear overview of current research on the fractionation and purification of this type of oil derivatives is missing. Moreover, separation efficiencies and therefore the upscaling possibilities of the applied technologies are not always consistently mentioned in literature. Therefore, this review focuses on the key opportunities and challenges in the versatile field of fractionation of FAAE in view of efficiency and possible upscaling processes. Oleochemicals are an important type of oil derivatives and insight into new implementation opportunities in the present market is of interest for both academia and industry. A critical overview of the available fractionation research performed in the last decade will provide the reader with clear information on large-scale implementation possibilities, technology readiness levels and scientific challenges.

KEYWORDS: fatty acid alkyl esters, large-scale separation, fractionation.

1. Introduction

1.1. Historical development and focus shift towards sustainable renewables

The fractionation of fatty acids (FA) and their derivatives has been an important research topic already since the beginning of the 20th century [1]. Early on, fatty acid separation of the olein and stearin fraction was performed using decantation, crystallization and/or filtration [2-4]. Solvent extraction and distillation was used for the fractionation of high and low molecular-weight FAs [5]. Later, this expanded to long chain fatty acids and esters [6-9]. Chromatography was only used for analytical purposes [10]. In the second half of the 20th century, the research focussed on plant oils and animal fats and different new separation methods were introduced including enzymatic reactions, urea complexation and supercritical fluid extraction [11-13]. Since then, chromatography was also used on a large scale for fatty acid refining [14].

By the end of the 20th century and the beginning of the 21st century, sustainable development became a hot topic [15, 16]. During this period, the research on fatty acid methyl ester (FAME) fractionation for biodiesel applications increased significantly [17].

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The two examples given by Metzger and Eissen, which show that renewable raw materials are more sustainable, are also based on the substitution of petrochemicals with plant-based triglycerides [16]. For applications in the food industry, the health aspects of processed fats and oils were investigated. This research revealed the health benefits of highly unsaturated FA and the health risk of saturated and *(E)-isomer* fatty acids [18]. Although triglycerides are seen as the most natural way of oil uptake by the human body, FA and fatty acid alkyl esters (FAAE) can also be processed by our biological system [19]. Moreover, FAAE are less susceptible to deterioration (e.g. autoxidation processes), compared to FA, making their separation from oil sources more interesting for industry. Currently, the most widely investigated FAAE are docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), α-linolenic acid (ALA) (omega-3 fatty acids) and arachidonic acid, linoleic acid and γ-linolenic acid (GLA) (omega-6 fatty acids). The fractionation of fish oil at large scale is currently performed using urea complexation and molecular distillation. For the purification of DHA and EPA ethyl esters, supercritical fluid chromatography is used at large scale already since the 1990s by KD Pharma [20].

Using fractional distillation, the FAAE mixtures can be fractionated and then hydrolysed into FA in high purity. The fractionation of FAAE is preferred to FA fractionation due to their lower boiling point. Alternatively, they can be converted to fatty acid alcohols. From the latter, a wide range of base compounds for industrially-relevant organic syntheses can be accessed; saturated fatty alcohols of 8 to 10 carbons are typically used in the production of high-boiling esters for plasticizers, whereas fatty alcohols of 12 to 18 carbons are used in high quantity to produce detergents. Another application is the hardening of oil, which is used for producing cooking fats and oleomargarine. Here, cheap oils including cottonseed oil, corn oil and soybean oil are used. For drying oils, used in paints and varnishes, linseed oil and tung oil are most important [21].

In recent years, awareness towards the importance of sustainable development has increased significantly. In 2015, the United Nations presented 17 sustainable development goals (SDG) [22]. These include the shift from fossil-based building blocks towards renewable sources (SDG no. 12). This shift not only includes fossil fuels, but also other (side-)products originating from fossil sources which are currently being used for health care products, pharmaceutics, soaps, etc. [23, 24].

The SDG also include the shift towards a circular economy. Therefore, the separation of FA from waste streams has become increasingly important [25]. However, this approach is now rendered far more challenging as the mixture, derived from streams, is much more complex. Consequently, current separation techniques require further development towards these types of oil streams. To-date, several research groups are continuously investigating the omega-3 concentration processes to achieve higher yield and purity at lower costs [26]. The new oil-based streams and their importance in sustainable development create new opportunities for both old and new separation techniques. In that context, this review will focus on the current opportunities and challenges in both recent and well-documented separation techniques concerning the fractionation of mixtures of FAAE.

These FAAE are part of the fatty acid ester (FAE), oleochemicals, specialty oleochemicals, omega-3 fatty acids (derivatives) and FAME market. For the FAE market the main commercial uses are food processing, personal care, cosmetics, surfactants and detergents as shown in Figure 1-1 [27]. For oleochemicals, the main market segments are fatty acids, fatty alcohols and glycerol. Fatty acid esters are known to be an intermediate in the production of fatty acids and fatty alcohols [28]. In the specialty oleochemical market, the applications are most diverse as shown in Figure 1-2 and are mainly industrial, personal care, cosmetics, paints and inks [29]. For Omega-3 fatty acids, Figure 1-3 shows that the commercial use is focused on supplements and functional food, pharmaceuticals and infant formulas [30]. Based on end-use applications in the FAME market, Figure 1-4 shows that commercial uses are mainly in the fields of fuel, metal-working fluids and lubricants [31].

1.2. Overview of the current state-of-the-art

A concise overview of the different separation and fractionation steps of FFA and FAAE is given in Figure 1-5. Starting from rich oil streams in the

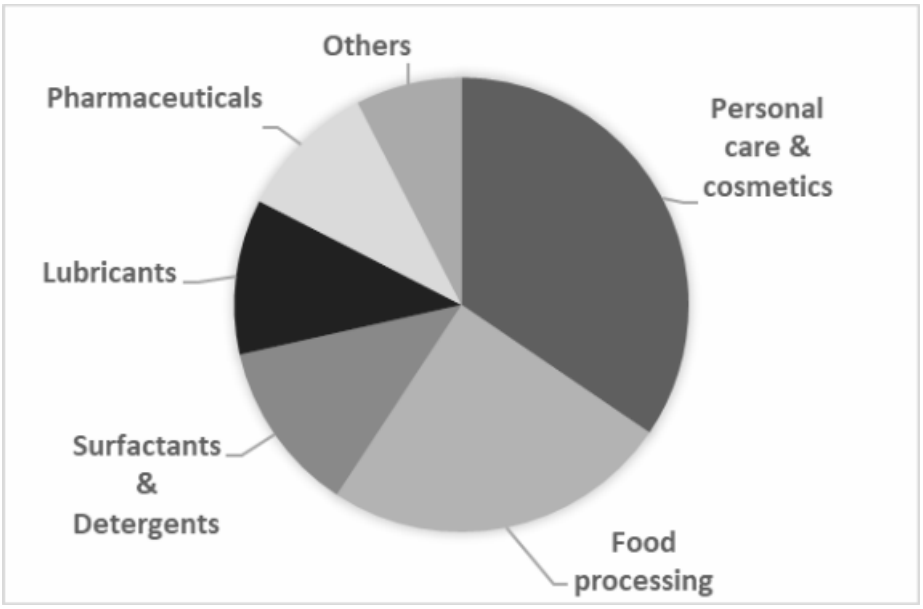

Figure 1-1. Global fatty acid ester market share, by application, 2014. Adapted from ref. [27].

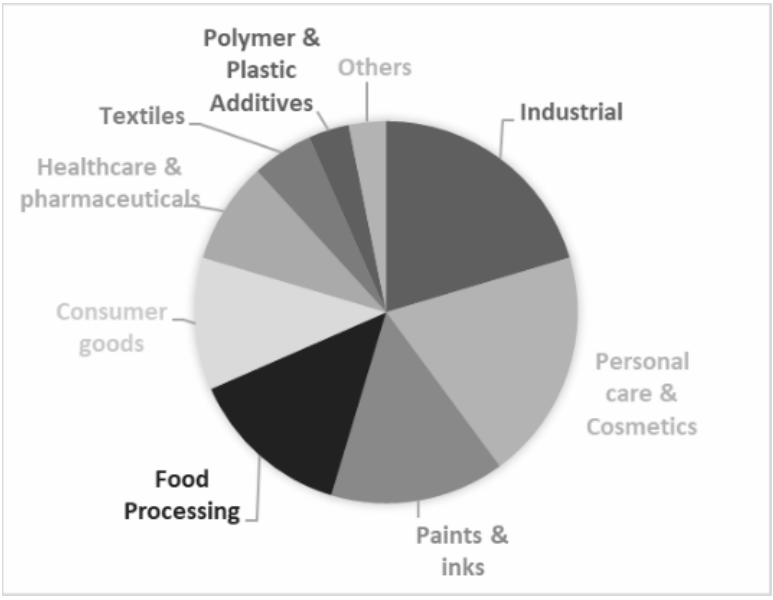

Figure 1-2. US specialty oleochemical market share, by application, 2014. Adapted from ref. [29].

upper left corner of the figure, the triglyceride-containing source can be oil seeds, fish waste, animal fats, tall oils or side products from oil processes. In the first separation step (separation 1), the raw oil is separated from other fractions. Oil-containing seeds are first mechanically pressed before solvent extraction, usually with hexane. After this step, the raw oil is refined in different steps (not shown in Figure 1-1) including degumming, acid treatment, bleaching and deodorizing to eventually obtain a triglyceride fraction of high purity (separation 2) [32]. Thereafter, two pathways are possible. First, the triglyceride mixture itself can be separated (separation 3) into a saturated

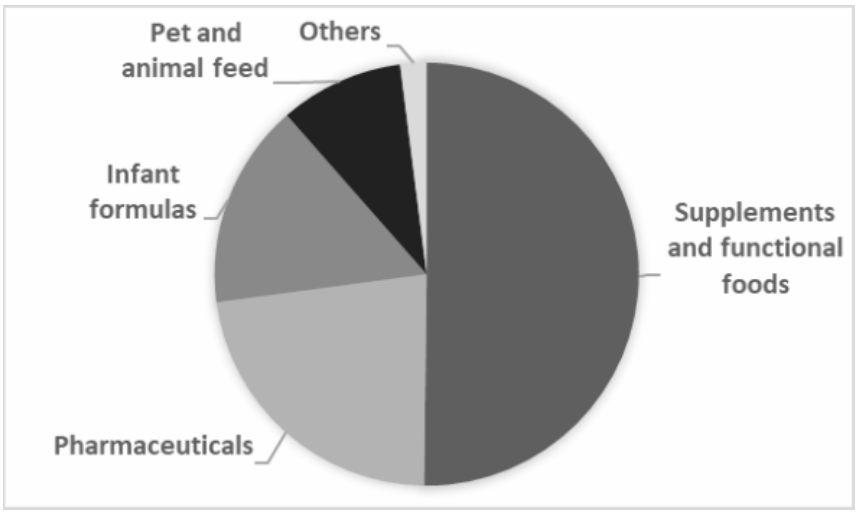

Figure 1-3. US Omega-3 market, by application, 2014. Adapted from ref. [30].

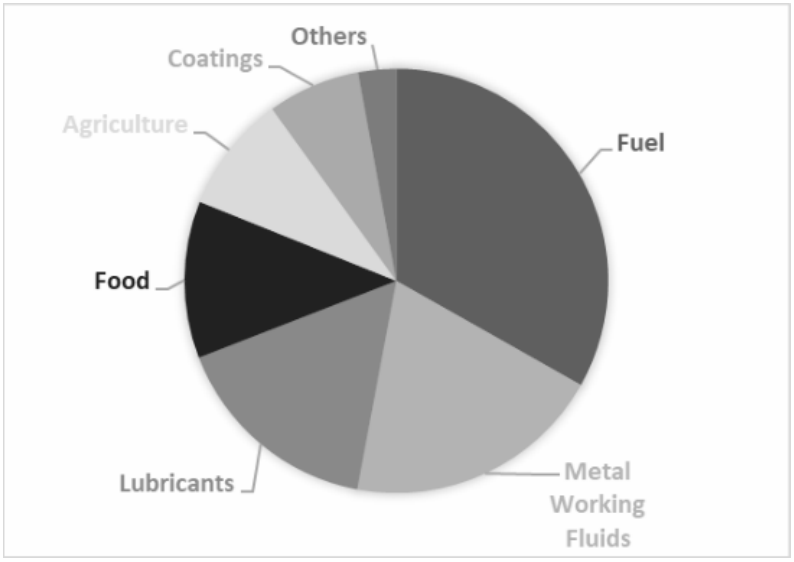

Figure 1-4. Global FAME market, by application, 2017. Adapted from ref. [31].

and unsaturated fraction (separation based on saturation degree) or into a short-chain and long-chain fraction (separation based on carbon chain length). Second, the triglycerides can be further processed before the separation step. Here, three pathways are possible. First, the triglycerides can be hydrolyzed chemically or be transesterified and the FFA fraction can be separated from the glycerol (separation 4 and 5). Second, the triglyceride mixture can be transesterified into an FAAE and glycerol, after which the glycerol is separated (separation 6). After both reactions, the glycerol is separated from the FA(AE) mixture (separation 4 and 6) and the FFA and FAAE mixtures are separated based on saturation degree or carbon chain length (separation 5 and 7). Third, specific enzymatic cleavage can yield an FA(AE) mixture after removing the enzyme and glycerol (separation 8). After separating the desired FA fraction (separation 5 and 8), the fraction can be esterified to attain FAAE (arrow A & B).

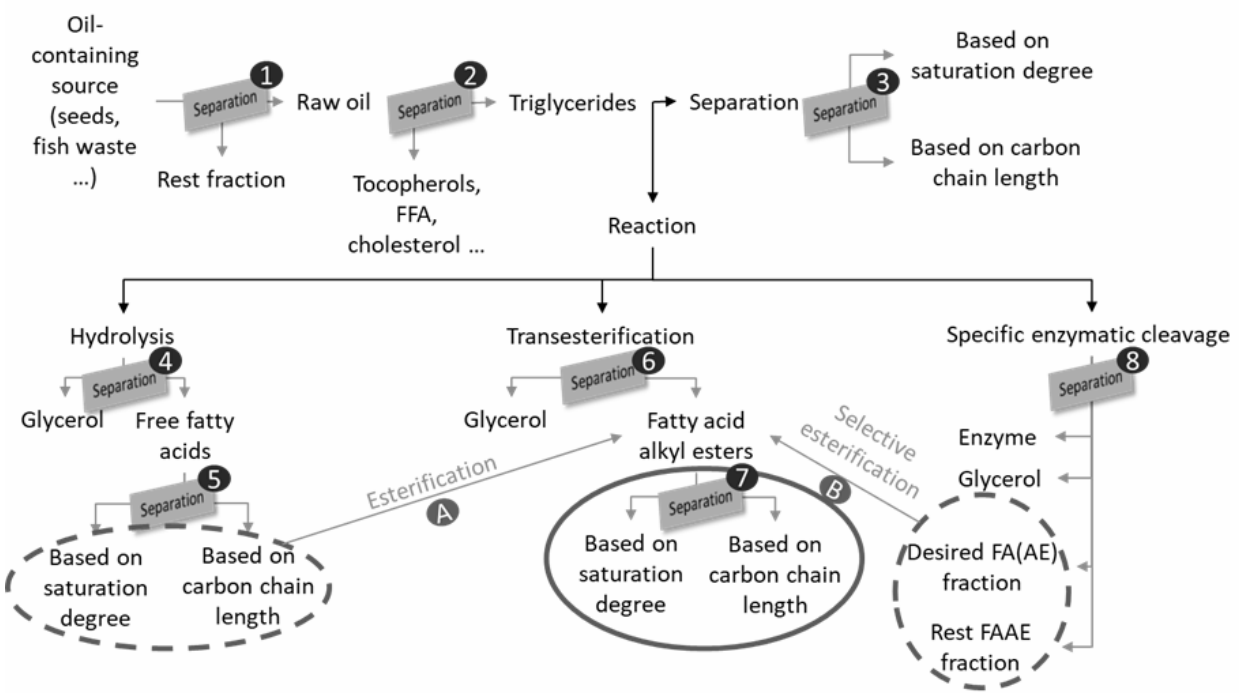

Figure 1-5. Overview of the different separation steps involving free fatty acids (FFA) and FAAE.

Various reviews on FAAE separation have already been written. However, it needs to be emphasized that not all the literature regarding separation of FAAE covers the fractionation of FAAE into their individual compounds. For instance, Savaliya *et al.* reviews current separation techniques for FAME, but focusses on the types of transesterification reactions and the purification processes of the FAME fraction [33]. The most recent review including FAAE fractionation is that of Wanasundara *et al.* [34]. This review addresses different separation techniques for the separation of FAME, mainly for biodiesel production. However, they do not focus on fractionating FAAE mixtures and only include chromatography applications for identification purposes. Other reviews, including that of Temelli, and Montañes and Tallon, focus on a single technique and cover a broad range of lipids [35, 36]. Other reviews, which cover the purification of polyunsaturated FAAE (PUFAE), only mention recent separation techniques [26]. For instance, Kahveci *et al.* demonstrated clearly the new possibilities for PUFAE purification using enzymes [37]; the 1,3-specific lipase reacts with PUFAE and concentrates this type of FA by interesterification with an existing triglyceride. Other techniques, which are used for FA separation (e.g. physical vapor deposition), have not (yet) been investigated for separation of FAAE [38]. Lembke gives a clear overview of the different techniques for PUFAE concentration in his book chapter [39]. These techniques, together with other procedures applied to FAAE fractionation found in literature, will be discussed to a broader extent within this review. As currently the most extensively investigated FAAE are DHA, EPA, α-linolenic acid, γ-linolenic acid, arachidonic acid and linoleic acid esters, this review will focus on the alkyl esters of these FA.

Besides the separation of FA on large scale, there are numerous literature reports regarding the analysis of FA mixtures [40-42] from both GC [43-49] and LC [50-52] perspectives. As analysis techniques are outside of the scope of this review, the reader is referred to these sources for more detailed information.

The previously mentioned reviews can be assigned to different steps in the oil separating process. This review concerns the fractionation of mostly FAAE into their individual compounds and is indicated in Figure 1-1 with a bold circle. It should

be noted that pathways involving hydrolysis and re-esterification (chemical and enzymatic) are omitted from this review (shown with dashed circles). However, in some cases, when combining different techniques, it is possible that an "in between" esterification reaction takes place. Furthermore, the separation techniques after selective esterification by lipases, which is not shown in Figure 1-1, are also mentioned in this review.

2. Properties of fatty acid esters

Triglycerides are the natural components of fats and oils. Triglycerides consist of three FA bonded to a glycerol backbone. Typically, the FA are alkyl chains of various lengths and can be saturated (no C-C double bonds) or unsaturated (1 to 6 C-C double bonds in the *E/Z* isomer form). The alkyl chains can contain different functional groups. Plant oils and animal fats generally consist of approximately 20 different FA. The composition of FA depends highly on the oil source and this determines which separation technique can be best used. The reader is referred to the data published by Lide *et al*. for more details on the FA composition of common oils and fats [53]. Global consumption of fats and oils in 2015 reached a total of 202 million tonnes. Besides their major use in food (76%), fatty acids are converted to biodiesel and oleochemicals [54]. The oleochemical market size was USD 18.6 billion in 2016 and is expected to grow to USD 26.8 billion by 2022 [55].

FAAE are the esterified form of fatty acids. The majority is produced *via* transesterification of plant, animal and tall oils. The reaction can be performed *via* different synthesis routes. Generally, a strong acid (H2SO4) or base (NaOH or KOH) is mixed with an alcohol (typically methanol or ethanol) and reacts with the triglyceride (TG) at temperatures ranging from 60 to 80 °C [56]. Enzymatically, a lipase catalyst can be used. Here, the reaction takes place at moderate temperatures (30-40 °C) and the direction of current research is towards lipase activity that can be controlled to work for a specific type of FA, easing the purification process [57]. After reaction, the glycerol, the remaining alcohol and catalyst need to be separated from the FAAE fraction. This separation is cumbersome, especially when the resulting FAAE mixture needs to meet biodiesel standards. An overview of a typical transesterification reaction is given in Figure 2-1.

Based on the amounts of saturated or unsaturated FA in the mixture, the FAAE phase can be solid or liquid at room temperature. Triglyceride mixtures of plants containing large quantities of unsaturated FA are liquid at room temperature (olive, rapeseed) and those containing large amounts of saturated FA are solid at room temperature (coconut, palm). This is due to the differences in melting points between saturated and unsaturated FAAE. Other chemical and physical properties of typical FAAE are shown in Table 2-1. As shown in Table 2-1, not only the presence and number of double bonds influences FAAE characteristics, but also alkyl chain length and ester chain length. Therefore, their separation is so challenging: fractionation based on differences in one chemical property is often not enough to generate high yields and high recoveries of individual FAAE.

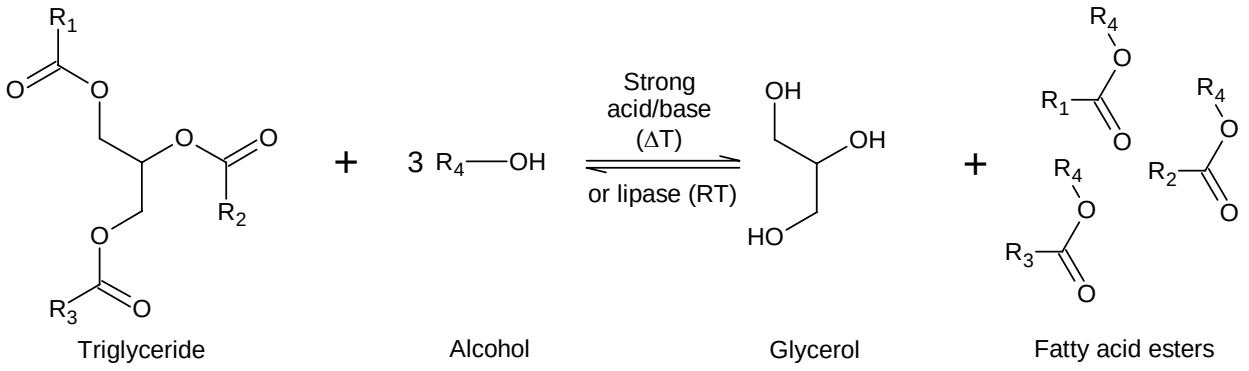

Figure 2-1. General transesterification reaction.

Table 2-1. Properties of typical FAAE. Data from Bruno *et al.* [58].

Compound	Molecular formula	Acid code	Melting point (°C) [A]	Boiling point (°C) [A]
Methyl butanoate	$C_5H_{10}O_2$	C4:0	-85.8	102.8
Ethyl butanoate	$C_6H_{12}O_2$	C4:0	-98	121.3
Methyl octanoate	$C_9H_{18}O_2$	C8:0	-40	192.9
Ethyl octanoate	$C_{10}H_{20}O_2$	C8:0	-43.1	208.5
Methyl decanoate	$C_{11}H_{22}O_2$	C10:0	-18	224
Ethyl decanoate	$C_{12}H_{24}O_2$	C10:0	-20	241.5
Methyl laurate	$C_{13}H_{26}O_2$	C12:0	5.1(0.2)	268(2)
Ethyl laurate	$C_{14}H_{28}O_2$	C12:0	-1.8(0.4)	276(3)
Methyl myristate	$C_{15}H_{30}O_2$	C14:0	19.0(0.5)	295(10)
Ethyl myristate	$C_{16}H_{32}O_2$	C14:0	12.3(0.8)	308(3)
Methyl palmitate	$C_{17}H_{34}O_2$	C16:0	29.6(0.5)	324(6)
Ethyl palmitate	$C_{18}H_{36}O_2$	C16:0	24.2(0.4)	334(7)
Methyl palmitoleate	$C_{17}H_{32}O_2$	C16:1 9c	-33.7(0.6)	325(6)
Ethyl palmitoleate	$C_{18}H_{34}O_2$	C16:1 9c	-36(1)	355.5(21.0)[B]
Methyl stearate	$C_{19}H_{38}O_2$	C18:0	38.7(0.6)	353(8)
Ethyl stearate	$C_{20}H_{40}O_2$	C18:0	33.1(0.7)	356(6)
Methyl oleate	$C_{19}H_{36}O_2$	C18:1 9c	-19.7(0.5)	347(5)
Ethyl oleate	$C_{20}H_{38}O_2$	C18:1, 9c	-21(2)	357(9)
Methyl linoleate	$C_{19}H_{34}O_2$	C18:2 9c,12c	-36.6(0.5)	347(5)
Ethyl linoleate	$C_{20}H_{36}O_2$	C18:2 9c,12c	-55(5)	351(10)
Methyl c9,t11-CLA	$C_{19}H_{34}O_2$	C18:2 9c,11t	/	378.5(21.0)[B]
Ethyl c9,c11-CLA	$C_{20}H_{34}O_2$	C18:2 9c,11t	/	/
Methyl t10,c12-CLA	$C_{19}H_{34}O_2$	C18:2 10t,12c	-12	376.1(11.0)[B]
Ethyl t10,c12-CLA	$C_{20}H_{34}O_2$	C18:2 10t,12c	/	/
Methyl linolenate	$C_{19}H_{32}O_2$	C18:3 9c,12c,15c	-49(4)	348(8)
Ethyl linolenate	$C_{20}H_{34}O_2$	C18:3 9c,12c,15c	/	357(10)
Methyl GLA	$C_{19}H_{32}O_2$	C18:3 6c,9c,12c	/	385.4(0)[B]
Ethyl GLA	$C_{20}H_{34}O_2$	C18:3 6c,9c,12c	/	/
Methyl ALA	$C_{19}H_{32}O_2$	C18:3 9c,12c,15c	-49	348
Ethyl ALA	$C_{20}H_{34}O_2$	C18:3 9c,12c,15c	/	/
Methyl pinolenate	$C_{19}H_{32}O_2$	C18:3 5c,9c,12c	/	375.7(31.0)[B]
Ethyl pinolenate	$C_{20}H_{34}O_2$	C18:3 5c,9c,12c	/	/
Methyl stearidonate	$C_{19}H_{30}O_2$	C18:4 6c,9c,12c,15c	/	374.5(31.0)[B]
Ethyl stearidonate	$C_{20}H_{32}O_2$	C18:4 6c,9c,12c,15c	/	389.4(31.0)[B]
Methyl arachidate	$C_{21}H_{42}O_2$	C20:0	46.4(0.3)	371(15)
Ethyl arachidate	$C_{22}H_{44}O_2$	C20:0	41.7(0.5)	389(20)
Methyl *cis*-11-eicosenoate	$C_{21}H_{40}O_2$	C20:1	-34(2)	378(10)
Methyl eicosapentaenoate	$C_{21}H_{32}O_2$	C20:5 5c,8c,11c,14c,17c	/	115-125[C]
Ethyl eicosapentaenoate	$C_{22}H_{34}O_2$	C20:5 5c,8c,11c,14c,17c	/	417.0(34.0)[B]
Methyl behenate	$C_{23}H_{46}O_2$	C22:0	53.3(0.4)	402(16)
Methyl erucate	$C_{23}H_{44}O_2$	C22:1	-1.1(0.5)	100(9)
Methyl docosapentaenoate	$C_{23}H_{36}O_2$	C22:5 7c,10c,13c,16c,19c	/	215[D]

Table 2-1 continued…

Ethyl docosapentaenoate	$C_{24}H_{38}O_2$	C22:5 7c,10c,13c,16c,19c	/	444.6(24.0)[B]
Methyl docosahexaenoate	$C_{23}H_{34}O_2$	C22:6 4c,7c,10c,13c,16c,19c	/	429.9(24.0)[B]
Ethyl docosahexaenoate	$C_{24}H_{36}O_2$	C22:6 4c,7c,10c,13c,16c,19c	/	443.5(24.0)[B]

[A]Number in brackets is the combined expanded uncertainty. Melting and boiling points were measured at 760 mmHg.
[B]Source: SciFinder. Data is predicted. Melting and boiling points were measured at 760 mmHg.
[C]Source: SciFinder. Data is predicted. Boiling point was measured at 0.005 mmHg.
[D]Source: SciFinder. Data is predicted. Boiling point was measured at 15 mmHg.

3. Available techniques: possibilities and challenges for scale-up

An overview of recent research for isolating FAAE and the technology readiness level (TRL) in the respective article is given in Table 3-1.

In each of the following sections, a brief explanation is given on how the technique is employed to separate FAAE mixtures. Then, an overview of literature over the past decade is given, together with the scale at which the FAAE separation was employed (if data is present in the article) and what currently hampers their upscaling. Where possible, yield, purity and recovery of the fractionated FAAE are given.

3.1. Crystallization

3.1.1. Research and methodology

Crystallization, sometimes also described as winterization, uses the difference in melting point and solvent solubility of oil fractions to accomplish a separation. As can be seen from Table 3-1, the melting points between saturated and unsaturated FAAE differ greatly: with increasing double bonds, the melting point decreases. The same trend can be observed with decreasing carbon chain length. Crystallization takes advantage of these properties to separate FAAE mixtures. Generally, a mixture of FAAE, with or without solvent, is cooled at a fixed cooling rate. During the crystallization process the more saturated fraction undergoes selective nucleation and subsequent crystal growth while being strictly controlled in cooling rate and gentle agitation. The cooling rate and the final temperature depend on the oil source and the desired FAAE fraction [135]. Then, the solid and liquid fractions are separated using a filtration or centrifugation process. For vegetable oil separation, the example of palm oil fractionation *via* crystallization is used to explain different process types. Figure 3-1 shows the three fractionation processes that have been used since the 1970s for this oil stream: dry fractionation, solvent fractionation and detergent fractionation. With dry fractionation, the pure oil is used. The oil is cooled gradually (temperature depending on the desired FAAE fraction) and the saturated fraction solidifies, whereas the unsaturated fraction remains liquid. Afterwards, both fractions can be separated using a filter. With solvent fractionation, the oil is mixed with a specific solvent (often acetone or hexane) that favours the solubility of specific FAAE. This increases the separation efficiency. When using detergent fractionation, or Lanza fractionation, a surface-active agent is added to increase the selectivity [134]. A well-known example of detergent fractionation is the Lipofrac process, which was developed by Alfa Laval.

Currently biodiesel purification is the most investigated application of FAAE crystallization. Both dry and solvent fractionation crystallization have been used at large scale for this application. The purpose here is to improve the cold flow properties (CFP) which consists in lowering the pour point and cloud point of an oil mixture by decreasing the amount of saturated FAME [65]. It is however essential that not all saturated FAME are removed, as this fraction has a high caloric value and ignition quality for the fuel [136, 137]. The solvents used for solvent fractionation are methanol, acetone, chloroform and hexane with a preference for methanol, as it can be integrated more easily in industrial biodiesel processes [60].

3.1.2. Biodiesel applications

Recent research shows the principle direction of investigations being towards the use of new oil streams. Examples include peanut oil [60], waste

Table 3-1. Overview of the reviewed articles listed chronologically per technique. "NA" = Not Applicable. TRL level was assigned based on the information in ref. [59].

Separation technique	Oil type	Application	Desired FAAE fraction	TRL	Ref.
Crystallization	Peanut oil	Biofuel	Unsaturated FAMEs	3-4	[60]
Crystallization	Modified soybean oil	Functional lipids, clinical trials	SDA-EE	3-4	[61]
Crystallization	Waste cooking oil	Biofuel	Unsaturated FAMEs	3-4	[62]
Crystallization	Beef tallow	Biofuel	Unsaturated FAMEs	3-4	[63]
Crystallization	*Jatropha curcas* oil, WCO	Biofuel	SFA-ME, unsaturated FAME	3-4	[64]
Crystallization	Animal tallow	Biofuel	Saturated and unsaturated FAMEs	3-4	[65]
Crystallization	Palm, canola, and corn oils	Biofuel	Unsaturated FAMEs	3-4	[66]
Crystallization	Model mixture	Biofuel	Unsaturated FAMEs	3-4	[67]
Crystallization	Model mixture	Biofuel	Unsaturated FAMEs	3-4	[68]
Urea complexation	Corn oil	Biofuel	Unsaturated FAMEs	3-4	[69]
Urea complexation	Fish oil	Human diseases, human health	EPA-EE, DHA-EE	3-4	[70]
Urea complexation	Palm oil	Oleochemicals	Unsaturated FAMEs	3-4	[71]
Urea complexation	Soybean oil	Plasticizer, resin, biofuel	Unsaturated FAMEs	3-4	[72]
Urea complexation	Sunflower, Echium oil, fish oil	Human nutrition, disease prevention	Polyunsaturated FAMEs	3-4	[73]
Urea complexation	Microalgae oil	Food supplement, infant formula	DHA-EE	3-4	[74]
Urea complexation	Echium oil	Functional ingredients, human health	GLA-EE, SDA-EE	3-4	[75]
Urea complexation	Echium oil	Functional lipids, nutraceuticals	SDA-EE	5-6	[76]
Urea complexation	Seal oil	Human health	EPA-EE, DPA-EE, DHA-EE	3-4	[77]
Distillation	Milk fat and coconut oil	Functional lipids	SCFA-EE, MCFA-EE	3-4	[78]
Distillation	Squid oil	Disease prevention	EPA-EE, DHA-EE	5-6	[79]
Distillation	Squid oil	Disease prevention	EPA-EE, DHA-EE	5-6	[80]
Distillation	Fish oil	Food supplements	EPA-ME, EPA-EE, DHA-ME, DHA-EE	3-4	[81]
Distillation	Coconut oil	Oleochemicals, biofuel, surfactants	Methyl laurate	3-4	[82]
Distillation	Fish waste	Biofuel, nutraceuticals	EPA-EE, DHA-EE and SFA-EE	3-4	[83]

Table 3-1 continued..

Liquid-liquid extraction	Model mixture	Food additives, medical supplies	DHA-EE	3-4	[84]
Liquid-liquid extraction	Model mixture	Pharmaceuticals	DHA-EE	3-4	[85]
IL	Fish oil	Food	EPA-ME, DHA-ME	3-4	[86]
IL	Model mixture	Food	PUFA-ME	3-4	[87]
IL	Fish oil	Food	PUFA-ME	3-4	[88]
IL	Fish oil	Food	PUFA-ME	3-4	[89]
IL	Soybean oil	Biofuel	PUFA-ME	3-4	[90]
IL	Fish oil	Human disease, human health	PUFA-EE	3-4	[91]
IL	Model mixture	Biodiesel	EPA-ME, DHA-ME, methyl linolenate	3-4	[92]
Molecular sieve adsorbents	Model mixture	Biological and nutritional applications	*cis*-FAME	3-4	[93]
Countercurrent chromatography	Fish oil	Healthy food	Omega-3 PUFA-EE	3-4	[94]
RPLC	Model mixture	Disease treatment, epidemiologic and clinical trials	EPA-EE and DHA-EE	5-6	[95]
Ag^{+}-chromatography	Sardine oil	Food and pharmaceutical products	Long-chain PUFA-ME	3-4	[96]
Molecular sieve adsorbents	Fruit kernel oil	Biofuel	Methyl octanoate, methyl decanoate, methyl laurate	3-4	[97]
HPLC	Model mixture	Investigation of physiological effect on humans	EPA-EE, DHA-EE	3-4	[98]
Countercurrent chromatography	Fish oil	Food	DHA-EE	3-4	[99]
SFE	Butter oil	Functional lipids	SCFA-EE and MCFA-EE	3-4	[100]
SFE	Model mixture	Human health	CLA-EE	3-4	[101]
SFE	Fish oil	Pharmacology, functional ingredients	Omega-3 PUFA-EE	3-4	[102]
SFC	Fish oil, algae oil	Human diet	EPA-EE and DHA-EE	3-4	[103]
SFE	Fish oil	Biopharmaceutical, nutraceutical and food sector	Omega-3 PUFA-EE	5-6	[104]
SFE	Fish oil	Nutraceutical and pharmaceutical industry	PUFA-EE	3-4	[105]

Table 3-1 continued..

SFE	NA	NA	NA	3-4	[106]
SFE	Fish oil	Biofuel, nutraceuticals, fish proteins	Omega-3 PUFA-EE	5-6	[107]
SFE	Fish oil	Disease treatment	EPA-EE, DHA-EE	3-4	[108]
Enzymatic methods	Modified soybean oil	Food	SDA	3-4	[109]
Enzymatic methods	Tuna oil	Pharmaceuticals	DHA	3-4	[110]
Enzymatic methods	Tuna oil	Pharmaceuticals	DHA	3-4	[111]
Enzymatic methods	Evening primrose oil	Pharmaceuticals, food	GLA	3-4	[112]
Enzymatic methods	Echium oil	Health	SDA	3-4	[113]
Enzymatic methods	Tuna oil	Health	DHA	3-4	[114]
Enzymatic methods	Commercial CLA oil	Disease treatment	CLA	3-4	[115]
Enzymatic methods	Tuna oil	Health	DHA	3-4	[116]
Enzymatic methods	Sardine oil	Food industry	EPA	3-4	[117]
Enzymatic methods	Commercial CLA oil	Health	CLA	3-4	[118]
Enzymatic methods	Echium oil	Structured bioactive lipids	SDA, GLA	3-4	[119]
Enzymatic methods	Fish oil	Nutritional supplements, personal care, pharmaceuticals	EPA, DHA	3-4	[120]
Dialysis	Waste fat	Lubricants	SFA-ME	3-4	[121]
Membranes	Model mixture	Industrial applications	Unsaturated FA	3-4	[122]
Membranes	Model mixture	Biofuel	SFA-ME	3-4	[123]
Urea complexation, MD	Sardine oil	Health food, infant formulas	EPA-EE, DHA-EE	3-4	[124]
Enzymatic reaction, urea complexation	Pine nut oil	Health benefits	Pinolenic acid LE	3-4	[125]
Enzymatic reaction, urea complexation	Menhaden oil	Functional food, pharmaceuticals	Omega-3 PUFA-EE	3-4	[126]
Urea complexation, Ag^+ silica gel chromatography	Tuna oil	Health benefits	EPA-ME, DPA-ME, DHA-ME	3-4	[127]
Distillation, crystallization	Beef tallow	Biofuel, human consumption	PUFA-ME	3-4	[128]

Table 3-1 continued..

Winterization, urea complexation, Ag^+-chromatography	Liver Shark oil	Health benefits	EPA-ME, DHA-ME	3-4	[129]
Winterization urea complexation, preparative HPLC	Fish oil	Biodiesel, food and industrial feedstock	PUFAME, SFAME	3-4	[130]
Urea complexation, MD	Ray oil	Human nutrition, human health	EPA-EE, DHA-EE	3-4	[131]
Enzymatic reaction, MD	Fish oil	Health care, medicine	EPA-EE, DHA-EE	3-4	[132]
Urea complexation, MD, preparative HPLC	Seal oil	Human nutrition, disease prevention	DPA-EE	3-4	[133]

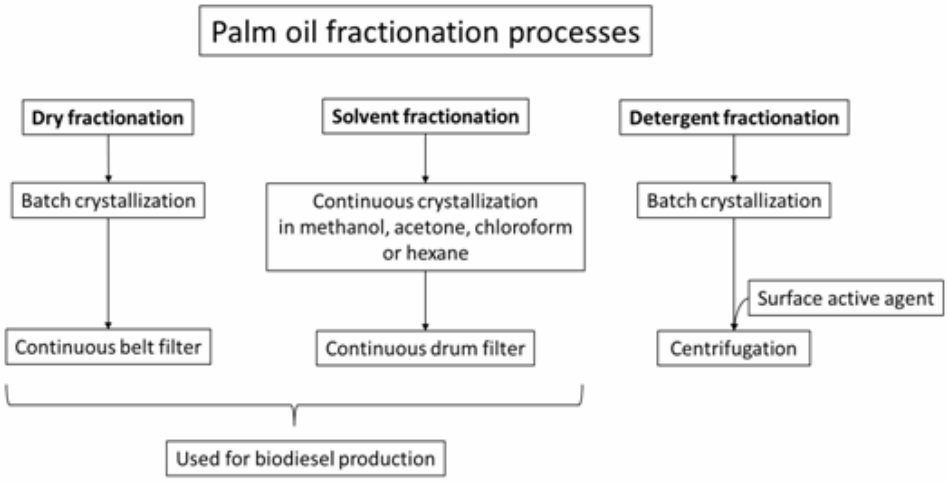

Figure 3-1. The three fractionation processes of palm oil that are applied in industry. Information taken from ref. [134].

cooking oil (WCO) [62], beef tallow oil [63, 67] and *Jatropha curcas* oil [64]. In most reports, different processes are investigated to lower the CFP using dry fractionation. Processes ranging from solvent fractionation with methanol detergent fractionation and (nonionic) surfactant fractionation to blending with petro diesel and kerosene have been explored. With surfactant fractionation, the use of polyglycerol ester showed the lowest cold filter plugging point (CFPP) at 17 °C with a yield of 73.1% (wt. liquid WCO phase/wt. total WCO phase) [62]. The studies with non-ionic surfactants used sorbitan monopalmitate and sorbitan monostearate as additives. Here, the results showed that separation was feasible with a factor of 15.6 (see Eq. 1) for methyl oleate at temperatures above 0 °C. A successive scale-up study showed that stirring agitation did not improve the separation recoveries [67, 68]. In the study where blending was investigated, this method was preferred over winterization to increase the oxidative stability and overall yield [63]. Other investigations consisted of developing a method to predict the necessary winterization temperature for a given reduction ratio of the saturated fraction [66].

$$SF = \frac{mass\ fraction\ of\ C18:1\ in\ liquid\ phase\ /\ mass\ fraction\ of\ FAME\ in\ liquid\ phase}{mass\ fraction\ of\ C18:1\ in\ solid\ phase\ /\ mass\ fraction\ of\ FAME\ in\ solid\ phase} \quad \text{Eq. 1}$$

3.1.3. Oleochemical applications

Strohmeier *et al*. optimized the solvent crystallization temperature (-22 °C), time (4 h) and solvent/oil ratio (10:1 v/w) for efficient separation between saturated and unsaturated FAME [65]. Methyl oleate was purified to 69.8%. Total unsaturated FAME purity increased from 49% to 87.2%, with a yield of 56.0% (%m/m) (calculation not given in paper) and a 99% recovery. The optimized method showed equal or slightly less efficiency for other FAAE derivatives.

3.1.4. Food applications

Vázquez *et al*. investigated the ideal solvent crystallization properties for FAEE and FA mixtures of modified soybean oil to obtain high purity stearidonic acid (SDA) [61]. SDA is a metabolic intermediate in the conversion of ALA to EPA [138]. Results showed that crystallization of FAEE was most successful when using solvents with high polarity.

3.2. Urea complexation

3.2.1. Research and methodology

Urea is an additive which is widely used for solvent crystallization of oil mixtures and derivatives thereof. It is an organic compound that crystallizes differently when mixed with an alcohol in an oil mixture, as shown in Figure 3-2. In the presence of straight

alkyl chains, urea crystallizes in a hexagonal structure, forming a spiral-shaped channel.

With this new structure, the aliphatic chains can be captured in the urea complex and be separated from other branched and/or highly unsaturated fractions [140]. Therefore, urea complexation is often perceived as the most suitable method for omega-3 PUFA enrichment. Already in the 1950s, it was demonstrated that urea complexation can be used to fractionate FA and derivatives from seed, fish and other oils [141]. The separation is more efficient compared to crystallization; large amounts of oil mixtures can be processed using standard equipment, and relatively cheap solvents – e.g. methanol, ethanol and hexane – can be used. In addition, the process does not require cooling to temperatures below 0 °C and the overall cost is lower. It should be noted that the urea complexation protects the omega-3 PUFA fraction from autoxidation [142, 143]. A drawback is the formation of alkyl carbamates, which are possible carcinogenic compounds [144, 145]. According to Guil-Gerrero *et al.*, the PUFA recovery is maximal at about 4 °C, a temperature particularly suitable for SDA and DHA concentration. For EPA purification, crystallization at around 20-28 °C is preferred [142]. On the other hand, Wille *et al.* [146] reported that SDA and DHA are concentrated more efficiently in the filtrate when crystallization occurs at −5 °C, As for total omega-3 content and EPA this temperature is 10 and 15 °C, respectively.

Urea complexation is mostly used in combination with other techniques which are more suitable for the purification of a specific type of FAAE. Ratnayake *et al*. presented, already in 1988, a pilot plant scale for omega-3 PUFA ethyl ester concentrates using urea complexation and short-path distillation. Another example is the patent by KD Pharma where urea complexation and liquid-liquid chromatography are used for FA and FAAE fractionation [147]. In general, the technique is used for FAAE purification for biodiesel and food applications. In the context of food applications, a significant amount of research has been performed on SDA, EPA and DHA purification in fish oils. Nowadays, new research focusses mainly on the purification of new types of oil streams not only in biodiesel and food applications, but also in the oleochemical and industrial application fields. The formation of ethyl carbamate during the urea complexation process was also investigated [73].

3.2.2. Biodiesel applications

Bin *et al*. studied the urea complexation of corn oil [69]. Here, optimal process parameters were 1:1 (w/w) urea/FAME ratio, 5:1 (w/w) methanol/ FAME ratio, 20 °C crystallization temperature and 2 h crystallization time. The research showed

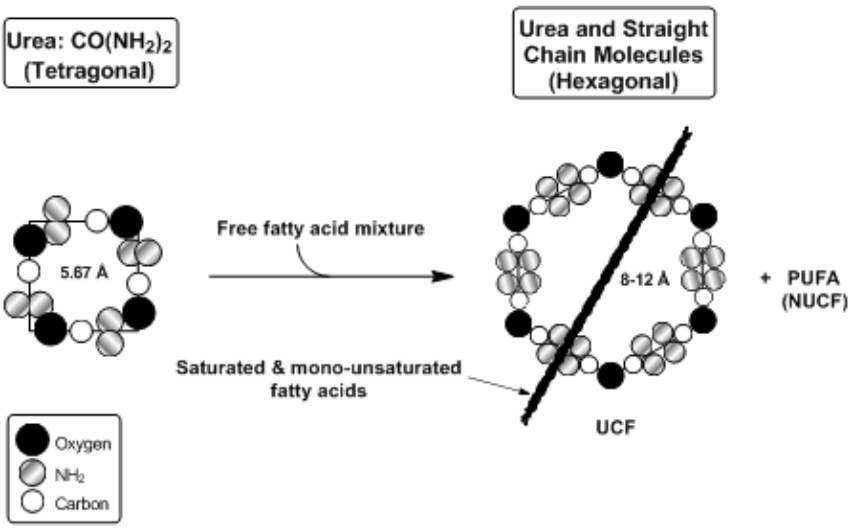

Figure 3-2. Visualization of urea complexation. UCF = Urea Complexation Factor, NUCF = Non-Urea Complexation Factor. Image reprinted from Shahidi, F. and Wanasundara, U. N. 1998, Trends Food Sci. Tech., 9, 230 with permission from Elsevier.

that sufficient crystallization occurred already at 20 °C. Interestingly, the yield of unsaturated FAME from the non-complexing mixture decreases significantly with increasing urea/FAME ratio. The maximum yield was 53% and the purity of unsaturated FAME increased from 74.6 to 98.8%.

3.2.3. Food applications

Zhang *et al*. determined the optimal urea/FAEE ratio (0.75:1), ethanol/urea ratio (2:1) and complexation temperature (65 °C for 30 minutes) for optimal EPA ethyl ester (EPA-EE) and DHA ethyl ester (DHA-EE) recovery from fish oil [70]. Yield was 56.4% and the PUFA purity increased from 30.0% to 60.6%.

Wu *et al*. optimized the urea complexation process parameters for the concentration of DHA from microalgae [74]. These parameters include a 5.8:1 urea/FAEE ratio, a crystallization temperature of 6.0 °C and crystallization time of 38.2 h. DHA purity increased from 35.1 to 76.0% with a recovery of 41.2%. Response surface methodology was used for process optimization and the model was successfully experimentally verified.

Zheng *et al*. investigated the optimal conditions for urea complexation of seal oil ethyl esters [77]. Optimal parameters were 2.38:1 urea/FAEE, 15 °C and 2.5 h of crystallization temperature and time. Yield of PUFA-EE, which consisted mainly of EPA-EE, docosapentaenoic acid ethyl ester (DPA-EE) and DHA-EE, was 71.4% with 82.6% recovery. Although no scale was mentioned in the paper, the authors claim that the seal oil has interesting EPA, DPA and DHA compositions for use at large scale. It should however be noted that the large-scale usage of seal oil can raise ethical questions.

Rincón-Cervera *et al.* investigated the concentration of γ-linolenic acid and SDA in their FFA and FAEE form from *Echium* seed oils [75]. Method performance was only stated by using the concentration factor (this is defined as the %FAAE in the concentrate divided by the %FAAE in the original oil mixture). The concentration factor for γ-linolenic acid ethyl ester was 2.35 and for SDA ethyl ester (SDA-EE) 2.65.

3.2.4. Oleochemical applications

Strohmeier *et al*. (see section 3.1) also included urea complexation of tallow oil FAAE [65]. Their results showed no satisfactory fractionation of unsaturated FAME. According to the authors, it is due to the high number of saturated FA in tallow oil as other urea complexation research is performed on oil sources with lower amounts of saturated FA. The purification of unsaturated FA from waste cooking oil for use as oleochemicals and biochemicals, was investigated by Idris *et al*. [71]. Results showed a purity increase of 55.8 to 88.0% for unsaturated FAME at FAME/urea ratio of 1:0.75.

3.2.5. Industrial applications

Jiang *et al*. optimized the urea complexation parameters of unsaturated FAME from soybean oil [72]. These parameters included urea/FAME ratio, solvent/FAME ratio, crystallization temperature and time. The parameters were further optimized using Box-Behnken design and response surface methodology (RSM). The results followed the same trends as the results of Bi *et al*. [69]. The optimal yield and purity were 58.8% and 98% respectively at a complexation temperature of 0 °C and a urea/FAME ratio of 1.23:1.

As for the investigation on ethyl carbamate formation during the urea complexation process, Vázquez *et al*. investigated sunflower, *Echium* and fish oil [73]. Herein, the application field was again food, together with the nutraceutical and the pharmaceutical industries. Urea complexation was performed at room temperature and at elevated temperatures. With all three oil sources, the production of ethyl carbamate was higher when high temperature complexations were performed. Nevertheless, the ethyl carbamate could be sufficiently washed out using two water-washing steps. In a successive study, the process was further optimized using *Echium* oil [76]. Pilot scale testing (see Figure 3-3) at room temperature showed that SDA concentration increased from 14.3 to 29.2% with a 78% yield (100 g of final product) without the presence of ethyl carbamate impurities due to sufficient washing.

3.3. Vacuum and molecular distillation

3.3.1. Research and methodology

Vacuum distillation and, in particular, short-path distillation are useful separation techniques for the purification of thermally unstable and high-boiling compounds including TG, FA and their derivatives.

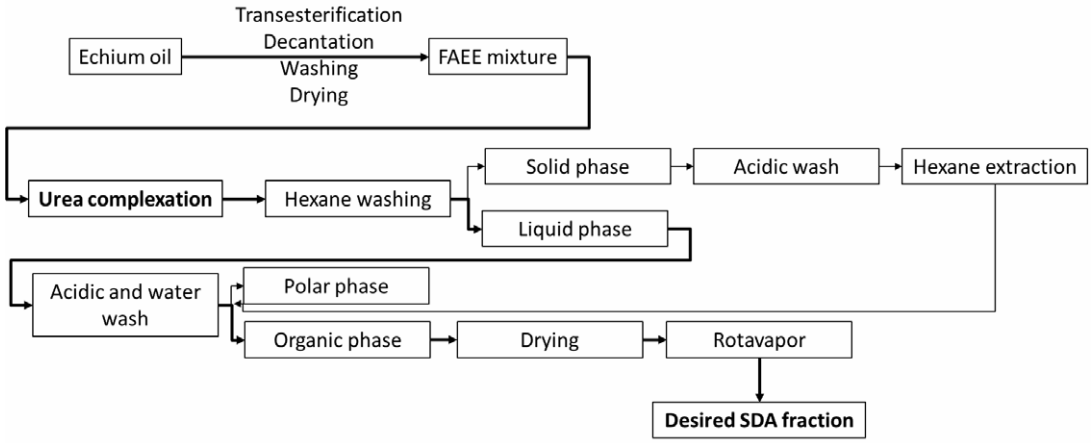

Figure 3-3. Example of a urea complexation flow chart for SDA purification using Echium oil. Information taken from ref. [76].

At vacuum pressures from 10 to 0.1 Pa, the necessary temperature for FAAE fractionation decreases to the range of 60 to 120 °C compared to normal distillation. Due to the high vacuum, FAAE reach the surface of the condenser in a short period of time with a high evaporation velocity, minimizing the thermal effect on the FAAE [148]. Distillation of FAAE has several advantages compared to FA distillation. First, FAAE are less corrosive, have lower boiling points and follow Raoult's law more closely, thus lowering equipment and process costs. The FAAE are also less susceptible to colour formation, decarboxylation and degradation [149]. When lower vacuum is applied (<0.9 Pa), the technique is also called molecular distillation.

Short-path and molecular distillation have already been extensively used for oil fractionation [150]. To date, fractional vacuum distillation of methyl esters is the most widely used distillation process [34]. The technology was stated by European Pharmacopoeia as necessary to remove side products formed during FA(E) fractionation [151]. It is generally used in combination with other technologies like urea complexation and selective enzymatic reactions. However, there are also recent examples using solely short-path distillation steps [152]. There is a clear focus on EPA and DHA from fish oils, as the properties of this technique are particularly suitable for the separation of long-chain PUFAEs from other FAAEs. In the book chapter of Cermak *et al*., reactive distillation is mentioned as an efficient and vital approach to produce biodiesel [149], with an interesting example being a pilot plant for the production of methyl decanoate [153].

Present research involves the purification of specific types of saturated FAAE, originating from coconut oil, with broad application possibilities. As distillation is an established technology, most of the present research concerns theoretical modelling for process optimization of EPA and DHA purification from fish oils for food applications.

3.3.2. Coconut oil purification

Vázquez *et al*. investigated the separation of short chain FA ethyl esters (FAEE) and medium chain FAEE from a mixture of coconut oil and diary fat [78]. Possible applications were structured lipids, nutraceuticals or functional lipids, antimicrobial lipids and emulsifiers. Using the cyclic short-path distillation method, at 60-65 °C and a feed flow of 300-500 g/h, purity and yield of the short and medium chain FAEE mixture were 94% and 45%, respectively. By adapting process conditions, yield could be improved to 80-85%, with a purity of 82-84%. Sitompul *et al*. separated methyl laurate (C12:0) from coconut cream oil using a batch vacuum distillation unit [82]. The purity of methyl laurate increased from 49.1 to 68.8% with a yield of 48.6%.

3.3.3. Theoretical modeling

Rossi *et al*. investigated the optimal distillation temperature in a two stage molecular distillation process using phenomenological modelling [79].

The optimal model gave a purity increase from 29.3 to 80.4% of EPA-EE and DHA-EE with a recovery of 47.8%. The same data set was used for a different model type; artificial neural network modelling or ANN [80]. Although this type of modelling can be a useful tool, it needs a great amount of experimental data and, according to the authors, does not give information about a determined effect. They suggest a combined method with both the phenomenological modelling and the ANN, as this incorporates the advantages of both models. Lancu *et al*. developed a four-step distillation method (two steps vacuum, two steps molecular distillation), as shown in Figure 3-4 [81]. Maximum purity was obtained at 110 °C for FAME (96%) and at 120 °C for FAEE (98.5%). The same technique was used by Enascuta *et al*. on fish oil waste for the separation of omega-3 PUFA ethyl esters (PUFA-EE) from monounsaturated fatty acid ethyl esters (MUFA-EE) and saturated fatty acid ethyl esters (SFA-EE) [83]. Purity was 93% (34.0% and 40.2% for EPA-EE and DHA-EE, respectively) for the PUFA-EE fraction. This fraction was transesterified with glycerol to obtain TG and the SFA-EE fraction could be used as biodiesel.

3.4. Extraction

Extraction is a separation process which is based on differences in solubility. This technique selectively dissolves one or more solutes into an appropriate solvent, separating them from other materials (non-dissolved in the same solvent). Two types of extraction can be distinguished: solid-liquid extraction and liquid-liquid extraction [154]. With oil purification, liquid-liquid extractions are principally used. Here, a solute is extracted from a solution in a certain solvent by using another solvent. Applications range from sample preparation for analytical research to large scale separation. Extraction is mostly used for extracting the oil content from plant seeds. Research on FAAE fractionation with the aim of large-scale separation *via* non-supercritical extraction conditions is limited and relies on the ability of unsaturated FAAE to form complexes by coordinating to silver ions [155]. Recent research comprises the liquid-liquid extraction of FAEE in microdevices and in ionic liquids, which are both not widely used technologies. Both techniques rely on the presence of silver ions in the extraction phase.

For the use of omega-3 FAAE in food applications and medical supplies, Kamio *et al*. investigated the kinetic parameters and modelled the use of slug flow prepared by a microreactor for the purification of DHA-EE as shown in Figure 3-5 [84]. As only pure DHA-EE samples were used, no yield or purity could be calculated. The authors suggested a parallel set-up of different microreactors for a continuous process in a microchemical plant. In a second report, Kamio *et al*. compared the use of slug flow with emulsion in a microreactor [85]. As the extraction rate was identical in both cases, the slug flow was preferred as it did not require an emulsion stabilizer.

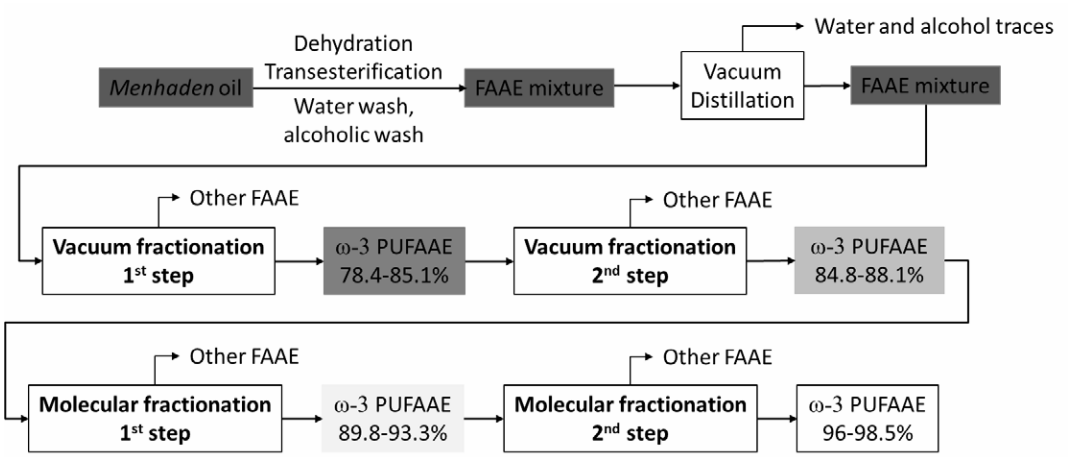

Figure 3-4. Example of a process for EPA and DHA concentration using vacuum and molecular distillation on pilot plant scale. Information taken from ref. [81].

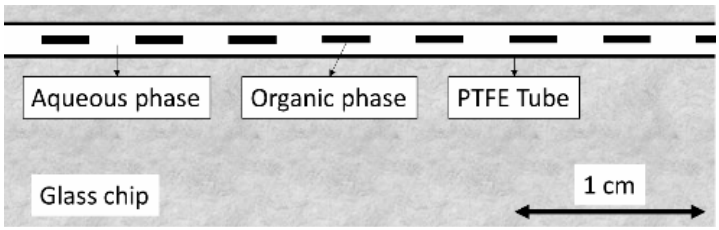

Figure 3-5. Picture of the slug flow in the microreactor. Picture adapted from ref. 84.

3.4.1. Ionic liquids

Ionic liquids (IL) are salts that are liquid at room temperature. They can act as a solvent for both inorganic and organic compounds, making a solution of otherwise immiscible reagents possible. Conveniently, they are immiscible with numerous organic solvents which make it an interesting polar alternative for two-phase systems. In addition, their extremely low volatility is considered a green property [156]. However, when looking at the manufacturing process of ionic liquids, the large number of steps and the non-renewable reagents necessary for their synthesis, several questions can be raised on the "greenness" of these solvents [157].

Despite these drawbacks, IL as extraction solvents for FAAE separation have recently been extensively investigated by Li M. *et al*. They published several reports on the use of (silica supported) ionic liquids, coated with silver for the purification of EPA methyl ester (EPA-ME), DHA methyl ester (DHA-ME), methyl linoleate and methyl linolenate for food, pharmaceutical and biodiesel applications. Their equilibrium studies showed that the extraction capability of IL containing silver salts was higher than the extraction capability of a water-silver salt system [87]. First, they purified EPA-ME and DHA-ME from cod liver oil using an IL-containing AgBF4. Purity of this fraction increased from 18 to 82.1% [86]. Second, the IL was immobilized onto silica which gave higher extraction capacities and improved reusability when compared to the same process without silver. An example of this silica coated IL is shown in Figure 3-6. EPA-ME and DHA-ME recovery was 93.5% at small scale (9.3 mg in 1 mL hexane) and 82.6% at larger scale (875 mg in 30 mL hexane) [88]. Third, they improved the IL and the extraction method. These adaptions increased the EPA-ME and DHA-ME

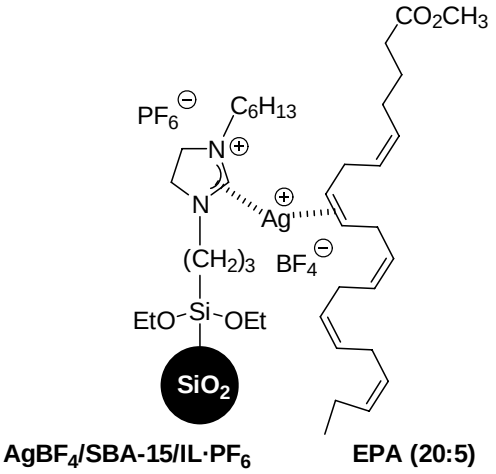

Figure 3-6. Schematic drawing of a silica-coated IL interacting with a PUFAME. Structure reprinted from Li, M., Pham, P. J., Pittman, C. U. and Li, T. 2009, Microporous Mesoporous Mater., 117, 436 with permission from Elsevier..

purity from 18 to 90.6% with a recovery of approximately 60% [89].

To improve the biodiesel quality, extraction of methyl linoleate and methyl linolenate from soybean-derived biodiesel was investigated by the same research group [90]. Solid phase extraction was used to remove the PUFA methyl esters (PUFA-ME) from the biodiesel mixture. After desorbing the PUFA-ME using 1-hexene the purity of methyl linoleate increased from 8.5% in the biodiesel mixture to 90% in the stripping solvent with a recovery of 95.3%.

Noteworthy, the removal of silver after extraction and its cost need to be considered. Silver is an expensive element and its presence can be an undesired component in the final product. Therefore, Cheong *et al*. investigated the possibility to use IL containing aromatic rings without silver for

omega-3 PUFA ethyl ester (PUFA-EE) enrichment of salmon oil for food applications [91]. Their research showed an increase of selectivity from 2.6 to 10, based on the ratio of the distribution coefficient of omega-3 compounds to non-omega-3 compounds. The extraction efficiency was, however, enhanced by adding AgBF4 to the IL, together with increasing the volume ratio of IL/solvent and the usage of 1-hexene as stripping solvent. Purity increased from 82% to 89% in the optimized method using multi-step reverse extraction. Extraction of PUFA-ME, using IL without silver salts, was also investigated by Li X. *et al*, for biodiesel applications [92]. Here the selectivity was 11.7 and this was highly influenced by the IL counter-anions. For upscaling, fractional extraction was simulated. The simulation showed no negative influence of the extraction stages (8 in total) on the purity and recovery.

3.5. Chromatography

3.5.1. Research and methodology

Chromatography technologies use differences in molecular affinity and solubility for fractionation purposes. The separation is based on three different set-ups: gas-liquid, solid-liquid and liquid-liquid separations. Gas-liquid, which covers gas chromatography analysis, is not used for large-scale production of FAAE and will therefore not be discussed here. Solid-liquid and liquid-liquid chromatography separations are used for analytical purposes, semi-preparative and large-scale implementations. For the two latter applications, an overview of recent developments is given in this review. As this technology is versatile and has a broad range of process set-ups, only a brief overview is provided without in-depth explanation.

Chromatographic techniques such as counter-current chromatography (CCC), reversed phase RP (RP-HPLC) using a C18 column, silver-ion HPLC (Ag^+-HPLC), silver-ion solid-phase extraction (Ag^+-SPE), Ag^+-silica gel TLC (Ag^+-TLC) and Ag^+-silica gel open column chromatography (Ag^+-CC) have been used for separation and purification of individual long-chain PUFA-ME [158]. Most of these techniques have also extensively been investigated for FAAE fractionation. Already in 1978, Teshima *et al*. [159] developed a method for the separation of EPA-ME and DHA-ME using silver chromatography and Yamamura *et al*. [160] proved in 1997 the HPLC concentration of DHA and DPA at industrial scale (Figure 3-7).

Also, in 1980, Lubsen and Maag published a patent for the purification of C16 and C18 saturated and unsaturated FAME using a silver-coated resin [161]. For large-scale applications, focus lies mainly on supercritical fluid chromatography (see section 3-6). As for non-supercritical processes, current research focusses on optimization of process parameters and possible new set-ups for PUFA separation of fish and algae oil.

3.5.2. Solid-liquid separations: molecular sieve adsorbents

Lykakis *et al*. investigated the use of a novel type of stationary phase, i.e. silver exchanged zeolites, for the semi-preparative separation of *E/Z* FAME [93]. For biological and nutritional applications,

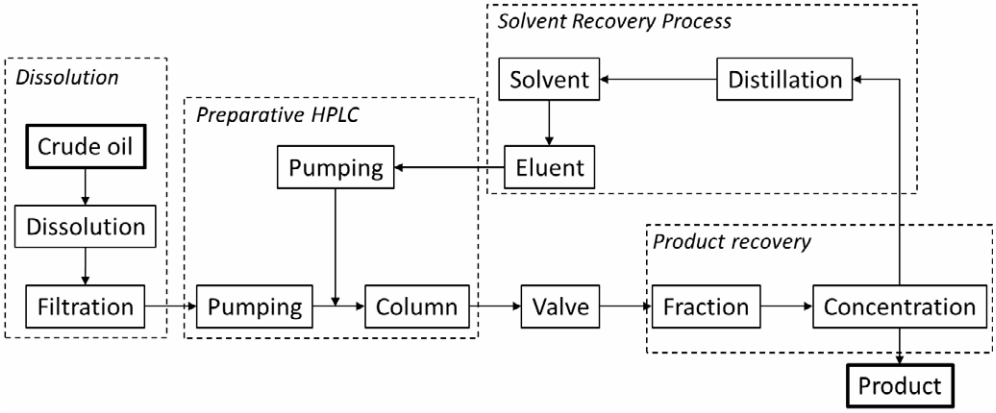

Figure 3-7. A flow diagram of the purification of DPA-EE and DHA-EE. Adapted from ref. [160].

model mixtures of mono- and di-unsaturated FAME were investigated. At small scale (3 mg FAME in 1.6 mL solvent) methyl oleate and methyl elaidate were adsorbed at 50 °C with 30 mg of Ag-coated zeolite for 5 h. Purity of the methyl oleate increased from 50 to 63%. In order to facilitate further characterization in the upscaling process, the authors suggest the use of silver zeolite cartridges in a pre-concentration step. Another example of molecular sieve adsorbents for FAAE separation is the selective adsorption of short-chain FAME from a biodiesel mixture [97].

3.5.3. Liquid-liquid separations: process set-ups

Chakraborty *et al*. used normal phase silver chromatography for the enrichment of methyl linoleate, EPA-ME and DPA-ME from fish oil for food and pharmaceutical products. They also investigated possible improvements of the oxidative stability of the PUFA fraction using seaweed extracts [96]. Their purity increased from ± 74 to ± 96% with a final recovery of only ± 0.6 – 4.4% using argentated silica as stationary phase. Addition of 1% seaweed extracts increased oxidation stability significantly.

The drawback of the aforementioned process is that the operation is performed in batch-mode with low productivity and large solvent consumption. Therefore, Li M. *et al*. studied the possibility of applying a pilot-scale simulated moving bed (SMB) unit for high purity EPA-EE and DHA-EE production [95]. The aim was to use the purified FAEE for clinical trials. The stationary phase was a C18 column, the mobile phase was pure methanol and a model mixture of EPA and DHA was used. With this method, they were able to produce 13.1 g EPA-EE and DHA-EE/L adsorbent/h with a purity of >99%. Solvent consumption was 0.46 L/g product and feed concentration was 100 g/L. Three years later, Dong *et al*. investigated a different stationary phase for separating EPA-EE and DHA-EE using a SMB unit [98]. Methanol was chosen as the best mobile phase, with a resolution of 2.75. A particle diameter of 20 µm is ideal for large-scale preparations with a column temperature of 40 °C. Purity of EPA-EE and DHA-EE was 91.6% and 93.6%, respectively, with a respective recovery of 97.0% and 91.6% and a productivity and solvent requirements of 5.97 g/L.h and 1.52 L/g, respectively.

Another possible process set-up for liquid-liquid separations is counter-current chromatography or CCC. According to Wanasundara *et al*. this technique is synonym for centrifugal partition chromatography [34]. On the other hand, according to the review of Friesen *et al*. [162], these are two different techniques of centrifugal countercurrent separations and these are separate techniques from liquid-liquid chromatography. However, no countercurrent separations of oil mixtures are mentioned in the review of Friesen. In short, CCC is the countercurrent distribution of a solute mixture between two immiscible liquid phases, without the use of a solid support [34].

Li *et al*. described the use of high-speed CCC for the fractionation of FAME from fish oil for food applications [94]. Besides the further investigation of possible scale-up, the isolation of an uncommon FAME (C16:4 *n-1*) is investigated. At a scale of 500 mg FAME, only 420 mL solvent was used for a purity of almost 99%.

In order to increase the throughput and enhance the productivity for scale-up possibilities, Müller *et al*. developed two variants on a multiple injection mode [99]. These alterations in the process were proven to increase the purity of a furan acid methyl ester from a purified DHA-EE fraction (0.9 g to 7.2 g) with lower mobile phase consumption (129 mL/g to 106 mL/g) and lower time necessary for separation (131 min/g to 30 min/g). The authors emphasize the need of a multiple injection mode option in CCC instruments. It should be noted that a lot of research is performed on CCC method development and that although its model molecules are not FAAE, this separation technique might be applicable to FAAE fractionation [162].

3.6. Supercritical fluid extraction and chromatography

3.6.1. Research and methodology

Supercritical fluid extraction (SFE) is an extraction method that uses solvents in their supercritical state. Generally, CO_2 is used as solvent as this is an inexpensive, low-toxicity, non-flammable solvent. By using supercritical CO_2 (SC-CO_2), the fractionation process occurs at low temperatures in inert atmosphere, eliminating possible oxidation

reactions when applied to oil fractionation. Afterwards, the oil fraction can be easily separated from the solvent by depressurizing the system. This avoids expensive evaporation costs after separation. Disadvantages of the technique are the limited selectivity and high investment costs [39].

Supercritical fluid chromatography (SFC) is a liquid chromatography where supercritical CO_2 is used as the mobile phase. Thus, the use of toxic organic solvents as mobile phase is avoided. The high selectivity of SC-CO_2 and the stationary phase can be combined which highly increases the separation efficiency. At large scale, the economic challenges are the specialized stationary phases, decreased separation efficiency during scale-up and throughput. As separation efficiency is highly influenced by particle size and pressure drop across column, the special coated packings for SC-CO_2 are too expensive to use at industrial scale. Therefore uncoated silica is currently used, decreasing the separation performance [163]. The technology works at moderate temperatures generally between 40 and 50 °C. Again, the low temperature reduces the thermal stress on oxidation-sensitive compounds like EPA and DHA. The low viscosity of SC-CO_2 enables the use of long columns with highly selective packing material. Compared to SFE, SFC can concentrate pure FA up to 99%, since the separation is conducted based on chain length and saturation degree [39].

These techniques are used for a broad range of FA separations including oleic acid from squalene [164], triglycerides [165], separation of FAMEs from acylglycerols [166] and GLA from plant oil [167]. Furthermore, supercritical fluids are widely investigated as solvents in the (bio)catalytic reaction from oil to FAAE, for biodiesel production [168]. As the separation efficiency is mainly based on the solubility of the oil fractions, numerous articles cover the solubility of different oil mixtures in supercritical CO_2 at different temperatures and pressures [106, 169-171].

For FAAE separation, KD Pharma patented in 1994 a large-scale industrial SFC technology for the enrichment of omega-3 fatty acids namely the kd-pür® technology, which is shown in Figure 3-8 [172]. In a review by Sahena in 2009, a pilot plant for the separation of EPA and DHA esters in fish oil using SC-CO_2 was described [173]. Here, an example of FAEE separation using SC-CO_2 in a octadecylsilane-grafted silica column was given, showing a total cost of 550 USD/kg DHA and EPA ester concentrate. Another example where SFC is used at industrial scale is that of Bioibérica in Spain offering DHA and EPA with purities >99% [35].

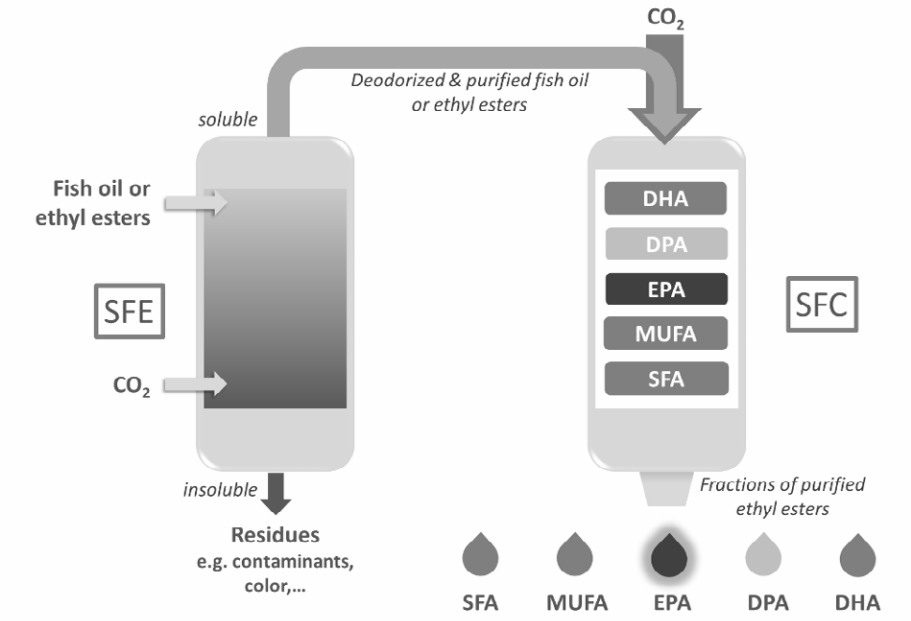

Figure 3-8. Graphic representation of the kd-pür® technology from KD Pharma. Adapted from ref. [174].

Recent research includes mainly the modelling of SFE processes and the model optimization. The modelling is based on the fractionation of fish oil ethyl esters to omega-3 concentrates for the use in pharmaceutical, nutraceutical and food industry. Part of the research comprises the purification of short-chain and medium-chain FA (SCFA and MCFA) from dairy products for food applications. Methods covering the SFC technique were already patented in the 1990s [172] and current research focusses mainly on optimizing key parameters of that process.

3.6.2. Process optimization

Chen *et al*. purified conjugated linoleic acid ethyl ester (CLA-EE) from biotechnological unpurified CLA-EE using SFE [101]. Influence of pressure and temperature on the yield was investigated and maximum purity (90%) and yield (± 95%) was obtained at 10 MPa and a temperature gradient of 11 °C (46 – 35 °C) at an extraction time of ± 3 h and a sample loading of 100 g.

Montañés *et al*. developed a one-step SFC process without co-solvent use for semi-preparative isolation and purification of PUFA-EE from fish and algae oils [103]. Different process conditions were investigated, together with stationary phases and their particle size. Results showed nearly 100% purity for EPA-EE from fish oil and 96.6% purity from algae oil. DHA-EE purity was 83.3% with 75% yield. Particle size of the stationary was found to be the most important parameter to consider for upscaling.

3.6.3. Dairy products' purification

Torres *et al*. investigated the separation of SCFA and MCFA (C4-C14) from long-chain fatty acids or LCFA (C14-C18) originating from butteroil using SFE [100]. The optimum conditions were 8.9 MPa and 48 °C or 10.1 MPa and 60 °C with a sample volume of 100 mL. The highest yield was obtained with C6 (56.6-77.7%) with a purity of 15.5-19.8%. Total average purity of the short and medium chain fraction was 63%. Another example is that of Lubary *et al*. who integrated the synthesis of SCFA and MCFA with their extraction from milk fat using SFE [175]. Focus lies on the industrial applications for these types of FAEE, obtained from natural sources, to replace the petroleum-based products. A good balance between a recovery of 77% and a selectivity of 9.7 (see Eq. 2) with a purity increase of 24.5 to 76.3 mol% of SCFA ethyl esters (SCFA-EE) was obtained at 9.1 MPa and 42 °C.

$$Selectivity = Recovery\ of\ SCFA\ fraction / Recovery\ of\ LCFA\ fraction \quad \text{Eq. 2}$$

3.6.4. Model optimization

Maschietti and Pedacchia examined and modelled the possibility of using an internal reflux in a continuous countercurrent fractionation process. The application of the purified omega-3 ethyl esters from fish oil is mainly aimed towards nutraceuticals and pharmaceuticals [105]. The simulation results showed that fractionation of fish oil ethyl esters is possible at lower temperature (50 vs. 70 °C) and lower pressure (13.3 vs. 16.7 MPa) with similar theoretical stages and solvent/feed ratio. DHA and EPA recovery was 95% and purity increased from 29 to 95 mass%.

To lower the workload of modeling, Pieck *et al*. developed a new simplified equilibrium-stage model for the fractionation of omega-3 ethyl esters from fish oil [108]. The viability of the model was checked by using a fractionation column with SC-CO2 at 60 °C and 14.5 MPa at a fixed solvent feed rate of 12 kg/h in continuous mode. Final omega-3 purity was 74%. Influence of solvent/feed ratio on the yield of omega-3 FAEE and on its distribution between raffinate and extract was investigated for use in future economic analyses.

Fiori *et al*. wrote multiple papers on the modelling of omega-3 purification from trout by-products for applications in food, (bio)pharmaceutics and nutraceuticals. Frist, they simulated and designed a multistage continuous fractionation process at preliminary level [102]. Second, they developed a model, based on data in previous papers covering SFE fractionation modeling [102, 176-179], in

Aspen Plus™ which offers a reproducible model for other researchers [104]. Plant design and economic feasibility were investigated. Optimum operation conditions were: column temperature 80 °C, column pressure 19.5 MPa, solvent/feed ratio 63:1 and flux ratio 0.92:1. Purity increased from 18 to 85 wt.% with a yield of 19.0% and recovery of 88.9% for EPA-EE and DHA-EE. The process cost was € 2.3 to € 2.5/kg. Lastly, Fiori *et al*. reported a case study of a fish waste biorefinery concept [107]. A simplified overview of the process is shown in Figure 3-9. The case study was based on a trout processing company in Italy, which produced 870 tons fish waste per year. The omega-3 fraction and fish proteins were purified, and the SFA-EE, SCFA-EE and glycerol were used as biodiesel. When the biodiesel would be used for a combined heat and power unit, the biorefinery could cover the total electricity need and 45% of the thermal energy need. Additionally, an income of € 1,3 million/year from the omega-3 rich oil and € 0.27 million/year from fish proteins was possible. With a total investment cost of € 3.34 million, the study demonstrated that the biorefinery concept could be applicable for the valorization of fish waste.

3.7. Enzymatic methods

3.7.1. Research and methodology

Research on the fractionation of FAAE using enzymatic methods differs somewhat compared to the former discussed technologies. Previously, the transesterification reaction of an oil mixture using a strong base or acid catalyst is performed prior to FAAE fractionation. With enzymatic methods, the catalyst is a lipase, an enzyme specific for lipid compounds. With lipases, the esterification specifically targets (un)desired FA, yielding a mixture of esterified and unesterified FA. FA can be cleaved from TG by selective esterification or hydrolysis, where the desired compounds, long-chain PUFA, can remain bonded to the glycerol backbone (in case of hydrolysis, Figure 3-10) or be present in the FA(E) fraction (in case of selective esterification, Figure 3-11). With both reactions, the desired FA is rarely present in the ester form. This is because the desired FA is often the least easy hydrolysed/ esterified by the lipase. Therefore, with hydrolysis of TG, the FA of interest remains bonded to glycerol, yielding an FAE in the acylglycerol form, as shown in Figure 3-10. Afterwards, it is easy to bind other FA on the glycerol and so forming a

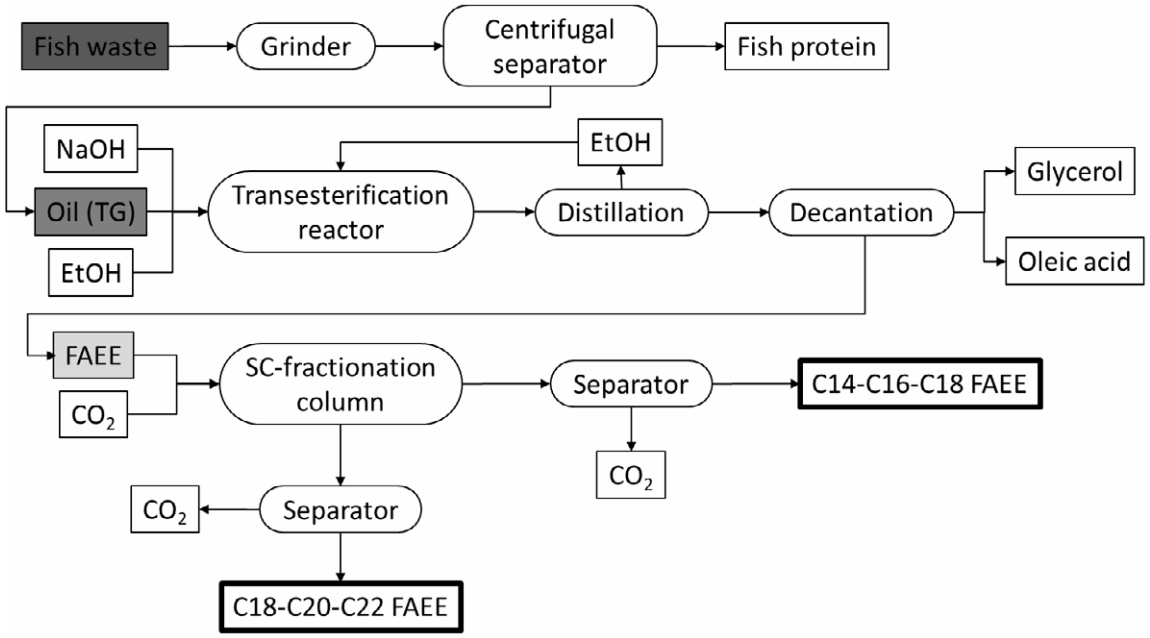

Figure 3-9. Simplified overview of the biorefinery concept for fish waste valorization. Scheme based on information from ref. [107].

reconstituted TG product. This is the most common used technology for omega-3 purification using lipases [39]. The technique holds several advantages compared to the chemical catalysts as it does not require elevated temperatures and no undesired by-products are produced [180].

With enzymatic methods involving FAAE, first the oil is saponified and washed yielding an FFA mixture. Then, the FFA mixture is selectively esterified using an alcohol where the FA of interest remains most often unreacted in the reaction mixture. An overview is given in Figure 3-12. The most common lipase used is that of *Candida rugosa*. As this enzymatic method involves the selective esterification to FAAE, only this type of reactions and their use for FA purification will be discussed here.

The commercial use of lipases is a billion-dollar business that consists of a broad range of applications. These applications include the detergent, food and flavor industries, biocatalytic resolution of pharmaceuticals, esters and amino acid derivatives, making of fine chemicals, agrochemicals, use as biosensor, bioremediation and use in cosmetics and perfumery [181].

CLA isomers, shown in Figure 3-13, are a class of FA with several health benefits, which differ according to the type of isomer. Therefore, isolation of one type of isomer can be interesting for the nutraceutical and pharmaceutical industry. With this aim Jafari *et al*. optimized five factors affecting the esterification of *c*9, *t*11-CLA from commercial CLA oil. The reaction with menthol was catalyzed by Lipase AY-30 from *Candida rugosa* in a solvent-free system [115]. The optimal factors were 23.1 h reaction time, 32.7 °C reaction temperature, 135.4 µmol/min or U enzyme loading, 1:1.7 CLA-oil/menthol ratio and pH 7.7. Although no clear data is given on the final purity of L-menthyl *c*9, *t*11-CLA, the authors stated that 'the lowest purity of c9, t11-CLA in free fatty acid fraction was 8.6%'.

To optimize the esterification process, immobilization of lipases onto a solid support can be used to increase the long-term stability. Therefore Yu *et al*. immobilized *Candida rugosa* lipases onto a MSU-H type mesoporous silica for the selective esterification of CLA with ethanol in isooctane [118]. Optimum conditions were 45 °C reaction temperature, CLA/ethanol 1:1 molar ratio, 6% lipase and 13.3 nm pore size of the silica support. The results showed that the lipase had high

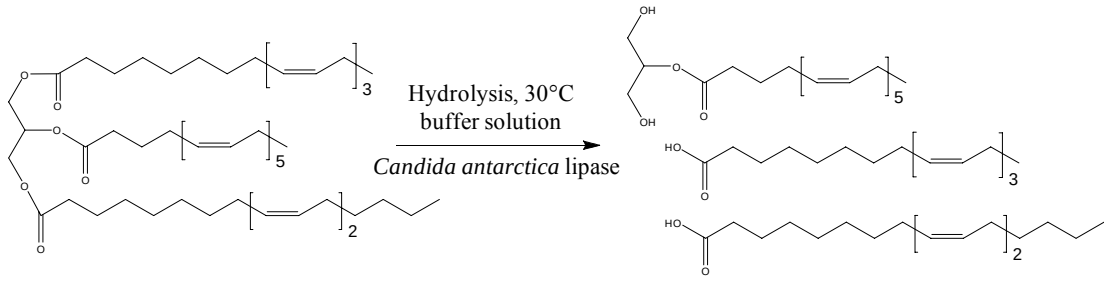

Figure 3-10. Example of a hydrolysis for omega-3 purification using a lipase.

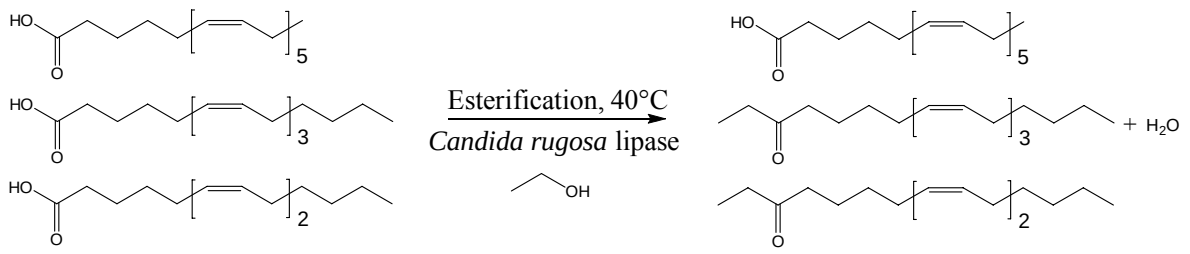

Figure 3-11. Example of a selective esterification for omega-3 purification using a lipase.

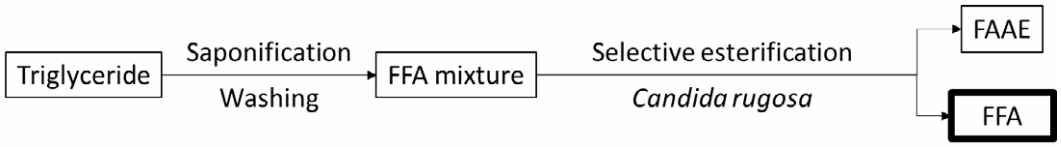

Figure 3-12. Example of a flow scheme using selective esterification for FA purification. In most research, the FFA fraction contains the desired FA compound.

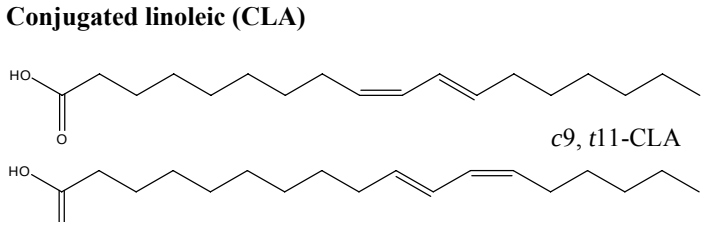

Figure 3-13. Two most common CLA isomers (C18:2). Top: cis-9, trans-11-CLA; bottom: trans-10, cis-12-CLA.

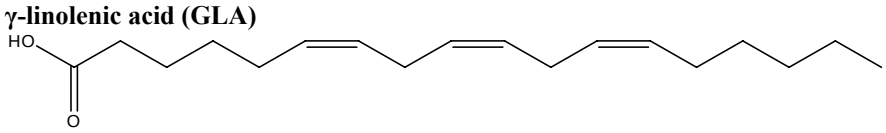

Figure 3-14. γ-linolenic acid (C18:3), an omega-6 linolenic acid.

selectivity towards *c*9, *t*11-CLA with a high operational stability over 48 h.

γ-Linolenic acid, shown in Figure 3-14, is a physiologically valuable FA. In 1998, Shimada *et al*. published a large-scale purification set-up for GLA [182]. Recent research involves the use of new oil streams [112] and TG as starting material for the selective esterification instead of FFA [119]. For the former, Baeza-Jiménez used evening primrose oil for the purification of GLA with one-step esterification [112]. Optimum conditions were 30 °C reaction temperature, 10% enzyme loading from *Candida rugosa*, FFA/butanol ratio 1:10 and 10% molecular sieves added after 36 h. GLA purity increased from 8,87 to 83.7% with a yield of 82.2%. For the latter, Corzo-Martínez selectively purified GLA and SDA from *Echium* oil *via* alcoholysis using *Rhizopus oryzae* lipase [119]. Reaction conditions were 30 °C, shaking 400 rpm, molar ratio 10:1 ButOH/Echium oil and 2.5 % (w/w) water addition. This resulted in a purity increase of GLA from 11 to 18% and SDA purity increase of 14 to 22%.

SDA, shown in Figure 3-15, is a metabolic intermediate in the conversion of ALA to EPA and DHA. It has a lower degree of unsaturation compared to EPA and DHA, making it less susceptible to undesired side reactions and is therefore more interesting for industrial processing. Only recently its purification has been investigated using lipases. Vázquez *et al*. developed a new method for the purification of SDA from modified soybean oil for application in the food industry [109]. Here, 5% (w/w) of *Candida rugosa* lipase was used together with dodecanol in a molar ratio of 1:1 dodecanol/FFA and a reaction time of 4 h. This yielded SDA in the FFA form with a purity increase from 23.7 to 57.8% and a 94% recovery. *Echium* oil is an interesting source of SDA as it contains significantly higher levels of SDA compared to other plant oils. Baik *et al*. therefore developed an enzymatic method for the purification

Stearidonic acid (SDA)

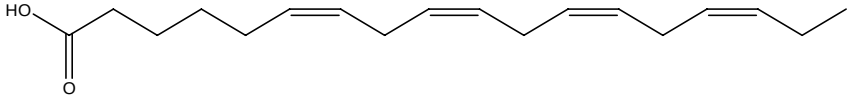

Figure 3-15. Stearidonic acid (C18:4), an omega-3 PUFA.

EPA and DHA

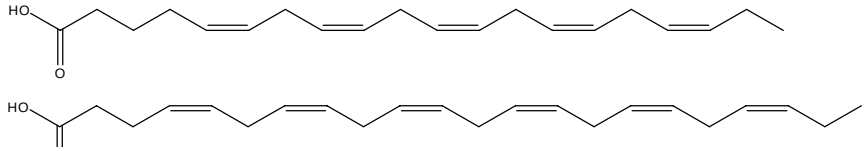

Figure 3-16. EPA (C20:5) and DHA (C22:6), two omega-3 PUFA.

of SDA from *Echium* oil also using *Candida rugosa* lipase [113]. Using a two-step lipase-catalyzed esterification at 30 °C with a water loading of 0.25%, enzyme loading of 2%, SDA could be purified from 14.3 to 54.1% with 74.8% yield.

Industrial applications of the purification processes of EPA and DHA (molecular structure shown in Figure 3-16) are already established and therefore current research focuses on method optimization to improve purity, modelling of the reaction, and the use of other lipases.

Bhandari *et al*. studied the use of *Rhizopus oryzae* for the purification of DHA in the FFA form [110]. The selective esterification was conducted at pH 7, temperature of 35 °C, reaction time 24 h, shaking speed 800 rpm and a 1:1.32 fatty acid/ solvent (isooctane) ratio. The DHA purity increased from 26 to 86.9% with 80% recovery. In a successive study, Bhandari *et al*. modelled this reaction using the Prazeres model, which fitted better the results compared to previous models used [111]. Another lipase, that was investigated for the first time for EPA purification in the FFA form, was *Thermomyces lanuginose* lipase studied by Moreno-Pérez *et al*. [117]. Here, the micro-organism was immobilized hydrophobically onto C18 Sepabeads and reaction occurred at 25 °C for 3 h in ethanol. The EPA purity increased to 80% with a selectivity (molar ratio between synthesized EPA-EE and synthesized DHA-EE) of over 20. To enhance DHA purity, Hong *et al*. used a continuous reactor system [114]. Lipase from *Rhizomucor miehei* was used. Parameters were a temperature of 20 °C, a molar ratio of 1:5 fatty acid/ethanol, a water content of 1.0 % and residence time 1.5 h. This resulted in a DHA purity increase from 21 to 70 wt% and a recovery of 87 wt%. Another way of optimizing the process was investigated by Ma *et al*., which used near SC-CO2 as solvent and also *Rhizomucor miehei* lipase [116]. Near SC-CO2 was used, prepared at 25 °C and 8.3 MPa, together with 0.2 wt% water, enzyme loading of 5 wt% and a reaction time of 18 h. Here, the DHA purity increased from 20.9% to 75.8 wt% and with an 81 wt% yield. Cao *et al*. produced a new lipase from *Trichosporon* sp. and investigated its application in EPA and DHA enrichment [120]. Here, 100 U of the lipase reacted with crude fish oil containing 20 mM Tris-HCl buffer at pH 8.0, reaction temperature 40 °C and continuous stirring. Results showed that the purity of EPA increased from ±18 to 22.9 mol% and DHA purity increased from ±12.5 to 25.4 mol%.

3.8. Membranes

Membranes offer a low-cost, low-energy separation process. The process does not require high temperatures and is therefore ideal for the use of separating thermal labile compounds such as unsaturated FAE. Membrane processes can be divided according to the driving force, the membrane pore size and the MWCO (the molecular weight of the compounds that are retained for 90% by the membrane, shown in Figure 3-17). The driving force can be a concentration difference over the membrane (dialysis) or a pressure

difference over dense membranes (nanofiltration or reverse osmosis, NF and RO respectively).

Until recently, the use of membrane technology for FAAE fractionation was limited to the use of large pore membrane filters after crystallization or urea complexation. Here, the membranes were merely used for solid-liquid separation. New applications of membranes are based on differences in molecular size of the TG, FA and FAE. For example, Ghasemian *et al*. showed several applications on the separation of FA, showing the opportunities of membranes for FAAE separation [183-185].

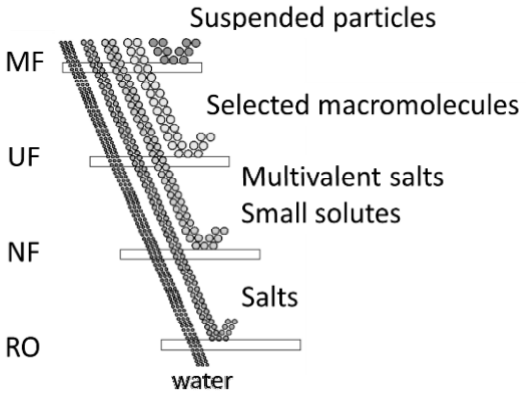

Figure 3-17. Different types of membranes, based on MWCO. (MF = microfiltration and UF = ultrafiltration).

Their implementation to membrane separation is currently limited since all separation processes should be evaluated individually for each solute, solvent and membrane material together with processing parameters and membrane types. However, despite these drawbacks, their application is still increasing [186]. Furthermore, new research is focussing on coupling supercritical fluid extraction with membrane separations [187,188].

Recent literature contains two examples of membrane use for FAAE fractionation, but its research is still at laboratory scale. The first example is that of Wichmann *et al*., who performed a feasibility study for the fractionation of FAME from waste fats [121]. The dialysis equipment contained 30 g of oil, where saturated fatty acids (SFA) were enriched from 28 to 51% in three steps, while reducing greatly the free fatty acid content. For scale-up, the authors stated that the effects are still far from a practical industrial application, due to the low flux and high solvent recovery needed after separation. The second example is that of Gupta *et al*, who. separated stearic acid from unsaturated FA by complexing with triisobutylamine before filtration using polydicyclopentadiene membranes [122]. Graphical representation of this separation is shown in Figure 3-18. Later, the same research group developed epoxy membranes

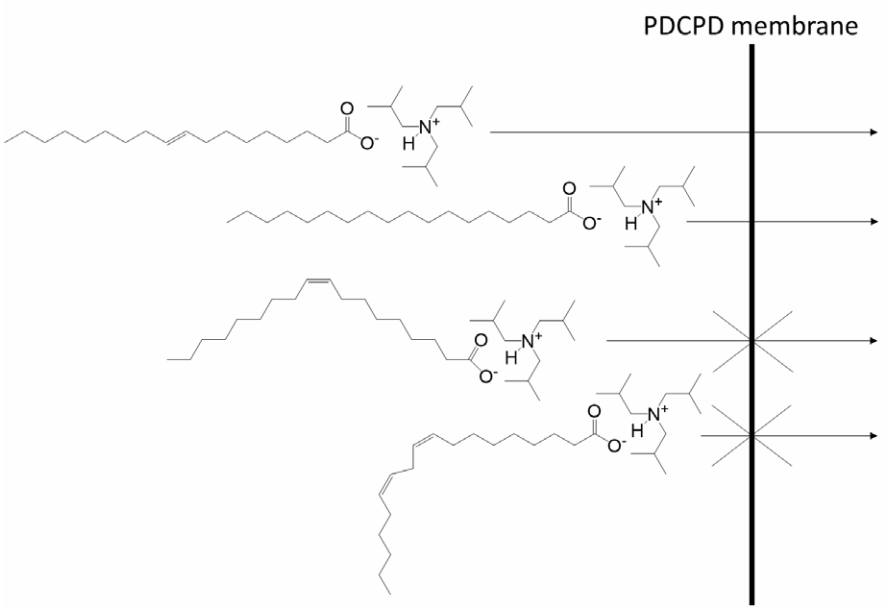

Figure 3-18. Graphic representation of FA separation using membranes. Adapted from ref. [122]

for the separation of SFA from FAME, based on their difference in molecular size [123].

3.9. Combination of different technologies

3.9.1. Research and methodology

FAAE are difficult to fractionate when using a single technique and when the separation is based on only one physical or chemical property. Therefore, the consecutive use of two or more techniques can give higher separation efficiencies. Even with sample preparation for FA profiling, a combination of different technologies can be used [189]. An example of a combination of technologies is shown in Figure 3-19. In the past decade, research on the combination of different technologies focused on alternative sources of FA with nutritional value, interesting fuel properties and pharmaceutical application.

Numerous articles cover the use of marine oils for PUFA concentration, with a focus on EPA, DPA and DHA purification. If possible, the remaining saturated and monounsaturated FAME fraction is further purified for biodiesel use. When combining different separation techniques there is a clear preference for winterization, molecular distillation and especially urea complexation. A possible explanation for this is the easy scale-up of these processes and the fact that their largest application is the production of biodiesel. Recently, a method has been patented by Dubois *et al*. for the selective transesterification of ricinoleic acid to a light alcohol with subsequent separation steps of distillation, centrifugation, decanting, liquid-liquid extraction and/or chromatographic separation of the light ricinoleic acid ester [190].

3.9.2. Fish oil application

Lin *et al*. combined urea complexation and molecular distillation to separate EPA-EE and DHA-EE from sardine oil [124]. Optimal urea complexation conditions were 1.9:1 urea/FAEE ratio and -1 °C crystallization temperature. For molecular distillation, FAEE containing 30% EPA-EE and DHA-EE were processed at 75 °C and 307 rpm for a final EPA-EE and DHA-EE purity of 83.5%. Another example of marine oils for EPA-EE, DPA-EE and DHA-EE concentration is given by Magallenes *et al*. [131]. Here, ray liver oil ethyl esters are fractionated using urea complexation and molecular distillation. The purity of the three FAEE increased from 31.7 to 95.1% with a recovery of 12.0%. Optimal urea complexation conditions were 74 °C dwell temperature, 18 h and a 1:1 FAEE/urea ratio. The molecular distillation process was performed in two stages: at 120 °C and at 140 °C with a feed flow of 1 mL/min, pressure at 0.05 mbar and 200 rpm rotor speed.

The two-step process, investigated by No *et al*. [125], was also used by Kim and Kim for the production of omega-3 FAEE from menhaden oil [126]. The lipase used was *Proteus Vulgaris* K80 which is immobilized onto a methyl methacryl divinylbenzene resin. 86% conversion of menhaden oil to omega-3 PUFA-EE was obtained. The reaction product was further purified to 92% using urea complexation.

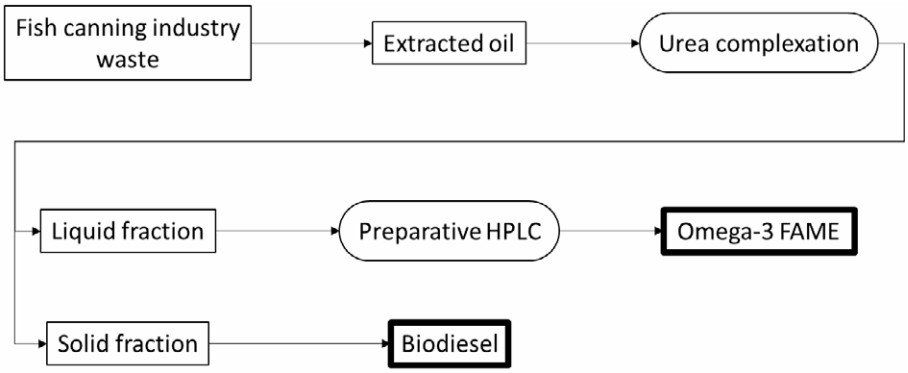

Figure 3-19. Example of a combination of different separation technologies for FAAE fractionation. Adapted from ref. [130]

Yan *et al*. used selective esterification using lipases combined with molecular distillation for the purification of EPA-EE and DHA-EE from low-grade fish oil feedstocks [132]. The two-step process was performed twice: first, NS81006 lipase was used for selective transesterification of the SCFA fraction of 100 g fish oil to ethanol. Then, molecular distillation was used for the separation of the SCFA-EE fraction and the unreacted DHA- and EPA glycerides. With the raffinate, Novozyme 435, immobilized on an acrylic resin, was used for selective transesterification of DHA to ethanol, which was further enriched in a second molecular distillation step. Final conversion ratio was 80 to 100% with a yield of 85% for the DHA-EE fraction.

The FAEE purity can be further enhanced using preparative chromatography. Therefore, Mu *et al*. combined urea complexation with argentated silica gel chromatography [127]. Here, the FA from tuna oil were separated using 3 cycles of urea complexation at a FA/urea ratio of 1:1.6; this increased the PUFA content. Subsequently, the FA fraction was esterified, and the FAME were further purified using argentated silica gel chromatography. Their purity increased from 7.32 to 91.9% for EPA-ME, 3.09 to 48.4% for DPA-ME and 27.3 to 99.5% for DHA-ME. Recoveries were 47.8% for EPA-ME, 66.7 and 70.7 for the two isomers of DPA-EE and 56.7% for DHA-ME. Joseph *et al*. purified long-chain PUFA from shark liver oil using winterization (4 °C with acetonitrile), urea complexation (1:3 FA/urea ratio) and argentated silica chromatography [129]. Long-chain PUFA-ME purity increased from 25.6% to 99.9%. Lopes de Silva *et al*. used the same method for the valorisation of fish canning industry by-products [130]. Both PUFA-ME and biodiesel feedstock (SCFA-ME and MUFA-ME) fractions were obtained. PUFA purity increased from 42.4 to 99.4%. The authors stated that the high market value of the PUFA fraction justifies the costs for upscaling. Lastly, Zheng *et al*. fractionated DPA-EE from seal oil using urea complexation, molecular distillation, silver complexation and preparative HPLC [133]. DPA-EE purity increased from 3.85 to 97.6% with a total yield of 44.8%.

3.9.3. Other oil sources

Dugan *et al*. investigated the use of beef tallow for PUFA-ME concentration combining molecular distillation and solvent crystallization [128]. The study focuses on the separation of SCFA-ME and the concentration of PUFA-ME biohydrogenation products. The latter include rumenic acid methyl ester and vaccenic acid methyl ester, which have potential health benefits, despite the presence of *(E)*-isomer bonds in their alkyl chain. PUFA-ME purity increased from 45.9 to 75.8%. Optimal molecular distillation conditions were 90 drops/min feed rate at 90 °C and 9.3 Pa. For crystallization, a methanol/FAME ratio of 10:1 was used and the mixture was cooled from room temperature to -20 °C at 0.5 °C/min for 4 h. The authors stated that before scale-up further research on the bio-activity of these compounds is necessary to confirm their added value for food/nutraceutical potential.

No *et al*. investigated the enrichment of pinolenic acid (PLA) from pine nut oil by selective esterification using *Candida rugosa* lipase with subsequent urea complexation 125. In this two-step process, PLA was selectively esterified with lauryl alcohol and purity increased from 13 mol% in the pine nut oil to 43 mol%. For the urea complexation, PLA lauryl ester was purified to 100% with a final yield of 8.7%. Optimal conditions for esterification were 0.1% enzyme loading, 10% additional water at 15 °C, followed by urea complexation using 5:1 urea/fatty acid ratio.

4. Conclusions

There are different available techniques for FAAE fractionation and each technique holds its own possibilities and challenges for large-scale separation.

- Both dry and solvent fractionation crystallization are interesting for improving biofuel properties, as it has no high selectivity towards specific FAE. For solvent fractionation, methanol is preferred as it can simply integrate in the biodiesel production process. Other applications areas are oleochemicals and food applications.
- Urea complexation is often combined with other techniques e.g. distillation and chromatography. Recent FAAE fractionation research comprises the purification of new oil streams for biofuel, food, oleochemical and industrial applications. Research on the formation of alkyl carbamates is growing. Due to the use of this technique on new oil sources, the TRL level of most current research is low.

- Distillation applications have already a high TRL level for EPA-EE and DHA-EE fractionation for use as nutraceuticals. When applied to fish waste, the research is still done on a small scale. Present research seems to focus on theoretical modelling for process optimization of EPA and DHA purification from fish oils.
- Non-supercritical extraction processes with the use of ionic liquids are still at low TRL, although some modelling has already been performed. For non-supercritical chromatography, focus lies on the optimization of process parameters and new set-ups for PUFA separation from fish and algae.
- Chromatography for large-scale separation is currently focusing on EPA and DHA esters for healthy food production from fish oils. Emphasis lies on countercurrent chromatography and supercritical fluid chromatography, while research on process set-up optimization for scale-up purposes is still ongoing.
- Supercritical fluid extraction and chromatography is currently the largest research area for FAAE fractionation and is specialized in omega-3 PUFA fractionation from fish oils for use in human food. Recent research includes modelling and modelling optimization of the process. Part of the research comprises the purification of SCFA and MCFA from dairy products for food applications.
- Commercial use of lipases is a large and highly profitable research area. Current research on enzymatic methods focuses on selective esterification of FFA mixtures, whereby the desired compound remains in the FFA phase. Therefore, current research is broad in terms of oil type and desired FA fraction.
- Application of membrane technology for oil fractionation constitutes a small research area; however, it is still growing. Current efforts are focusing mainly on membrane fabrication and coupling with supercritical fluid extraction.
- As seen in this review, when combining different technologies, there is a preference for crystallization, molecular distillation and especially urea complexation. Applications focus on EPA and DHA esters fractionation from fish oil for use as nutraceuticals. Side-streams of these processes are often purified for use as biofuel. Current research at large scale is limited when new types of oils streams are being tested.

With this review, we hope that we have clearly summarized the different techniques and challenges affecting the field of fractionation of FAAE in terms of efficiency and possible upscaling processes. It is clear that there is much potential in optimizing and upscaling all the described technologies, and even though some show more promising results than others, it is our opinion that the way forward will always be with a combination of two or more of these technologies regardless of the targeted product (Table 3-2).

ACKNOWLEDGEMENT

The authors would like to thank Dr. Winnie DeJonghe and Dr. Kathy Elst for proof-reading of manuscript text.

This publication was prepared within the Macbeth project. A European Union funded the project under the H2020-NMBP-Spire-2019 call, grant agreement no. 869896.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

Table 3-2. Overview of the reviewed technologies and their application fields.

Technology	Application field	Focus of recent research	Remarks
Crystallization	Biofuel, oleochemicals, food	Biofuel	Preference for methanol in solvent fractionation
Urea complexation	Biofuel, food, oleochemicals, industrial applications	Purification of new types oil streams	Mostly used in combination with other techniques
Vacuum and molecular distillation	Nutraceuticals, food applications	Theoretical modelling, coconut oil purification	Focus on EPA and DHA purification from fish oils

Table 3-2 continued…

Extraction	Food applications, pharmaceuticals	Microdevices, use of ionic liquids	Non-supercritical research is limited
Chromatography	Food applications	Optimization of process parameters, new process set-ups	Non-supercritical research is limited
Supercritical fluid extraction and chromatography	Pharmaceutical, nutraceutical and food industry	Modelling, modelling optimization, optimization of process parameters	Highest TRL level for current research
Enzymatic methods	Nutraceutical and pharmaceutical industry	New oil streams, novel lipases, method optimization, reaction modelling	Focus on FFA/reconstituted TG product
Membranes	Industrial applications, biofuel	Membrane fabrication	Research at small scale
Combination of different technologies	Nutraceuticals, food, biofuel	Fish oil purification	Preference for urea complexation
Crystallization	Biofuel, oleochemicals, food	Biofuel	Preference for methanol in solvent fractionation
Urea complexation	Biofuel, food, oleochemicals, industrial applications	Purification of new types oil streams	Mostly used in combination with other techniques
Vacuum and molecular distillation	Nutraceuticals, food applications	Theoretical modelling, coconut oil purification	Focus on EPA and DHA purification from fish oils
Extraction	Food applications, pharmaceuticals	Microdevices, use of ionic liquids	Non-supercritical research is limited
Chromatography	Food applications	Optimization of process parameters, new process set-ups	Non-supercritical research is limited
Supercritical fluid extraction and chromatography	Pharmaceutical, nutraceutical and food industry	Modelling, modelling optimization, optimization of process parameters	Highest TRL level for current research
Enzymatic methods	Nutraceutical and pharmaceutical industry	New oil streams, novel lipases, method optimization, reaction modelling	Focus on FFA/reconstituted TG product
Membranes	Industrial applications, biofuel	Membrane fabrication	Research at small scale
Combination of different technologies	Nutraceuticals, food, biofuel	Fish oil purification	Preference for urea complexation

ABBREVIATIONS

ALA : Alpha-Linolenic Acid
ANN : Artificial Neural Network
CC : Column Chromatography
CCC : Counter-Current Chromatography
CFP : Cold Flow Properties
CFPP : Cold Filter Plugging Point
CLA : Conjugated Linoleic Acid
CLA-EE : CLA Ethyl Ester
DHA : Docosahexaenoic Acid

DHA-EE	:	DHA Ethyl Ester
DHA-ME	:	DHA Methyl Ester
DPA	:	Docosapentaenoic Acid
DPA-ME	:	DPA Methyl Ester
DPA-EE	:	DPA Ethyl Ester
EPA	:	Eicosapentaenoic Acid
EPA-EE	:	EPA Ethyl Ester
EPA-ME	:	EPA Methyl Ester
FAAE	:	Fatty Acid Alkyl Ester
FAE	:	Fatty Acid Ester
FAEE	:	Fatty Acid Ethyl Ester
FAME	:	Fatty Acid Methyl Ester
FFA	:	Free Fatty Acid
GLA	:	Gamma-Linolenic Acid
IL	:	Ionic Liquid
LCFA	:	Long-Chain FA
MCFA	:	Medium-Chain FA
MD	:	Molecular Distillation
MF	:	Microfiltration
MUFA-EE	:	Monounsaturated FA Ethyl Ester
MWCO	:	Molecular Weight Cut-Off
NF	:	Nanofiltration
NUCF	:	Non-Urea Complexation Factor
PLA	:	Pinolenic Acid
PUFA	:	Polyunsaturated FA
PUFAE	:	Polyunsaturated FA Ester
PUFA-EE	:	Polyunsaturated Ethyl Ester
RO	:	Reverse Osmosis
RP-HPLC	:	Reversed Phase HPLC
SC-CO_2	:	Supercritical CO_2
SCFA	:	Short-Chain FA
SDA	:	Stearidonic Acid
SDG	:	Sustainable Development Goal
SFA	:	Saturated FA
SFA-EE	:	Saturated FA Ethyl Ester
SFA-ME	:	Saturated Fatty Acid Methyl Ester
SFC	:	Supercritical Fluid Chromatography
SFE	:	Supercritical Fluid Extraction
SMB	:	Simulated Moving Bed
SPE	:	Solid Phase Extraction
TG	:	Triglyceride
TLC	:	Thin Layer Chromatography
UCF	:	Urea Complexation Factor
UF	:	Ultrafiltration
WCO	:	Waste Cooking Oil
TRL	:	Technology Readiness Level

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