

Integrated Supercritical Fluid Extraction and Bioprocessing

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ABSTRACT

Supercritical fluids are increasingly being used and promoted at a laboratory and pilot scale to produce high value, natural bioactives from biologically based raw materials. Supercritical CO₂ is overwhelmingly the solvent of choice for these operations, but is largely limited to the processing of dry raw materials and the extraction of low polarity, low molecular weight compounds. The use of co-solvents and the use of alternative 'near-critical' extraction fluids such as dimethyl ether show potential to mitigate these limitations. Commercialisation of new supercritical extraction processes has arguably been limited because the supercritical extraction process has been developed in isolation of other processing steps necessary to achieve a successful product. This study reviews recent developments in integrated processing that incorporate the use of supercritical fluids for bioseparations and in particular process schemes that produce high value natural bioactives. Integrated processes include prior operation (fermentation, extraction, enzyme pre-treatment, physical fractionation or size reduction) followed by supercritical extraction or fractionation and processes in which operations are carried out in situ in supercritical fluids (supercritical chromatography, enzymatic conversion, precipitation and coating of solutes). The use of co-solvents and alternative extraction solvents in these processes is discussed. Prospects for future developments are also discussed.

Keywords: Supercritical Fluid, Extraction, Fractionation, Fermentation, Enzymatic Conversion, Integrated Processing

1. INTRODUCTION

Supercritical fluid extraction using CO₂ is now an established industrial process for the production of high value natural products. Typical products include hop extracts, decaffeinated tea and coffee, herb and spice extracts, medicinal herb extracts and seed and marine oils (King and Bott, 1992; Martinez, 2008). More recent examples include extraction processes where an undesirable component is removed, such as cork taint from corks for the wine industry (Taylor *et al.*, 2000) and pesticides from medicinal herbs such as ginseng (Quan *et al.*, 2004). CO₂ is the solvent of choice for all these processes. In general, CO₂ is a relatively poor solvent, but is highly selective for non-polar lipophilic

compounds. The solvent properties of CO₂ towards classes of natural products have been described extensively elsewhere (Gupta and Shim, 2006). Solubility generally decreases for a class of compound with increasing molecular mass and at fixed molecular mass decreases with increasing polarity of a compound. To process compounds that are more polar, a wide range of polar co-solvents can be used, although for products that are used in dietary and cosmetic applications, the range of options is generally restricted to non-toxic food-acceptable solvents such as ethanol and propanol. It is possible to carry out extractions using other liquefied gases, such as propane and dimethyl ether (DME), under conditions close to their critical point. These gases, when liquefied, are good solvents for lipophilic compounds

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with substantially greater solubility limits than CO₂. In the case of DME it will extract polar lipids (Catchpole *et al.*, 2008; 2009a; 2010) and to some extent water (Yano *et al.*, 1978; Catchpole *et al.*, 2007a; Pozo and Streett, 1984), making it a flexible solvent for many bioprocessing operations. Both propane and DME are suitable for food applications and are recognized as approved solvents for use in some countries including New Zealand and Australia. Use of liquefied gases, or near-critical fluids, has some similarities to use of supercritical fluid CO₂, including low temperature operation, easy separation of solvent from product due to the large difference in volatility, high solvent recyclability and low solvent residues.

1.1. The Need for Integrated Bioprocessing

Despite the benefits and flexibility of supercritical and near-critical solvent systems in bioprocessing it is advantageous and in many cases necessary, to carry out additional processing operations either before or during supercritical fluid processing and to consider it as a whole integrated process. This approach allows the overall process to be tailored towards better overall fractionation, targeting of specific compounds or product properties and utilization of the supercritical fluid solvent state to enhance or create new processing opportunities that could not be realized in conventional solvent systems.

Integrated bioprocessing operations are divided into four categories here:

- Upstream fractionation. In this case biological materials are pre-processed by solvent extraction or physical separations to produce a partial concentration of desired product compounds, to remove compounds (for example carbohydrates) that may inhibit downstream processing, or simply to dry or mill material to a suitable form
- Production of tailored feedstocks by micro-organisms. This is a rapidly growing field for renewable and high intensity production of a wide range of biochemicals for health, dietary, materials and also fuel applications. This new field has associated bioprocessing challenges, particularly with extraction and separation of compounds from dilute aqueous fermentation biomass
- Pre-processing by chemical or enzymatic reaction. Biological compounds of interest are often strongly bound to cellular structures and chemical or enzymatic treatment can be an effective way of facilitating extraction. Some compounds of interest can also be processed more effectively in a different

form, for example omega-3 fatty acids must be separated from the glycerol backbone if they are to be processed individually and ester forms of fatty acids are generally easier to solubilise

- In situ processes. This includes adsorption or reaction operations that are carried out under supercritical (or near-critical) solvent conditions. The supercritical fluid solvent acts as a high mass and heat transfer solvent system with controllable solvent density ideal for many adsorption/desorption operations. In the case of enzymatic processing, continuous processing is possible and continuous removal of reaction products gives potential for more complete reaction to occur

A description and examples of these types of integrated operation is given in this study.

1.2. Supercritical Fluid Systems for Integrated Bioprocessing

Figure 1 shows typical flow diagrams that could be applied to an integrated process. Pre-processed feed materials are either liquid solutions, fluids with suspended solids, or dry materials. Three supercritical fluid contacting arrangements are shown.

Arrangement (i) is commonly used in the extraction of solid feed materials. A pressure vessel is loaded with a basket or canister that contains the solid feed material but allows the passage of liquid or supercritical solvent to pass through the bed of solids until the bed is depleted of soluble material. The loaded solvent phase passes optionally to a further processing step, or is depressurized through one or more stages to separate the solvent from extracted material. Solvent flow can be either up flow or down flow through the bed. Co-solvents can be premixed with the solvent stream as required. Multiple vessels can be operated in sequence to achieve semi-continuous batch processing, or with some modification a fully continuous solids transfer system may be suitable for some low pressure applications (Waibel *et al.*, 2008).

Arrangement (ii) is used for processing liquid or slurry feed solutions. The contacting can be carried out as either a stirred tank or a counter current column with or without internal packing. The feed solution is typically dissolved in a solvent that has partial or complete miscibility in the supercritical fluid. When the feed solvent has limited solubility and is fed at a sufficiently high ratio to the supercritical fluid flow, then two liquid phases will develop and a continuous recovery of a liquid raffinate phase may be possible. If under-

saturated conditions are used the solvent phase will mix completely with the supercritical fluid, causing solutes that are not soluble in the supercritical fluid to precipitate. In either case, the supercritical fluid is typically a non-solvent for at least one solute in the feed solution and the process is referred to as an 'antisolvent' process. It is a very versatile processing operation. The solvent and supercritical fluid phases can be selected to give high solubility in the feed stream and rapid contacting in the supercritical fluid gives a high process throughput. Very high supersaturation can be achieved in operations involving nucleation and precipitation of solid solutes, known as the Supercritical Antisolvent (SAS) process. Simultaneous fractionation of compounds can also be achieved if some solutes are soluble in the supercritical fluid + solvent phase that is formed (Catchpole *et al.*, 2009b).

Arrangement (iii) is used for loading a feed solution onto a packed bed using a supercritical fluid as a carrier phase or as an eluting phase. This can be used for contacting a solute laden solvent phase with a solid packing for adsorption or chromatography, or the solid phase could be an enzymatic, ion exchange, or catalyst bed. Multiple vessels can be used for batch processes like adsorption/desorption to achieve semi-continuous operation.

These contacting arrangements can potentially be used in sequence. The feed stream to an adsorption bed, for example, could itself be a supercritical fluid solution from a previous packed bed extraction step.

The right hand side of **Fig. 1** shows a three stage separation process. Because a supercritical fluid has a continuously variable density with pressure it is possible to reduce the pressure (and density) in stages and fractionate some compounds from a mixture. Larger molecules and slightly polar compounds that are poorly soluble in CO₂, will separate at high pressures. At medium pressures shorter chain molecules can be recovered and finally co-solvents and volatiles like plant essential oils can be recovered at the lowest pressure. After the third stage the supercritical fluid solvent is in a gas state and is usually condensed and directly recycled. Extracts recovered from the separators will contain some dissolved supercritical fluid which could be recovered in a secondary depressurization step, or simply flashed to atmosphere. Co-solvents recovered from the separator may be able to be directly recycled to the process in some applications.

2. UPSTREAM FRACTIONATION OF BIOMASS

The efficacy of supercritical extraction for recovery of valuable compounds from large volume and/or feed

material with low concentrations can be greatly improved by combination with an effective upstream fractionation process. This can include very simple operations such as removing husks, shells, or skins, that do not contain the desired solutes and straining, pressing, or centrifuging high water content materials to give an aqueous phase and a residual solid biomass which may be concentrated in oils and low polarity compounds. Drying is also important to many extraction processes. The type of drying method used can affect the stability and quality of the product. Higher temperature operations such as vacuum evaporation or spray drying are suitable in some cases. Freeze drying is an alternative to higher temperature evaporation for compounds that are thermally labile but it is an expensive process to carry out and some protein based substrates, or live microorganisms, may still be damaged.

More complex upstream fractionation, followed by supercritical processing, can include any combination of chemical and physical processing operations. Five process technologies are described below: physical processing (drying, pressing and centrifuging) of wet feed materials; solvent extraction of biomass followed by supercritical antisolvent fractionation of the solution to yield a broad range of compounds; supercritical antisolvent production of salts and proteins from aqueous solutions, use of a solvent-based urea fractionation to concentrate Polyunsaturated Fatty Acids (PUFA) followed by supercritical antisolvent fractionation to recover the PUFA and physical processing of dairy streams to concentrate desired lipids, followed by supercritical extraction or fractionation to recover the lipids.

2.1. Drying, Pressing and Centrifuging

CO₂ is a hydrophobic solvent, which, with a few notable exceptions such as the decaffeination of green coffee beans (Lack and Seidlitz, 1992), cannot be used to extract wet biomass. Therefore most raw materials need to be dried and then ground before they can be extracted. While both operations are simple, they are critical in obtaining a high yield of extract and to minimization of degradation. Surprisingly little effort is put into optimizing either or both of these operations at an industrial scale. Drying technologies can be categorized as thermal (use of hot air and/or heated surfaces at atmospheric pressure, e.g., spray drying, oven drying, refractance window), vacuum (e.g., freeze-drying) and chemical (use of a dehydrating solvent, including use of CO₂ or DME).

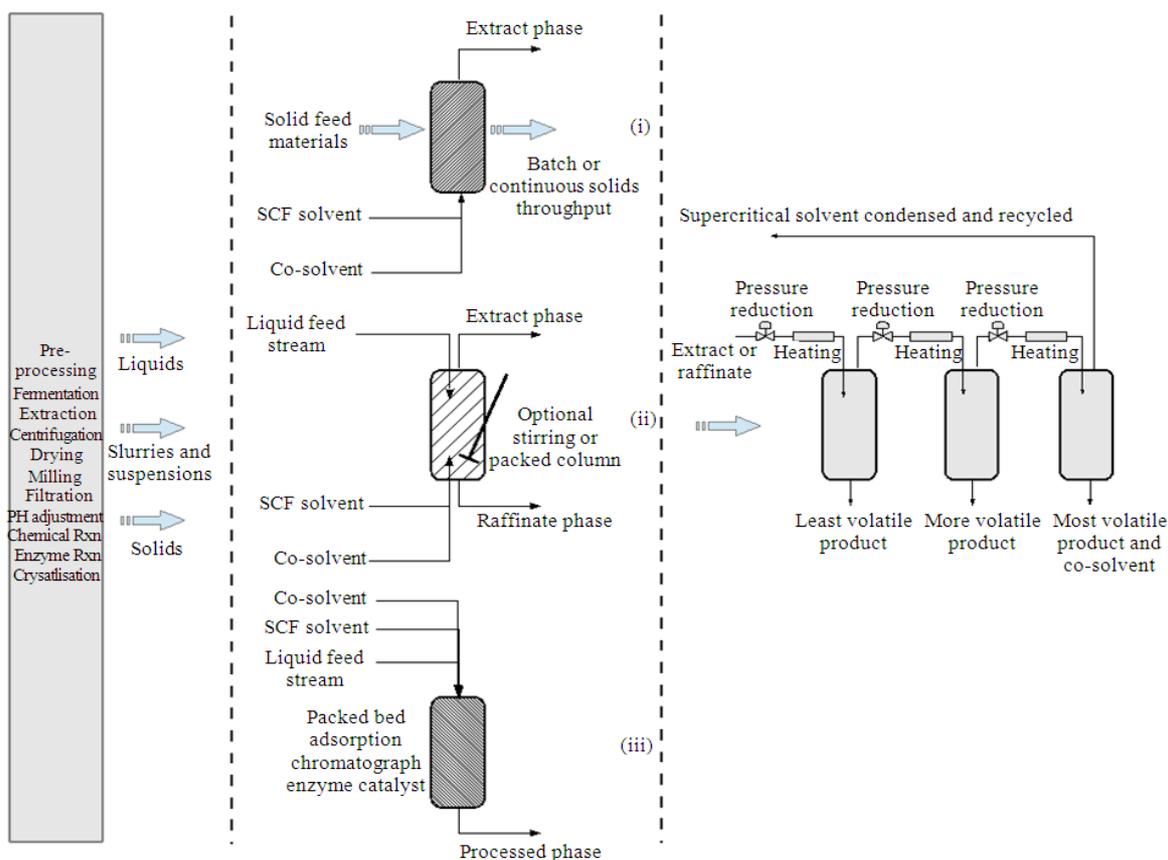


Fig. 1. Supercritical fluid processing scenarios that can be integrated with other bioprocessing operations

Freeze-drying is the most energy and capital intensive means of drying, but results in the lowest degradation of compounds that are sensitive to heat, light and/or oxygen. It is therefore the method of choice for drying materials that have high levels of polyunsaturated lipids or lipophilic antioxidants. The freeze-dried material must be packaged as quickly as possible after production, as the porous nature of the dried product allows very good access to oxygen. The dried material is usually very low density and must be ground before extraction.

Spray drying is the most commonly practiced drying technology and is used at a very large scale to produce powdered dairy products. The particles produced are in general not porous and the amorphous outside shell of the particle can be a strong barrier to mass transfer, although in many cases the powder produced can be extracted without further processing. Many plant materials are air dried, or are naturally low in water content, such as seeds.

The extraction of compounds from wet biomass using CO₂ is possible when the solutes are highly soluble in CO₂ (e.g., flavours and fragrances) and the water content has been reduced to the point where it does not provide a barrier to mass transfer. The acidic nature of CO₂ when dissolved in water needs to be taken into account if acid-labile materials are to be extracted. The dissolution of CO₂ into the aqueous phase of plant materials with a very high water content can also cause the material to become mucilaginous and for the material to ‘cook’ when heated in CO₂ above room temperature.

We have overcome some of these issues by developing a “squeeze and extract” process which has been applied to a number of herbs and vegetables to make them amenable to CO₂ extraction. The plant material is firstly coarsely ground using a mincer or knife mill and is then passed through a dejuicing screw press or alternatively is centrifuged. The juice phase normally has little to no CO₂ extractable material. The residual partially dewatered plant material is then

extracted with CO₂. This process has been applied to the extraction of oleoresin from celery and oregano (Catchpole *et al.*, 2004a); sulphur-rich compounds from onions; oleoresin and essential oil from sage and gingerols and essential oil from ginger root.

2.2. Supercritical Antisolvent Fractionation Technology (SAFT)

SAFT is an extremely versatile process. In general terms, an upstream process takes place using an organic solvent system consisting of a water-miscible organic solvent and water and the solution resulting from this operation is then processed using a super-, or near-critical solvent. The water content can range from zero to ~100% and is optimized according to the range of compounds to be extracted and the supercritical fluid used to fractionate the mixture thus obtained. Examples demonstrating the breadth of the possibilities that we have investigated are shown in **Table 1**. They include solvent extraction of a plant or animal material followed by SAFT (Catchpole *et al.*, 2009b; 2006); urea fractionation of fatty acids (or derivatives thereof) followed by polyunsaturated fatty acid (PUFA) recovery (Eltringham and Catchpole, 2008; Catchpole *et al.*, 2002) fractionation of propolis tincture (Catchpole *et al.*, 2004b), fractionation of phospholipids (Catchpole and Tallon, 2006) processing of aqueous streams containing protein and lipid (Catchpole *et al.*, 2007a; Fletcher *et al.*, 2003) and processing of algae in water or ethanol/water with DME extraction of lipids.

The principles of operation are simple (Catchpole *et al.*, 2009b). The organic solvent system is chosen such that it is capable of dissolving a broad spectrum of components and for practical purposes often consists of

ethanol/water mixtures. The solvent mixture itself is usually only partially miscible with the near-critical fluid. The portion of the solvent that does dissolve in the near-critical fluid acts as a co-solvent, increasing the solvent power of the near-critical fluid. The most polar compounds that were previously dissolved in the organic solvent phase are precipitated from solution, along with at least part of the water present in the solvent phase. If supercritical CO₂ is used as the near-critical fluid, the solutes dissolved in the near-critical phase can then potentially be separated into two or more extracts. Further modifications to the general process include a water-wash step, which can be used to wash medium polarity solutes out of the near-critical phase for CO₂ based processes and a temperature change step to remove water from DME as the solubility of water in DME decreases with decreasing temperature (Pozo and Streett, 1984).

2.2.1. Hydroalcoholic Extraction of Plant Antioxidants: Recovery of Diterpenes from Sage and Rosemary

The extraction and recovery of diterpene antioxidants (carnosic acid, carnosol and methyl carnosate) is an exemplar process using this technology. The initial extraction of rosemary or sage leaf is carried out with an ethanol/water mixture. Optimal conditions for carrying out this extraction using a stirred tank have been reported elsewhere (Durling *et al.*, 2007a). Briefly, the solvent is 70:30 mass ratio of ethanol to water and a solvent to dry ground leaf feed ratio of 4:1 is used. Extraction is carried out at 40°C.

Table 1. Examples of supercritical antisolvent fractionation applications and the fractions produced

Upstream process	Feed	Near-critical fluid		Extract 1	Extract 2	Ref ^b
			Raffinate			
Urea fractionation	Hydrolysed fish oils	CO ₂	urea, water	PUFA ethyl esters or free fatty acids	oxdn. products, ethanol	SA3,4
Solvent extn.	Rosemary, sage	CO ₂	phenolics	antioxidants	essential oil	SA1,2
Solvent extn.	Olive leaf	CO ₂	flavonoids	oleuropein	vitamin E	SA1,2
Solvent extn.	Propolis	CO ₂	phenolic oligomers	aglycone flavonoids	cinnamic acids	SA5
Solvent extn.	Phospholipids	CO ₂	phosphatidylserine, phosphatidylinositol	phosphatidylcholine, sphingomyelin	neutral lipids ^a	SA8
Solvent extn.	Berries	DME	sugars, acids	anthocyanins		SA4
Dairy process	Serum, whey	DME	proteins, lactose	lipids	water ^a	SA6,7
Reconstitution	Egg yolk powder	DME	proteins	lipids	water ^a	SA6
Slurry with solvent	Algae	DME	alginate	lipids	water ^a	

a; Co-extracted with Extract 1

b; See text for reference details

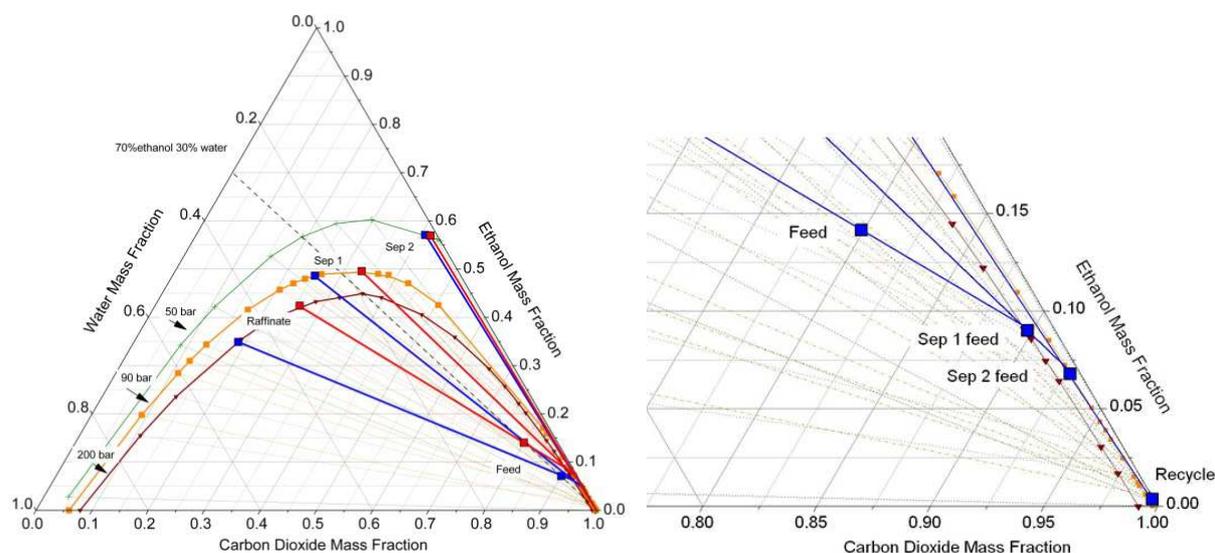


Fig. 2. Phase equilibrium compositions for antisolvent, first and second separator conditions at 10 and 20% feed solution to CO₂ mass ratios

The solution obtained is filtered and then processed using SAFT. Previous phase equilibrium measurements for CO₂/ethanol/water (Durling *et al.*, 2007b), coupled with experiments at a pilot scale, were used to establish suitable conditions for the supercritical antisolvent stage and also for the 2 or 3 stages of separation. **Figure 2** shows the predicted phase equilibrium for CO₂/ethanol/water at 200 bar for the antisolvent step at feed rates of 10 (blue) and 20% (red) by mass feed to CO₂ mass ratio. At the lower feed rate, the raffinate has a higher amount of water and thus lower loss of ethanol and similarly the first extract also has a higher amount of water which is co-extracted by the CO₂ + ethanol. Interestingly, this water content of the extract phase results in some extraction of the polar antioxidant, rosmarinic acid. As the feed solution to supercritical fluid ratio is increased, the loss of ethanol into the raffinate increases and the efficiency of separation of rosmarinic acid from diterpenes begins to decrease. However, the process economics improve because of the more rapid throughput of feed solution.

The recovery of Rosmarinic Acid (RA), diterpene antioxidant compounds carnosic acid, carnosol and methyl carnosate (CT) and essential oil/oleoresin (EO) is shown as a function of feed to CO₂ flow rate ratio in **Fig. 3** for the raffinate, first and second separators and the concentration of these same compounds in the same product streams is shown in **Fig. 4**. Also shown in **Fig. 4** is the concentration of these compounds in the feed

solution. It is possible to generate second separator products containing greater than 30% by mass diterpenes. In recent work, 3 stages of separation were applied, which enabled very good separation to be achieved between triterpenes (oleanolic acid + ursolic acid) and chlorophyll (first separator); diterpenes (second separator) and essential oil and oleoresin compounds (third separator). The diterpene concentration in the second separator can exceed 50% by mass.

2.2.2. Supercritical Antisolvent Precipitation of Solutes

The supercritical antisolvent process can also be used to generate particulate materials and reviews of the process and applications are given by Shoyele and Cawthorne (2008); Jung and Perrut (2001); Jessop and Subramaniam (2007); Tallon and Catchpole (2008) and Fages *et al.* (2004) among others. Because of the flexible density of the antisolvent phase, a range of particle morphologies can be produced as well as encapsulated particles or solid dispersions. The low fluid viscosity and high rates of mass transfer that can be achieved enable high levels of supersaturation and rapid nucleation of very fine particles. Control over the physical form of particles is useful in many applications where the physical form of the product affects its active properties. The particle size, for example, can affect the rate of solubilization and also bioavailability of some pharmaceuticals and nutritional foods.

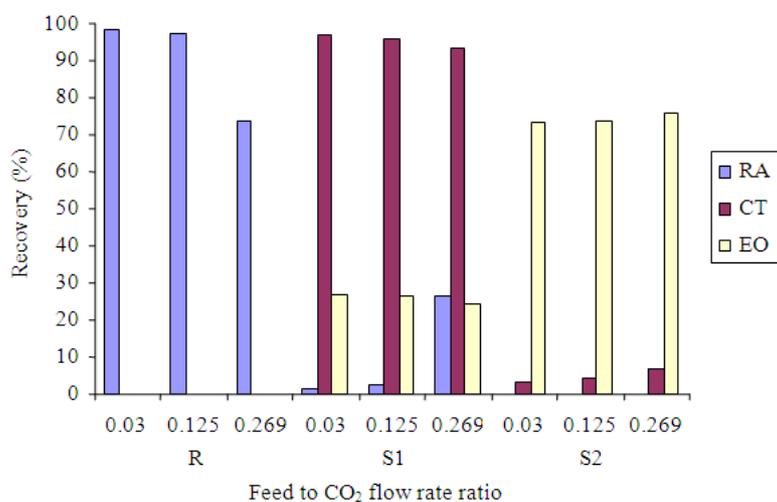


Fig. 3. Recovery of key sage extract components in raffinate (R), first separator (S1) and second separator (S2) products. RA = rosmarinic acid; CT = carnosic acid type (diterpene) compounds, EO = essential oil and oleoresin

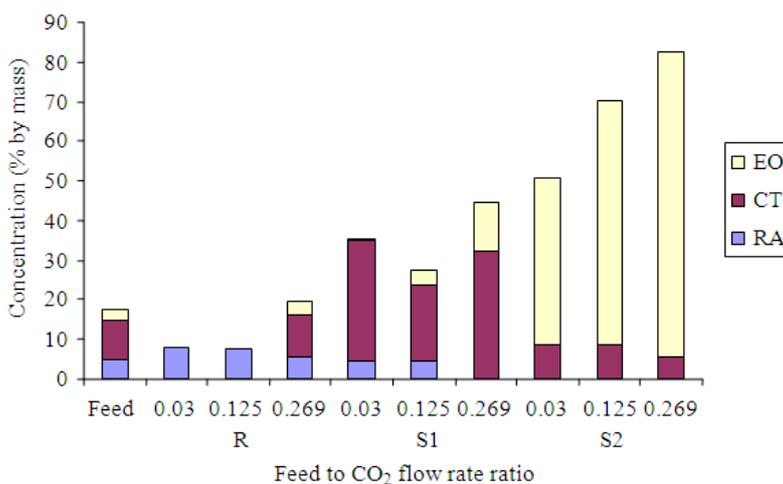


Fig. 4. Concentration of key sage extract components in raffinate (R), first separator (S1) and second separator (S2) products. RA = rosmarinic acid; CT = carnosic acid type (diterpene) compounds, EO = essential oil and oleoresin

Physical form also affects stability, handling, aerodynamics of inhalable products and even perceptions of taste and texture. Supercritical antisolvent processing can generally be carried out at low temperature, which is important for many thermally labile compounds to prevent degradation or conformational changes. The low processing temperature of supercritical fluid systems and the low solvent residues that can be obtained give an advantage in some cases over alternative approaches such as milling, drying and liquid solvent or temperature change induced crystallization.

There are two main classifications of supercritical antisolvent precipitation process depending on which phase is continuous and which is dispersed. When the supercritical fluid phase is continuous and the solvent feed stream is dispersed into it, the process is commonly known as Supercritical Antisolvent (SAS). The solvent phase needs to be at least partially miscible with the antisolvent and the process is generally operated so that solvent is completely and rapidly removed into the antisolvent phase leaving a mostly solvent free precipitated product. Under conditions where the mixture

of the solvent and antisolvent are still in a supercritical fluid region, particles will form without interference from surface tension associated with liquid-gas boundaries. Examples of compounds processed include insulin, hydrocortisone, lysozyme, albumin, (Shoyele and Cawthorne, 2008). Plant and dairy products (Catchpole *et al.*, 2007a; Tallon *et al.*, 2005) have also been processed. **Figure 5** shows an example of particles of potassium bicarbonate formed by this process, by precipitation (and simultaneous reaction) of potassium hydroxide with carbon dioxide from a solution in ethanol.

When the supercritical antisolvent forms the dispersed phase the process is often called Gas Antisolvent (GAS). The solution forms the continuous phase and the supercritical antisolvent is introduced, gradually decreasing the solvent capacity of the solution until particles precipitate. Further washing with the supercritical fluid can remove the majority of the solvent from the system. This has been used for many systems, including proteins, but is a batch process with more limited control over the particle size.

The antisolvent particle formation process has some flexibility in choice of the upstream solvent used to dissolve the solute, although this choice may be limited for applications that require non-toxic solvents. Suitable solvents for use with CO₂ include alcohols, but solvents such as dichloromethane and dimethylsulfoxide (DMSO), have also been used. Coating and excipient compounds that are poorly soluble in CO₂ can be processed using the right solvent, including a range of polymers, cyclodextrins, lactose and chitosan, as well as inorganics such as magnetite (Chattopadhyay and Gupta, 2002) for use in magnetic field directed drug delivery. Compounds including insulin and DNA have been successfully encapsulated and stabilized using these methods (Tandya *et al.*, 2007). CO₂ antisolvent systems are often used with DMSO or methylene chloride as the solvent and residues of these solvents in some products may be undesirable.

Use of other supercritical, or near-critical, fluids is possible, including ethane and ammonia (Shoyele and Cawthorne, 2008). Dimethyl ether has also been used to precipitate compounds directly from aqueous solution (Tallon *et al.*, 2005). An example from our work is the precipitation of bovine serum albumin from solution in water by dispersing the solution into a countercurrent flow of liquid dimethyl ether. Resulting particles are shown in **Fig. 6** for processing at 40 bar and 40°C. Co-precipitation of proteins with a secondary matrix is also possible. **Figure 7** shows composite particles produced by co-precipitation of a lipase (Amano) with β-

cyclodextrin from solution in water. Greater than 90% of the enzymatic activity was retained during processing.

Variations of these particle formation processes can be employed to form more complex systems, including the use of emulsions and sonication to aid phase dispersion. Surfactants and stabilizers can be added to limit re-agglomeration or deterioration of particles that are formed. Considerable research has also been carried out using supercritical fluids for particle coating applications. In organic systems this is largely driven by applications in drug delivery, where the aim is to develop a stable highly bio-available product with a controlled release rate, or to incorporate the drug within compounds that enable targeted delivery (Ginty *et al.*, 2005). Applications in food, cosmetics and agricultural products also exist (Taki *et al.*, 2001).

A wide range of other particle formation, encapsulation and impregnation applications exist using supercritical fluids as a solvent rather than an antisolvent. The supercritical solution is typically depressurized to remove the solvent and precipitate the solute. These processes are largely independent of upstream and downstream processing steps and are not discussed further here.

2.2.3. PUFA Concentration by Urea Complexing and Supercritical Antisolvent Fractionation

The recovery of Polyunsaturated Fatty Acids (PUFA) or esters from an upstream urea fractionation process using supercritical antisolvent fractionation technology is another exemplar process. Urea fractionation has been known since the 1950s as a way to separate unsaturated alkyl chains from saturated chains. Urea dissolved in solution can form a solid clathrate around hydrocarbon chains (around 4:1 urea:compound mass ratio) which is then insoluble in the solvent. The equilibrium constant for the formation of the clathrate depends on the temperature, degree of unsaturation, position of sites of unsaturation, hydrocarbon chain length and ratio of urea to hydrocarbon-containing molecules (Eltringham and Catchpole, 2008). The stability, or tendency to form clathrates increases with increasing saturation and chain length and decreasing temperature. The selectivity of the process is highly dependent on the ratio of urea to feed material and the temperature. The molecules containing the hydrocarbon moieties can at most be mono-substituted by non-alkyl groups-fatty acids and fatty acid esters can form complexes but triglycerides and phospholipids cannot for example.

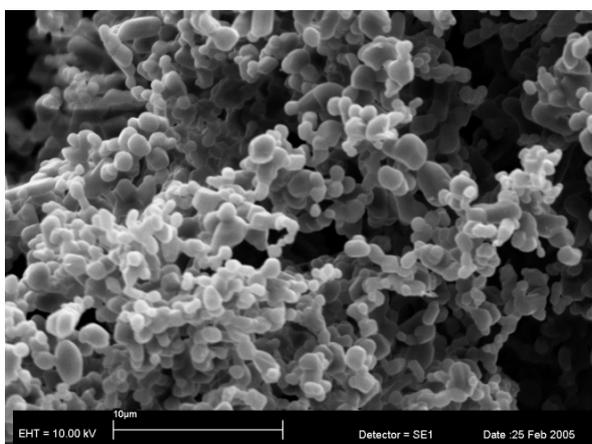


Fig. 5. Potassium bicarbonate formed by SAS precipitation of potassium hydroxide from solution in ethanol into supercritical CO₂

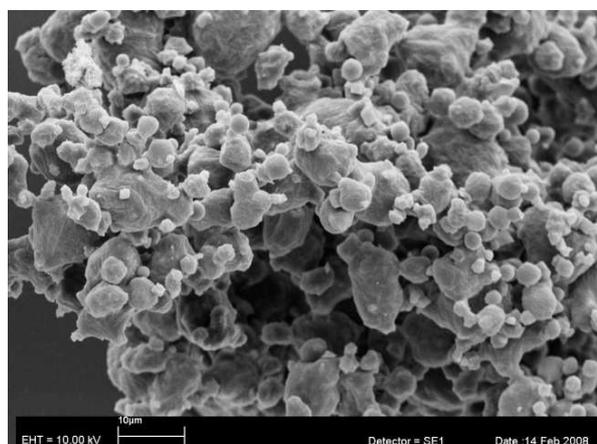


Fig. 7. Composite β -cyclodextrin and Amano Lipase precipitated from aqueous solution using dimethylether as antisolvent.

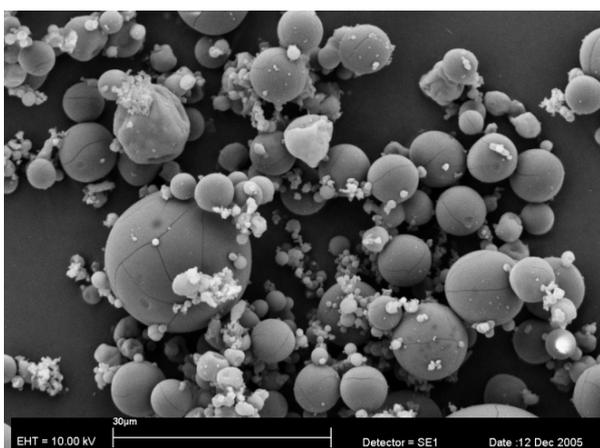


Fig. 6. Bovine serum albumin particles from aqueous solution using dimethylether as antisolvent

The most practiced form of the technology is to use urea dissolved in ethanol at high temperature and a mixture of ethyl esters as the feed material to form a miscible solution which is slowly cooled to induce clathrate formation. Unsaturated fatty acid esters stay in solution and are recovered by hexane extraction and water partitioning from the solution and the clathrates containing saturated and mono-unsaturated fatty acid esters are removed by filtration. This is a suboptimal process as the urea has limited solubility in the ethanol (approximately 10% by mass at 80°C), which then entails high use of ethanol and low throughput of lipid, the urea clathrate is difficult to re-use and the ethanol and hexane are expensive to recover by distillation.

The process can be improved by adding water to the solution to increase the urea solubility, exchanging fatty acids for ethyl esters, which are more soluble in ethanol/water mixtures and exchanging the hexane/water partitioning process for supercritical antisolvent fractionation (Catchpole *et al.*, 2002).

The supercritical antisolvent process can be applied to recovery of fatty acids from the clathrate phase, after it has been reheated in solvent to break up the complex and release the fatty acids. It can also be applied to the recovery of fatty acids from base-catalysed ethanol/water hydrolysate solutions of fish or plant oils. Here, the base salt is converted to potassium bicarbonate and the fatty acid ion is converted directly to free fatty acid through the decrease in pH engendered by CO₂ dissolving in the aqueous ethanol phase. Salts, water and glycerol are precipitated in the raffinate and the fatty acids are recovered in the first separator.

A schematic of the urea separation process is shown below in **Fig. 8**. The solvent phase is filtered to remove the clathrate and is then compressed to the operating pressure before being contacted with CO₂ using a static mixer placed inside the antisolvent fractionation vessel. Typically, the antisolvent vessel is operated at 200-300 bar and 313-333 K. Urea and water in the feed solution are precipitated into the antisolvent vessel and can be recovered from a valve at the bottom of the vessel. Polymerised fatty acids and long-chain oxidation products are also collected in this fraction if low grade oils are used as the feed material. If the solution is ethanol only, urea is precipitated inside the vessel as a solid.

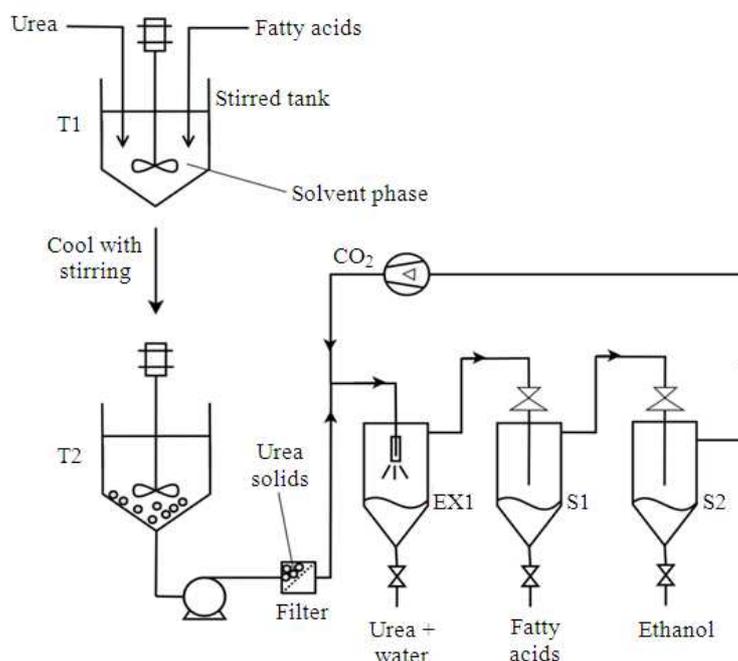


Fig. 8. Process schematic for the urea fractionation of fatty acids followed by supercritical antisolvent fractionation. T1 is the initial high temperature, T2 is the final temperature of the urea fractionation, EX1 is the antisolvent spray chamber, S1 is the first separator, S2 is the second separator

Optionally, a water-wash stream may be added, to improve the recovery of urea. The CO_2 dissolves the majority of the ethanol present along with the desired polyunsaturated fatty acids. A small amount of urea is co-extracted in this phase and so preferentially, a water wash stream is added to the CO_2 just prior to the pressure reduction valve of the first separator. The urea co-extraction prompted us to determine the solubility of urea in CO_2 + ethanol co-solvent (Catchpole *et al.*, 2005). Surprisingly, the solubility of urea in CO_2 + ethanol is almost independent of pressure within the range studied, but highly dependent on the co-solvent concentration. The water phase containing all the co-extracted urea (and some ethanol) separates out from the free fatty acids inside the separator and can be collected using the valve at the bottom of the separator separately from the free fatty acids. The remainder of the ethanol and short chain odour compounds and oxidation products are recovered in the second separator product.

The filter cake containing the clathrate of urea and PUFA can also be reprocessed using the supercritical antisolvent fractionation process. Solvent is added to the cake and the mixture is heated and stirred until completely dissolved in solution and the fatty acids are liberated. The hot solution is then processed using SAFT

under the same conditions. Again, the urea and water are precipitated and collected from the antisolvent let down vessel valve, the fatty acids are collected in the first separator and a minor fraction is collected in the second separator. The process has been tested on a wide variety of seed and fish oils (Catchpole *et al.*, 2002) in which the aim has been to concentrate γ -linolenic acid, or EPA and DHA, respectively. **Figure 9** shows the concentration of total PUFA, EPA and DHA from low grade Hoki oil which initially has 5% EPA and 9% DHA. After two cycles of the process, EPA has been increased to 20%, DHA to 40% and total PUFA to > 90%.

2.3. Concentration and Extraction of Phospholipids from Dairy Streams

Milk is a complex mixture of mainly proteins, lactose and lipids dispersed in water. The lipids are predominantly neutral lipids, with less than 0.1% by mass phospholipids. However, the phospholipids that are present are high in sphingomyelin and phosphatidylcholine, which are of interest in a variety of health and wellness applications. The complex lipid fraction in milk is also a rich source of gangliosides, lactosyl- and glucosyl-ceramide.

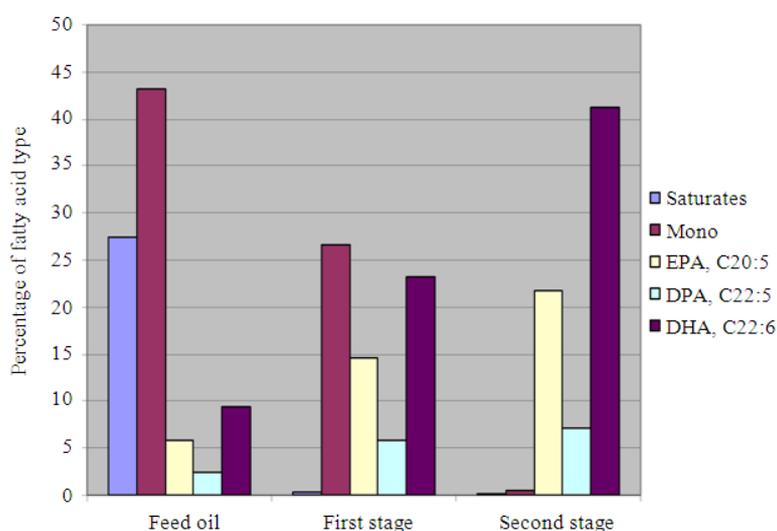


Fig. 9. Concentration of saturated and mono-unsaturated fatty acids, eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) in feed oil, first stage SAFT product and second stage SAFT product

There are two dairy streams that are produced in which the phospholipids have been substantially concentrated—these are beta serum, which is produced as a side stream from the production of anhydrous milk fat and skim milk (Catchpole *et al.*, 2008; Fonterra, 2012) and High Fat Whey Protein Concentrate (HFWPC), which is produced from cheese making and casein precipitation operations. Current production methods for Whey Protein Isolates (WPI) from HFWPC, which typically contain at least 90% protein and less than 1% lipid on a powder basis, involve either membrane processing or ion exchange (Zydney, 1998; Daultani *et al.*, 2003). Both methods result in considerable yield loss and so supercritical extraction was considered as an option for the removal of lipids.

Dairy complex lipid products produced by solvent extraction are available commercially (Fonterra, 2012). Supercritical extraction as an alternative to solvent extraction, was investigated to see if the defatted proteins could also be recovered in an undenatured state. In our study, spray dried Whey Protein Concentrate (WPC) powders have been extracted with both CO₂ and DME but in both cases lipid yields were very low, indicating that the lipids are strongly bound to the whey proteins (Catchpole *et al.*, 2007a). Liquid whey protein concentrates were also extracted by supercritical antisolvent fractionation using DME. It was found that high lipid yields could be obtained, but elevated temperatures of up to 333 K were required and the process resulted in some denaturation of protein. The process was also been used to extract High Fat Whey

Protein Concentrates (HFWPC) which already contain partly denatured proteins. The lipids are generally around 70% neutral and 30% phospholipids, consisting mainly of phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM) and phosphatidylserine (PS) with lower levels of phosphatidylinositol (PI).

Supercritical antisolvent fractionation using DME produced a lipid yield of between 60 and 80 wt% of the lipids present (Catchpole *et al.*, 2007a). Higher extraction yields were obtained with a HFWPC containing a higher 13.2 wt% lipid content (type A), while lower yields were obtained from powder with a 7.0 wt% lipid content made by a different process (type B). The residual non-extracted lipid levels were comparable for both feedstocks. The lowest extraction yield was for an acidified HFWPC feed stream which had a significantly higher viscosity. The effect of solids concentrations between 5 and 20 wt% was mixed, depending on the specific manufacturing process employed and the balance of proteins present. A comparison between the extract loading for egg lipids and for dairy lipids from HFWPC is shown in **Fig. 10**. The completeness of extraction is high when the lipid feed loading is low and declines at high lipid loadings. This may be due to neutral lipid solubility limitations in the DME + water solvent system. The solubility of neutral lipids in DME decreases with increasing water content, but the solubility of complex lipids is not affected, or in some cases even improves (Tallon and Catchpole, 2004).

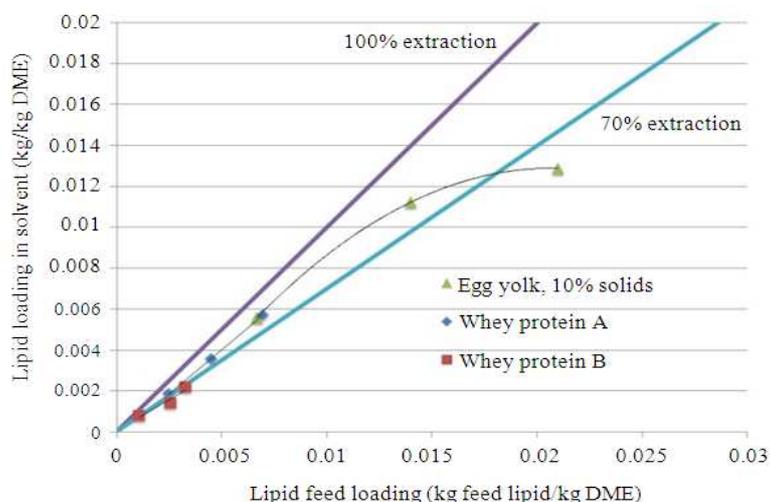


Fig. 10. Extraction of lipids from aqueous whey protein concentrates and egg yolk by co-current extraction with dimethyl ether

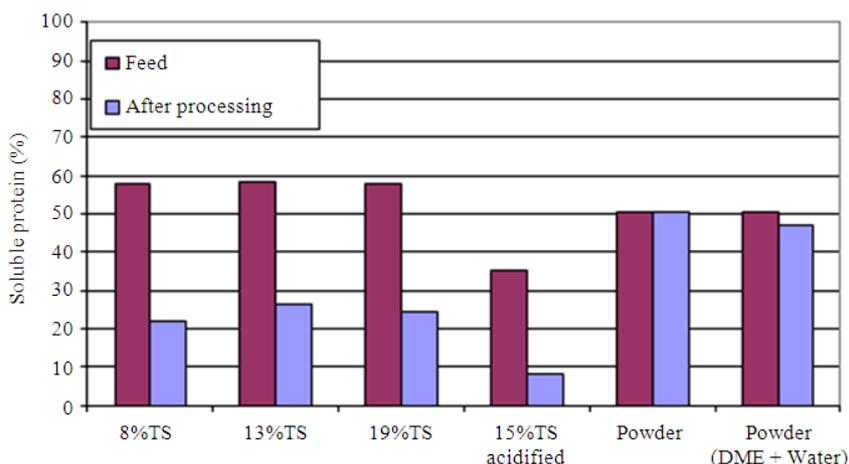


Fig. 11. Protein solubility before and after antisolvent processing of whey protein concentrate. TS = solids content by mass. Powder = spray dried

The decline in extraction efficiency may also be due to mass transfer limitations due to the short contacting time in the static mixer. Packed countercurrent flow columns are an alternative to overcome contacting limitations, but precipitated proteins can cause fouling and flooding of the column. It is advantageous to use a short contact time, because precipitated protein can be continuously removed as long as it does not become excessively dehydrated (which occurs to a greater extent with longer contact time) whereas the protein collects in co-precipitated water in a co-current configuration and can be recovered in a raffinate stream. Analysis of the phospholipids in the extract by ³¹P NMR (Mackenzie *et al.*,

2009) shows good extraction of all the main classes of phospholipid present, but with slightly better extraction of PC, PE and SM compared to PI and PS.

Decreases in the solubility of proteins were observed to varying degrees following antisolvent processing. For the dairy streams, which consist largely of whey proteins, changes in the percentage of soluble protein after processing are shown in **Fig. 11** for type A reconstituted solutions. Also included for comparison are the changes in protein solubility for type A powder extracted with DME and DME + water as a co-solvent. There is a reduction in protein solubility for antisolvent runs (liquid feed) as shown in **Fig. 11**, but very little

change following the extraction of powders, even when using water as a co-solvent with DME. Unfortunately, the extraction yield of lipids is also low from powders. It is predominantly the β -lactoglobulin and to a lesser extent, α -lactalbumin that becomes denatured and thus insoluble after processing. These whey proteins are particularly affected when processing lower pH feeds.

Changes in the solubility of the freeze dried fresh egg yolk proteins have also been studied as a function of the water loading during processing (Tallon *et al.*, 2005). The protein solubility of freeze dried yolks was measured to be 22% by mass. Extracting lipids from egg yolk directly with a DME antisolvent process gave solubilities between 7 and 19 wt% depending on the solids content in the feed and the processing temperature. A greater reduction in solubility was observed for higher processing temperatures and for runs where the amount of water in the system was close to the limit of the water solubility in DME. Processing with either a substantial excess of water, or with a shortage of water, results in a smaller degree of solubility reduction. Extraction of freeze dried powder gives very little reduction in protein solubility.

3. PRODUCTION OF BIOMASS BY MICRO-ORGANISMS

The ability of microorganisms (algae, bacteria, fungi and yeasts) to produce a wide range of biochemicals under controlled conditions is increasingly being recognized for its commercial potential. Microorganisms can produce large amounts of biomass rapidly, can metabolize and convert a wide range of feed materials from sugars through to carbon monoxide and can be engineered to produce high concentrations of specific target compounds. Microorganism derived consumer products are favourable to some sectors of the market as alternatives to animal derived products and the production process is sustainable compared to other feedstocks derived from wild harvest. Recovery of minerals and water is however important for full lifecycle sustainability. Target products are often medium to high value petrochemical replacements or bioactive compounds for dietary, cosmetic and pharmaceutical applications (Certik *et al.*, 2012), although an increasing body of work is also targeted at low value, high volume, biofuel production from algae (Ratledge and Cohen, 2008; Yuan *et al.*, 2011).

3.1. Dietary Applications

Dietary applications include production of lipids that are highly enriched in the unsaturated fatty acids

eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), γ -linolenic acid (GLA) and arachidonic acid (ARA) (Ratledge and Cohen, 2008; Olaizola, 2003; Catchpole *et al.*, 2010; Wen and Chen, 2005; Mendes, 2008) and microorganisms that produce lipophilic pigments (Mendes, 2008; Catchpole *et al.*, 2010; Krichnavaruk *et al.*, 2008; Lim *et al.*, 2002; Schonemann *et al.*, 2008). The production of lipids from micro-organisms via industrial fermentation has been commercialized by Martek Biosciences Corporation and others for infant formula and other specialized dietary needs (Wynn and Ratledge, 2007).

An example from our work is the heterotrophic fermentations of a wild-type strain of the fungus *Mortierella Alpina* (MA) to produce lipids highly enriched in ARA. **Figure 12** shows the fatty acid composition evolving during a 13 day growth period in a 250 L pilot scale fermentation during which palmitic and linoleic acids are converted primarily to ARA and to a small extent, EPA. At the end of the fermentation ARA makes up approximately 30% of total fatty acids.

The commercial scale extraction of lipids from fermentation systems has so far been limited to use of hexane (Wynn and Ratledge, 2007). The use of CO₂ or CO₂ + co-solvents for extraction has been investigated and has been reviewed by Mendes (2008); Wisniak and Korin (2005) and Catchpole *et al.* (2010). The challenges to successful commercial extraction of lipids include the high cost of fermentation, costly drying of the biomass before extraction for many solvent systems and difficulties obtaining a good yield. Poor yields are due to a combination of factors, including difficulties in breaking open dry cell walls and the cells containing complex lipids that are not soluble in non-polar solvents (Mendes, 2008; Catchpole *et al.*, 2010). Extraction methods that avoid the need for drying such as sonication, enzymatic digestion, pH adjustment and high shear flows have been investigated with varying degrees of success.

To overcome the high costs of drying and the poor yields due to the form or type of lipid present, we have developed a processing technology using DME to extract wet biomass (Catchpole *et al.*, 2010; 2007b). The extraction of lipids from wet MA biomass is shown in **Fig. 13** and from freeze-dried biomass in **Fig. 14**, with comparison against CO₂ extraction. The extraction of lipids from freeze-dried *Mortierella* species using supercritical fluids (Sako *et al.*, 1989; Sakaki *et al.*, 1990) and using CO₂ (Dalai *et al.*, 2011; Nisha *et al.*, 2012) and organic solvents has been previously reported.

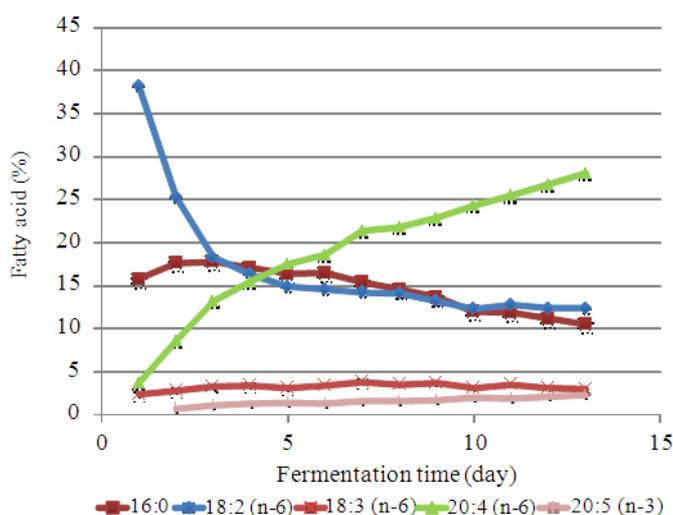


Fig. 12. Fatty acid composition of *Mortierella alpina* fermentation biomass as a function of fermentation time. Key to fatty acid type: 16:0 = palmitic acid; 18:2 = linoleic acid; 18:3 (n-6) = γ -linolenic acid; 20:4 (n-6) = arachidonic acid; 20:5 (n-3) = eicosapentaenoic acid

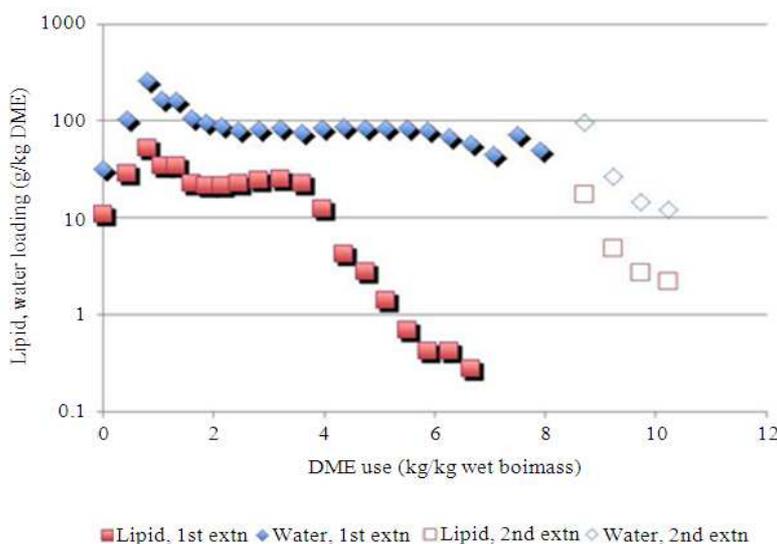


Fig. 13. Lipid and water loadings in DME from the extraction of wet *Mortierella alpina* biomass as a function of DME usage for two stages of extraction

The batch extraction of water from a bed of MA with DME takes place under almost steady state conditions, as shown in **Fig. 13**, whereas the extraction of lipids is steady for a period until a point where the rate drops off. Inspection of the residual bed of material revealed significant bypassing and aggregation of the bed into wet patches (poorly extracted) and dry patches (complete extraction).

A secondary extraction after repacking the bed of material resulted in near-complete extraction of the remaining lipids, **Fig. 13**. The poor extraction behaviour from the bed is at least partly explained by the coating of biomass with ‘sticky’ carbohydrates. These tend to cause clumping and aggregation and a barrier to mass transfer. The benefits of pre-processing of biomass by enzymatic or chemical methods to remove this sticky exudate and

improve extraction are discussed later in this study. In contrast, freeze-drying of the biomass gives a porous structure in which the lipids are easily accessible to the solvent. Extraction of freeze dried powder using DME gives almost complete lipid extraction using around 2 kg DME/kg dry biomass, shown in **Fig. 14** (square symbols), with minimal additional extraction after this point. In contrast, more than 40 kg CO₂/kg dry biomass is required to extract all the neutral lipids (diamonds). Dalai *et al.* (2011) also reported low CO₂ yields compared with solvent extraction.

The residual biomass after CO₂ extraction was re-extracted with DME to give a complex lipid-rich extract **Fig. 14** (triangles). The extraction yields are higher using DME than using CO₂ and the yield is close to the total quantity of lipid present measured by exhaustive chloroform/methanol extraction. The higher yield using DME is largely due to the extraction of both neutral and complex lipids. The solubility of lipids in DME is also substantially higher than in CO₂ and extractions are more rapid.

3.2. Biofuel Applications: Extraction of Butanol from Fermentation Broth

An example of a liquid biofuel application is the production of biobutanol by *Clostridium* bacteria. 1-Butanol is widely used in the production of butyl

acrylate and methacrylate and is also added to plastics, hydraulic fluids and detergent formulations (EPA, 1994). More recently it has been recognised as a potential biofuel (Jin *et al.*, 2011; Ranjan and Moholkar, 2012). It has a high energy content and can be directly used in internal combustion engines, is less volatile and explosive and has a lower vapour pressure (Garcia *et al.*, 2011; Ranjan and Moholkar, 2012) than ethanol. The Acetone-Butanol-Ethanol (ABE) fermentation process using *Clostridium* bacteria allows production of biobutanol from renewable, low cost lignocellulosic biomass, with an A:B:E ratio of 3:6:1 and a typical butanol concentration around 12 g L⁻¹ (Kumar and Gayen, 2011). The butanol production level is self-limiting, as it is toxic to the microorganism above these levels. Distillation is the traditional method to recover butanol from the fermentation broth, but several alternatives have also been investigated (Oudshoorn *et al.*, 2009). The high pressure phase equilibrium of butanol:CO₂ (Secuianu *et al.*, 2004; Chen *et al.*, 2002) and butanol:water:CO₂ (Panagiotopoulos and Reid, 1986; Chen *et al.*, 2002; Winkler and Stephan, 1997) have been reported, indicating that butanol extraction from fermentation broth could be feasible. Over 99% separation of butanol from a 5 wt% butanol feed has been reported using CO₂ in a mechanically agitated Oldshue-Rushton column (Laitinen and Kaunisto, 1999).

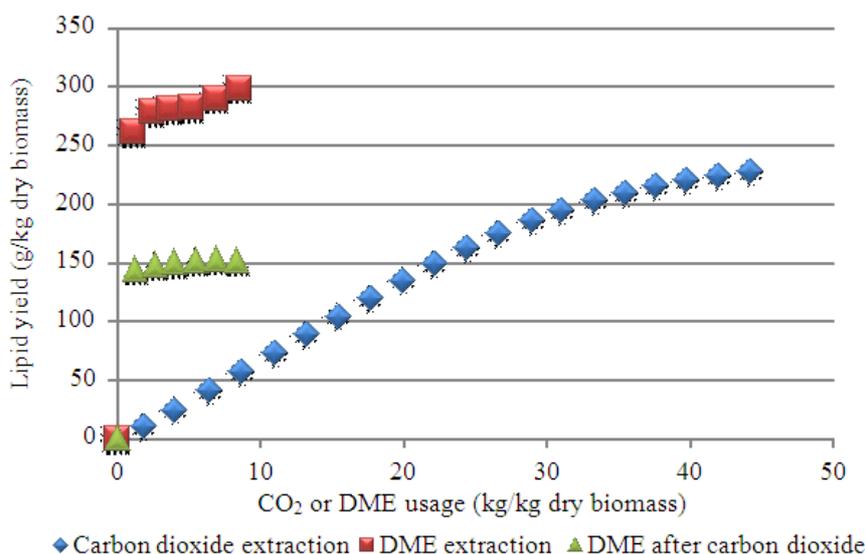


Fig. 14. Lipid yield from the extraction of freeze-dried *Mortierella alpina* as a function of dimensionless solvent usage. Symbols: diamond = extraction of MA with CO₂ at 300 bar, 313 K; squares = extraction of MA with DME at 40 bar and 316 K; triangles = re-extraction of residues after CO₂ extraction with DME at 40 bar and 317 K

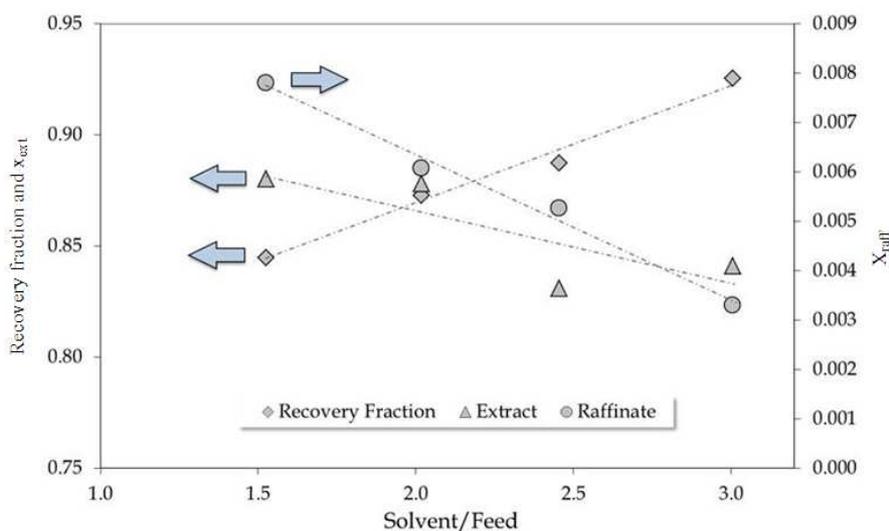


Fig. 15. Composition of raffinate and extract, and recovery fraction of 1-butanol, for different CO_2 -to-feed ratios and 5 wt% concentration of 1-butanol in the feed. Pressure 100 bar

We have carried out CO_2 extraction of model 1-butanol solutions and actual ABE fermentation broths using a fully recycled CO_2 flow. **Figure 15** shows the percentage of feed butanol recovered and the butanol concentration in extract and raffinate after separation of a 5 wt% butanol solution in a countercurrent flow column (20 mm ID, 1200 mm length, 16mm diameter Pall ring packing) operated at 100 bar and 40°C . Recovery of butanol was better at higher solvent to feed ratios, as expected for a stripping operation, but greater quantities of water were also co-extracted resulting in a lower butanol concentration in the extract. The extract however is still substantially enriched, up to 90% butanol and the residual water could be removed by conventional drying methods. Results obtained without the Pall ring packing were similar indicating no added benefit was obtained in this case. Smaller packing with a higher surface area was also tested but had a flooding limit lower than the minimum flow rate we could operate on the equipment used.

A trial biobutanol solution was produced by anaerobic fermentation in a 20 L bioreactor using *Clostridium acetobutylicum* ATCC 824. Temperature was controlled at 37°C and the broth harvested after 68 h. After centrifuging the solid biomass was discarded. The broth contained a 1-butanol concentration of 1.2 wt%. After separation using CO_2 as described above, 82% recovery of butanol was achieved, with a concentration in the extract of 72 wt%.

4. PRE-PROCESSING BY PHYSICAL, CHEMICAL OR ENZYMATIC REACTION

Many plant, animal and microbial-based feed materials contain relatively low levels of desired bioactives such as phospholipids and glycolipids, flavours and fragrances, pigments, flavonoids and correspondingly large amounts of water and also carbohydrates that may hinder supercritical extraction. Generally, CO_2 extraction cannot be used on biomasses containing high amounts of water, due to the immiscibility of water and CO_2 and so the processing strategy is to first dry and grind the biomass to a small particle size and then carry out supercritical extraction. Recently, we and others have been investigating the use of pre-treatment processes to increase the yield of bioactives in the feed material by reducing the amount of non-extractable material. These processes include enzyme pre-processing (using proteases, carbohydrases) and acid-base treatments. Enzymes can also be used to convert lipids to free fatty acid or ester forms to make them more easily extractable, or to cleave selected fatty acids and enable fractionation of fatty acids and transesterification reactions to be carried out. These processes can lend themselves to supercritical extraction either post reaction, or even in situ.

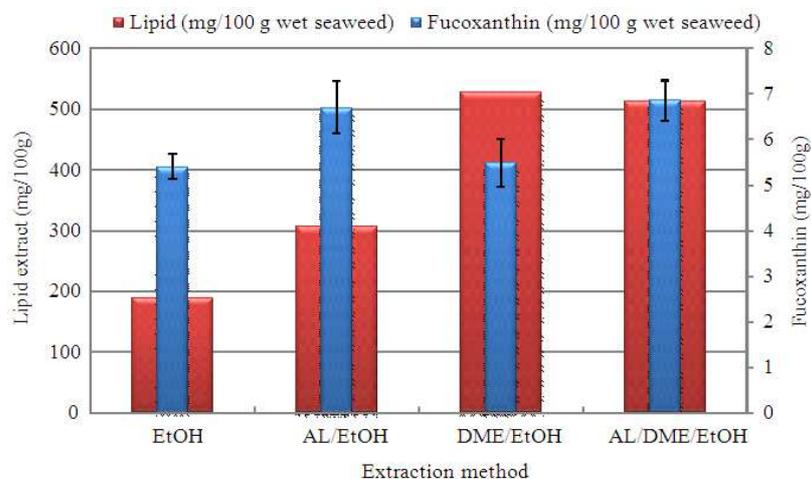


Fig. 16. Extraction of lipids and fucoxanthin from *Undaria Pinnatifida* seaweed with different methods. EtOH-ethanol extraction (90% ethanol); AL/EtOH-alginase treatment followed by ethanol extraction (90% ethanol); DME/EtOH-dimethylether extraction with 10% ethanol co-solvent; AL/DME/EtOH-alginase treatment followed by dimethylether extraction with 10% ethanol co-solvent

4.1. Size Reduction

Drying and/or milling of feed materials are simple and widely used pre-processing operations, but the importance of these steps to the success of downstream processing is sometimes overlooked, leading to inefficient use of processing plant, handling difficulties, or changes in product composition or stability.

The effect of particle size on the extraction rate of solid substrates is complex, depending also on the quantity and distribution of extractable material, as well as variations in density, porosity and composition of the non-extractable matrix (Mukhopadhyay, 2000). Extractions using CO₂ often have an initial period of constant solubility limited extraction, due to the high diffusivity of CO₂ compared to liquid solvents and the relatively low solubility of solutes in CO₂. After this initial period, extraction may become mass transfer limited and it is important to have a sufficiently small particle size to avoid a long extraction 'tail'. The effect of particle size on yield and extraction time has been reported for sage leaf, coriander and celery seed (Catchpole *et al.*, 1996; 1997) and Totara wood (Catchpole *et al.*, 2003). Mukhopadhyay (2000) observed that particle size less than about 0.3 mm was sufficient for good extraction from a selection of plant materials.

For materials with a hard shell or epithelium it can also be useful to break this by milling, or other means, to improve solvent access to the substrate. Conversely it is

also important not to reduce the particle size more than necessary. This can lead to unnecessary handling difficulties or an increase in pressure drop that limits the overall capacity of processing plant.

The choice of milling operation depends on the material to be milled. High oil content materials, such as some seeds, are more suited to low shear rolling or crushing. Soft plant materials can also be processed in a low shear device including mincers and juicers unless, like microalgae or ova, they have natural small scale barriers that require a high shear rate to break, in which case jet milling or a mill with a cutting blade may be suitable. In some cases milling may be combined with a chemical treatment. Harder materials, or materials with low moisture or oil content, are well suited to blade or impact mills.

4.2. Enzymatic Pretreatment of Macroalgae

An exemplar process is described below for the pre-treatment of a macro-algae, *Undaria pinnatifida* using an enzyme, followed by extraction of fucoxanthin using either ethanol or DME. *Undaria pinnatifida* is a seaweed that grows naturally in Japan (known as Wakame) but is an invasive species in New Zealand and Australia. It produces the valuable lipophilic pigment fucoxanthin and also a small amount of lipids rich in Stearidonic Acid (SDA) (C18:4 n-3) and EPA. Roh *et al.* (2008) have investigated the extraction of fucoxanthin from *Undaria pinnatifida* using CO₂ and co-solvent. Enzymatic pre-treatment to improve the yield of fucoxanthin from *Undaria* in an ethanol extraction process has been

investigated (Sho and Sugiyama, 2010), but this has not been combined with supercritical fluid extraction.

The use of enzymes to assist the extraction and recovery of bioactives from seaweeds has been reviewed in detail by Wijesinghe and Jeon (2012). The alginase lyase enzyme selected for this work hydrolyses specific polysaccharides containing β -D-mannuronate residues in seaweed cell walls. The effects of pH, reaction temperature, enzyme to feed ratio and reaction time on fucoxanthin yield were investigated for their effect on fucoxanthin stability. The incubation time and temperature were the most important parameters. Typical enzyme pre-treatment conditions were 5% (w/v) solids, pH 6.2, reaction temperature of 37°C, reaction time of 2 h and 0.05 wt% enzyme using continuous mixing. Water soluble hydrolysis products from the reaction were separated from the residual seaweed biomass by centrifugation at 6000g for 20 min. Removal of the hydrolysis products improved the recovery of lipids and fucoxanthin and also reduced the amount of residual biomass that needed to be processed by around 40-50% prior to extraction.

Yields for unprocessed and enzyme processed feed material are shown in **Fig. 16** and images of the processed seaweed fractions are shown in **Fig. 17**. Alginase pre-treatment increased the total lipid yield and yield of fucoxanthin for extraction with either ethanol or liquid DME. The DME extraction runs shown here were carried out using a packed bed extraction and, 10% ethanol co-solvent was used to help maintain a uniform extraction bed throughout the extraction.

The enzyme pre-treatment, followed by removal of the aqueous phase containing water soluble compounds, reduced by 50% the mass of material that required further processing. The water soluble material contained some lipids (10-15% of total lipids in the seaweed) but a negligible amount of fucoxanthin. The enzyme pre-treatment improves the overall lipid yield by >10% and fucoxanthin yield by >20%.

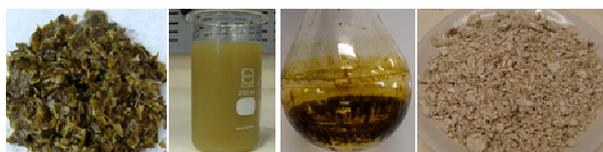


Fig. 17. Photos of seaweed extraction process. From left: close-up of wet, chopped seaweed; aqueous phase after enzyme treatment; crude extract from DME + EtOH cosolvent extraction; residual biomass after extraction

DME extraction with an ethanol co-solvent extracts some non-lipid material. Without enzyme pre-treatment the non-lipid material was approximately 50% of total extract. Ethanol solvent extracts contained even higher amount of non-lipid materials. After enzyme pre-treatment the non-lipid content reduced to <20% for DME+ethanol co-solvent extraction.

5. IN SITU PROCESSES

The controllable density and mass transport properties of supercritical fluids lend themselves well to fully integrated reaction and adsorption processing using the supercritical fluid as the primary processing medium. Carrying out chemical reactions, for example enzymatic hydrolysis, under supercritical conditions allows continuous stripping of any soluble reaction products that are produced. Adsorption and/or desorption processing is aided by low fluid viscosity which allows high flow through packed beds of particles without excessive pressure drop. In some cases a switch between sorption and desorption conditions can be induced simply by changing the operating pressure or temperature.

5.1. Supercritical Fluid Phase Chromatography

Supercritical chromatography is now an accepted method for the analysis of a wide range of raw materials, particularly lipophilic drug candidate molecules including enantiomeric isomers. Analytical scale chromatography is reviewed in detail elsewhere (Taylor, 2009) and is not covered here.

Supercritical chromatography also has great potential for preparative separations. The benefits over conventional liquid chromatography include the significant reduction in pressure drop that can be achieved because of the low viscosity of the solvent, meaning that higher surface area stationary phases can be used and/or higher solvent and product throughput achieved. Disadvantages of supercritical chromatography at a production scale include some limitations in options for on-line detection and the high overall capital costs for high pressure processing equipment.

Some studies on the separation of high value polyunsaturated ethyl esters, particularly EPA and DHA with supercritical fluid chromatography have been carried out. Perrut *et al.* (1998) achieved EPA and DHA fractions with a purity of 92% and 85%, respectively and yields of 99% starting from a feed material containing

50% EPA and 30% DHA. Higashidate *et al.* (1990) used a silver nitrate-loaded silica gel column at a laboratory scale to separate fatty acid extracts from the CO₂ extract of esterified sardine oil and obtained EPA and DHA-rich fractions with purities of 93% and 82%, respectively. Nilsson *et al.* (1988) used pressure and temperature gradients for fractionation of urea-crystallized fish oil ethyl esters to recover 85% of the EPA and DHA from the feed with 90% purity. Snoey-Elich (2001) was also able to concentrate fish oil EPA ethyl esters from a purity of 50% to > 95%. This was achieved using an industrial column of porous silicon dioxide impregnated with (3-aminopropyl)-triethoxysilane as the stationary phase. The economic feasibility of producing industrial-scale quantities of EPA and DHA using SFC was investigated by Alkio *et al.* (2000). Pettinello *et al.* (2000) carried out pilot scale trials using quantities of feed materials on the order of hundreds of grams and achieved a 93% pure EPA rich fraction at 25% yield. So far the only known commercial scale operation to produce highly pure ethyl esters is carried out by KD-Pharma (2012).

We have carried out a range of optimisation studies at semi-preparative scale to evaluate a range of proprietary column packings and operating conditions, for separation of EPA and DHA from marine and algal oils (Montanes *et al.*, 2012), juniperonic acid from *Biota orientalis* seed oil and xymenynic acid from Sandalwood seed oil. Oils were first converted to ethyl esters or free fatty acids before being loaded at levels of approximately 100-1000 µL on columns with approximately 50 g of silica. We have been able to achieve >95% separation and good recovery in most cases using CO₂ without co-solvent and with both modified and unmodified silica.

More recently we have commissioned a pilot plant, shown in **Fig. 18**, holding up to 4 by 10 L columns, up to 4 separation stages, on-line UV absorbance measurement and supercritical or liquid mobile phase flow rates up to 100 kg h⁻¹. The plant is mobile and rated for use with flammable solvents. Results for trial separation of fish oil ethyl esters using a coarse grade of normal phase silica (60-200 µm) are shown in **Fig. 19**. EPA and DHA peaks are not completely separated in this example, but reasonable concentration of EPA was still able to be achieved using relatively coarse silica.

We are also investigating the isolation and purification of a range of other highly unsaturated fatty acids and the fractionation of complex lipids and other polar compounds.



Fig. 18. Photo of 4 by 5 kg column mobile pilot plant recently commissioned

5.2. PUFA Enrichment by Selective Fatty Acid Esterification

Fish oil and more recently microalgae, have been the traditional source for EPA and DHA, with PUFA usually found as triglycerides (TAG) and mainly located at the sn-2 position of the glycerol backbone (Ando *et al.*, 1992). Vegetable oils such as borage, evening primrose or blackcurrant seed oils are particularly rich in γ -Linolenic Acid (GLA, 18:3 n-6), which is preferentially located at the sn-2 and to a lesser extent in the sn-3 positions (Lawson and Hughes, 1988). Several options are available to obtain PUFA concentrates (Rubio-Rodriguez *et al.*, 2010; Eltringham and Catchpole, 2008), but the use of specific enzymes, i.e. lipases, has attracted attention recently due to their high stereospecific activity and the mild reaction conditions they require, which prevents degradation of the highly labile oils. These position specific enzymes are commercially available in free or immobilized form and have a high regiospecificity for the sn-1 and sn-3 positions of the glycerol backbone, which allows the enrichment of PUFA in the sn-2 position either by hydrolysis (Linder *et al.*, 2002) or by transesterification processes (Munio *et al.*, 2008; Haraldsson *et al.*, 1997).

By adjusting extraction conditions, supercritical CO₂ allows selective removal of the Free Fatty Acids (FFA) or Fatty Acid Ethyl Esters (FAEE) produced in the enrichment enzymatic reaction, leaving a PUFA enriched partial glyceride fraction in the raffinate. It is also possible to carry out the enzymatic reaction under CO₂ conditions (Knez, 2009) and the reaction step can be easily integrated with the extraction post-processing, minimizing the number of handling and processing steps and limiting degradation of both the oil and the enzyme itself.

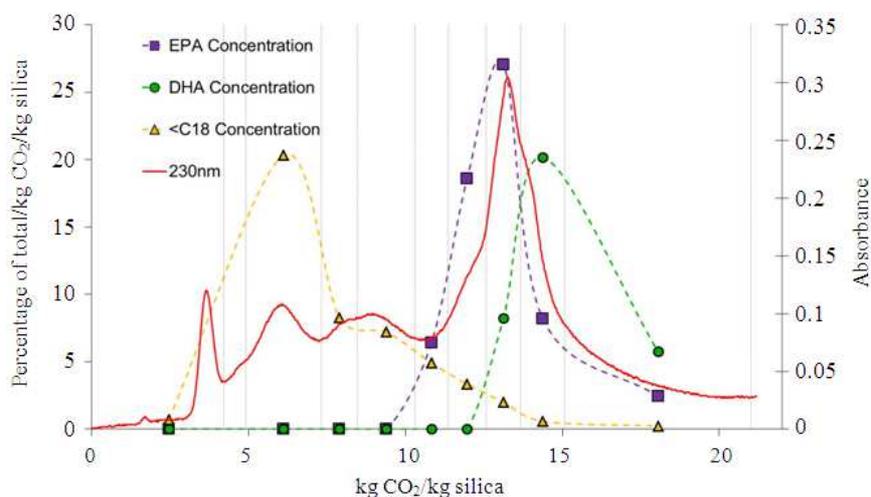


Fig. 19. Separation of fish oil ethyl esters on a 5 kg silica column using CO₂ at 170 bar. Red line shows absorbance at 230 nm. Dotted lines show recovery of EPA, DHA, and fatty acids <C18, as measured by gas chromatography from samples taken during the run

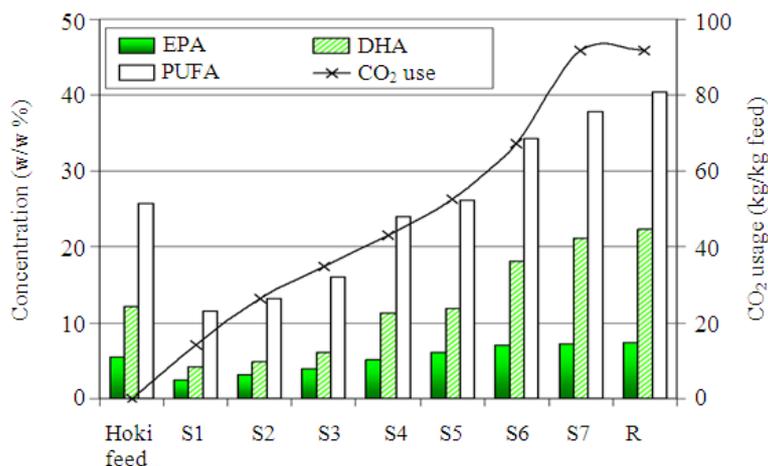


Fig. 20. EPA, DHA and total PUFA profiles of the feed oil, Separator (S) and Raffinate (R) fractions from stirred tank processing of hoki liver oil. Reaction conditions: 3:1 ethanol:oil molar ratio, 1:10 enzyme:oil weight ratio

Enzymatic reactions in supercritical fluids often show higher reaction rates than those carried out under classical conditions due to favourable mass transfer kinetics (Knez, 2009). An in situ supercritical fluid extraction process allows continuous transesterification with immediate product recovery and may help drive the reaction to completion.

We have carried out enrichment of PUFA from refined hoki liver oil using both a stirred tank and a packed column method at 40°C (Vagi *et al.*, 2009; Weber *et al.*, 2008). **Figure 20** shows the lipid

composition of different fractions from a batch experiment in which Hoki liver oil was esterified using Lipozyme® RM-IM (NovozymesA/S, Denmark) under CO₂ conditions at 150 bar in a stirred reactor. CO₂ and ethanol were continuously passed through the reactor and the extracts (S1 to S5) contained the highly soluble fatty acid ethyl esters from the sn-1 and sn-3 positions which are concentrated in saturated fatty acids. Further extraction at 300 bar (S6 and S7) was then carried out to recover the partial glycerides from the reaction. This fraction is enriched in EPA and DHA as shown in **Fig. 20**.

The residual raffinate fraction also contains partial glycerides as well as unreacted triglyceride and is enriched in EPA and DHA. Fractions S6, S7 and R contained enhanced levels of DHA and total PUFA over the feed (1.8 and 1.6 fold increase for DHA and total PUFA, respectively) and a small enrichment in EPA.

The high solvent usage required (92 kg CO₂/kg oil), is not practical for industrial scale-up and we have further investigated a semi-continuous, packed column method, which produced a >60% PUFA product and a FAEE rich byproduct (>95%) using only one-fourth of the solvent required in the stirred tank experiments. Moreover, no deactivation was observed after 9 cycles of packed column processing and it is believed that keeping the enzyme under a CO₂ atmosphere can help prevent enzyme deactivation.

The enrichment of borage seed oil was also studied using the packed column method, obtaining a GLA content in the PUFA rich fraction of 35-40% (cf. 20% in the feed). Ethanol-to-oil ratios up to 9 were studied and no detrimental effect was observed on enzymatic conversion or GLA content. Continuous flow pilot trials were carried out in a 2L enzyme packed bed, during which an oil mass of greater than 60 times the mass of enzyme was processed over a period of several days. Consistent enrichment and no significant loss of enzyme activity during processing was observed. Conversion of triglycerides was greater than 80% throughout the run.

6. CONCLUSION

General awareness and understanding of supercritical and liquefied gas processing technology is increasing, as is understanding of where this technology best fits for processing of a wide range of emerging biological products. This awareness is being matched by an increase in industrial processing capacity and it is becoming increasingly viable to consider a pressurized solvent extraction process as a standard unit operation in development of new products and processes.

The integrated processing operations described here are some examples of the range of processing opportunities that are being investigated, or are yet to be fully developed, but they give an indication of some important emerging trends. In particular, the use of microbiological systems for the primary production of chemicals has much promise for high volume sustainable production of dietary, medical and biochemical products. Associated with this is the need for development of efficient processes for separation of organic compounds from dilute aqueous systems and for the isolation and

concentration of target compounds. Also of increasing importance is for products to be formulated for efficacy and stability. In all of these areas supercritical fluid processing should be investigated, along with other processing alternatives, to determine the most favourable overall integrated process.

7. REFERENCES

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