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Analytical data

Echium oil is extracted from the seeds of *Echium plantagineum* by crushing. The crude oil obtained from the crushing operation is refined using a process optimized to achieve high purity natural oils by removing polar impurities without altering the chemical composition.

Fatty acid Profiles:

Fatty acid profiles for unrefined and refined Echium oil are presented in Appendix-1, Tables 1 & 2.

Echium oil is mainly composed of α -linolenic (30 – 33%), linoleic (14 – 18%), γ -linolenic (10 – 13%), stearidonic (13 – 15%), oleic (14 – 17%), and palmitic (6 – 7%) acids. We have presented the data on fatty acids that constitute 0.05% or more of the total fatty acids in the oil.

The methods for the lipid analysis are given in Appendix 2. The results of analysis in Appendix 1 clearly demonstrate the fact that the refining process does not significantly alter the fatty acid profile of the oil. Minor variations in the lipid composition of different batches of oil are due to the effect of seasonal changes in temperature, light intensity, etc. The lipid profile of both the unrefined and refined oil remains within the specified ranges. The lipid profiles presented in Appendix 1 cover the period between 1999 and 2003, confirming that the information represents the characteristics of the oil of the species.

Erucic acid is present at less than 0.5% in the oil. In the analysis presented in Appendix 1, the levels of erucic acid are between 0.1 to 0.3% of total fatty acids.

The fatty acids in Echium oil are mainly present as triglycerides with traces of di- and monoglycerides. Analysis of one large scale production batch (lot number 010803-N) confirmed levels of triglyceride as 97.9%, diglycerides as 2.1% and monoglycerides as less than 0.005%.

Non-Fatty acid components:

Unsaponifiable Matter:

Like other vegetable oils, Echium oil contains small amounts of unsaponifiable matter. Unsaponifiable matter is composed of hydrocarbons, sterols and other non-fatty acid compounds. The amount of unsaponifiable matter is less than 2.0% in Echium oil while other vegetable oils may contain unsaponifiable matter from a low of 0.2% in refined canola oil to up to 5% in rice bran oil (3).

Unsaponifiable matter in the production batch number 010803-N was 0.91%. A copy of the analytical report from the contract lab is appended in Appendix 3 while the analytical procedure used to isolate the unsaponifiable fraction is included in Appendix 4a.

Capillary Gas Chromatography (GC) analysis was carried out on the isolated unsaponifiables from batch 010803-N. Of total unsaponifiable matter, campesterol was 15.71%, beta-sitosterol was 12.53%, stigmaterol was 0.55%, and others were 33.52%, accounting for 62.31%. Tocopherols constituted 8.37% of total unsaponifiable matter, and consisted of alpha- (0.53%), gamma- (6.92%) and delta- (0.92%) tocopherols. Other material constituted 29.32% of total unsaponifiable matter. No attempt was made to identify these compounds. These unidentified compounds may be heavy molecular weight components that do not pass through the GC column. Similar levels for recovery of unsaponifiable compounds are experienced with oils such as sesame, soya and oilseed rape. The method for analysis of sterols and tocopherols is attached in Appendix 4b.

Analysis for potential contaminants:

Heavy Metals:

Total heavy metal content for different batches was less than 10ppm. The level of Iron was 0.45 ppm in unrefined oil (batch number EO 010803 –CF) and 0.14 ppm in refined oil (batch number EO 010803–N). The level of Copper was less than 0.05 ppm for both batches. A copy of the contract laboratory certificate of analysis for each batch is included in Appendix 5.

Pyrrrolizidine Alkaloids:

Members of Boraginaceae family are known to contain pyrrrolizidine alkaloids in seeds and leaves. *Echium plantagineum* [4, 5] also contains these alkaloids. Unsaturated pyrrrolizidine alkaloids are of concern because of their hepato-toxic effects [6]. In addition, they may damage the lung, kidney and other organs and they also possess mutagenic, teratogenic and carcinogenic properties [6]. Chronic liver disease was observed at dietary levels of 2ppm with the pyrrrolizidine alkaloid monocrotaline [6]. A no

effect level of 1ppm in the diet has been hypothesized for monogastric animals such as pigs, poultry and rats [6].

Analysis of several plant samples of *Echium plantagineum* from New South Wales revealed a total alkaloid content of about 0.3% [6]. The maximum level of total alkaloid measured was 0.9% [6].

Pyrrolizidine alkaloids are not lipophilic therefore they would not be expected to be present in the oil. This was confirmed in an analysis of the alkaloid content of the *Echium plantagineum* meal, the crude seed oil and the refined oil (*Echium* oil). The pyrrolizidine alkaloids were extracted in 0.5 M sulfuric acid and converted to the free base by reduction with zinc powder. The basified solution underwent solid-liquid extraction before being analyzed using HPTLC method with a detection limit of 4 µg/kg oil. The *Echium plantagineum* meal contained 120 µg total alkaloids. Crude seed oil contained 8 ppb (µg/kg) of these alkaloids per kg of oil while the refined oil contain 4 ppb (µg/kg) of oil. The detection limit for this method was 4 µg/kg.

A copy of the test results as supplied by the contract testing laboratory is included in Appendix 6.

Allergens:

Pollens of *Echium plantagineum* can induce allergic reaction in hypersensitive individuals. Cytochrome C allergens have been isolated from the pollen of *Echium plantagineum* [7]. In a rural area of Australia 60% of subjects with respiratory allergy were found to give positive skin test reactions to *Echium plantagineum* pollen extract and a similar number gave positive radioallergosorbent (RAST) tests [8]. In a case of allergic rhinitis to *Echium plantagineum* symptoms developed on exposure to both the flowering and dried plants [9]. Challenge tests with pollen and particulate plant debris including plant hairs also produced symptoms [9].

The refining process used in the production of *Echium* oil will filter out any pollen or particulate plant debris in the oil. Cytochrome C allergens isolated from the pollen of *Echium plantagineum* were characterized as proteins with a molecular weight of 12,800 [7]. It was assumed that all the protein present in the oil will be allergen. To test for the absence of allergens, we tested the protein content of the oil using the AOCS method (see Appendix 7) at a contract laboratory and also using bovine serum albumin as the standard in our in-house laboratory. The contract laboratory reported an absence of protein in the oil sample. In-house method also failed to detect any protein in the oil sample.

To confirm the absence of Cytochrome C allergens in the refined oil a total protein test has been performed using Bradford Reagent. The absorbance at 595 nm of the colored product of the reaction of protein and Bradford Reagent was measured.

Herbicide and Pesticides:

Herbicides and pesticides are the most common agrochemical products that are used in cultivation of crops. These products could potentially be used at two stages within the production cycle of *Echium*, pre-drilling for weed control or as a pre-harvest desiccant.

Weed control strategies for *Echium* are fundamentally based on drilling into what is termed a stale seedbed. In this technique, as many weed seeds are stimulated to germinate and grow as possible and are subsequently controlled, thereby leaving a minimal weed seed burden to cause problems in the following *Echium* crop. The control of germinated weeds can either be by cultivation methods or by the use of herbicides. Due to there being no crop present when applied, these herbicides can be non-selective and either systemic or contact in action. Products used are commonly based around actives such as Glyphosate, and Glufosinate-Ammonium. As these are broken down on contact with the soil, residues are not a problem for the following *Echium* crop which is not drilled at the time of application.

The *Echium* crop needs to be desiccated prior to combining not only to remove moisture from the crop but also to promote evenness of seed maturity. Mechanical swathing has been shown to be by far the best method of enhancing maturity and harvest for *Echium* to the extent that it is the only practice that is being utilized by contract growers for Bioriginal.

Chemical desiccation is achieved in several other crops using either contact or systemic broad spectrum herbicides to finally "kill off" the maturing plant. These products are based on two active ingredients, Glyphosate, Glufosinate-Ammonium. All of these products are readily and quickly broken down on contact with the soil and hence will not cause any future residue problems with following crops. Residue levels in the oils from treated crops can be considered a negligible risk for two reasons. Firstly these herbicides are not soluble in oil and secondly oil extraction and refining techniques used will breakdown or remove possible residues. Bioriginal does not recommend the use of any herbicide on Echium crops.

To make sure that the oil is free from herbicides and pesticides contamination, we tested several lots of oils. The results are presented in Appendix 8. The oil was found to contain less than detection limits of these herbicide/pesticides.

Based on the analysis of several batches of Echium seed oil, the complete specifications have been developed and are included in Appendix 9. These specifications are based on major fatty acids.

A peroxide value of 5 maximum is included on the product specification. The peroxide value for batches analyzed varies from 0.00 to 4.82. Peroxide value is an indicator of primary oxidation products in the oils containing unsaturated fatty acids. As the Echium oil is rich in unsaturated fatty acids, it is prone to oxidation. Peroxide values will indicate the level of oxidation/rancidity of the oil.

Anticipated use

Echium oil is a rich source of omega-6 and omega-3 polyunsaturated fatty acids. It contains stearidonic acid and γ -linolenic acid in addition to linoleic acid and α -linolenic acids. Gamma linolenic acid and stearidonic acids are produced in the human body from the desaturation of linoleic and alpha-linolenic acid respectively, by a rate limiting reaction catalyzed by enzyme delta-6-desaturase. Due to these fatty acids, it will be used as a dietary supplement of omega-3 and omega-6 fatty acids. The dietary supplement of Echium oil could be offered as soft gelatin capsules, bottles oils and/or oral emulsions. They may or may not contain antioxidants like vitamin E [10].

Oils rich in omega-6 fatty acids currently available on the market include soybean oil, safflower oil, blackcurrant seed oil, borage oil, and evening primrose oil. Soybean and sunflower oils are commonly used as cooking oils while borage, black currant and evening primrose oils are used as dietary supplements due to their content of gamma linolenic acid. Oils rich in omega-3 fatty acids currently available on the market include flax oil, perilla oil, herring oil, mackerel oil, menhaden oil, sardine oil and tuna oil. Omega-3 rich oils have been incorporated into breakfast cereals, milk, margarine, spreads, bread, cheese, yogurt, cocoa, soft drinks, tea, confectionery, cookies and infant foods [11-13]. Omega-3 enriched products are currently marketed in Japan, Korea, Taiwan and Europe including the United Kingdom and Scandinavia. docosahexaenoic acid (DHA) enriched eggs are being marketed in the USA and Canada. These are produced by enriching the diets of hens with either flaxseed or a DHA-enriched product [11, 12].

An analysis of seventeen brands of encapsulated fish oil products purchased in the USA, UK and Canada during 1984-1988 identified eicosapentaenoic acid levels of between 80 – 302 mg/gram and docosahexaenoic acid levels of between 78 - 254 mg/gram [14].

A similar analysis of encapsulated evening primrose oil products identified gamma linolenic acid levels of between 1.9 – 10.5 expressed as percentage weight of total fatty acids and linoleic acid levels of between 60.1 – 75.8 [15].

Several brands of omega-3/omega-6 fatty acid blends are currently marketed in the USA. These include The Total EFA™ (capsules and oil), Essential Max™ (capsules and bottled oil blend), Essential oils™ (capsules) and Omega 3,6,9™ (capsules). An omega-3 / omega-6 fatty acid blend which is currently marketed [Total EFA™] in the form of capsules provides 400 mg of α -linolenic acid, 160 mg of γ -linolenic acid, 144 mg of eicosapentaenoic acid and 90 mg docosahexaenoic acid per serving of 2 capsules. The omega-3 / omega-6 fatty acid blend is provided by combining borage oil, flax oil and a marine fish oil. Essential Max™ liquid provides 3 g alpha linolenic acid, 6 g linoleic acid and 1.8 mg gamma-linolenic acid per tablespoonful. The recommended dose is one and a half tablespoonful per 100 lbs body weight which translates to 6.75 g alpha-linolenic acid, 13.5 g of linoleic acid and 4.05 mg of gamma linolenic acid per day for an adult of average body weight of 150 lbs. Omega Twin™ liquid is a blend of flax oil and borage oil that provides 510 to 1020 mg gamma linolenic acid, 2.35 to 4.70 g linoleic acid and 5.20 to 10.40 gram alpha linolenic acid per day.

In comparison 1000 mg capsules based solely on Echium oil would provide 115 mg of γ -linolenic acid, 325 mg α -linolenic acid and 145 mg of stearidonic acid.

We do not intend to sell Echium oil direct to consumers. Echium oil will be sold to dietary supplement manufacturers throughout the USA as an alternative to existing oils and fats rich in omega-6 or omega-3 polyunsaturated fatty acids. We consider that the main application for Echium oil will be as a dietary supplement of essential fatty acids for adults and children above 12 years of age. This will be in capsule form or a blend with other nutritional oils with a likely level of consumption of either 500 mg to 2000 mg per day. Echium oil will be marketed as possessing the benefits of both omega-3 and omega-6 essential fatty acids.

Nutritional data

Total fat intake:

Dietary fat is essential for health and the FAO/WHO expert consultation on fats and oils in human nutrition have recommended that fat should constitute between 15% - 35% of energy intake [16]. Adequate dietary fat intakes are considered particularly important prior to and during pregnancy and lactation [16]. The FAO/WHO joint expert consultation recommended that women of reproductive age should consume at least 20% of their energy from fat [16]. A calorific fat intake of approximately 20% is normally used clinically in hospitalized patients who are infected or at risk of becoming so [17]. The American Heart Association recommends that total fat intake should be no more than 30% of total calorific intake.

The American Heart Association recommends saturated fat intake to be between 7 – 10% of total calories, a monounsaturated fat intake of up to 15% of total calories and polyunsaturated fat intake of up to 10% of total calories. The American Heart Association also recommends cholesterol intake should be less than 300 mg per day.

Americans are estimated to consume fats and oils at a level of 34 to 37% or more of their daily calories [18]. The average number of calories consumed per person per day is 2500 [18]. Since 1 gram of fat produces 9 calories, this amounts to 110 g of fat per person per day [18]. Levels of fat consumption reported for developed countries include: Denmark 160 g per day, New Zealand 155 g per day, United Kingdom 142 g per day and Canada 142 g per day [18]. The 1979 figures for the United States estimated fat consumption to be around 168 g per day of which 34% was saturated, 40% monounsaturated and 15% polyunsaturated [18].

Echium oil contains on average 11.1% of saturated fatty acids. This compares to levels of saturated fatty acids in omega-6 rich vegetable oils of 8.3% blackcurrant seed oil, 13.6% borage oil, 9% evening primrose oil, 16% soybean oil and 10.1% safflower oil [19]. The level of saturated fatty acids in herring oil is 26.1% and in mackerel is 27.5% [19].

Omega-6 fatty acids:

About 1% of daily calories (an average of 3 g) linoleic acid is enough to relieve the symptoms of deficiency of this essential fatty acid and therefore represents a minimum daily requirement [18]. The optimum dose of linoleic acid is considered to be between 3-6% (9-18 g on average) [18]. The FAO/WHO expert consultation on fats and oils in human nutrition have recommended that linoleic acid should provide between 4-10% of energy [16]. Recently, the Institute of Medicine, in its recent report, recommended an adequate daily intake of 17 g of linoleic acid for adult men and 12 g for adult women [20].

Echium oil contains on average 15.4% of linoleic acid and 11.5% of its metabolite gamma linolenic acid. Omega-6 rich vegetable oils such as blackcurrant seed oil, borage oil, evening primrose oil, soybean oil and safflower oil all provide significantly higher levels of linoleic acid. Gamma linolenic acid levels vary greatly from 0% for safflower oil to 10% for evening primrose oil and 20.68% for borage oil [19].

Omega-3 fatty acids:

The daily requirement and optimum dose of alpha linolenic acid is not known [18]. The Institute of Medicine, in its recent report recommended an adequate daily intake of 1.6 g of α -linolenic acid for men and 1.1 g for women [20]. A level of 0.54% of daily calories was required to reverse symptoms of alpha linolenic acid deficiency in a 6 year old girl [18]. An optimum dose is hypothesized for alpha-linolenic acid of 6 g per day [18]. It is estimated that 95% of affluent people would benefit from dietary supplementation with omega-3 fatty acids [18].

The average western diet contains lower quantities of omega-3 than omega-6. The dietary intake of total omega-3 fatty acids in the United Kingdom was estimated to be 250 mg per capita in 1992 [13]. Data from 1985 on the US national food supply indicates a level of 50 mg per capita per day of eicosapentaenoic acid and 80 mg per capita per day for docosahexaenoic acid [10].

Echium oil contains on average 30.7% of alpha-linolenic acid and 12.8% of its metabolite stearidonic acid. In comparison the total omega-3 fatty acid content of fish oils is 7.46% herring and 19.83% mackerel [19]. Although vegetable oils on the market such as corn and sunflower oil contain high levels of omega-6 fatty acids they usually have very low levels of omega-3 fatty acids [21]. Blackcurrant seed oil is an exception in that it contains 11.4% of alpha-linolenic acid and 3.02% of stearidonic acid.

Omega-6:omega-3 ratio:

The *delta*-6- desaturase step is considered to be rate limiting and the incorporation of high levels of linoleic or alpha-linolenic acid does not seem to raise the levels of their corresponding metabolites [21]. However, administration of the metabolites of linoleic and alpha linolenic acid usually raises the levels of that metabolite and its elongation products in human plasma [21].

Dietary supplementation with oils rich in linoleic acid, such as safflower oil, did not increase omega-6 fatty acid content of human milk [21]. Whereas oils rich in gamma linolenic acid such as evening primrose oil and black currant seed oil increased the levels of dihomogamma linolenic acid in human milk two fold [21].

The occurrence of eicosapentaenoic acid in the liver and plasma was two fold higher for rats whose diet was supplemented with the ethyl ester of stearidonic acid than with the ethyl ester of alpha-linolenic acid [22].

In a comparison of various combinations of omega-3 and omega-6 methyl ester mixtures it was demonstrated that gamma linolenic acid and its metabolites were incorporated more favorably into liver phospholipids than stearidonic acid and its metabolites [23]. Switching the omega-6 content from linoleic to gamma-linolenic increased the omega-6:omega-3 ratio two fold [23], whereas switching the omega-3 content from alpha linolenic to stearidonic acid decreased the omega-6:omega-3 ratio by 30% [23].

The enzymes that convert omega-6 and omega-3 fatty acids are slower by a factor of four in the case of omega-3 fatty acids [18]. However, detailed kinetic analysis of prostaglandin biosynthesis from omega-6 and omega-3 fatty acids indicated a four fold difference in favour of omega-6 [24].

A ratio of linoleic to alpha-linolenic acid of between 5:1 and 10:1 is recommended in the diet [16]. The FAO/WHO expert consultation on fats and oils in human nutrition have recommended that linoleic acid should provide between 4-10% of energy [16]. Therefore alpha-linolenic acid should provide between 0.4%-2% of energy depending on the amount of linoleic acid in the diet. The Institute of Medicine recommends intake of linoleic acid at 5 – 10% of total energy while that for α -linolenic acid at 0.6 to 1.2% [20]

The average western diet contains lower quantities of omega-3 than omega-6. The dietary intake of total omega-3 fatty acids in the United Kingdom was estimated to be 250 mg per capita in 1992 which represents only 0.09% of dietary energy [13]. Analysis of the diet of healthy 40 year old men in Edinburgh indicated that linoleic acid intake was low but still represented 3% of energy levels [21]. It is estimated that 95% of people would benefit from dietary supplementation with omega-3 fatty acids [18].

Echium oil offers high levels of both omega-6 (43.5%) and omega-3 (26.9%) fatty acids in a single oil of plant origin. It is rich in the metabolites of linoleic (11.5%) and alpha linolenic acid (12.8%) that are not affected by the rate limiting *delta*-6-desaturase step. The activity of the *delta*-6-desaturase enzyme is known to be inhibited by a number of factors, including diabetes, stress, excess saturated fats, high alcohol intake, smoking and viral infections. This can lead to deficiencies in the levels of the various essential fatty acids [25].

Microbiological data

Echium oil is an anhydrous system and therefore will not support microbiological growth. Processing conditions of manufacturing will act to filter out any microbial organisms. The absence of microbiological contamination has been confirmed by testing a sample of the oil (see Appendix 9).

Toxicological data

Echium plantagineum:

Echium plantagineum and its products have not hitherto been used for human consumption to a significant degree. Human exposure to the plant is limited to its use in the manufacture of honey. Experimental and field evidence is available on the effects in animals of ingestion of Echium plantagineum.

Echium plantagineum occurs over significant areas of farmland in Australia [6]. The young growth is eaten readily by livestock [6]. The plant is considered a weed in good pastures while on poor country it is considered a reserve fodder [5]. Measurements of herbage dry matter content, nitrogen content and digestibility of Echium plantagineum indicate that it would be nutritious forage for grazing animals [26]. However the presence of pyrrolizidine alkaloids in the plant means that there is a risk that grazing animals will be poisoned [6]. The level of pyrrolizidine alkaloids is normally between 0.1-0.3% of the dry weight of the whole plant but levels as high as 0.9% have been reported [27].

Field evidence strongly indicates that horses, pigs and to a lesser extent sheep are all affected [6]. Experimental evidence includes a study by the New South Wales Department of Agriculture in which young pigs were fed 15% Echium plantagineum in the diet [6]. All developed the typical chronic liver damage within 5 months and one animal died within 4 months [6].

Echium plantagineum was fed as the sole diet to crossbred sheep with or without a history of previous access to the plant in a pen feeding trial [28]. Compared to a control group receiving a diet of lucerne chaff and oats, sheep on the Echium diet lost weight and deaths occurred [28]. Histological examination produced evidence of excessive copper accumulation in the liver and biochemical evidence of liver toxicity and was usually accompanied by pyrrolizidine alkaloid damage [28]. It was concluded that Echium plantagineum alone was not suitable fodder for sheep [28].

There were no mortalities involving pyrrolizidine alkaloid poisoning in crossbred sheep grazing pasture for 19 months where Echium plantagineum constituted a considerable portion of the available forage [29]. Histological evidence of moderately severe liver damage associated with high liver copper concentrations was found in at least one sheep [15]. Sheep on the Echium plantagineum diet were significantly lighter and grew less wool as compared with sheep on Echium free pasture [29].

Young rats fed 40% Echium plantagineum for two weeks suffered 70% mortality within 5-13 weeks [30]. Young rats fed 20% Echium plantagineum for alternate two week periods with a control feed had 50% mortality in 21 weeks [30]. Adult rats fed Echium plantagineum continuously all died within 7-16 weeks at the 40% level and 37-40 weeks at the 20% level [30]. The rats died with a mixture of acute and chronic liver damage [30]. Tumors, 3 benign and one malignant, of a type observed in carcinogenesis experiments with other pyrrolizidine alkaloids developed in survivors of the study on adult rats fed 20% Echium plantagineum [6]. The number of tumors was below the significance level [6].

Honey bees collect nectar from Echium plantagineum flowers to produce honey [6]. It is estimated that honey from Echium plantagineum constitutes about 10-15% of total Australian production [6]. The honey is sold mainly as blends with other honey. Honey prepared from Echium plantagineum has been shown to contain between 0.27 – 0.95ppm alkaloids [31]. In a report published by the International Program on Chemical Safety, it is estimated that individuals may consume up to 80 g honey/day with a corresponding alkaloid intake of 80 µg/day, if only the Echium honey were used. No reports of human toxicity through this source are available [32]. The possible intakes of pyrrolizidine alkaloids from this source are considered to be very low [6].

All the toxicological findings reported are consistent with pyrrolizidine alkaloid poisoning. Pyrrolizidine alkaloids are not lipophilic therefore they would not be expected to be present in refined Echium plantagineum oil. An analysis of the alkaloid content of the crude and refined oil and the Echium plantagineum meal has been carried out. The meal contained 0.12 mg/g total alkaloids. Refined oil demonstrated only 4 ppb (4 µg/kg of oil) content of the alkaloids as tested with the method with the detection limit of 4 µg/kg; it is a non-significant amount. German health authorities have recommended a daily intake of not more than 1 µg of unsaturated pyrrolizidine alkaloids per day from the registered products and 0.1 µg/day from unregistered products [33]. To consume 0.1 µg of pyrrolizidine alkaloids from Echium oil, one will have to consume at least 25 kg of oil per day. This amount is impossible to consume as it is 12 to 25 thousand times the recommended dose of 1 to 2 g per day. Hence, there

is no concern regarding toxicity due to pyrrolizidine alkaloids from consumption of Echium oil at the recommended dose as a dietary supplement of essential fatty acids, or even in cases of accidental overdose.

Component fatty acids:

The lipid profile for Echium oil is similar to that of Borage oil and Blackcurrant oil [Appendix 10]. Both borage oil and blackcurrant oil are widely used as ingredients of cosmetics, pharmaceuticals, foods and food supplements [34], [35]. The major fatty acids found in Echium oil are as follows:

Palmitic acid is the most widely occurring saturated fatty acid and is present in most commercial oils [19]. It is found in large quantities in fish oils (10-30%) and tropical fats such as coconut (6.9%), palm kernel (6.5–11%) and palm (32–59%) oils [18, 19]. Echium oil contains on average 6.5% palmitic acid.

Stearic acid is found in abundance in tallow (5-30%), cocoa butter (30-36%) and shea nut butter (44%) [18, 19]. Echium oil contains on average 4.0% stearic acid.

Oleic acid is the most widely occurring natural fatty acid and is found in practically all lipids [19]. It is found in large quantities in olive (43.7-83%), almond (65-70%) and peanut (37.9%) oils [19]. Oleic acid is also manufactured in the body [18, 19]. Echium oil contains on average 15.2% oleic acid.

Linoleic acid is found in safflower (75.3%), sunflower (68.5%), soybean (53%) and sesame (45%) oils [19]. Echium oil contains on average 15.4% linoleic acid.

Linolenic acid is the major fatty acid found in plant leaves, stems and roots and other photosynthetic organisms [19]. Flax seed is the richest source of ALA with over 50%, Chia and kukui (candlenut) contain about 30%, hemp seed around 20% [18]. Pumpkin seed oil may have up to 15%, canola up to 10% and walnut between 3-11% [19]. Soybean oil normally contains 5-7% [19]. Echium oil contains on average 30.7% ALA.

The richest source of GLA is borage oil (22%) followed by black currant seed oil (15%) and evening primrose oil (9%) [18]. Echium oil contains on average 11.5% GLA.

Stearidonic acid is found in fish oils such as mackerel (2.47%), herring (1.1-2.8%), sardine (2.9%) and menhaden (0.8-3.6%) [19]. The most well known plant source of stearidonic acid is black currant seed oil (3%) [18]. Echium oil is characterized by higher levels of alpha-linolenic and stearidonic acids than observed in other plant oils such as borage oil and blackcurrant seed oil. Echium oil contains on average 14.1% stearidonic acid.

Omega-6 & omega-3 fatty acids:

Echium oil is considered to be substantially equivalent to existing oils and fats on the market which are rich in essential fatty acids. Essential fatty acids is a term used to describe fatty acids which are needed in order to manufacture body lipids, biological membranes and hormone like substances such as prostaglandins but which cannot be synthesized in the body and therefore must be obtained from the diet [36, 37]. Only two fatty acids are truly essential, linoleic acid and α -linolenic acid, the remaining polyunsaturated fatty acids are derived from these by a sequence of desaturation and elongation steps. Linoleic acid is the precursor for the omega-6 series of fatty acids which are found primarily in plant oils whereas *alpha*-linolenic acid is the precursor for the omega-3 series of fatty acids which occur mainly in green leafy vegetables and oily fish [Appendix 11] [37].

Both series of essential fatty acids are the starting materials for the manufacture of a group of complex hormone like compounds known collectively as eicosanoids which include the prostaglandins, leukotrienes, prostacyclins and thromboxanes. The eicosanoids have profound physiological activity even at extremely low concentrations. They are implicated in the functions of the nervous, cardiovascular and immune systems and can also affect the function of both the endocrine and exocrine glands.

The correct balance between the various eicosanoids is required in order to maintain good health. The ratio of omega-6:omega-3 in the body is about 1:1 in the brain, 5:1 in fat tissue and 4:1 in other tissues [18]. The levels of the eicosanoids can vary during different stages in the development of the body, with age and during the menstrual cycle. In addition the activity of delta-6-desaturase, an enzyme system involved in the metabolism of essential fatty acids, is known to be inhibited by a number of factors, including diabetes, stress, excess saturated fats, high alcohol intake, smoking and viral infections. This can lead to deficiencies in the levels of the various essential fatty acids [25]. The same enzymes are used to metabolize both the omega-3 and the omega-6 series of essential fatty acids

and it is believed that the metabolites of *alpha*-linolenic acid will compete for these enzymes with the metabolites of linoleic acid.

A number of diseases exhibit deficiencies in the various essential fatty acids and this has led to research into the pharmacological effects of omega-6 and omega-3 fatty acids as outlined below:

Cardiovascular disease:

The benefits of long chain polyunsaturated fatty acids in the prevention of cardiovascular disease has long been recognized. Modest supplementation of the diet with fish oil has a dramatic effect in reducing coronary death and doses of as little as 150 mg of eicosapentaenoic acid per day inhibit platelet aggregation [25]. Research into the effects of omega-3 fatty acids on the cardiovascular system indicates that these effects are small but cumulative resulting in a dramatic reduction in the risk of coronary heart disease, especially when used alongside standard therapy and lifestyle changes [38]. The combination of beneficial effects involved include, increased nitric oxide formation, a reduction in platelet-activating factor, thromboxane and fibrinogen levels, reduction of high blood pressure (both systolic and diastolic) and anti-arrhythmic effects [38]. Patients with glycogen storage disease type-1, taking fish oil supplements showed improvements in hypertriglyceridemia and hypercholesterolemia after three months [39]. Withdrawal of fish oil for a further three months resulted in a return to pretreatment abnormalities in plasma lipid and lipoprotein levels [39]. Omega-3 triglyceride treatment was associated with epistaxis in 8 out of 11 patients and prolonged bleeding time was noted in 3 patients [40].

Triglycerides of essential fatty acids have been utilized in pharmaceutical compositions for oral and parenteral application in the treatment and prevention of diseases caused by platelet aggregation such as thrombotic inflammation and arterial sclerosis [41]. The UK Medicines Committee has licensed the use of certain fish oil preparations in patients at risk of ischemic heart disease and/or pancreatitis and for the treatment of hypertriglyceridemia.

Gamma-linolenic acid and dihomo-gamma-linolenic acid also have a number of beneficial effects on the cardiovascular system including inhibition of platelet aggregation, reduction of blood pressure, vasodilation, lowering of cholesterol levels as well as inhibition of vessel wall smooth muscle and fibrous tissue proliferation [25]. There is evidence that low tissue and dietary linoleic acid content is associated with high incidence of coronary heart disease [21]. In patients with total cholesterol levels of >300 mg/decilitre the risk factor for atherosclerosis dropped from 6.34 to 3.48 with a daily dose of 6 x 450 mg capsules of black currant seed oil for 12 weeks [42, 43].

Osteoporosis:

Both omega-3 and omega-6 essential fatty acids appear to work synergistically to increase calcium absorption from the gut, reduce its excretion in urine and promote its deposition in bone rather than kidneys and arterial walls [25]. The essential fatty acids may therefore prove useful in the treatment of osteoporosis [25].

Diabetes:

Impairment of the metabolism of essential fatty acids in diabetic animals is believed to be responsible for a number of long term complications including damage to the eyes, nerves and kidneys [25].

There is conflicting evidence regarding the effects of omega-3 fatty acids in diabetic patients [44]. It is generally agreed that glucose control is not hampered in patients with insulin dependent diabetes [44]. Some studies have shown deterioration in glucose homeostasis for patients with non-insulin dependent (adult onset) diabetes [44]. However some of these studies had not corrected for the high energy content of the fish oil [44].

Gamma-linolenic acid is able to prevent diabetes induced reduction in nerve conduction velocity and reverse diabetic nerve damage in animals and humans [45, 46]. The exact mechanism is not clear but it is proposed to act by altering nerve blood flow [25]. Studies in which gamma linolenic acid was provided in combination with eicosapentaenoic acid and docosahexaenoic acid produced slightly but not significantly better results [47]. In combination with antioxidants (alpha lipoic acid), gamma linolenic acid was shown to act synergistically in prevention of a reduction in diabetic nerve conduction velocity deficit [46].

Kidney Disorders:

Animal studies have indicated potential benefits for fish oil therapy in the treatment of kidney disorders [48]. Fish oils are reported to delay the onset of nephritis and reverse proteinuria, prevention of nephrotoxicity and reversal of

dyslipidemia from cyclosporine has also been demonstrated [Ref 46]. Flax oil, a source of α -linolenic acid, has also been shown to prevent/treat nephritis.

Asthma:

In a double blind study in 12 asthmatic subjects a 23% increase in forced air volume was observed after 9 months of consuming 1 gram of eicosapentaenoic acid and docosahexaenoic acid per day [49]. In a study on 8,960 current or former smokers there was an inverse relationship between omega-3 fatty acid intake and risk of obstructive pulmonary disease [49].

Rheumatoid Arthritis:

Fish oil supplements containing essential fatty acids are reported to have beneficial effects on the symptoms of rheumatoid arthritis while offering few, if any, side effects at the levels used [25]. Modest clinical improvements usually emerge after 12 weeks of treatment and peak around 18 to 24 weeks [38]. The levels of eicosapentaenoic acid and docosahexaenoic acid required to produce a beneficial effect is around 90 mg/kg of bodyweight per day [49]. Stearidonic acid was as effective as eicosapentaenoic acid in inhibiting 5-lipoxygenase when tested in vitro [50]. The use of stearidonic acid and oils rich in this acid such as black currant seed oil and certain fish oils in anti-inflammatory pharmaceuticals administered orally, rectally, enterally or parenterally is patented in the US [51].

Gamma-linolenic acid has also proved effective in treating human rheumatoid arthritis [25, 52]. Both gamma-linolenic acid and dihomo-gamma-linolenic acid have demonstrated anti-inflammatory effects in animals and humans although these effects are subtly different from those demonstrated by eicosapentaenoic acid [25]. Acute and chronic inflammatory response in the rat was markedly reduced when fed with diets containing 15% borage seed oil (equivalent to 23% gamma-linolenic acid) [53]. Patients given six 500 mg capsules per day of black currant seed oil (equivalent to 525 mg of gamma linolenic acid) for six weeks reported modest clinical improvement compared to a control group taking sunflower seed oil [54]. In a double blind study on human patients of rheumatoid arthritis, GLA given as a free fatty acid in the dose of 2.8 g per day afforded significant improvement in the symptoms [55]. In another clinical trial, gamma linolenic acid given in the dose of 1.4 g per day as borage oil showed significant improvement in symptoms (joint tenderness, joint swelling, morning stiffness, grip strength, and ability to do daily activities) of arthritis over placebo [56].

Skin Disorders:

Mild to moderate improvement in psoriatic lesions was reported in 8 out of 13 patients consuming 60 g of fish oil (equivalent to 10.8 g of eicosapentaenoic acid per day) [49]. Patients with atopic eczema given 10 g of fish oil daily for 12 weeks showed significant improvement with regard to scaling, itching and subjective assessment of overall severity in comparison with a control group receiving olive oil [21].

Polyunsaturated fatty acids have demonstrated a protective effect against damage to muscle by free-radicals. As free-radicals are implicated in skin damage and skin cancer the effects of fish oil supplements on skin exposed to UV has been investigated. Levels of 10 g/day of an omega-3 triglyceride containing 18% eicosapentaenoic acid and 12% docosahexaenoic acid reduced the amount of sunburn which occurred in volunteers exposed to UV [57]. A reduction in the sensitivity to UV provocation of a papular response was also observed at this level in light sensitive patients suffering from moderate or severe polymorphic light eruption [58].

Treatment of atopic eczema with gamma-linolenic acid has shown modest beneficial effects [25]. An evening primrose oil formulation containing 8-9% gamma-linolenic acid and 71-74% linoleic acid has been approved by the UK Department of Health for the treatment of atopic eczema [21]. Studies using a mixture of 80% evening primrose oil and 20% fish oil have been reported to give better results than evening primrose alone in the treatment of atopic eczema [47]. In a pilot double blind study infantile seborrheic dermatitis cleared up in all the children treated with a cream containing 40% borage oil [38]. Nine children out of the placebo group showed no improvement and the remaining six members of the placebo group showed slight improvement ascribed to the mild keratolytic effects of the cream base [38]. Six out of nine patients with biliary pruritus showed significant improvement in symptoms when given 8 x 500 mg capsules of an evening primrose oil preparation for 12 weeks [21]. Patents have been applied for covering the use of essential fatty acid derivatives in the treatment of skin disorders such as psoriasis [59].

Anti-inflammatory Properties:

Echium oil contains stearidonic acid and gamma linolenic acid. Both of these fatty acids have been shown to exert anti-inflammatory activities [49-56, 60]. Stearidonic acid has been shown to possess anti-inflammatory properties

[50, 51, 60]. Ishihara et al. (2002) recently compared the anti-inflammatory activity of stearidonic acid with other omega-3 fatty acids (α -linolenic acid and eicosapentaenoic acid). They observed similar depression of tumor necrosis factor α (TNF- α) in whole blood from mice compared to the mice fed controlled diet [60].

Cancer:

Omega-3 essential fatty acids in vitro and in vivo are also reported to slow malignant cell proliferation, kill malignant cells and enhance susceptibility to conventional cytotoxic agents without harming normal cells [25]. The methyl ester of linolenic acid and the polyunsaturated fatty acids derived from it demonstrated anti-mutagenic activity on busulfan induced genotoxicity in Chinese hamsters [61].

There is abundant data demonstrating higher incidence of tumors of the mammary gland, intestine, skin and pancreas in animals fed high fat diets compared to animals fed low fat diets [16]. In experimental animal models of cancer diets high in omega-6 fatty acids produced the greatest incidence and size of tumors [49]. However in human populations the total fat content of the diet rather than the type of fat appears to have greater influence on incidence of cancer [16]. Inter-country studies indicate that levels of omega-6 fatty acids of around 4-8% of energy are not correlated with breast cancer [16].

Gamma-linolenic acid and dihomo-gamma-linolenic acid in vitro have been reported to kill 40 different human cancer cell lines within 5-7 days at concentrations which do not harm normal cells [25]. In addition, gamma-linolenic acid reduces the motility and invasiveness of cancer cells within hours [25]. The methyl ester of linoleic acid demonstrated anti-mutagenic activity on busulfan induced genotoxicity in Chinese hamsters [61]. Gamma linolenic acid in the form of evening primrose oil was found to inhibit the growth of R3230AC transplantable and of dimethylbenzanthracene induced mammary tumors in rats [47].

Psychiatric Disorders & Neuropathies:

Co-administration of arachidonic acid and docosahexaenoic acid in the form of free fatty acids or esters is patented for the treatment of the negative syndrome of schizophrenia by oral, enteral, parenteral, topical, rectal and vaginal routes [62]. A study in the USA indicated that approximately 40% of children suffering from Attention Deficit Hyperactivity Disorder (ADHD), which is characterized by behavioral, learning and health problems, exhibit low blood levels of omega-3 fatty acids [63].

Evening primrose oil has been reported to reduce hyperactivity in some children [21]. When given to children with mood disorders, 67% showed some improvement with evening primrose oil compared to a placebo of olive oil [21]. In a placebo controlled trial patients with Alzheimer's disease receiving evening primrose oil showed significant improvements in several tests of cerebral function [47]. In a double blind placebo controlled trial, schizophrenics receiving evening primrose oil showed moderately better Simpson scores for tardive dyskinesia and significantly improved psychosis scores [64].

Other areas of brain function in which the effects of omega-6 and omega-3 fatty acids have been investigated include alcoholism, depression, aggression, dyslexia, Huntingdon's Chorea, memory loss and dementias [38, 65-68].

Premenstrual Syndrome & Endometriosis:

A combination of 80% evening primrose oil and 20% fish oil was found to reduce the severity of symptoms in a significant number of women suffering from endometriosis [69].

Several studies have demonstrated that gamma linolenic acid in the form of evening primrose oil is better than placebo in the treatment of premenstrual syndrome and breast pain [47]. Patents have been applied for covering the use of essential fatty acid derivatives in the treatment of premenstrual syndrome [70].

Multiple Sclerosis:

Oils rich in essential fatty acids, such as evening primrose oil, have been investigated in the treatment of multiple sclerosis [71]. Multiple sclerosis patients taking safflower oil supplements for two years had less frequent and less severe relapses compared to a control group taking olive oil [Ref 17]. Improved mitogenic response was noted in lymphocytes from multiple sclerosis patients receiving evening primrose oil supplements for 85 days [Ref 17].

Wound Healing & Infection:

Wound healing in infants fed for long periods with fat-free parenteral nutrition was found to be defective and could be corrected by introducing essential fatty acids into the diet [72]. The influence of total parenteral nutrition of blackcurrant seed oil compared to soy oil on the metabolic response of acute operatively stressed guinea-pigs to endotoxin has been investigated [73]. No beneficial effects were observed with gamma-linolenic acid at the levels used in this study [73]. Arachidonic acid significantly enhanced human neutrophil antiparasitic activity to *Plasmodium falciparum* asexual blood forms [74].

Fish oil has been shown to improve survival of Guinea-pigs exposed to endotoxin [19, 49]. Substitution of half the safflower oil administered to burn patients with fish oil led to a reduction in wound infection and mortality and a shorter stay in the hospital [49]. Eicosapentaenoic acid and docosahexaenoic acid enhanced human neutrophil antiparasitic activity to *Plasmodium falciparum* asexual blood forms [74].

At the end of a 3 month trial 85% of myalgic encephalomyelitis (ME) patients receiving a combination of 80% evening primrose oil and 20% fish oil reported themselves better compared to 17% receiving a placebo [50]. AIDS patients taking a combination of evening primrose oil and fish oil showed significantly increased CD4 lymphocytes and on average gained weight and reported reduced fatigue and diarrhea [75].

Infant Development:

Lipids in the fetus rise from 0.1% at 24 weeks to 3-5% at 28 weeks and 15-16% at term [12]. After birth, infants gain on average 9-10 g of fat per day and 40-50% of their energy requirement is supplied by fat [12]. Human milk contains substantial quantities of essential fatty acid metabolites and therefore their role in infant development has been investigated [25].

Bottle fed premature and full-term infants demonstrated increased visual acuity when their diet was supplemented with eicosapentaenoic acid and docosahexaenoic acid [25]. In an 8 year follow-up study, breast-fed premature babies demonstrate higher IQ than formula-fed infants and this was believed to be due to docosahexaenoic acid which is present in breast milk but not in infant formula [16]. This theory is supported by animal studies in which docosahexaenoic acid levels in the brain correlated directly with performance in learning and intelligence tests.

Supplementation with a marine oil having an eicosapentaenoic acid: docosahexaenoic acid ratio of 2:1 was found to compromise arachidonic acid levels which adversely affected growth [16]. Arachidonic acid appears to play an important role in fetal growth since the levels of this acid correlate with head circumference [25]. However, supplementation with a marine oil low in eicosapentaenoic acid did not compromise weight gain and raised Bayley mental development scores at 12 months [16].

A supplement based on four parts tuna oil and one part evening primrose oil added to infant formula improved neurological visual evoked responses in preterm and term babies [76].

Guidelines offered by FAO/WHO for formula for preterm babies is 700 mg linoleic acid, 50 mg α -linolenic acid, 60 mg arachidonic acid and 40 mg of docosahexaenoic acid per kilogram bodyweight [16]. This is equivalent to 5.6% of energy as parent essential fatty acids and 0.8% as long chain polyunsaturated fatty acids [16]. In addition linoleic acid should not exceed 10% of total energy [16].

Total parenteral nutrition in the premature infant usually includes 2-4 g of soybean emulsion per kilogram bodyweight per day [21]. The soybean oil emulsion most commonly used for parenteral nutrition contains around 50% linoleic acid (omega-6) and 9% alpha-linolenic acid (omega-3) [21].

For full term infants the corresponding FAO/WHO recommended values per kilogram bodyweight are 600 mg of linoleic acid, 50 mg of alpha-linolenic acid, 40 mg of arachidonic acid and 20 mg of docosahexaenoic acid [16]. The guidelines recommend that the essential fatty acid composition of infant formulae and foods for infants up to the age of two should be similar to breast milk [16]. The critical role of both omega-6 and omega-3 fatty acids is recognized [16]. The ratio of linoleic to alpha-linolenic acid is recommended to be between 5:1 and 10:1 [16].

The FAO/WHO joint expert consultation recommend that the maternal diet should provide an additional 3-5 g per day of essential fatty acids during lactation [16].

Reproductive Effects:

High levels of omega-3 fatty acids in fish oil have been reported to prolong gestation and impair parturition in rats [48]. However omega-3 fatty acid supplements of 200 mg (omega-3:omega-6 = 0.8) did not alter any of the indices of gestational performance [17]. Omega-3 fatty acid supplements of 480 mg (omega-3:omega-6 = 2.8) did not significantly alter length of gestation although dam bodyweight and average pup weight was decreased [17]. There is evidence that high fish intakes are associated with longer gestation, higher birth weight and reduced incidence of premature birth [16].

Gastrointestinal Disturbances:

Significant clinical improvement was noted in patients with ulcerative colitis, an inflammatory bowel disease, receiving fish oil supplements for 12 weeks [49]. Administration of fish oil is reported to protect against damage to the gastro-duodenal mucosa caused by aspirin [77].

Slight side effects of nausea and eructation have been reported with high doses of the triglycerides of omega-3 fatty acids [40]. Other reports of adverse effects include loose motions and unabsorbed oil in stools with a purified ethyl ester of eicosapentaenoic acid [40].

Acute studies with linoleic acid have shown a protective effect on the gastric mucosa against challenge with ethanol but not aspirin [40]. Investigations carried out in animals have demonstrated a protective effect for gamma linolenic acid in gastric ulceration [47]. In a three way trial evening primrose oil performed better than fish oil and both performed better than a placebo in the treatment of ulcerative colitis [47].

Occasional mild gastrointestinal upsets such as eructation and loose bowel motions have been reported in clinical trials involving evening primrose oil rich in omega-6 fatty acids [40].

Allergic Reactions:

A 68 year old woman taking omega-3 triglycerides developed fever, myalgia, sore throat and tender lymphadenopathy lasting 3 weeks; symptoms recurred 48 hours after restarting treatment [40].

Mortality:

The effects of dietary polyunsaturated fatty acids on mortality have been investigated in the Multiple Risk Factor Intervention Trial (MRFIT) [78]. The Multiple Risk Factor Intervention Trial was a randomised clinical trial in coronary heart disease primary prevention involving 12,866 middle-aged men determined to be at high risk [78]. Subjects were assigned to either a Special Intervention (SI) or Usual Care (UC) group [78]. Only data on the UC group (6,250 subjects) were analysed [78]. PUFA intakes were determined by dietary recall interviews at baseline and follow up years 1, 2 and 3 [78]. The study has been ongoing since 1979 and the following analysis is based on deaths up to 1985 representing up to 7.7 years of follow up [78]. Four mortality outcome groups were established: 1) coronary heart disease (CHD), 2) cardiovascular disease (CVD), 3) cancer (CA); and 4) all causes (AC) which represented 75 other causes of mortality in addition to CHD CVD and CA. The data was evaluated by proportional hazards regression and quintile analysis controlling for age, race, baseline diastolic blood pressure, high and low density lipoprotein cholesterol levels, smoking and alcohol [78].

There were no significant changes in mortality associated with linoleic acid (C18:2n-6) for any mortality group [78]. A significant inverse relationship was noted with C18:3n-3 and CHD, CVD and AC mortality when expressed as percentage of total kilocalories and for AC mortality when expressed in g [78]. Results for the combined fatty acids normally found in fish oil showed significant inverse associations with CHD, CVD and AC mortality expressed in percentage of total kilocalories and with CHD and CVD when expressed in g [78]. A significant inverse relationship was observed with CA mortality and 18:3n-3:18:2n-6 ratios or total n-3:n-6 ratios [78].

Conclusion

Dietary fat is essential for health. An optimum dose as recommended by the Institute of Medicine for linoleic acid is 17 g per day for men and 12 g per day for women for alpha-linolenic acid of 1.6 g per day for men and 1.2 g per day for women [20]. Echium oil offers high levels of both omega-3 (43.5%) and omega-6 (26.9%) fatty acids in a single oil of plant origin. It is rich in the metabolites of linoleic (11.5%) and alpha linolenic acid (12.8%) that are not affected by the rate limiting delta-6-desaturase step. The activity of the delta-6-desaturase enzyme is known to be inhibited by a number of factors, including diabetes, stress, excess saturated fats, high alcohol intake, smoking and viral infections. This can lead to deficiencies in the levels of the various essential fatty acids [25].

Echium oil is intended to be sold as a dietary supplement for adults and children over 12 years of age either in the form of an oral liquid, emulsion or capsules. The likely level of consumption will be between 500 to 2000 mg per day.

Echium plantagineum and its products have not hitherto been used for human consumption to a significant degree. Human exposure to the plant is limited to its use in the manufacture of honey. Experimental and field evidence is available on the effects in animals of ingestion of Echium plantagineum foliage. All the toxicological findings reported are consistent with pyrrolizidine alkaloid poisoning. The refined oil (item of commerce) contains only 4 parts per billion of pyrrolizidine alkaloids. At this level, no toxicity is expected.

Triglycerides are consumed every day in any normal mixed diet. Triglycerides of fatty acids derived from edible sources are considered to have no acute toxic effects at practicable dosage levels. Coronary heart disease, stroke and certain cancers (breast, colon, prostate and skin cancers) have been linked to high fat diets. However the evidence indicates that it is the saturated fat content which is the major factor in increasing risk of cardiovascular disease. For cancer, the total fat content of the diet rather than the type of fat appears to have more influence.

The major fatty acids found in Echium oil are palmitic, stearic, oleic, linoleic, alpha linolenic, gamma linolenic and stearidonic acids. All these fatty acids are normal components of the human diet. The lipid profile for Echium oil is similar to that of borage oil and blackcurrant oil which are rich in omega-6 fatty acids. Both borage oil and blackcurrant oil are widely used as ingredients of cosmetics, pharmaceuticals and food supplements. However, Echium oil is characterized by higher levels of omega-3 fatty acids than observed in these oils.

Echium oil is expected to possess physiological properties similar to those of fish oils which are rich in omega-3 fatty acids and plant oils rich in omega-6 fatty acids. A number of diseases exhibit deficiencies in essential fatty acids and this has led to research into the effects of omega-6 and omega-3 fatty acids. Omega-3 and omega-6 fatty acids have demonstrated prophylactic effects in cardiovascular disease, diabetes, kidney disorders, asthma, rheumatoid arthritis, eczema, psoriasis, psychiatric disorders and neuropathies, multiple sclerosis, premenstrual syndrome, endometriosis, ulcerative colitis, wound healing and infection. The critical role of both omega-6 and omega-3 fatty acids in infant development is recognized. The UK Medicines Committee has licensed the use of certain fish oil preparations in patients at risk of ischemic heart disease and/or pancreatitis and for the treatment of hypertriglyceridemia. Echium oil has been shown to possess anti-inflammatory properties.

High levels of omega-3 fatty acids in fish oil have been reported to prolong gestation and impair parturition in rats. There is evidence that high fish intakes are associated with longer gestation, higher birth weight and reduced incidence of premature birth. However omega-3 fatty acid supplements of 200 mg did not alter any of the indices of gestational performance. Omega-3 fatty acid supplements of 480 mg did not significantly alter length of gestation although dam bodyweight and average pup weight was decreased. Slight side effects of nausea and eructation have been reported with high doses of the triglycerides of omega-3 fatty acids. Occasional mild gastrointestinal upsets such as eructation and loose bowel motions have been reported in clinical trials involving evening primrose oil rich in omega-6 fatty acids. As noted under "Allergic Reactions", page 15 of this application, a 68 year old woman taking omega-3 triglycerides developed fever, myalgia, sore throat and tender lymphadenopathy lasting 3 weeks, with symptoms recurring 48 hours after restarting treatment. There have been no other reports of allergic reactions to omega-3 triglycerides.

5) The signature of the person designated by the manufacturer or distributor of the dietary supplement that contains a new dietary ingredient.

Signature:

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Appendix 1 – Fatty acid profile of Echium seed oil

Table 1: Fatty acid profile of Echium seed oil extracted and refined in the laboratory

Fatty Acid	Content in oil (Area % of total fatty acids)			
	Lot number			
	EO-010803-CF Unrefined	EO-010803-N Refined	EO-010803-RB Lab Refined	Seed oil (extracted in lab)
C16:0 Palmitic	6.76	6.61	6.58	6.91
C16:1n7 Palmitoleic	0.12	0.11	0.11	0.12
C18:0 Stearic	3.82	3.59	3.61	3.72
C18:1 Oleic	15.59	15.24	15.28	15.91
C18:2 Linoleic	15.30	14.91	14.91	14.68
C18:3n6 γ -Linolenic	11.12	11.15	11.11	12.11
C18:3n3 α -Linolenic	32.30	32.59	32.51	30.81
C18:4 Stearidonic	13.24	14.03	14.08	14.00
C20:0 Arachidic	0.11	0.09	0.10	0.12
C20:1n9 Eicosenoic	0.73	0.81	0.75	0.76
C22:0 Behenic	0.05	0.05	0.06	0.05
C22:1n9 Erucic	0.13	0.14	0.15	0.14
C24:1 Tetracosanoic	0.14	0.13	0.14	0.13

Table 2: Fatty acid profile of Echium seed oil extracted and refined at full production scale.

Fatty Acid	Content in oil (Area % of total fatty acids)			
	Lot number			
	8160-E03	8160-E01	8160-F01	8160-D02
C16:0 Palmitic	6.6	6.9	6.5	6.8
C16:1n7 Palmitoleic	0.1	0.1	0.1	0.2
C18:0 Stearic	3.7	3.4	3.6	3.8
C18:1 Oleic	14.7	16.4	15.1	14.8
C18:2 Linoleic	14.2	17.8	14.7	15.2
C18:3n6 γ -Linolenic	11.3	10.8	11.1	10.8
C18:3n3 α -Linolenic	32.7	30.2	32.5	32.7
C18:4 Stearidonic	14.6	13.1	14.1	13.2
C20:0 Arachidic	0.1		0.1	0.1
C20:1n9 Eicosenoic	0.7	0.8	0.7	0.7
C22:1n9 Erucic	0.2	0.4	0.2	
C24:1 Tetracosanoic	0.1	0.1	0.2	0.1

Appendix 2: Test method for analysis of Fatty acid profile

SAMPLING AND ANALYSIS OF COMMERCIAL FATS AND OILS

AOCS Official Method Ce 1e-91
Revised 2000

Determination of Fatty Acids in Edible Oils and Fats by Capillary GLC

DEFINITION

This method is for the determination of fatty acid methyl esters by capillary gas-liquid chromatography.

SCOPE

This method is applicable to the determination of fatty acid methyl esters obtained from vegetable oils and fats according to AOCS Official Method Ce 2-66, IUPAC Method 2.301, AOAC 969.33 or other accepted standard method.

APPARATUS

The instructions given apply to normal equipment used for gas-liquid chromatography employing capillary columns and flame-ionization detection.

1. Gas-liquid chromatograph.
2. Injection system.

Note—The injection system should be specially designed for use with capillary columns. It should be of the split type or on-column (see Notes, 1).

3. Oven.

Note—The oven should be capable of heating the column to at least 220°C and of maintaining the desired temperature to within 0.1°C (see Notes, 2).

4. Capillary column, with following specifications—fused silica or glass; length 25–60 m with 0.20–0.35 mm i.d.
5. Stationary phase—moderate polarity, mainly of the type: polyglycol (polyethylene glycol 20,000), polyester (butanediol polysuccinate) or polar polysiloxane (cyanosilicones), e.g., Carbowax, Durabond 225, FFAP, Silar 5 CP or Supelcowax. The coating should be 0.2–0.25 µm in thickness.
6. Assembly and conditioning of the column:
 - (a) Observe the normal precautions for assembling capillary columns—arrangement of the column in the oven (support), choice and assembly of joints (leak tightness), positioning of the ends of the column in the injector and the detector (reduction of dead spaces). Place the column under a carrier gas flow (e.g., 0.3 bar for a 25-m column of 0.3 mm i.d.).
 - (b) Condition the column by temperature programming the oven at 3°C/min from ambient temperature to a temperature 10°C below the decomposition limit of the stationary phase. Maintain at this temperature for 1 hr or until the baseline stabilizes. Return to 180°C to work under isothermal conditions.
7. Syringe—maximum capacity of 10 µL graduated in 0.1 µL.
8. Any suitable recorder, integrator or data processor may be used.

REAGENTS

1. Carrier gas—either helium or hydrogen (see Notes, 3).
2. Auxiliary gas—hydrogen 99.9% min, free from organic impurities, air or oxygen.

3. Reference standards—a mixture of methyl esters of fatty acids (see Notes, 4).

PROCEDURE

Selection of Optimum Operating Conditions

See IUPAC Method 2.304, capillary column gas-liquid chromatography of fatty acid methyl esters, section 5.1.1.

The determination of efficiency and resolution as carried out for packed columns (Ce 1-62) is no longer necessary, as commercially available capillary columns usually exhibit excellent performance. Instead, a system suitability test can be carried out by analyzing a certified reference material. In doing so, the operating conditions, the efficiency and resolution of the column can be checked at the same time.

System Suitability Test

Reference standards of known fatty acid composition (containing, e.g., C16:0, C18:0, C18:1, C18:2, C18:3 and C20:0 methyl esters) or certified reference material (e.g., CRM 162) are used to check the performance of the system (Notes, 4). If the quantitative results obtained correspond to the theoretical values or the certified values, the system can be regarded as suitable.

Sample Analysis

1. Use the syringe (Apparatus, 7) to take 0.1–1 µL of the solution of 1 to 5% of methyl esters prepared according to AOCS Official Method Ce 2-66, IUPAC Method 2.301 or AOAC 969.33 (see Notes, 1).
2. Operate the oven isothermally at a temperature of 180–210°C or operate by linear heating from 80–220°C (see Notes, 5).
3. Set injector and detector temperatures 30–50°C above the column temperature (see Notes, 7).

CALCULATION AND EXPRESSION OF RESULTS

Identification of Peaks

Analyze a reference standard mixture of known composition under the same operating conditions as those employed for the samples and determine the retention times (Notes, 4). Identify the peaks from the sample using the retention times of the reference standards.

Ce 1e-91 • Determination of Fatty Acids in Edible Oils and Fats

Quantitative Analysis

- (a) Apart from exceptional cases, assume that all of the components of the sample are represented on the chromatogram, so that the total of areas under the peaks represents 100% of the constituents (excluding the solvent peak).
- (b) For samples in which significant amounts of components below C12 are absent, calculate the content of a particular constituent (expressed as percent of methyl ester) by determining the percentage represented by the area of the corresponding peak relative to the sum of the area of all the peaks.

Area percent of the component *i* expressed as methyl ester =

$$\frac{A_i}{\sum A_i} \times 100$$

Where—

$\sum A_i$ = sum of the areas under all the peaks

In the presence of significant amounts of components below C12, the areas obtained from the gas chromatogram have to be multiplied with correction factors in order to convert area percentages into mass percentages.

Correct the area of each peak to compensate for the flame ionization detector (FID) response for each component. The FID correction factors are calculated from the molecular weight of the FAME as follows:

$$FID_x = \frac{MW_x}{(n_x - 1)(AW_C)(FID_{16:0})}$$

Where—

FID_x = the FID factor for component *x*

MW_x = molecular weight of component *x*

n_x = the number of carbon atoms in the FAME of component *x*

AW_C = the atomic weight of carbon (12.01)

$FID_{16:0}$ = the FID correction factor for 16:0 (1.407)

All other FID correction factors used in the calculation are relative to $FID_{16:0}$. For example, the correction factor for 10:0 becomes 1.10. FID correction factors are listed in Table 3.

Calculate the (relative) percentage *x* of each component by determining the corrected area of the corresponding peak relative to the sum of the corrected areas of all the peaks, as follows:

$$x = \frac{A_x}{A_t}$$

Where—

A_x = the corrected area of the peak corresponding to component *x*

A_t = sum of the corrected areas under all the peaks, excluding the solvent peak

PRECISION

The results of interlaboratory studies, organized at the international level, gave the statistical results (evaluated in accordance with International Organization for Standardization (ISO) 5725-1986) which are summarized

in Tables 1 and 2 (References, 2). See also—Tables 2.303.1 and 2.304.1 (References, 1).

Repeatability—When the mean of the values obtained from two single determinations, carried out in rapid succession by the same operator using the same apparatus under the same conditions for the analysis of the same test sample, lies within the range of the mean values cited in Tables 1 and 2, the difference between the two values obtained should not be greater than the repeatability value (*r*) for the level of fatty acids comparable to those cited in Tables 1 and 2.

Reproducibility—When the values for the final result, obtained by operators in different laboratories using different apparatus under different conditions from the analysis of the same laboratory sample, lie within the range of mean values cited in Tables 1 and 2, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility (*R*) for the level of fatty acids comparable to those cited in Tables 1 and 2.

NOTES

1. On-column injection is preferred because it generally gives less discrimination with oil-column injection mode. Dilute the solution to 0.05% before injection. An automatic injection system is recommended because it improves the precision of results. If a split injector is used, adjust the split ratio to approximately 1:100.
2. Apparatus equipped with a temperature programmer is recommended.
3. Either helium or nitrogen may be suitable as a carrier gas, but these may increase helium elution times with respect to hydrogen. Hydrogen, which is used only with capillary columns, can double the speed of analysis, but it is hazardous. Safety devices are available and should

Table 3
List of FID response factors.

FAME	MW	n - 1	FID factor	Correction factor
4:0	102.13	4	2.126	1.51
6:0	130.19	6	1.807	1.28
8:0	158.24	8	1.647	1.17
9:0	172.27	9	1.594	1.13
10:0	186.30	10	1.551	1.10
11:0	200.32	11	1.516	1.08
12:0	214.35	12	1.487	1.06
13:0	228.37	13	1.463	1.04
14:0	242.40	14	1.442	1.02
15:0	256.42	15	1.423	1.01
16:0	270.46	16	1.407	1.00*
17:0	284.49	17	1.393	0.99
18:0	298.52	19	1.370	0.97
19:0	312.52	19	1.370	0.97
20:0	326.57	20	1.360	0.97
21:0	340.57	21	1.350	0.96
22:0	354.62	22	1.342	0.95
23:0	368.62	23	1.334	0.95
24:0	382.68	24	1.328	0.94

*Reference.

be used. Oxygen must be removed from the carrier gas by suitable filters.

4. Standards of most known fatty acids are available from suppliers such as: Nu-Check-Prep, Inc., P. O. Box 172, Elysian, MN, USA; Bast of Copenhagen V, Denmark; Lanodon Fine Chemicals AB, Limhamnsgränd 9, S21616, Malmö, Sweden; Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178, USA. Otherwise oils with specific fatty acids should be used as standards, such as blackcurrant seed oil containing C18:3 (n-6) (γ -linoleic acid)* and C18:4 (n-3) (stearidonic acid)**.

* systematic name, 6,9,12-octadecatrienoic acid.

** systematic name, 6,9,12,15-octadecatetraenoic acid. Alternatively, certified reference material (e.g., CRM 162) from BCR, Joint Research Center, Institute for Reference Materials and Measurement, Reijseweg, B-2440, Belgium, can be used.

5. Operating the oven by linear heating increases the speed of fatty acid elution but does not improve or reduce the resolution of the gas chromatograms. A suitable heating program is—

(a) Start at an oven temperature of 80°C; hold for 2 min.

(b) Heat up at a rate of 20°C/min until 125°C; hold at 125°C for 1 min.

(c) Continue to increase temperature at a rate of 3°C/min until 220°C.

(d) Hold at 220°C for at least 5 min until all high carbon number fatty acid methyl esters are eluted.

6. In the abbreviated symbols C18:2 and C18:3, C18 stands for the number of carbon atoms (18) whereas :2 and :3 indicate the number of double bonds in the fatty acid chain, e.g., :0 means saturated fatty acids.

7. For on-column injection, set the injector and oven approximately 10°C beneath the boiling point of the solvent and start with heating 1 minute after injection (boiling point 60°C for hexane).

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Appendix 3: Analysis of non-triglyceride components (unsaponifiables)

To: Bioriginal Food & Science Corp.
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Jeanette Fusick

Project No: 03-905
Report Date: 21/11/03
Lab Group ID: 111303163542
Lab Number: AA01394

ANALYTICAL REPORT

Sample Description: Echium Oil Lot #EO-010803-N

PO#: 177145

Analysis Results:

Analyte	Result	Units	
Unsaponifiable Matter	0.91	%	
Monoglycerides	<0.005	%	
Diglycerides	2.10	%	
Triglycerides	97.9	%	
Campesterol	143	mg/100g	
Stigmasterol	5.0	mg/100g	
B-sitosterol	114	mg/100g	
Others	305	mg/100g	
Protein	0	%	Nx 6.25
alpha tocopherol	4.8	mg/100g	
gamma tocopherol	63.0	mg/100g	
delta tocopherol	8.4	mg/100g	

Laurie Forseille
Analytical Services Coordinator

SAMPLING AND ANALYSIS OF COMMERCIAL FATS AND OILS

AOCS Official Method Ca 6b-53

Reapproved 1997 • Revised 2001

Unsaponifiable Matter

DEFINITION

Unsaponifiable matter includes those substances frequently found dissolved in fats and oils that cannot be saponified by the usual caustic treatment, but are soluble in ordinary fat and oil solvents. Included in this group of compounds are higher aliphatic alcohols, sterols, pigments and hydrocarbons.

SCOPE

Applicable to fats and oils containing higher levels of unsaponifiable matter than usually found in normal tallows and greases. This method is especially suited for marine oils, but is also applicable to vegetable oil deodorizer distillates and sludges. This method does not apply to feed-grade fats.

APPARATUS

1. Extraction cylinder—graduated, with glass stopper, capacity about 200 mL (see Notes, 1).
2. Erlenmeyer flasks—narrow mouth, 250-mL capacity, with $\frac{3}{4}$ " outer joint.
3. Separatory funnels—250 mL.
4. Glass siphon—see Procedure, 3 and Notes, 1.
5. Condensers—with $\frac{3}{4}$ " joint to fit Erlenmeyer flasks. Either water- or air-cooled condensers may be used.
6. Beakers—250 mL.
7. Erlenmeyer flasks, or flat-bottom extraction flasks—50 mL.

REAGENTS

1. Ethyl alcohol, 95%—USSD formulas 30 and 3A are permitted (see Notes, *Caution*).
2. Aqueous potassium hydroxide (KOH), 50% by weight—prepared by dissolving 60 g of reagent-grade KOH in 40 mL of distilled water with cooling (see Notes, *Caution*).
3. Aqueous potassium hydroxide (KOH) solution, 0.5 N, prepared by dissolving 30 g of reagent grade KOH in water, cooling and diluting to 1 liter (see Notes, *Caution*).
4. Sodium hydroxide (NaOH) solution, 0.02 N—accurately standardized. See AOCS Specification H 12-52.
5. Phenolphthalein indicator solution—1.0% in 95% ethyl alcohol.
6. Diethyl ether—reagent grade, free from peroxides (see Notes, *Caution*).
7. Acetone—reagent grade (see Notes, *Caution*).

PROCEDURE

1. Accurately weigh about 2.0–2.5 g \pm 0.1 mg of well-mixed sample into a 250-mL Erlenmeyer flask with ground-glass joint. Add 25 mL of 95% ethyl alcohol and 1.5 mL of 50% KOH solution. Boil gently but steadily under reflux, with occasional swirling, for 30 min or until completely saponified. Complete saponification is essential. No loss of alcohol should occur during saponification.
2. Transfer while warm to the extraction cylinder (see Notes, 1), using a total of 50 mL of water. Wash the flask with 50 mL of diethyl ether and add to the cylinder. Cool the contents of the cylinder to room temperature (20–25°C).
3. Insert the stopper and shake vigorously for at least 1 min, and allow to settle until both layers are clear. Use a glass siphon to remove the upper layer as completely as possible without including any of the lower portion (see Notes, 1). Transfer the diethyl ether layer to a 250-mL separatory funnel.
4. Repeat the extraction two more times, using 50-mL portions of diethyl ether each time and shaking vigorously with each extraction (see Notes, 2).
5. Rotate the combined diethyl ether extracts gently with 20 mL of water. Violent agitation at this step may result in emulsions that are difficult to break. Allow the layers to separate completely and draw off the lower aqueous layer. Wash the diethyl ether layer two more times, using 20 mL of water each time, shaking gently each time and discarding the lower aqueous layer after separation.
6. Wash the combined extracts in the separatory funnel three times, using 20-mL portions of 0.5 N KOH, shaking vigorously. Follow each alkali washing by washing with 20 mL of water. If an emulsion forms during this washing procedure, allow to separate as much as possible, discard the clear aqueous layer and proceed with the next step, leaving any emulsion in the separatory funnel with the diethyl ether layer. After the third washing with 0.5 N KOH, wash the diethyl ether with successive 20-mL portions of water until the washings are no longer alkaline to phenolphthalein.
7. Transfer the diethyl ether extract to a tared beaker, rinsing the separatory funnel and its pouring edge with diethyl ether and adding the rinsings to the solution in the beaker. Evaporate to dryness in a water bath, using a gentle stream of clean, dry nitrogen. When almost all of the diethyl ether has been evaporated, add 2–3 mL of acetone and remove all traces of solvent with the aid of a stream of nitrogen. Complete the drying to constant weight in a vacuum oven at 75–80°C and an internal pressure of not more than 200 mm of mercury. Cool in a desiccator and weigh. The result becomes "A" in the calculations.
8. After weighing, take up the residue in 2 mL of diethyl ether and then add 10 mL of 95% alcohol, containing phenolphthalein indicator and previously neutralized to the phenolphthalein end point. Titrate with 0.02 N NaOH to the same final color. Correct the weight of the residue (see Notes, 3) for free fatty acid content, using

SAMPLING AND ANALYSIS OF COMMERCIAL FATS AND OILS
Ca 6b-53 • Unsaponifiable Matter

the following relationship: 1 mL of 0.02 N NaOH is equivalent to 0.0056 g of oleic acid. The grams of fatty acid determined by this titration become "B" in the calculations. A reagent blank correction should be determined.

- Correct for any reagent blank by conducting the unsaponifiable matter procedure without any fat or oil present. The blank determined by this procedure becomes "C" in the calculations.

CALCULATIONS

$$1. \text{ Unsaponifiable matter, \%} = \frac{A - (B + C)}{\text{mass of sample, g}} \times 100$$

Where—

- A = mass of residue, g
- B = mass of fatty acid, g
- C = mass of blank, g

PRECISION

For vegetable oil deodorizer distillates and sludges—

- Two single determinations performed in the same laboratory should not differ by more than 2.6%.
- Two single determinations performed in different laboratories should not differ by more than 3.8%.

For other fats and oils see Numbered Notes, 4.

NOTES

Caution

Ethyl alcohol (ethanol) is flammable. Use a fume hood when heating or evaporating this solvent.

Potassium hydroxide, like all alkalis, can burn skin, eyes and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials, such as potassium hydroxide, always add pellets to water and not the reverse. Alkalis are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Acetone is highly flammable. It may form explosive peroxides with oxidizing agents. Use effective fume-removal device. Do not mix with chloroform.

NUMBERED NOTES

- Alternately, a 500-mL separatory funnel may be substituted for the extraction cylinder, eliminating the need for the siphon. If a separatory funnel is substituted, draw off the lower aqueous layer into another separatory funnel, retaining the diethyl ether extract in the

Table 1
 Test organized by FOSFA International in June 1995.

	Soybean oil ^a	
	A	B
No. of participating laboratories after eliminating outliers ^b	49	50
Mean value, % (by mass)	0.58	0.69
Repeatability standard deviation, s_r , %	0.025	0.07
Repeatability limit r ($2.8 \times s_r$), %	4.3	0.027
Coefficient of variation of repeatability, %	0.08	3.9
Reproducibility standard deviation, s_R , %	0.22	0.62
Reproducibility limit R ($2.8 \times s_R$), %	37.9	0.24
Coefficient of variation of reproducibility, %	0.67	34.7

^aSample A is refined, bleached, deodorized soybean oil. Sample B is dried, crude water-degummed soybean oil.

^bCollaborative test involving 51 laboratories in 16 countries.

Table 2
 Test organized by the FOSFA International.

	Fish oil
No. of participating laboratories after eliminating outliers ^a	37
Mean value, % (by mass)	0.81
Repeatability standard deviation, s_r , %	0.02
Repeatability limit r ($2.8 \times s_r$), %	0.06
Coefficient of variation of repeatability, %	2.46
Reproducibility standard deviation, s_R , %	0.29
Reproducibility limit R ($2.8 \times s_R$), %	0.81
Coefficient of variation of reproducibility, %	35.8

^aCollaborative test involving 43 laboratories in 17 countries.

first funnel. Repeat the diethyl ether extraction of the aqueous phase, as noted in Procedure, 4, combining all of the diethyl ether extracts in the first funnel.

2. Some fats high in unsaponifiable matter, especially those of marine origin, may require more than three extractions for complete removal of the unsaponifiable matter. This is best judged by making another extraction and separately evaporating this extract as noted in Procedure, 7. There should be no unsaponifiable matter in this extract. If there is, dissolve in a small volume of diethyl ether and add back to the combined extracts. Continue with the extractions until no unsaponifiable matter remains in the extract.
3. The titration correction for extractable free fatty acids and other extractable unsaponifiable impurities (both reported as oleic acid) will tend to increase as the crude nature of the sample increases. For example, high-energy fats (used in animal feeds), feed fats, tall oil and foots would be expected to give a higher free fatty acid titration than relatively pure refined, bleached and deodorized (RBD) oil.
4. ISO 3596 recommends the use of samples up to 5.0 g and adjusting solution volumes to 50 mL KOH/ethanol and doubling the volumes of diethyl ether and washing solutions. Precision values using these conditions are presented in Tables 1-3.

Table 3
Test organized by IUPAC between 1976 and 1997.

	Refined soybean oil	Refined tallow	Crude rapeseed oil
No. of participating laboratories after eliminating outliers ^a	10	10	10
Mean value, % (by mass)	0.630	0.253	1.432
Repeatability standard deviation, s_r , %	0.032	0.069	5.0
Repeatability limit r ($2.8 \times s_r$), %	0.024	0.067	9.3
Coefficient of variation of repeatability, %	0.068	0.19	24.7
Reproducibility standard deviation, s_R , %	0.140	0.397	22.3
Reproducibility limit R ($2.8 \times s_R$), %	0.154	0.435	60.9
Coefficient of variation of reproducibility, %	0.137	0.389	9.6

^aInternational collaborative test involving 10 laboratories.