

Bioriginal

Bioriginal Food & Science Corp.

RECEIVED

Aug. 15, 2001

102 Melville Street
Saskatoon, Saskatchewan
Canada S7J 0R1
Ph 306 975 1166
Fx 306 242 3829
www.bioriginal.com

August 13, 2001

Division of Standards and Labeling Regulations
Office of Nutritional Products, Labeling, and Dietary Supplements (HFS-820)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, SW
Washington, DC 20204
Telephone Number: (202) 205-4168

COPY

Attention: Rhonda Kane

Dear Rhonda:

Re: New Dietary Ingredient Notification for Crosssential SA14

As required under 21CFR190.6, I am submitting one original and two copies of the New Dietary Ingredient Notification on Crosssential SA14 (Echium oil). Unless otherwise directed, Bioriginal Food and Science Corp. plans to market this product in the U.S.A. subsequent to the requisite 75-day waiting period enforced after receiving your acknowledgement of the receipt of this application.

If you have any question, please let me know by email, fax or phone. Contact information is provided below.

Thank you.

Sincerely,



for: Rakesh Kapoor, Ph.D.
Product Development Manager
Ph.: (306) 975 9265
Fax: (306) 242 3829
Email: rkapoor@bioriginal.com

Premarket notification for Crossential SA14 for use in dietary supplements in the US (*Federal Register*, 62 (184), p49886-49892)¹

- 1) *The name and complete address of the manufacturer or distributor of the dietary supplement that contains a new dietary ingredient, or of the new dietary ingredient.*

Distributor: Bioriginal Food & Science Corp
102 Melville Street, Saskatoon
SK, S7J 0R1, Canada
Ph: (306) 975 1166
Fax: (306) 242 3829
Email: business@bioriginal.com

- 2) *The name of the new dietary ingredient that is the subject of the premarket notification, including the Latin binomial name (including the author) of any herb or other botanical.*

Crossential SA14

Taxonomy (Linnaeus):
Division: Spermatophyta
Subdivision: Angiospermae
Class: Dicotyledonae
Family: Boraginaceae
Genus: Echimium
Species: plantagineum

Boraginaceae is a large plant family with approximately 100 genera and 2500 species that are widely distributed throughout the Northern Hemisphere [Ref 1]. The family is well known to herbalists and gardeners because it includes many ornamental plants [Ref 1].

The genus Echimium contains about 30 species distributed across Europe, the Mediterranean region, Madeira, the Canaries and the Azores [Ref 2].

Echimium plantagineum is an erect biennial 20-60cm high, softly hairy, with one or many flowering stems [Ref 2]. The basal leaves are ovate with prominent lateral veins and soft appressed setae [Ref 2]. The cauline leaves are oblong to lanceolate, the uppermost being more or less cordate at the base [Ref 2]. Inflorescence usually branched [Ref 2]. Calyx 7-10mm at anthesis, up to 15mm in fruit [Ref 2]. Corolla 18-30mm infundibuliform blue becoming pink through purple, hairy on veins and margins only [Ref 2]. Two stamens exerted from corolla tube, the remaining stamens included or only slightly exerted [Ref 2]. Stigmae distinctly bifid [Ref 2].

Echimium plantagineum is also known by the common names of Purple Vipers Bugloss, Paterson's Curse and Salvation Jane.

- 3) *A description of the dietary supplement or dietary supplements that contain the new dietary ingredient including:*
- (i) *The level of the new dietary ingredient in the dietary supplement;*

¹ After the 90th day, all information in the notification will be placed on public display, except for any information that is trade secret or otherwise confidential commercial information.

Crossential SA-14 will be sold as a bulk oil to encapsulators/Dietary supplement manufacturers, or as soft gelatin capsules containing 500 or 1000 mg Crossential SA14 or blend with other nutritional oils rich in essential fatty acids and/or vitamins/minerals, herbal extracts to dietary supplement manufacturers and distributors.

- (ii) *The conditions of use recommended or suggested in the labeling. If no conditions of use are recommended or suggested in the labeling of the dietary supplement then the ordinary conditions of use of the supplement.*

Crossential SA14 will be marketed as a dietary supplement of essential fatty acids of omega 3 and omega 6 series. As it is enriched in gamma-linolenic acid (omega-6 fatty acid) and stearidonic acid (omega-3 fatty acid), it will bypass the need for delta-6-desaturase, a rate limiting enzyme in the metabolism of linoleic acid (omega-6 essential fatty acid) and alpha-linolenic acid (omega-3 essential fatty acid).

4) The history of use or other evidence of safety establishing that the dietary ingredient when used under the conditions recommended or suggested in the labelling of the dietary supplement will reasonably be expected to be safe, including any citation to published articles or other evidence that is the basis on which the distributor or manufacturer of the dietary supplement that contains the new dietary ingredient has concluded that the new dietary supplement will reasonably be expected to be safe Any reference to published information offered in support of the notification shall be accompanied by reprints or photostatic copies of such references. If any part of the material submitted is in a foreign language it shall be accompanied by an accurate and complete English translation.

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Analytical data

Crossential SA14 is a pure triglyceride obtained by Super Refining® oil extracted from the seeds of *Echium plantagineum*. The oil is extracted from the seed of *Echium plantagineum* by crushing. The oil is then processed using a patented commercial scale chromatographic technique developed to achieve high purity natural oils by removing polar impurities without altering the chemical composition [Ref 3].

Lipid profiles for unrefined oil extracted from *Echium plantagineum* seed are presented in Appendix-1 Tables 1 & 2. Each row in Tables 1 & 2 relates to the lipid profile for a single representative sample of unrefined oil extracted from *Echium plantagineum* seed. Where there is more than one sample for a single year this relates to different seed accessions (seed sourced from different locations). The size of the seed accession varies from a few grams to several kilograms. Data in Table 1 was provided by Scotia, data in Table 2 was provided by Croda Universal, Hull.

Lipid profiles for Super Refined® *Echium plantagineum* oil (Crossential SA14) are presented in Appendix 1 Table 3. Each row in Table 3 relates to the lipid profile for a single representative sample of Crossential SA14. Where there is more than one sample for a single year this relates to different laboratory batches of the Super Refined® oil. The methods for the lipid analysis are given in Appendix 2.

It can be seen from the tables in Appendix 1 that the lipid constituents of the Super Refined® oil are not significantly different from those of the natural oil. Although there are minor variations in oil composition from season to season due to changes in temperature, light intensity, etc, the lipid profile of both the unrefined and Super refined® oil remains within the specified ranges. The information covers several years' trials work, and is therefore considered an accurate representation of this species.

A full lipid profile for two production volume batches is included in Appendix 3. All fatty acids at a percentage composition of 0.1 and above have been identified conclusively. We have no data to suggest the presence of cyclopropenoid and epoxy fatty acids. These acids are either not present or are at a level of 0.05% or less of the total fatty acid composition.

Erucic acid is present at trace levels ie not more than 0.5% in the Super Refined oil. The results from analysis of production batches PP4/572 and PP4/774 were 0.1% and 0.5% erucic acid respectively.

Purified triglycerides normally contain traces of mono and di-glycerides, free fatty acids, plant sterols, coloring matter (chlorophyll / carotenes) and oxidation products [Ref 3]. All of these compounds are relatively polar and are removed extremely efficiently by Super Refining® [Ref 3].

Gel Permeation Chromatography (GPC) analysis of a laboratory sample of the Super Refined oil batch CW/014 identified the levels of triglyceride as 99.7% and oligomer as 0.3% of the oil. The oligomer may be defined as oxidized triglyceride, this is not a natural component of the oil. Oxidation of the triglyceride is initiated during processing and storage and is kept to a minimum by storing in drums under nitrogen. Commercial samples of vegetable oils contain less than 1.0% oligomers while fish oil can contain up to 3% oligomers.

The remaining component of the oil, the unsaponifiable matter, was not detected by GPC analysis. The unsaponifiable matter is a natural component of the oil and could contain hydrocarbons, sterols and other non-fatty acid compounds. The amount of unsaponifiables isolated from the oil represents a level of 0.7%. A copy of the procedure used to isolate the unsaponifiable fraction is included in Appendix 4.

Gas Chromatography (GC) analysis was carried out on 14.5 milligrams of the extracted unsaponifiables. Sterols accounted for 3.2 milligrams and lower molecular weight compounds of

unknown identity, some of which may be hydrocarbons, accounted for 2.1 milligrams. This leaves 9.2 milligrams unaccounted for by GC which amounts to 0.44% of the Super Refined oil. 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. A copy of the GC chromatogram is included in Appendix 4. The chromatogram includes a cholesterol peak. The unsaponifiables do not naturally contain cholesterol, the cholesterol was added as an internal standard for quantification.

Total heavy metal content for batches PP4/572 and PP4/774 was less than 10ppm. A copy of the Contract laboratory certificate of analysis for each batch is included in Appendix 5. The level of Copper was less than 0.1milligrams/litre for both batches. The level of Iron was 4.6ppm for batch PP4/572 and 11.4ppm for batch PP4/774. A copy of the Contract laboratory certificate of analysis for each batch is included in Appendix 5.

A peroxide value of 5 maximum is included on the product specification. The peroxide value for batch PP4/472 was 2.6 and for batch PP4/744 was 4.1.

Pyrrolizidine alkaloids are known to occur in certain species of the family Boraginaceae and have been isolated from *Echium plantagineum* [Ref 4][Ref 5]. Pyrrolizidine alkaloids are of concern because they cause acute and chronic liver disease [Ref 6]. In addition to the liver they may damage the lung, kidney and other organs, they also possess mutagenic, teratogenic and carcinogenic properties [Ref 6]. Chronic liver disease was observed at dietary levels of 2ppm with the pyrrolizidine alkaloid monocrotaline [Ref 6]. A no effect level of 1ppm in the diet has been hypothesized for monogastric animals such as pigs, poultry and rats [Ref 6].

Analysis of several plant samples of *Echium plantagineum* from New South Wales revealed a total alkaloid content of about 0.3% [Ref 6]. The maximum level of total alkaloid measured was 0.9% [Ref 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 Super Refined® oil (Crossential SA14) was carried out by Prof Dr Michael Wink at Ruprecht-Karls-Universität Heidelberg. The pyrrolizidine alkaloids were extracted in 0.5 M hydrochloric acid and converted to the free base by reduction with zinc powder. The basified solution underwent solid-liquid extraction before being analyzed using capillary GLC and GLC-MS (EI and CI-MS). Senecionine [CAS number 130-01-8] was used as an external standard for quantification. The *Echium plantagineum* meal contained 0.1milligrams/gram total alkaloids. No alkaloids were detected in the crude seed oil or the Super Refined® oil (Crossential SA14). The detection limit for this method was <1micrograms/gram.

Additional testing carried out on two production batches of Crossential SA14 identified the pyrrolizidine alkaloid content as less than 4 ng per gram of oil. A copy of the test results supplied by the contract testing laboratory are included in Appendix 6.

Cytochrome C allergens have been isolated from the pollen of *Echium plantagineum* [Ref 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 test (RAST) tests [Ref 8]. In a case of allergic rhinitis to *Echium plantagineum* symptoms developed on exposure to both the flowering and dried plants [Ref 9]. Challenge tests with pollen and particulate plant debris including plant hairs also produced symptoms [Ref 9].

The chromatographic technique used in Super Refining® will act to filter out any pollen or particulate plant debris in the oil. To confirm the absence of Cytochrome C allergens in the Super Refined® oil a total protein test has been performed using Bradford Reagent. The absorbance at 595nm of the colored product of the reaction of protein and Bradford Reagent was measured.

Cytochrome C allergens isolated from the pollen of echium plantagineum were characterized as proteins with a molecular weight of 12,800 [7].

It can be assumed that the maximum Cytochrome C allergen concentration is equivalent to the total protein content. Using standards a total protein content of less than 19.85µg/gram of oil was determined. However problems associated with the low solubility of echium oil in the aqueous reagent meant that the aqueous reagent phase and echium oil (dissolved in hexane) phase had to be mixed vigorously before measurement of the sample solution using UV spectrometry at a wavelength of 595nm. This is not ideal and therefore a visual inspection of the sample, standards and blank was carried out. A total and recordable protein content (and therefore a Cytochrome C allergen content) of less than 1µg/gram of oil was ascertained by this means.

Agrochemical 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. This is essentially one in which as many weed seeds have been 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 their 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, Paraquat, Diquat and Glufosinate-Ammonium. As all 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.

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 Kings recommend their growers to use.

Specification

<u>Parameter</u>	<u>Product Specification</u>
Acid value	2 maximum
Hazen colour	200 maximum
Iodine value	160 (min)
Peroxide value	5 maximum
Saponification value	160 (min)

Anticipated use

Crossential SA14 is a vegetable oil rich in both omega-6 and omega-3 polyunsaturated fatty acids. It is intended to be incorporated into dietary supplements.

Dietary supplements of omega-6 and omega-3 fatty acids are normally offered in the form of gelatin capsules or oral emulsions. The addition of Vitamin E or other antioxidants to these formulations is recommended in accordance with the rules in effect in country of sale [Ref 10].

Oils rich in omega-6 fatty acids currently available on the market include blackcurrant seed oil, borage oil, evening primrose oil, soybean oil and safflower oil. Oils rich in omega-3 fatty acids currently available on the market include flax 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 [Ref 12][Ref 13][Ref 14]. Omega-3 enriched products are currently marketed in Japan, Korea, Taiwan and Europe including the United Kingdom and Scandinavia. In addition a docosahexaenoic acid enriched product has been fed to chickens in order to produce DHA enriched eggs [Ref 12][Ref 13]. These eggs are marketed in several countries including Germany, Spain, Portugal, Belgium, Luxembourg, Norway and Andorra, USA and Canada.

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 - 302milligrams/gram and docosahexaenoic acid levels of between 78 - 254milligrams/gram [Ref 15].

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 [Ref 16].

Several brands of omega-3/omega-6 fatty acid blends are currently marketed in USA. These include Efamarine™ (capsules and emulsion), Essential Max™ (capsules and bottled oil blend) and Omega Twin™ (oil blend). An omega-3 / omega-6 fatty acid blend which is currently marketed [Efamarine™] in the form of capsules and an oral emulsion provides 68milligrams of gamma linolenic acid, 34 milligrams of eicosapentaenoic acid and 22 milligrams docosahexaenoic acid per daily intake. The daily intake of 2 gelatin based capsules contains in addition 20 milligrams of vitamin E (as D alpha tocopheryl acetate). The daily intake of 1 tsp (5ml) of oral emulsion based on high oleic acid sunflower oil contains in addition 10 milligrams vitamin E (as DL-alpha tocopheryl acetate) The omega-3 / omega-6 fatty acid blend is provided by combining evening primrose 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 500milligrams capsules based solely on Crossential SA14 would provide 58 milligrams of gamma linolenic acid and 64milligrams of stearidonic acid.

We do not intend to sell Crossential SA14 direct to consumers. Crossential SA14 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 Crossential SA14 will be as a dietary supplement. This will be in capsule form or a blend with other nutritional oils with a likely level of consumption of either 500 mg to 3000 mg per day. Crossential SA14 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 [Ref 17]. Adequate dietary fat intakes are considered particularly important prior to and during pregnancy and lactation [Ref 17]. The FAO/WHO joint expert consultation recommended that women of reproductive age should consume at least 20% of their energy from fat [Ref 17]. A calorific fat intake of approximately 20% is normally used clinically in hospitalized patients who are infected or at risk of becoming so [Ref 18]. 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 milligrams per day.

Americans are estimated to consume fats and oils at a level of 34 to 37% or more of their daily calories [Ref 19]. The average number of calories consumed per person per day is 2500 [Ref 19]. Since 1 gram of fat produces 9 calories this amounts to 110 grams of fat per person per day [Ref 19]. Levels of fat consumption reported for developed countries include, Denmark 160 grams per day, New Zealand 155 grams per day, United Kingdom 142 grams per day and Canada 142 grams per day [Ref 19]. The 1979 figures for the United States estimated fat consumption to be around 168 grams per day of which 34% was saturated, 40% monounsaturated and 15% polyunsaturated [Ref 19].

Crossential SA14 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 [Ref 20]. The level of saturated fatty acids in herring oil is 26.1% and in mackerel is 27.5% [Ref 20].

Omega-6 fatty acids:

About 1% of daily calories (an average of 3 grams) linoleic acid is enough to relieve the symptoms of deficiency of this essential fatty acid and therefore represents a minimum daily requirement [Ref 19]. The optimum dose of linoleic acid is considered to be between 3-6% (9-18 grams on average) [Ref 19]. The FAO/WHO expert consultation on fats and oils in human nutrition have recommended that linoleic acid should provide between 4-10% of energy [Ref 17].

Crossential SA14 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 [Ref 20].

Omega-3 fatty acids:

The daily requirement and optimum dose of alpha linolenic acid is not known [Ref 19]. A level of 0.54% of daily calories was required to reverse symptoms of alpha linolenic acid deficiency in a 6 year old girl [Ref 19]. An optimum dose is hypothesized for alpha-linolenic acid of 6 grams per

day [Ref 19]. It is estimated that 95% of affluent people would benefit from dietary supplementation with omega-3 fatty acids [Ref 19].

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 milligrams per capita in 1992 [Ref 14]. Data from 1985 on the US national food supply indicates a level of 50 milligrams per capita per day of eicosapentaenoic acid and 80 milligrams per capita per day for docosahexaenoic acid [Ref 10].

Crossential SA14 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 [Ref 20]. 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 [Ref 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 incorporation of high levels of linoleic or alpha-linolenic acid does not seem to raise the levels of their corresponding metabolites [Ref 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 [Ref 21].

Dietary supplementation with oils rich in linoleic acid, such as safflower oil, did not increase omega-6 fatty acid content of human milk [Ref 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 [Ref 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 [Ref 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 [Ref 23]. Switching the omega-6 content from linoleic to gamma-linolenic increased the omega-6:omega-3 ratio two fold [Ref 23]. Whereas switching the omega-3 content from alpha linolenic to stearidonic acid decreased the omega-6:omega-3 ratio by 30% [Ref 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 [Ref 19]. 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 [Ref 24].

A ratio of linoleic to alpha-linolenic acid of between 5:1 and 10:1 is recommended in the diet [Ref 17]. The FAO/WHO expert consultation on fats and oils in human nutrition have recommended that linoleic acid should provide between 4-10% of energy [Ref 17]. Therefore alpha-linolenic acid should provide between 0.4%-2% of energy depending on the amount of linoleic acid in the diet.

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 milligrams per capita in 1992 which represents only 0.09% of dietary energy [Ref 14]. 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 [Ref 21]. It is estimated that 95% of affluent people would benefit from dietary supplementation with omega-3 fatty acids [Ref 19].

Crossential SA14 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 [Ref 25].

Microbiological data

Crossential SA14 is an anhydrous system and therefore will not support microbiological growth. In addition the chromatographic technique used in Super Refining® will act to filter out any microbial organisms. The absence of microbiological contamination has been confirmed by testing a sample of the oil. Analytical report is attached in appendix 7.

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 [Ref 6]. The young growth is eaten readily by livestock [Ref 6]. The plant is considered a weed in good pastures while on poor country it is considered as a reserve fodder [Ref 5]. Measurements of herbage dry matter content, nitrogen content and digestibility of Echium plantagineum indicate that it would be nutritious forage for grazing animals [Ref 26]. However the presence of pyrrolizidine alkaloids in the plant means that there is a risk that grazing animals will be poisoned [Ref 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 [Ref 27].

Field evidence strongly indicates that horses, pigs and to a lesser extent sheep are all affected [Ref 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 [Ref 6]. All developed the typical chronic liver damage within 5 months and one animal died within 4 months [Ref 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 [Ref 28]. Compared to a control group receiving a diet of lucerne chaff and oats, sheep on the Echium diet lost weight and deaths occurred [Ref 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 [Ref 28]. It was concluded that Echium plantagineum alone was not suitable fodder for sheep [Ref 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 [Ref 29]. Histological evidence of moderately severe liver damage associated with high liver copper concentrations was found in at least one sheep [Ref 16]. Sheep on the Echium plantagineum diet were significantly lighter and grew less wool compared with sheep on Echium free pasture [Ref 29].

Young rats fed 40% Echium plantagineum for two weeks suffered 70% mortality within 5-13 weeks [Ref 30]. Young rats fed 20% Echium plantagineum for alternate two week periods with a control feed had 50% mortality in 21 weeks [Ref 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 [Ref 30]. The rats died with a mixture of acute and chronic liver damage [Ref 30]. Tumors, 3 benign and 1 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 [Ref 6]. The number of tumors was below the significance level [Ref 6].

Echium plantagineum is known to secrete nectar that is gathered by bees and it is used extensively by apiarists [Ref 6]. It is estimated that honey from Echium plantagineum constitutes about 10-15% of total Australian production [Ref 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 [Ref 31]. The possible intakes of pyrrolizidine alkaloids from this source are considered to be very low [Ref 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 Echium plantagineum oil. An analysis of the alkaloid content of the crude and Super Refined® oil and the Echium plantagineum meal has been carried out. The meal contained 0.1milligrams/gram total alkaloids. No alkaloids were detected in the crude or Super Refined® oils. The detection limit was <1micrograms/gram.

Component fatty acids:

The lipid profile for Crossential SA14 is similar to that of Borage oil and Blackcurrant oil [Appendix 9]. Both borage oil and blackcurrant oil are widely used as ingredients of cosmetics, pharmaceuticals, foods and food supplements [Ref 32], [Ref 33]. The major fatty acids found in Crossential SA14 are as follows:

Palmitic acid is the most widely occurring saturated fatty acid and is present in most commercial oils [Ref 20]. 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 [Ref 19][Ref 20]. Crossential SA14 contains on average 7.1% palmitic acid.

Stearic acid is found in abundance in tallow (5-30%), cocoa butter (30-36%) and shea nut butter (44%) [Ref 19] [Ref 20]. Crossential SA14 contains on average 4.0% stearic acid.

Oleic acid is the most widely occurring natural fatty acid and is found in practically all lipids [Ref 20]. It is found in large quantities in olive (43.7-83%), almond (65-70%) and peanut (37.9%) oils [Ref 19]. Oleic acid is also manufactured in the body [Ref 19][Ref 20]. Crossential SA14 contains on average 17.3% oleic acid.

Linoleic acid is found in safflower (75.3%), sunflower (68.5%), soybean (53%) and sesame (45%) oils [Ref 19][Ref 20]. Crossential SA14 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 [Ref 20]. Flax seed is the richest source of ALA with over 50%, Chia and kukui (candlenut) contain about 30%, hemp seed around 20% [Ref 19]. Pumpkin seed oil may have up to 15%, canola up to 10% and walnut between 3-11% [Ref 19]. Soybean oil normally contains 5-7% [Ref 19]. Crossential SA14 contains on average 30.7% ALA.

The richest source of GLA is borage oil (20%) followed by black currant seed oil (15%) and evening primrose oil (9%) [Ref 19]. Crossential SA14 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%) [Ref 20]. The most well known plant source of stearidonic acid is black currant seed oil (3%) [Ref 19]. Crossential SA14 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. Crossential SA14 contains on average 12.8% stearidonic acid.

Omega-6 & omega-3 fatty acids:

Crossential SA14 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 [Ref 34][Ref 35]. Only two fatty acids are truly essential, linoleic acid and *alpha*-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 10] [Ref 35].

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 [Ref 19]. 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 [Ref 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 150milligrams of eicosapentaenoic acid per day inhibit platelet aggregation [Ref 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 [Ref 36]. 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 [Ref 36]. Patients with glycogen storage disease type-I taking fish oil supplements showed improvements in hypertriglyceridemia and hypercholesterolemia after three months [Ref 37]. Withdrawal of fish oil for a further three months resulted in a return to pretreatment abnormalities in plasma lipid and lipoprotein levels [Ref 37]. Omega-3 triglyceride treatment was associated with epistaxis in 8 out of 11 patients and prolonged bleeding time was noted in 3 patients [Ref 38].

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 [Ref 39]. 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 [Ref 11].

Gamma-linolenic acid and dihomogamma-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 [Ref 25]. There is evidence that low tissue and dietary linoleic acid content is associated with high incidence of coronary heart disease [Ref 21]. In patients with total cholesterol levels of >300milligrams/decilitre the risk factor for atherosclerosis dropped from 6.34 to 3.48 with a daily dose of 6 x 450milligrams capsules of black currant seed oil for 12 weeks [Ref 40][Ref 41].

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 [Ref 25]. The essential fatty acids may therefore prove useful in the treatment of osteoporosis [Ref 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 [Ref 25].

There is conflicting evidence regarding the effects of omega-3 fatty acids in diabetic patients [Ref 42]. It is generally agreed that glucose control is not hampered in patients with insulin dependent diabetes [Ref 42]. Some studies have shown deterioration in glucose homeostasis for patients with non-insulin dependent (adult onset) diabetes [Ref 42]. However some of these studies had not corrected for the high energy content of the fish oil [Ref 42].

Gamma-linolenic acid is able to prevent diabetes induced reduction in nerve conduction velocity and reverse diabetic nerve damage in animals and humans [Ref 43], [Ref44]. The exact mechanism is not clear but it is proposed to act by altering nerve blood flow [Ref 25]. Studies in which gamma linolenic acid was provided in combination with eicosapentaenoic acid and docosahexaenoic acid produced slightly but not significantly better results [Ref 45]. 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 [Ref 44].

Kidney Disorders:

Animal studies have indicated potential benefits for fish oil therapy in the treatment of kidney disorders [Ref 46]. 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].

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 [Ref 47]. 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 [Ref 47].

Rheumatoid Arthritis:

Fish oil supplements containing essential fatty acids are reported to have beneficial effects on the symptoms of rheumatoid arthritis whilst offering few, if any, side effects at the levels used [Ref 25]. Modest clinical improvements usually emerge after 12 weeks of treatment and peak around 18 to 24 weeks [Ref 36]. The levels of eicosapentaenoic acid and docosahexaenoic acid required to produce a beneficial effect is around 90milligrams per kilogram of bodyweight per day [Ref 47]. Stearidonic acid was as effective as eicosapentaenoic acid in inhibiting 5-lipoxygenase when tested in vitro [Ref 48]. 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 [Ref 49].

Gamma-linolenic acid has also proved effective in treating human rheumatoid arthritis [Ref 25][Ref 50]. Both *gamma*-linolenic acid and dihomogamma-linolenic acid have demonstrated anti-inflammatory effects in animals and humans although these effects are subtly different from those demonstrated by eicosapentaenoic acid [Ref 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) [Ref 51]. Patients given six 500milligrams capsules per day of black currant seed oil (equivalent to 525milligrams of gamma linolenic acid) for six weeks reported modest clinical improvement compared to a control group taking sunflower seed oil [Ref 52]. 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 [Ref53]. 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 [Ref 54].

Skin Disorders:

Mild to moderate improvement in psoriatic lesions was reported in 8 out of 13 patients consuming 60 grams of fish oil (equivalent to 10.8 grams of eicosapentaenoic acid per day) [Ref 47]. Patients with atopic eczema given 10 grams 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 [Ref 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 10g/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 [Ref 55]. 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 [Ref 56].

Treatment of atopic eczema with *gamma*-linolenic acid has shown modest beneficial effects [Ref 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 [Ref 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 [Ref 45]. In a pilot double blind study infantile seborrheic dermatitis cleared up in all the children treated with a cream containing 40% borage oil [Ref 36]. 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 [Ref 36]. Six out of nine patients with biliary pruritus showed significant improvement in symptoms when given 8 x 500milligrams capsules of an evening primrose oil preparation for 12 weeks [Ref 21]. Patents have been applied for covering the use of essential fatty acid derivatives in the treatment of skin disorders such as psoriasis [Ref 57]

Anti-inflammatory Properties:

Stearidonic acid has been shown to possess anti-inflammatory properties [Ref 49]. Croda has investigated the anti-inflammatory activity of Crossential SA14 on the production of certain UVB induced inflammatory mediators. The reconstructed skin model ZK1301 was chosen since it is known to produce inflammatory mediators such as PGE₂ (Prostaglandin E₂) and IL-1 *alpha* (Interleukin 1 *alpha*). The model has been used previously to demonstrate the anti-inflammatory effects of indomethacin, a nonsteroidal anti-inflammatory drug [Ref 38], using ultraviolet radiation to artificially stimulate the skin. PGE₂ (Prostaglandin E₂) is a strongly pro-inflammatory eicosanoid, it is indicative of membrane perturbation events which activate the release of arachidonic acid IL-1 *alpha* (Interleukin 1 *alpha*) is a cytokine released in response to chemical insult or inflammatory stimuli.

The ZK1301 tissues were exposed to ultraviolet irradiation to induce an inflammatory response and the levels of a number of inflammatory mediators were used as endpoints. In the first study carried out the levels of PGE₂ (Prostaglandin E₂) and IL-1 *alpha* (Interleukin 1 *alpha*) were measured [Appendix 11]. Crosssential SA14 reduced the levels of UVB stimulated PGE₂ release.

In the second study carried out the levels of PGE₂ (Prostaglandin E₂) and TNF *alpha* (Tumor Necrosis Factor *alpha*) were measured [Appendix 12]. Crosssential SA14 reduced the levels of UVB stimulated PGE₂ release. The levels of TNF *alpha* induced were at or near the detection limit of the assay and therefore effects on the production of this cytokine could not be determined. Both studies demonstrated that Crosssential SA14 was non-cytotoxic and non-phototoxic, indeed Crosssential SA14 may possess UVB protective effects.

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 [Ref 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 [Ref 58].

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 [Ref 17]. In experimental animal models of cancer diets high in omega-6 fatty acids produced the greatest incidence and size of tumors [Ref 47]. 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 [Ref 17]. Inter-country studies indicate that levels of omega-6 fatty acids of around 4-8% of energy are not correlated with breast cancer [Ref 17].

Gamma-linolenic acid and dihomogamma-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 [Ref 25]. In addition, *gamma*-linolenic acid reduces the motility and invasiveness of cancer cells within hours [Ref 25]. The methyl ester of linoleic acid demonstrated anti-mutagenic activity on busulfan induced genotoxicity in Chinese hamsters [Ref 58]. *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 [Ref 45].

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 [Ref 59]. 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 [Ref 60].

Evening primrose oil has been reported to reduce hyperactivity in some children [Ref 21]. When given to children with mood disorders 67% showed some improvement with evening primrose oil compared to a placebo of olive oil [Ref 21]. In a placebo controlled trial patients with Alzheimer's disease receiving evening primrose oil showed significant improvements in several tests of cerebral function [Ref 45]. In a double blind placebo controlled trial schizophrenics receiving evening primrose oil showed moderately better Simpson scored for tardive dyskinesia and significantly improved psychosis scores [Ref 61].

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 [Ref 36][Ref 62][Ref 63][Ref 64][Ref 65].

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 [Ref 66].

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 [Ref 45]. Patents have been applied for covering the use of essential fatty acid derivatives in the treatment of premenstrual syndrome [Ref 67].

Multiple Sclerosis:

Oils rich in essential fatty acids, such as evening primrose oil, have been investigated in the treatment of multiple sclerosis [Ref 68]. 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].

Experimental Allergic Encephalomyelitis:

Croda have investigated the prophylactic effects of Crossential SA14 on the emergence and development of neurological Experimental Allergic Encephalomyelitis (EAE) in the Lewis rat [Appendix 13]. Rats were dosed with 500milligrams/kilogram bodyweight/day for 3 days prior to inoculation and 20 days post-inoculation. Treatment of EAE inoculated Lewis rats with Crossential SA14 before and after sensitization for disease did not alter the onset of neurological deficits or lessen the duration of the disease under these conditions. No adverse effects were reported other than the normal symptoms of the disease.

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 [Ref 69]. 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 [Ref 70]. No beneficial effects were observed with gamma-linolenic acid at the levels used in this study [Ref 70]. Arachidonic acid significantly enhanced human neutrophil antiparasitic activity to Plasmodium falciparum asexual blood forms [Ref 71].

Fish oil has been shown to improve survival of Guinea-pigs exposed to endotoxin [Ref 18][Ref 47]. 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 [Ref 47]. Eicosapentaenoic acid and docosahexaenoic acid enhanced human neutrophil antiparasitic activity to Plasmodium falciparum asexual blood forms [Ref 71].

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 [Ref 48]. 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 [Ref 72].

Infant Development:

Lipids in the fetus rise from 0.1% at 24 weeks to 3-5% at 28 weeks and 15-16% at term [Ref 13]. After birth infants gain on average 9-10 grams of fat per day and 40-50% of its energy requirement is supplied by fat [Ref 13]. Human milk contains substantial quantities of essential fatty acid metabolites and therefore their role in infant development has been investigated [Ref 25].

Bottle fed premature and full-term infants demonstrated increased visual acuity when their diet was supplemented with eicosapentaenoic acid and docosahexaenoic acid [Ref 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

[Ref 17]. 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 [Ref 17]. Arachidonic acid appears to play an important role in fetal growth since the levels of this acid correlate with head circumference [Ref 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 [Ref 17].

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 [Ref 73].

Guidelines offered by FAO/WHO for formula for preterm babies is 700milligrams linoleic acid, 50milligrams alpha-linolenic acid, 60milligrams arachidonic acid and 40milligrams of docosahexaenoic acid per kilogram bodyweight [Ref 17]. This is equivalent to 5.6% of energy as parent essential fatty acids and 0.8% as long chain polyunsaturated fatty acids [Ref 17]. In addition linoleic acid should not exceed 10% of total energy [Ref 17].

Total parenteral nutrition in the premature infant usually includes 2-4 grams of soybean emulsion per kilogram bodyweight per day [Ref 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) [Ref 21].

For full term infants the corresponding FAO/WHO recommended values per kilogram bodyweight are 600milligrams of linoleic acid, 50milligrams of alpha-linolenic acid, 40milligrams of arachidonic acid and 20milligrams of docosahexaenoic acid [Ref 17]. 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 [Ref 17]. The critical role of both omega-6 and omega-3 fatty acids is recognized [Ref 17]. The ratio of linoleic to alpha-linolenic acid is recommended to be between 5:1 and 10:1 [Ref 17].

The FAO/WHO joint expert consultation recommend that the maternal diet should provide an additional 3-5 grams per day of essential fatty acids during lactation [Ref 17].

Reproductive Effects:

High levels of omega-3 fatty acids in fish oil have been reported to prolong gestation and impair parturition in rats [Ref 46]. However omega-3 fatty acid supplements of 200milligrams (omega-3:omega-6 = 0.8) did not alter any of the indices of gestational performance [Ref 18]. Omega-3 fatty acid supplements of 480milligrams (omega-3:omega-6 = 2.8) did not significantly alter length of gestation although dam bodyweight and average pup weight was decreased [Ref 18]. There is evidence that high fish intakes are associated with longer gestation, higher birth weight and reduced incidence of premature birth [Ref 17].

Gastrointestinal Disturbances:

Significant clinical improvement was noted in patients with ulcerative colitis, an inflammatory bowel disease, receiving fish oil supplements for 12 weeks [Ref 47]. Administration of fish oil is reported to protect against damage to the gastro-duodenal mucosa caused by aspirin [Ref 74].

Slight side effects of nausea and eructation have been reported with high doses of the triglycerides of omega-3 fatty acids [Ref 38]. Other reports of adverse effects include loose motions and unabsorbed oil in stools with a purified ethyl ester of eicosapentaenoic acid [Ref 38].

Acute studies with linoleic acid have shown a protective effect on the gastric mucosa against challenge with ethanol but not aspirin [Ref 38]. Investigations carried out in animals have demonstrated a protective effect for gamma linolenic acid in gastric ulceration [Ref 45]. 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 [Ref 45].

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 [Ref 38].

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 [Ref 38].

Mortality:

The effects of dietary polyunsaturated fatty acids on mortality have been investigated in the Multiple Risk Factor Intervention Trial (MRFIT) [Ref 75]. 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 [Ref 75]. Subjects were assigned to either a Special Intervention (SI) or Usual Care (UC) group [Ref 75]. Only data on the UC group (6,250 subjects) were analysed [Ref 75]. PUFA intakes were determined by dietary recall interviews at baseline and follow up years 1, 2 and 3 [Ref 75]. 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 [Ref 76]. 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 [Ref 75].

There were no significant changes in mortality associated with linoleic acid (C18:2n-6) for any mortality group [Ref 75]. 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 grams [Ref 75]. 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 grams [Ref 75]. A significant inverse relationship was observed with CA mortality and 18:3n-3:18:2n-6 ratios or total n-3:n-6 ratios [Ref 75].

Conclusion

Dietary fat is essential for health. An optimum dose is hypothesized for linoleic acid of 9-18 grams per day and for alpha-linolenic acid of 6 grams per day. Crossential SA14 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 [Ref 25].

Crossential SA14 is intended to be sold as a dietary supplement either in the form of an oral liquid, or emulsion or capsules. The likely level of consumption will be between 500 to 1000 milligrams per day. The addition of Vitamin E to these formulations is recommended in accordance with guidance offered by the UK committee on medical aspects of food policy.

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. No pyrrolizidine alkaloids were detected in tests carried out on Crossential SA14.

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.

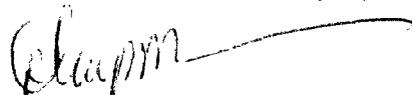
The major fatty acids found in Crossential SA14 are palmitic, stearic, oleic, linoleic, alpha linolenic, gamma linolenic and stearidonic acids. All these fatty acids are normal components of the human diet. The lipid profile for Crossential SA14 is similar to that of borage oil and blackcurrant oil which 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, Crossential SA14 is characterized by higher levels of omega-3 fatty acids than observed in these oils.

Crossential SA14 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. Crossential SA14 has been shown to possess anti-inflammatory properties. 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.

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 200milligrams did not alter any of the indices of gestational performance. Omega-3 fatty acid supplements of 480milligrams 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. 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. 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:



Rakesh Kapoor, Ph.D.

Product Development Manager, Bioriginal Food & Science Corp

102 Melville Street, Saskatoon, SK, S7J 0R1, Canada

Ph. 306 975 9265, Fax 306 242 3829

Email rkapoor@bioriginal.com

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Appendix 1 – Lipid profiles

Table 1: Lipid profiles of unrefined oil extracted from *Echium plantagineum* carried out by Scotia Plant Technology Centre, Writtle College, Chelmsford, Essex

Year	% oil ¹	Lipid constituents ²						
		16.0	18.0	18.1	18.2	18.3 ³	18.3 ⁴	18.4 ⁵
1991	28.0				14.0	37.0	9.1	14.6
1992	24.7				14.7	38.8	10.2	7.5
1992	23.1				13.1	34.9	9.8	16.2
1992	23.9				14.9	37.2	10.5	9.4
1993	25.5							14.4
1993	22.5							15.9
1993	28.9							14.5
1993	28.0							12.8
1993	27.0							13.8
1993	25.3							14.3
1993	27.2						9.5	15.4
1993	24.8						9.2	11.7
1995	24.6							11.2
1995	28.6							10.8
1995	37.8							11.6
1995	35.3							9.9
1995	39.8							11.7
1995	33.4							12.0
1995	41.5							11.5
1995	29.0							11.8
1995	28.4							10.8
1996	29.9	6.4	3.6	14.5	13.8	36.0	9.7	14.9
1996	28.7	6.6	2.6	13.6	14.8	37.3	10.0	13.8
1997	24.6						12.1	11.7
Mean	28.8	6.5	3.1	14.1	14.2	36.9	10.0	12.6
± SE	1.06	1.41	2.19	0.45	0.29	0.54	0.30	0.45

Each row relates to the lipid profile for a single representative sample of unrefined oil extracted from *Echium plantagineum* seed. Where there is more than one sample for a single year this relates to different seed accessions. The size of the seed accession varies from a few grams to several kilograms.

¹ The percentage of oil in the seed on a dry weight basis

² The first number represents the number of carbon atoms in the fatty acid analyzed, the second number represents the number of double bonds. Fatty acids not determined are left as blank entries.

³ alpha-Linolenic acid (ALA)

⁴ gamma-Linolenic acid (GLA)

⁵ Stearidonic acid (SA)

Appendix 1 – Lipid profiles

Table 2: Lipid profiles of unrefined oil extracted from *Echium plantagineum* carried out by Croda Universal Ltd, Hull, East Yorkshire

Year	% oil ¹	Lipid constituents ²						
		16.0	18.0	18.1	18.2	18.3 ³	18.3 ⁴	18.4 ⁵
1995	21.0	7.6	3.8	16.7	16.0	29.9	11.9	12.3
1995	26.3	7.4	4.1	18.9	16.6	28.5	11.4	11.3
1996	32.1	6.9	3.6	17.5	16.8	31.1	10.8	11.5
1996	26.1	7.3	4.1	17.2	16.2	30.5	10.9	11.9
1996	28.3	7.4	4.2	18.0	16.0	30.3	10.3	11.7
1996	30.9	7.1	3.6	15.8	16.5	33.6	9.8	11.9
1996	27.0	7.3	3.8	16.7	17.8	31.8	10.2	10.6
1996	32.4	6.3	3.5	17.5	14.0	32.0	11.2	13.9
Mean	25.5	7.2	3.8	17.3	16.2	31.0	10.8	11.9
± SE	3.61	0.14	0.09	0.33	0.38	0.54	0.24	0.34

Each row relates to the lipid profile for a single representative sample of unrefined oil extracted from *Echium plantagineum* seed. Where there is more than one sample for a single year this relates to different seed accessions. The size of the seed accession varies from a few grams to several kilograms.

¹ The percentage of oil in the seed on a dry weight basis

² The first number represents the number of carbon atoms in the fatty acid analyzed, the second number represents the number of double bonds. Fatty acids not determined are left as blank entries.

³ alpha-Linolenic acid (ALA)

⁴ gamma-Linolenic acid (GLA)

⁵ Stearidonic acid (SA)

Appendix 1 – Lipid profiles

Table 3: Lipid profiles of Super Refined® Echium plantagineum oil (Crossential SA14) carried out by Croda Universal Ltd, Hull, East Yorkshire

Year	Lipid constituents ¹						
	16.0	18.0	18.1	18.2	18.3 ²	18.3 ³	18.4 ⁴
1996	7.3	4.2	19.0	16.5	27.8	11.6	11.1
1996	7.1	4.2	18.6	16.4	28.7	12.0	12.2
1997	7.1	3.7	15.8	14.3	33.1	11.2	13.9
1997	6.8	3.8	15.9	14.5	33.2	11.1	13.8
Mean	7.1	4.0	17.3	15.4	30.7	11.5	12.8
± SE	0.10	0.13	0.86	0.59	1.43	0.21	0.67

Each row relates to the lipid profile for a single representative sample of Super Refined® Echium plantagineum oil (Crossential SA14). Where there is more than one sample for a single year this relates to different laboratory batches of the Super Refined® oil.

¹ The first number represents the number of carbon atoms in the fatty acid analyzed, the second number represents the number of double bonds. Fatty acids not determined are left as blank entries.

² alpha-Linolenic acid (ALA)

³ gamma-Linolenic acid (GLA)

⁴ Stearidonic acid (SA)

Appendix 2 – Test method for analysis of lipid profiles

2011/11/22

APPENDIX 2

Method No CU-RM-014
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5.0 PROCEDURES

PREPARATION OF METHYL ESTERS

- 5.1 Weigh 2-3g of the oil into the 100ml flask. Add 25ml of methanolic potassium hydroxide solution and reflux the mixture for 30 minutes on a sand bath.
- 5.2 To this solution add 25ml of methanolic sulphuric acid solution and reflux for 30 minutes.
- 5.3 Remove the flask from the sand bath, add 10ml of n-Heptane and then fill the flask with saturated salt solution such that the heptane solution of the methyl esters fills the neck of the flask.

GLC ANALYSIS OF METHYL ESTERS

- 5.4 The methyl esters prepared as above are then analysed by gas chromatography by an appropriate method. Two typical methods are outlined below.

5.5 PACKED G.C ANALYSIS

G.C type	:=	Unicam PU4550 or similar machine
Column length	:=	6 ft glass
diameter	:=	¼" OD
Packing	:=	20% diethylene glycol succinate on Chromosorb WAW
Carrier gas	:=	Nitrogen
Detector	:=	Flame ionisation
Temperature		
Oven	:=	200°C isothermal
Injector	:=	250°C
Detector	:=	300°C
Injector Volume	:=	0.3µl

5.6 CAPILLARY G.C ANALYSIS

G.C. type	:=	Perkin Elmer 8600 or similar machine
Column length	:=	60 metres
diameter	:=	0.36 mm

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Replaces.....
Date.....
Signed..... *B. Langford*

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Column type	:=	J & W / DB-23 0.15µm film thickness or similar column
Carrier gas	:=	Helium 1.0ml/minute
Detector	:=	Flame ionisation
Temperature		
Oven	:=	160 - 230°C at 6C/minute
Injector	:=	250°C
Detector	:=	300°C
Injection volume	:=	0.2µl
Injection split ratio	:=	150 to 1

5.7 The above two methods will encompass all normal G.C requirements. The packed column method is used for general routine analysis and where a detailed examination is required the capillary column method should be used.

6.0 REFERENCES

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- 6.2 Test Methods for Fatty Acids (AFAD) 1968 method 10
- 6.3 American Oil Chemists Society (AOCS) 1989 method Ce 1-62
- 6.4 Standard Methods for the Analysis of Oils, Fats and Derivatives (IUPAC) 1979 methods 2.301 and 2.302

**Q. A.
UNCONTROLLED
DOCUMENT**

Issue No.
Replaces.....
Date.....
Signed.....

Appendix 3 – Analytical data for production batches of Crossential SA14

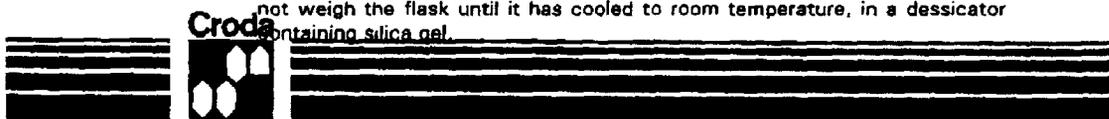
Parameter	PP4/886	PP4887	PP4/888	Method
Appearance	pale yellow	pale yellow	pale yellow	TMB31
Odour	odourless	odourless	odourless	-
Colour (hazen)	70	50	50	TMB27
Acid value	0.1	0.1	0.1	TMA1
Peroxide value	0.1	0.1	0.1	TMA10
Iodine value	200.2	197.2	197.9	TMA3
Saponification value	190.8	192.3	190.6	TMA4
Unsaponifiable matter	0.98	0.83	0.94	TMA13
Cold test (3 hours at 10C)	Clear	Clear	Clear	
Total protein (microgram/gram)	<1	<1	<1	
Glyceride profile:				TMB157
Glyceride oilgomer (%)	1.8	1.4	2.1	
Triglyceride (%)	98.1	98.5	97.9	
Fatty acid/fatty acid ester (%)	0.1	0.1	0.0	
Fatty acid profile:				TMA118/TMB86
C14:0 (%)	0.1	0.1	0.1	
C16:0 (%)	7.0	7.0	7.1	
C16:1 (%)	0.3	0.4	0.2	
C18:0 (%)	3.3	3.4	3.5	
C18:1 (%)	16.4	16.4	16.3	
C18:2 (%)	17.8	17.7	17.6	
C18:3 n-3 (%)	29.2	29.2	29.6	
C18:3 n-6 (%)	10.9	11.1	11.1	
C18:4 (%)	13.2	13.1	13.0	
C20:0 (%)	0.8	0.7	0.8	
C20:1 (%)	0.2	0.2	0.1	
C22:1 (%)	0.3	0.3	0.3	
C24:1 (%)	0.1	0.1	0.1	
Others (%)	>0.2	>0.2	>0.1	

Batch Number	PP4/572	PP4/744
Appearance	Colourless liquid	Pale yellow liquid
Odour	Odourless	Odourless
Colour (Hazen)	15/20	150
Acid Value (mg KOH/g)	0.20	0.25
Peroxide value (MeqO2/kg)	2.8	4.1
Iodine Value (g/100g)	199	201
Saponification Value (mg KOH/g)	192	186
Unsaponifiables (%)	0.71	0.57
Cold Test	Fail	Fail
Copper content (mg/litre)	0.1	awaiting
Iron content (mg/litre)	4.8	awaiting
Total heavy metals (ppm)	less than 10	awaiting
Pyrolizidine alkaloids (ng/gram)	less than 4	awaiting
Glyceride composition	99.71 % tri-glyceride	100 % tri-glyceride
Fatty acid distribution		
16:0 (%)	6.9	7.8
16:1 (%)	0.1	-
18:0 (%)	3.8	4.1
18:1 (%)	18.4	16.8
18:2 (%)	14.8	16.1
18:3 n-3 (%)	33.1	30.2
18:3 n-6 (%)	10.4	11.2
18:4 (%)	13.1	12.5
20:0 (%)	0.7	0.5
22:0 (%)	0.1	-
22:1 (%)	0.1	0.5
Cyclopropanoid and epoxy fatty acids	less than 0.05%	less than 0.05%
Total Protein (µg/gram)	awaiting	less than 1.0
Stability Trials		Currently being performed

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ISSUE	2
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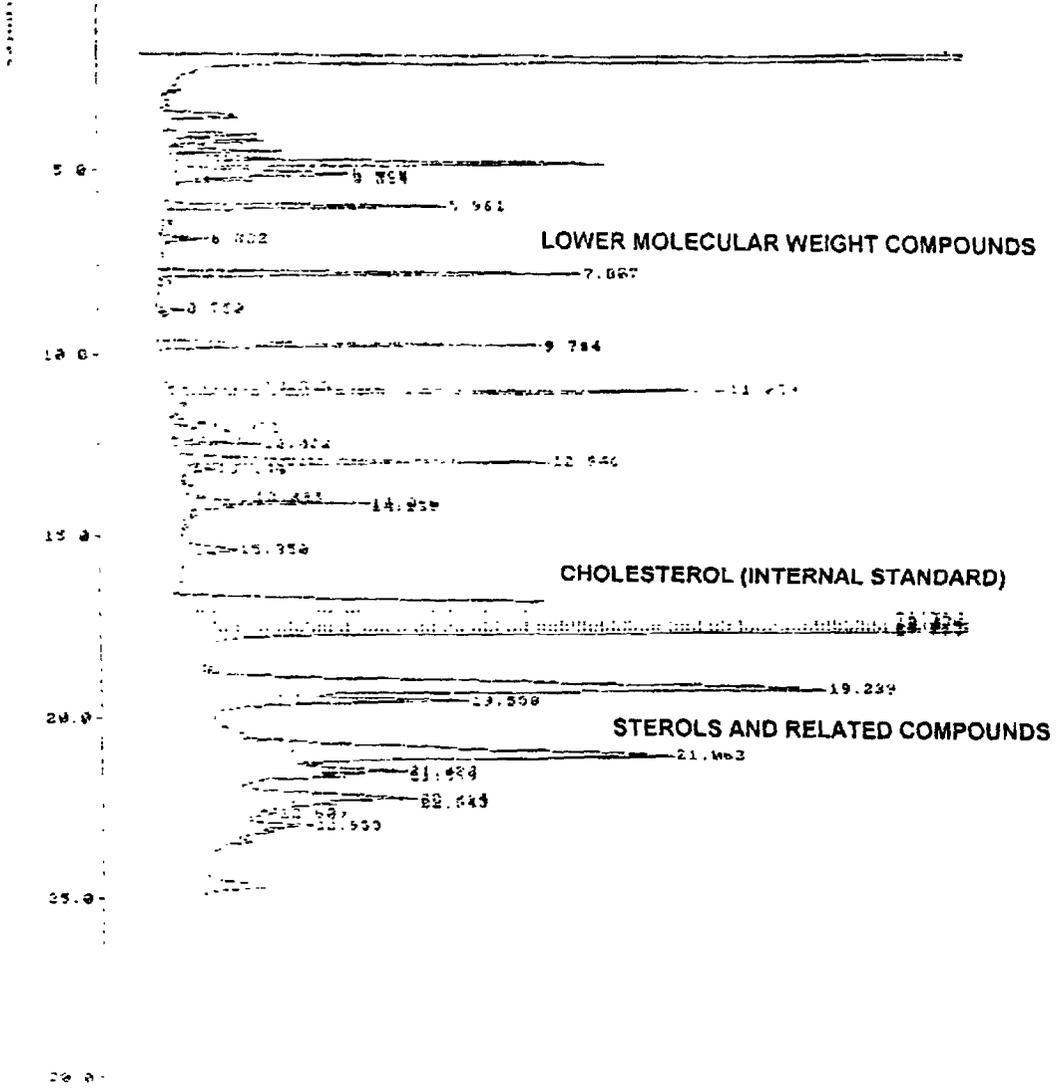
5.0 Procedure

- 5.1 Weigh accurately 2.0 to 2.2g of the oil or fat into a 250ml flask and add 25ml of the alcoholic potassium hydroxide solution. Attach the flask to reflux condenser and heat on a boiling-water bath for 1 hour, swirling at frequent intervals to ensure complete saponification.
- 5.2 Remove the flask from the bath, detach the condenser and transfer the contents of the flask to a 250ml separating funnel, washing the flask with 50ml of water. Rinse the flask with 50ml of diethyl ether and pour the ether cautiously into the funnel. Stopper the funnel, shake vigorously while the contents are still slightly warm, and allow the funnel to stand until the two layers of liquid separate and clarify. Draw off the aqueous alcoholic layer into the flask used for the saponification.
- 5.3 Transfer the ethereal layer from the funnel into a second 250ml separating funnel containing 20ml of water. Extract the aqueous alcoholic soap solution twice more, each time with 50ml of ether in the same manner, and combine the three extracts in the second funnel.
- 5.4 Rotate the ethereal extracts gently in the second funnel, without violent shaking, with the 20ml of water, and after allowing to separate, run off the water. Wash the ethereal solution twice with 20ml of water, shaking vigorously on each occasion.
- 5.5 Then successively wash the ethereal extracts with 20ml of 0.5N aqueous potassium hydroxide solution, 20ml of water, 20ml of 0.5N aqueous potassium hydroxide solution, 20ml of water and again with 20ml of 0.5N aqueous potassium hydroxide solution, and at least twice more with 20ml of water.
- 5.6 Continue washing with water until the wash no longer turns pink on addition of phenolphthalein indicator.
- 5.7 Transfer the ethereal solution to a weighed flask and evaporate to small bulk by means of a rota-evaporator under reduced pressure, or under a gentle current of nitrogen the flask being immersed in a water water bath.
- 5.8 Add 2 to 3ml of acetone to the remaining extract and completely remove the solvent by means of a gentle current of nitrogen, the flask being immersed in a boiling water bath.
- 5.9 Dry the flask and contents to constant weight at a temperature of 80°C. Do not weigh the flask until it has cooled to room temperature, in a dessicator containing silica gel.



Crode Leek Ltd Barnfield Road Leek Staffordshire ST25 5QJ England

UNSAPONIFIABLES OF ECHIUM SEED OIL



Appendix 5 – Heavy metal analysis

BRETBY ANALYTICAL
LABORATORIES LTD

Certificate of Analysis

CRODA LEEK LTD
Barnfield Road
Leek
Staffordshire
ST13 5QJ

Our Reference 44/154431
Date Received 30/10/98
Date Completed 10/11/98
Your Reference 48879

Sample: 391 SR Echium Seed Oil SR3940 BPO/744

Test Description	Ref No	391
Heavy Metals BP Limit Test	BPO08.P	< 10PPM
Copper ppm	IP009M.P	< 0.1
Iron ppm	IP010M.P	11.4

M F Scott
BSc, MSc Civil Microb FIMgt FIFST

T K Madden
BSc MSc MChemA EurChem CChem FRSC
Registered Analytical Chemist
Registered Professional Water Chemist

D K Buckley
MSc FIMgt FIFST CChem FRSC
Registered Analytical Chemist
Qualified Person

The suffix letter P indicates the test method is not NAMAS accredited

Page 1 of 1

Bretby Business Park, Ashby Road, Bretby, Leek, Staffordshire DE13 0GD
Tel: 01829 721111 Fax: 01829 721112

BRETBY ANALYTICAL CONSULTANTS LTD



Technical report

Computer index number 6883

Author Graham Atkinson
Author's reference R49/GMA/211
Laboratory Analytical Services Department

Date 23 April 1998
Enquiry no. CSE 954
Customer Internal
Country -

Keywords

Crossential SA14, Vegetable Glycerides derived from Echium Seed Oil, Herbicide analysis, Glyphosphate, Paraquat, Microbiological contamination.

Objective

To discover if it is possible for Crossential SA14 to be contaminated with any herbicides, pesticides or microorganisms as Crossential SA14 is to be submitted for novel food approval.

Abstract

Two herbicides are used in the growth of Echium Seed Oil (Crossential SA14). Theoretically it will be impossible to find levels of the Herbicides, Glyphosphate or Paraquat in Crossential SA14 due to the insolubility of the herbicides in fats or organic solvents. A leading Analytical Consultants Laboratory has confirmed this.

No evidence of microbiological contamination was found in the sample of Crossential SA14 submitted.

Further action

None.

Distribution

KC KL (Leek) KB KVP DC ARB JAN PM IM File

References: Expt nos.

Missing English Translation of pages 37 and 38

Chemisches Laboratorium Dr. Hermann Ulex Nachf.

Staubschlangenzug (Andreas 1763) als "Apotecke am Stubbenhuk" am Orte des Bräuerknechtgrabens

Inhaber: Hans Joachim Mierendorf

Royal Soc. / Food & Drug Res. London / Analyst (RST DR) / Intern. Soc. / Residue Anal. Wash. D.C. / (Publ. Anal. Chem. Soc. U.K.)
Deutscher Gewerbeverband / VDEh / (DIN 58341)

Chem. Laboratorium Dr. H. Ulex Nachf. Glasstr. 22 22531 Hamburg

Firma
Croda Leek Ltd
Barnfield Road

Leek ST135QJ

Chemisches Handelslaboratorium
Messstelle für Radioaktivität
Glasmoorstraße 23
22551 Hamburg-Norderstedt
Tel.: +49-40-529 587-0
Fax: +49-40-529 587-33
Mobil: +49-172-655 73 10
eMail: DrUlexHmb@aol.com

Hamburg-Norderstedt,
den 06. November 1998
Ulex-No.: 8X 30 02

ATTEST

Betr.: Echinum seed oil
C 392 Purchase order No. 46680

Sie übersandten uns o.g. Probe zur Bestimmung des Gesamtgehaltes an Pyrrolizidinalkaloiden (PA) vom Retronecintyp

Probenaufarbeitung:

Die Probe wurde in Gegenwart von Ethanol und Schwefelsäure mit Zinkpulver mehrere Stunden bei 40° C nach der Methode

"Bestimmung von Pyrrolizidinalkaloiden durch Dünnschichtchromatographie in Samenölen von Borago off. L." H.-J. Mierendorf, Fat. Sci. technol. 97 No. 1 (1995) 33-37"

hydriert zur Reduktion der N-Oxyde der PA zu freien Alkaloiden

Diese wurden durch mehrfache Säure/Basen-Extraktion unter definierten Bedingungen extrahiert und aliquote Anteile zur HPTLC-Analyse gebracht.

DC-Bedingungen:

Platte: Kieselgel KG 60 E Merck (No. 5563)

Laufmittel 1: Aceton, p.a., Merck

Laufmittel 2: Chloroform / Methanol, Ammoniak 80 / 10 / 1

Kammer, gesättigt

Detektion nach Marfcks (spezifisch für PA vom Retronecin-Melitridin-Typ)

Referenzsubstanzen: 7-Acetyllycopsamin / 7-Acetylintermedin

Innerer Standard: Monocrotalin

Resultat:

Keine PA nachweisbar, d.h. pro 100 µl Analysenlösung entsprechend

10g Öl < 20 ng PA

entsprechend (bei einem mittleren Respons von ca. 50-60%)

< 4 ng PA / g Öl (eng/g) (Nachweisgrenze)



Fachlaboratorium I
Allgemeines Handelslaboratorium

Lebensmittel, Gewürze, Speisefettsäuren, Fette, Öle,
Färbemittel, Düngemittel, Trinkwasser, Abwasser,
Kosmetika, Acetylcholinesterase,
Metallanalysen, Giftstoffe, Kontrollen, Umwelt-
untersuchung, Lebensmitteltechnische Beratung

Fachlaboratorium II
Inhaltsstoffe und
Pharmakologikum

Drogen, Heilmittel,
Arzneimittel, Zerkleinerung,
Pharmakologische
und Entwertung

Fachlaboratorium III
Analyse für Umwelt- und forensische Analytik

Mikro- und Ultramikroanalyse von Pflanzen- und Tierproben (Festproben)
Cytotoxizität, toxischen Gewebeschäden, Mykotoxinen / Mykotoxine, Giftstoffe etc.,
Arbeitsproben, Einzelproben, Analysen,
Umweltchemie, -physik und -biologie, Gaschromatographie, HPLC, DA-
ID, und Fluoreszenzspektroskopie, Atomabsorptionsspektroskopie (AAS),
Flammen- und Graphitrohr-AAS, Polarographie, Elektrophorese etc.

Geprüft nach DIN EN ISO 9001:2000 Hamburg. Die in der 2. Auflage des Allgemeinen deutschen Technischer Normen (DIN) mit dem Titel "Allgemeines
Verfahrensprinzipien (Teil 1) und 2", welche ausdrücklich zum Vertragsinhalt gehören. Bestmögliche Qualität, schnellstmögliche Lieferung.
DIN 45 536 - AT - Hamburg

154-111 Nr. 10 544 163 104

Chemisches Laboratorium Dr. Hermann Ulex Nachf.

Stammhaus gegr. Andreae 1763 als "Appotheker am Stubbenhuk" am Orde des Brauerknechtgrabens

Inhaber: Hans-Joachim Mierendorf

Royal Soc. f. Food a. Drug Res. London/Glasgow (RSFDR) • Intern. Soc. f. Residue-Anal. Wash., D.C./Palo Alto C.A. (ISRA)
Deutsche Ges. f. Fotlw. e.V. Münster/Westf. (DGF)

Chem. Laboratorium Dr. H. Ulex Nachf., Glasmoorstr 23, 22851 Norderstedt

Chemisches Handelslaboratorium
Meistelle fr Radioaktivitt
Accreditation EN 17026 in Progress

Firma
Bioriginal Food & Science Corp.
102 Melville Street S7J OR1

Saskatoon, SA-Canada

Glasmoorstrae 23
22851 Hamburg-Norderstedt

Tel. : +49-40-529 587- 0

Fax : +49-40-529 587-33

Mobil: +49-172-655 73 10

eMail: Dr.Ulex@gmx.de

Hamburg-Norderstedt,

den 30. Oktober 2000

Certificate of Analysis No. 0X 06 04

Betr.: Borage oil 23% bleached
Lot-No. 800741

We got the above sample fr analysing the content of Pyrrolizidinalkaloides (PA) of Rebronecintype.

Clean-up:

The sample was hydrated in presence of ethanol and sulfuric acid with zincpowder for 6 hours at 40°C by the method:

"Bestimmung von Pyrrolizidinalkaloiden durch Dnnschichtchromatographie in Samenlen von Borago off.L. : H.-J. Mierendorf, Fat Sci. technol. 97 No. 1 (1995) 33-37"

for reduction of the N-oxydes of the PA's to free alcaloids.

These alcaloides were extracted under defined conditions and aliquot parts of this solution were used for HPTLC-analysis.

DC-Conditions:

Platte: Kieselgel KG 60 E. Merck (No. 5563)

Laufmittel 1: Aceton, p.a., Merck

Laufmittel 2: Chloroform, Methanol, Ammoniak 80 / 19 / 1

Kammer: gestigt

Detektion nach Mallocks (spezifisch fr PA vom Rebronecin-/Heliotridin-Typ)

Referenzsubstanzen: 7-Acetyltycopsamin / 7-Acetylintermedlin

Innerer Standard: Monocrotalin

Result:

No PA's were detectible

< 4 µg PA / kg Oil (Limit of detection: 4µg/kg)

C:\Office52\user\TXT\PHARM\DIV-PHAR\pyrrolizidinalkengl.edw

Fachlaboratorium I: Allgemeines / Landwirtschaft

Lebensmittel, Gewrze, Genussmittel, Fette, le,
Futtermittel, Dngemittel, Trinkwasser, Abwasser,
Kosmetika, Bedarfsgegenstnde,
Betriebsberatung, laufende Kontrollen, Probe-
nahmen, Lebensmittelrechtliche Beratung

Fachlaboratorium II: Industrie und Pharmalaboratorium

Drugs, Chemikalien,
Arzneibuchwaren, Zerk-
nungen, Pharmaforschung
und Entwicklung

Fachlaboratorium III: Institut fr Umwelt- und Rckstandsanalytik

Mikro- und Ultramikroanalytik von Pflanzenschutzmittelrckstnden (Pestiziden),
Carcinogenen, toxischen Schwermetallen, Mykotoxinen (Aflatoxine), Giften etc.,
Aminosurepektren, enzymatische Analysen,
Dichtschicht-, Nitren- und Gaschromatographie, HPLC, UV-,
IR- und Fluoreszenzspektrophotometrie, Atomabsorptionsspektroskopie (Hydrid-,
Fluores- und Graphitrohr-AAS), Polarographie, Elektrophorese etc.

Gerichtsstand und Erfllungsort: Hamburg. Die in der 9. Auflage des Allgemeinen deutschen Gerichtsverfahrensverzeichnisses fr Chemiker abgedruckten allgemeinen
Vertragsbedingungen, insbesondere § 10, werden ausdrcklich zum Vertragsinhalt erhoben. Kostmuster siehe, soweit mglich, 30 Tage zur Verfeinerung

Hamburg

(ZfL-ID Nr.: DE 134 361 103)

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Appendix 7: Herbicide, Pesticide and Microbiological Results and Test Methods



Technical report

Computer index number 6883

Author Graham Atkinson
Author's reference R49/GMA/211
Laboratory Analytical Services Department

Date 23 April 1998
Enquiry no. CSE 954
Customer Internal
Country -

Keywords

Crossential SA14, Vegetable Glycerides derived from Echium Seed Oil, Herbicide analysis, Glyphosphate, Paraquat, Microbiological contamination.

Objective

To discover if it is possible for Crossential SA14 to be contaminated with any herbicides, pesticides or microorganisms as Crossential SA14 is to be submitted for novel food approval.

Abstract

Two herbicides are used in the growth of Echium Seed Oil (Crossential SA14). Theoretically it will be impossible to find levels of the Herbicides, Glyphosphate or Paraquat in Crossential SA14 due to the insolubility of the herbicides in fats or organic solvents. A leading Analytical Consultants Laboratory has confirmed this.

No evidence of microbiological contamination was found in the sample of Crossential SA14 submitted.

Further action

None.

Distribution

KC KL (Leek) KB KVP DC ARB JAN PM IM File

References.	Expt. nos.
	Reports
	Other

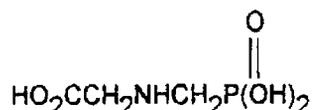
1.0 Introduction

Croda Oleochemicals are to submit Crossential SA14 for novel food approval. Information Services are preparing a dossier, which requires information to state if the material could be contaminated with either agrochemicals or micro-organisms.

2.0 Possible Pesticides

After consultation with Dave Coupland (Croda's agrochemicals expert) and Kevin Peacock, information showed that the farmer responsible for growing the crop had used only two "pesticides" on the land which could have come into contact with the Echium Seed Oil. The two herbicides which had been used in the growth of the plant were:-

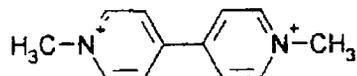
2.1 Glyphosphate (N-(phosphonomethyl) Glycine)



This herbicide is very insoluble in common organic solvents e.g. acetone, ethanol and xylene and hence is unlikely to be soluble in fat.

Environmental fate – usually stated as not being metabolised in plants but there is some evidence of metabolism in certain plants. The principle metabolite is aminomethylphosphoric acid.

2.2 Paraquat (1,1-dimethyl-4,4-bipyridium)



Also a herbicide and practically insoluble in most organic solvents and unlikely to be soluble in fat.

Environmental fate – on plant surfaces photochemical degradation occurs. Degradation products, which have been isolated, include 1-methyl-4-carboxypyridium chloride and methylamine hydrochloride.

3.0 Analysis of Glyphosphate and Paraquat

To prove that Crossential SA14 had not been contaminated with the two herbicides it was proposed that an external analyst be contacted to carry out the analysis. Bretby Analytical consultants were contacted. After being asked to analyse for the two herbicides in Crossential SA14 they stated that they could do the analysis but due to the insolubility of both herbicides and their metabolites in fat it would be highly unlikely to find anything. Over a period of years they have never seen these species in a fatty glycerine sample matrix. It was deemed unnecessary to carry out any analysis.

4.0 Possible Microbiological Contamination

A sample was submitted to the Rawcliffe Bridge microbiology lab for a total plate count. The tests revealed no evidence of microbiological contamination.

It should be noted that testing an isolated sample for micro-organisms does not on its own prove that Croda will be able to produce this material free from microbiological contamination. As microbiological contamination can come from three main sources:-

- i) Raw materials (including the packaging)
- ii) Environmental
- iii) Personnel

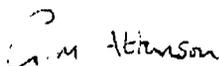
To gain novel load approval it should be demonstrated through documentation that contamination at Croda Leek in the production of this material is unlikely to occur (GMP type documentation should suffice with information about the housekeeping practices in place).

5.0 Conclusions

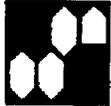
- 5.1 It is impossible for the two herbicides mentioned in this report to be found in Crossential SA 14.
- 5.2 No evidence of any microbiological contamination can be found in the sample of Crossential SA 14 submitted.
- 5.3 Leek's production documentation should give evidence of housekeeping practices in place that will prevent microbiological contamination of products occurring.

6.0 References

- 6.1 The Pesticide Manual 10th Edition (The Royal Society of Chemistry).
- 6.2 Practical Microbiology for the Cosmetics Industry (The Cosmetic Toiletry and Perfumery Association).



G M Atkinson
/jw



035

No G038-1

Date 3 April 1993

Total aerobic bacteria

1.0 Definition

The total aerobic bacteria plate count is the number of bacteria present in the test sample under the conditions of the determination.

2.0 Principle

A known mass of sample is dissolved in a suitable solvent, filtered through a membrane and cultured on nutrient agar. An estimate of total count of aerobic bacteria is obtained from a colony count after 3 or 5 days if the growth is poor.

3.0 Scope

The plate count method may be applied to give an overall picture of the extent of contamination due to viable microorganisms. For a viable count to be statistically correct between 5 and 300 colonies per plate should be achieved.

4.0 Revision Changes

4.1 Change of nutrient broth.



5.0 Apparatus

- 5.1 Sterile, single vent, disposable Petri dishes (10).
- 5.2 Cotton wool plugged sterile blow out pipettes (1 ml).
- 5.3 Beaker (250 ml).
- 5.4 Incubator set at $30^{\circ}\text{C} \pm 1^{\circ}\text{C}$
- 5.5 Autoclave (at 121°C and 15 psi).
- 5.6 Lamina flow cabinet.
- 5.7 Balance (to 2 decimal places).
- 5.8 Butane gas burner.
- 5.9 Aluminium foil.
- 5.10 Conical flask (250 ml).
- 5.11 Non absorbent cotton wool.
- 5.12 Membrane filter holder (sterile plugged).
- 5.13 Membrane filters (0.45 micron pore, type WCN sterile).
- 5.14 Buchner funnel.
- 5.15 Vacuum pump.
- 5.16 Test tubes (3).
- 5.17 Microscope (40 x magnification).

6.0 Reagents

- 6.1 Distilled water (fresh daily).
- 6.2 Nutrient agar.
- 6.3 Nutrient broth (Difco).
- 6.4 Isopropyl myristate.
- 6.5 99% Ethanol (IMS 74OP).
- 6.6 Hycolin solution (2%).

7.0 Procedure

- 7.1 Disinfect the lamina flow cabinet and all work areas with 2% Hycolin solution.
- 7.2 Preparation of agar plates.
 - 7.2.1 Weigh nutrient agar (4.6 g \pm 0.01 g) into a 250 ml conical flask.
 - 7.2.2 Add distilled water (200 ml) to the flask.
 - 7.2.3 Plug the flask with non absorbent cotton wool and cover with aluminium foil.
 - 7.2.4 Place the flask in the autoclave at 121°C and 15 psi for 15 minutes.
 - 7.2.5 Allow to cool to approximately $60 - 70^{\circ}\text{C}$ in the lamina flow cabinet and then pour approximately 20 ml of the medium into 10 Petri dishes.



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- 7.2.6 Using the butane burner carefully flame the surface of each plate to remove any air bubbles formed.
- 7.2.7 Allow the plates to set and then store them upside down at 30°C for 24 hours until they are dry.
- 7.3 Preparation of the broth.
- 7.3.1 Prepare nutrient broth (250 ml) according to the manufacturers instructions.
- 7.3.2 Warm on a hot plate to dissolve the broth and then pour into a 250 ml bottle.
- 7.3.3 Plug the bottle with non absorbent cotton wool and cover with aluminium foil.
- 7.3.4 Place the bottle in the autoclave at 121°C and 15 psi for 15 minutes.
- 7.3.5 Allow to cool to room temperature in the lamina flow cabinet.
- 7.4 Sample preparation
- 7.4.1 Sterilise the filtration unit with the top plugged with non absorbent cotton wool wrapped in cotton in aluminium foil.
- 7.4.2 Dissolve the test sample (1 g \pm 100 mg) in a suitable sterile solvent (9 ml) in each of three capped test tubes using a 1 ml sterile pipette for liquids or a spatula flamed in 99% ethanol for solids
- 7.4.2.1 Use distilled water for water soluble samples and isopropyl myristate for water insoluble samples.
- 7.4.3 With the aid of tweezers (sterilised by flaming in 99% ethanol) remove a membrane from its wrapping and place it on the sterile membrane filter holder.
- 7.4.4 Attach the Buchner funnel to the holder.
- 7.4.5 Flame the rim of the test tube, turn on the vacuum and as quickly as possible remove the plug from the funnel, pour in the sample and replace the plug.
- 7.4.6 When all the sample has filtered (15 - 60 seconds) flame the rim of the Letheen broth bottle and pour in approximately 20 ml to rinse the membrane. Replace the funnel plug.
- 7.4.7 When the filtration is complete turn off the vacuum pump and remove the membrane using sterile tweezers.
- 7.4.7.1 The funnel may be used again if the funnel is kept in a sterile place.
- 7.4.8 Place the membrane on the agar plate.



- 7.4.9 Repeat the process for the remaining two tubes of sample.
- 7.4.10 Incubate the nutrient agar plates at 30°C.
- 7.4.11 Examine the plates using a microscope after 48 and 72 hours and count any colonies arising.
- 7.4.12 Report the average number of colonies obtained after 72 hours.

8.0 Notes

- 8.1 After use all petri dishes and filter papers should be sterilised by autoclaving before disposal.

9.0 Health and safety

- 9.1 Refer to the health and safety data sheet of the test sample.
- 9.2 Hycolin contains synthetic phenolic derivatives. May cause irritation to eyes and skin. Exposure limit not assigned.
- 9.3 Nutrient broth is not a hazardous material. Exposure limit not assigned.
- 9.4 99% Ethanol (IMS 74 OP) is intoxicating if inhaled or ingested. Irritating to eyes. If ingested in undiluted form has a severe drying effect on mucous membranes of mouth and throat. Can be damaging if splashed in eyes. Exposure limit is 1900 mg/m³.
- 9.5 Isopropyl myristate may be harmful if ingested in quantity. Irritating to eyes. Can be absorbed through the skin. Exposure limit not assigned.

10.0 References

- 10.1 None.

11.0 Approval

Compiled by	Position	Signature	Date
M R Harrison	Technical Director	<i>M. R. Harrison</i>	4/3/93

Authorised by	Position	Signature	Date
K Backhouse	Assistant Chemist	<i>K Backhouse</i>	19/5/93
T J Bateman	QC Manager	<i>T J Bateman</i>	17/5/93.

Croda Chemicals Ltd



Cowick Hall Snaith Goole
North Humberside DN14 9AA UK
Telephone 0405 860551
Telex 57601 Fax 0405 860205

Crodaspec test method

Obs.

No G038-2

Date 2 June 1993

Yeasts and moulds

1.0 Definition

The yeasts and moulds plate count is the number of yeasts and moulds present in the test sample under the conditions of the determination.

2.0 Principle

A known mass of sample is dissolved in a suitable solvent, filtered through a membrane and cultured on potato dextrose agar. An estimate of total count of yeasts and moulds is obtained from a colony count after a minimum of three days.

3.0 Scope

The plate count method may be applied to give an overall picture of the extent of contamination due to viable yeasts and moulds. For a viable count to be statistically correct between 5 and 300 colonies per plate should be achieved.

4.0 Revision Changes

4.1 Change of nutrient broth.

Croda Chemicals Ltd



Cowick Hall Snaith Goole
North Humberside DN14 9AA UK
Telephone 0405 860551
Telex 57601 Fax 0405 860205

Crodaspec test method

No G038-2

Date 2 June 1993

5.0 Apparatus

- 5.1 Sterile, single vent, disposable Petri dishes (10).
- 5.2 Cotton wool plugged sterile blow out pipettes (1 ml).
- 5.3 Beaker (250 ml).
- 5.4 Incubator set at $30^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
- 5.5 Autoclave (at 121°C and 15 psi).
- 5.6 Lamina flow cabinet.
- 5.7 Balance (to 2 decimal places).
- 5.8 Butane gas burner.
- 5.9 Aluminium foil.
- 5.10 Conical flask (250 ml).
- 5.11 Non absorbent cotton wool.
- 5.12 Membrane filter holder (sterile plugged).
- 5.13 Membrane filters (0.45 micron pore, type WCN sterile).
- 5.14 Buchner funnel.
- 5.15 Vacuum pump.
- 5.16 Test tubes (3).
- 5.17 Microscope (x 40 magnification).

6.0 Reagents

- 6.1 Distilled water (fresh daily).
- 6.2 Potato dextrose agar.
- 6.3 Potato broth (Difco).
- 6.4 Isopropyl myristate.
- 6.5 99% Ethanol (IMS 74OP).
- 6.6 Hycolin solution (2%).

7.0 Procedure

- 7.1 Disinfect the lamina flow cabinet and all work areas with 2% Hycolin solution.
- 7.2 Preparation of agar plates.
 - 7.2.1 Weigh the potato dextrose agar ($7.8 \text{ g} \pm 0.01 \text{ g}$) into a 250 ml conical flask.
 - 7.2.2 Add distilled water (200 ml) to the flask.
 - 7.2.3 Plug the flask with non absorbent cotton wool and cover with aluminium foil.
 - 7.2.4 Place the flask in the autoclave at 121°C and 15 psi for 15 minutes.
 - 7.2.5 Allow to cool to approximately $60 - 70^{\circ}\text{C}$ in the lamina flow cabinet and then pour approximately 20 ml of the medium into 10 Petri dishes.



No G038-2

Date 2 June 1993

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- 7.2.6 Using the butane burner carefully flame the surface of each plate to remove any air bubbles formed.
- 7.2.7 Allow the plates to set and then store them upside down at 30°C for 24 hours until they are dry.
- 7.3 Preparation of the broth.
- 7.3.1 Prepare potato dextrose broth (250 ml) according to manufacturers instructions.
- 7.3.2 Warm on a hotplate to dissolve the broth and then pour into a 250 ml bottle.
- 7.3.3 Plug the bottle with non absorbent cotton wool and cover with aluminium foil.
- 7.3.4 Place the bottle in the autoclave at 121°C and 15 psi for 15 minutes.
- 7.3.5 Allow to cool to room temperature in the lamina flow cabinet.
- 7.4 Sample preparation
- 7.4.1 Sterilise the filtration unit with top plugged with non absorbent cotton wool wrapped in aluminium foil.
- 7.4.2 Dissolve the test sample (1 g ± 100 mg) in a suitable sterile solvent (9 ml) in each of three capped test tubes using a 1 ml sterile pipette for liquids or a spatula flamed in 99% ethanol for solids
- 7.4.2.1 Use distilled water for water soluble samples and isopropyl myristate for water insoluble samples.
- 7.4.3 With the aid of tweezers (sterilised by flaming in 99% ethanol) remove a membrane from its wrapping and place it on the sterile membrane filter holder.
- 7.4.4 Attach the Buchner funnel to the holder.
- 7.4.5 Flame the rim of the test tube, turn on the vacuum and as quickly as possible remove the plug from the funnel, pour in the sample and replace the plug.
- 7.4.6 When all the sample has filtered (15 - 60 seconds) flame the rim of the Letheen broth bottle and pour in approximately 20 ml to rinse the membrane. Replace the funnel plug.
- 7.4.7 When the filtration is complete turn off the vacuum pump and remove the membrane using sterile tweezers.
- 7.4.7.1 The funnel may be used again if it is kept in a sterile place.
- 7.4.8 Place the membrane on the potato dextrose agar plate.

Croda Chemicals Ltd



Cowick Hall Snarth Goole
North Humberside DN14 9AA UK
Telephone 0406 860651
Telex 57601 Fax 0405 860205

Crodaspec test method

No G038-2

Date 2 June 1993

-
- 7.4.9 Repeat the process for the remaining two tubes of sample.
- 7.4.10 Incubate the potato dextrose agar plates at 30°C.
- 7.4.11 Examine the plates using a microscope after 48 and 72 hours and count any colonies arising.
- 7.4.12 Report the average number of colonies obtained after 72 hours.

8.0 Notas

- 8.1 After use all petri dishes and filter papers should be sterilised by autoclave before disposal.

9.0 Health and Safety

- 9.1 Refer to the health and safety data sheet of the test sample.
- 9.2 Lethen broth is not a hazardous material. Exposure limit not assigned.
- 9.3 Potato dextrose agar is not a hazardous material. Exposure limit not assigned.
- 9.4 99% Ethanol (IMS 74OP) is intoxicating if inhaled or ingested. Irritating to eyes. If ingested in undiluted form has a severe drying effect on mucous membranes of mouth and throat. Can be damaging if splashed in eyes.
- 9.5 Isopropyl myristate is irritating to eyes. Frequent or prolonged contact with skin may cause dermatitis.

10.0 References

- 10.1 None.

11.0 Approval

Compiled by	Position	Signature	Date
M R Harrison	Technical Director	<i>M. R. Harrison</i>	2/6/93

Authorised by	Position	Signature	Date
K Backhouse	Assistant Chemist	<i>K Backhouse</i>	3/6/93
T J Bateman	QC Manager	<i>T J Bateman</i>	3/6/93

Croda Chemicals Ltd



Cowick Hall Snaith Goole
North Humberside DN14 9AA UK
Telephone 0405 860551
Telex 57601 Fax 0405 860205

Crodaspec test method

025

No G038-3

Date 18 March 1993

Gram negative bacteria

1.0 Definition

The Gram negative bacteria plate count is the number of Gram negative bacteria present in the test sample under the conditions of the determination.

2.0 Principle

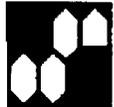
A known mass of sample is dissolved in a suitable solvent, filtered through a membrane and cultured on MacConkey's agar. An estimate of total count of aerobic bacteria is obtained from a colony count after a minimum of three days.

3.0 Scope

The plate count method may be applied to give an overall picture of the extent of contamination due to viable microorganisms. For a viable count to be statistically correct between 5 and 300 colonies per plate should be achieved.

4.0 Revision Changes

4.1 The temperature of incubation is changed to 30°C.



No G038-3

Date 18 March 1993

5.0 Apparatus

- 5.1 Sterile, single vent, disposable Petri dishes (10).
- 5.2 Cotton wool plugged sterile blow out pipettes (1 ml).
- 5.3 Beaker (250 ml).
- 5.4 Incubator set at $30^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
- 5.5 Autoclave (at 121°C and 15 psi).
- 5.6 Lamina flow cabinet.
- 5.7 Balance (to 2 decimal places).
- 5.8 Butane gas burner.
- 5.9 Aluminium foil.
- 5.10 Conical flask (250 ml).
- 5.11 Non absorbent cotton wool.
- 5.12 Membrane filter holder (sterile plugged).
- 5.13 Membrane filters (0.45 micron pore, type WCN sterile).
- 5.14 Buchner funnel.
- 5.15 Vacuum pump.
- 5.16 Test tubes (3).
- 5.17 Microscope (x 40 magnification).

6.0 Reagents

- 6.1 Distilled water (fresh daily).
- 6.2 MacConkey's agar (Difco).
- 6.3 Lethen broth.
- 6.4 Isopropyl myristate.
- 6.5 99% Ethanol (IMS 74OP).
- 6.6 Hycolin solution (2%).

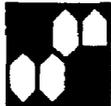
7.0 Procedure

- 7.1 Disinfect the lamina flow cabinet and all work areas with 2% Hycolin solution.
- 7.2 Preparation of agar plates.
 - 7.2.1 Weigh the MacConkey's agar ($10.0 \text{ g} \pm 0.01 \text{ g}$) into a 250 ml conical flask.
 - 7.2.2 Add distilled water (200 ml) to the flask.
 - 7.2.3 Plug the flask with non absorbent cotton wool and cover with aluminium foil.
 - 7.2.4 Place the flask in the autoclave at 121°C and 15 psi for 15 minutes.
 - 7.2.5 Allow to cool to approximately $60 - 70^{\circ}\text{C}$ in the lamina flow cabinet and then pour approximately 20 ml of the medium into 10 Petri dishes.



-
- 7.2.6 Using the butane burner carefully flame the surface of each plate to remove any air bubbles formed.
- 7.2.7 Allow the plates to set and then store them upside down at 30°C for 24 hours until they are dry.
- 7.3 Preparation of the broth.
- 7.3.1 Prepare MacConkey broth (250 ml) according to the manufacturers instructions.
- 7.3.2 Warm on a hotplate to dissolve the broth and then pour into a 250 ml bottle.
- 7.3.3 Plug the bottle with non absorbent cotton wool and cover with aluminium foil.
- 7.3.4 Place the bottle in the autoclave at 121°C and 15 psi for 15 minutes.
- 7.3.5 Allow to cool to room temperature in the lamina flow cabinet.
- 7.4 Sample preparation
- 7.4.1 Sterilise the filtration unit with the top plugged with non absorbent cotton wool wrapped in aluminium.
- 7.4.2 Dissolve the test sample (1 g \pm 100 mg) directly into a suitable sterile solvent (9 ml) in each of three capped test tubes using a 1 ml sterile pipette for liquids or a spatula flamed in 99% ethanol for solids
- 7.4.2.1 Use distilled water for water soluble samples and isopropyl myristate for water insoluble samples.
- 7.4.3 With the aid of tweezers (sterilised by flaming in 99% ethanol) remove a membrane from its wrapping and place it on the sterile membrane filter holder.
- 7.4.4 Attach the Buchner funnel to the holder.
- 7.4.5 Flame the rim of the test tube, turn on the vacuum and as quickly as possible remove the plug from the funnel, pour in the sample and replace the plug.
- 7.4.6 When all the sample has filtered (15 - 60 seconds) flame the rim of the Lethen broth bottle and pour in approximately 20 ml to rinse the membrane. Replace the funnel plug.
- 7.4.7 When the filtration is complete turn off the vacuum pump and remove the membrane using sterile tweezers.
- 7.4.7.1 The funnel may be used again if it is kept in a sterile place.
- 7.4.8 Place the membrane on the MacConkey's agar plate.

Croda Chemicals Ltd



Cowick Hall Snaith Goole
North Humberside DN14 9AA UK
Telephone 0405 860551
Telex 57801 Fax 0405 860205

Crodaspec test method

No G038-3

Date 18 March 1993

- 7.4.9 Repeat the process for the remaining two tubes of sample.
- 7.4.10 Incubate the MacConkey's agar plates at 30°C.
- 7.4.11 Examine the plates using a microscope after 48 and 72 hours and count any colonies arising.
- 7.4.12 Report the average number of colonies obtained after 72 hours.

8.0 Notes

8.1 After use all petri dishes and filter papers should be sterilised by autoclave before disposal.

9.0 Health and Safety

- 9.1 Refer to the health and safety data sheet of the test sample.
- 9.2 Lethen broth is not a hazardous material. Exposure limit not assigned
- 9.3 MacConkey's agar is not a hazardous material. Exposure limit not assigned.
- 9.4 96% Ethanol is intoxicating if inhaled or ingested. Irritating to eyes. If ingested in undiluted form has a severe drying effect on mucous membranes of mouth and throat. Can be damaging if splashed in eyes. Exposure limit is 1900 mg/m³.
- 9.5 Isopropyl myristate may be harmful if ingested in quantity. Irritating to eyes. Can be absorbed through the skin. Exposure limit not assigned.

10.0 References

10.1 None.

11.0 Approval

Compiled by	Position	Signature	Date
M R Harrison	Technical Director	<i>M. R. Harrison</i>	19/3/93

Authorised by	Position	Signature	Date
K Backhouse	Assistant Chemist	<i>K Backhouse</i>	3/6/93
T J Bateman	QC Manager	<i>T J Bateman</i>	3/6/93

Appendix 8: The revised long term arrangements for extension of use (2000)



PESTICIDES SAFETY DIRECTORATE

Masart House, Kings Pool, 3 Peasemere Green, York YO1 7PX
Switchboard: 01904 846500 GTN: 5138
Direct Dial: 01904 45 FAX: 01904 45
International: (+44) 1904 45 International Fax: (+44) 1904 45
Email: f.j.daly@psd.maff.gov.uk

To: All MAFF Approval Holders
cc: Interested Parties for Information

Our Ref: AAHL/23/99
PRD 2224

Date: 13 December 1999

Dear Sir/Madam

THE REVISED LONG TERM ARRANGEMENTS FOR EXTENSION OF USE (2000)

Since 1 January 1990 'The Long Term Arrangements for Extension of Use' have been in place to permit many professional pesticide products to be used for additional specific minor uses, subject to adherence to certain conditions. These arrangements were subsequently reviewed in December 1994, with a requirement for a further review by the 31 December 1999. The Pesticides Safety Directorate (PSD) has undertaken this review in consultation with the Advisory Committee on Pesticides and Government Departments responsible for the approval of pesticides. The new arrangements are attached at Appendix II and are valid until 31 December 2004.

PSD has considered whether the current extrapolations are still valid, relevant and in line with currently accepted EU residues data extrapolations. New extensions of use requested by growers have been considered and the restrictions and guidance have been refined and clarified. The amendments/additions that have been made are detailed in Appendix I.

As this review has resulted in various amendments to the arrangements, it is important that the new arrangements are read thoroughly before proceeding with pesticide uses that were previously allowed under 'The Revised Long Term Arrangements for Extension of Use' (1993).

The continuation of the Long Term Arrangements for Extension of Use will ensure that the UK industry has access to essential pesticides, whilst ensuring that risks to the operator, consumer and wildlife/environment are not increased.

Users are reminded that use of a pesticide under these arrangements is at all times undertaken at the user's choosing and the user accepts the commercial risk to the crop. The conditions of use relating to the approved label recommendations and/or specific off-label approval apply to the extension of use, unless superseded by the terms of these arrangements.



EXECUTIVE AGENCY OF THE MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

An Executive Agency of the Ministry of Agriculture, Fisheries and Food

Appendix I

AMENDMENTS

- (i) Red chard, white chard and yellow chard have been added to the extrapolation from spinach to beer leaves in 'Section V: Crops Used Partly or Wholly For Human or Animal Consumption.'
- (ii) The extrapolation to *Miscanthus spp* (Elephant Grass) from cereals/grass/maize has been added to 'Section F: Non-edible crops and Plants', as follows:
- 'Subject to the specific restrictions for extension of use set out above, herbicides approved for use on cereals, grass and maize may be used on commercial agricultural and horticultural holdings on *Miscanthus spp* (Elephant grass). Applications must not be made after the crop is 1 metre in height. The crop or products of the crop must not be used for food or feed.'
- (iii) Oilseed rape to *Echium vulgare/Echium plantaginum* has been added to 'Section V: Crops Used Partly or Wholly For Human or Animal Consumption', with a restriction that the extrapolation is for crops grown as an oilseed only and does not apply to seed treatments.
- (iv) The extrapolation in 'Section V: Crops Used Partly or Wholly For Human or Animal Consumption' from cereals to grass seed crops has been amended as follows:
- The phrase 'Treated crops must not be grazed or cut for fodder' has been amended to read: 'Treated crops must not be grazed or cut for fodder until 90 days after treatment'.
- The phrase 'Use of chlormequat-containing products is not permitted' has been added.
- (v) A section clarifying crops that are considered to be equivalent has been added to 'Section V: Crops Used Partly or Wholly For Human or Animal Consumption', as follows:
- Cobnuts and filberts are synonymous with hazelnuts
Navy beans are synonymous with French beans
Pickling pea/shelling pea/non-edible podded pea are synonymous with vining peas
Linols and flax are synonymous with linseed.
Durum wheat is considered to be wheat.
- (vi) The 'Farm Forestry and Rotation Coppicing' section now includes reference to 'reclaimed brownfield sites'.
- (vii) The extrapolation 'Rye and triticale from wheat and barley (Treatments applied before second node observable stage only)' has been amended to read:
- 'Barley to rye and triticale for treatments applied before first spikelet of inflorescence just visible'
'Wheat to rye and triticale.'

Appendix II

THE LONG TERM ARRANGEMENTS FOR EXTENSION OF USE (2000)

Please note that these extensions of use are at all times done at the user's choosing, and the commercial risk is entirely theirs.

SPECIFIC RESTRICTIONS FOR EXTENSION OF USE UNDER THESE ARRANGEMENTS

To ensure that the extension of use does not increase the risk to the operator, the consumer or the environment, the following conditions **MUST** be followed when applying pesticides under the terms of this scheme

GENERAL RESTRICTIONS

- 1 These arrangements apply to label and specific off-label recommendations for use of **ONLY** products approved for use as Agricultural/Horticultural pesticides.
- 2 All safety precautions and statutory conditions relating to use (which are clearly identified in the statutory box on product labels) **MUST** be observed. If extrapolation from a specific off-label is to be used then in addition to all safety precautions and statutory conditions relating to use specified on the product label, all conditions relating to use specified on the Notice of Approval for the specific off-label use **MUST** be observed.
- 3 Pesticides **MUST** only be used in the same situation (outdoor or protected) as that specified on the product label/specific off-label Notice of Approval for the use on which the extrapolation is to be based, specifically:

Pesticides must not be used on protected crops, i.e. crops grown in glasshouses, poly tunnels, cloches or polythene covers or in any other building, unless the product label/specific off-label Notice of Approval specifically allows use under protection on the crop on which the extrapolation is to be based. Similarly, pesticides approved only for use in protected situations must not be applied outdoors.

PLEASE NOTE: Unless specifically restricted to outdoor crops only, pesticides approved for use on tomatoes, cucumbers, lettuce, chrysanthemum and mushrooms are assumed to be approved for use under protection. For all other uses, if the label/specific off-label Notice of Approval does not specify a situation, then only extrapolation to an outdoor use is permitted.

APPLICATION METHOD RESTRICTIONS

- 4 The method of application must be as stated on the pesticide label and in accordance with the relevant codes of practice and requirements under COSHH 1994 (Control of Substances Hazardous to Health)

petal fall) unless otherwise permitted. Applications of such pesticides must also not be made when flowering weeds are present or where bees are actively foraging.

8. If there is an aquatic buffer zone restriction set for the on-label/off-label use, then where appropriate, users are also obliged to conduct a Local Environmental Risk Assessment for Pesticides (LERAP) for the extension of use.
9. All reasonable precautions **MUST** be taken to safeguard wildlife and the environment.

EXCLUSIONS

10. The following uses are **NOT PERMITTED** under these arrangements.
 - (a) Aerial applications
 - (b) Use in or near water (in or near water includes drainage channels, streams, rivers, ponds, lakes, reservoirs, canals, dry ditches, areas designated for water storage)
 - (c) Use in or near coastal waters.
 - (d) Use of rodenticides and other vertebrate control agents
 - (e) Use on land not intended for cropping, land not intended to bear vegetation, amenity grassland, managed amenity turf and amenity vegetation (this includes areas such as paths, pavements, roads, ground around buildings, motorway verges, railway embankments, public parks, turf, sports fields, upland areas, moorland areas, nature reserves, etc.).

EXTENSIONS OF USE

1. NON-EDIBLE CROPS AND PLANTS

- (a) Subject to the **SPECIFIC RESTRICTIONS FOR EXTENSION OF USE** set out above, pesticides approved for use on any growing crop may be used on commercial agricultural and horticultural holdings and in forest nurseries on the following crops and plants:
 - (i) hardy ornamental nursery stock, ornamental plants, ornamental bulbs and flowers and ornamental crops grown for seed where neither the seed nor any part of the plant is to be consumed by humans or animals;
 - (ii) forest nursery crops prior to final planting out
- (b) Subject to the **SPECIFIC RESTRICTIONS FOR EXTENSION OF USE** set out above, pesticides approved for use on any growing edible crop may be used on commercial agricultural and horticultural holdings on non-ornamental crops grown for seed where neither the seed nor any part of the plant is to be consumed by humans or animals. This extrapolation **EXCLUDES** use on

If hand held or broadcast air assisted use is required see paragraphs 5 and 6 respectively of the **SPECIFIC RESTRICTIONS FOR EXTENSION OF USE.**

IV. HOPS (*Humulus spp.*)

Subject to the **SPECIFIC RESTRICTIONS FOR EXTENSION OF USE** set out above, pesticides may be used on commercial agricultural and horticultural holdings on the following hop plants grown in the circumstance below:

- (a) Mature stock or mother plants which are kept specifically for the supply of propagation material.
- (b) Propagation of hop planting material- propagules prior to final planting out
- (c) "Nursery hops". First year plants not taken to harvest that year, in their final planting out position

PLEASE NOTE:

For a - c above, treated hops must **NOT** be harvested for human or animal consumption (including hilling) within 12 months of treatment.

If hand held or broadcast air assisted application is required, users must comply with paragraphs 5 and 6 respectively of the **SPECIFIC RESTRICTIONS FOR EXTENSION OF USE.**

V. CROPS USED PARTLY OR WHOLLY FOR HUMAN OR ANIMAL CONSUMPTION.

Subject to the **SPECIFIC RESTRICTIONS FOR EXTENSION OF USE** set out above, pesticides may be used on commercial agricultural or horticultural holdings on the crops listed in **TABLE ONE** and **TWO** below in the first column if they have been approved for use on the crop(s) listed opposite them in the second column.

HOWEVER, BEFORE USING ANY OF THE FOLLOWING EXTRAPOLATIONS (TABLES ONE AND TWO), THE USER MUST FIRST OBSERVE THE FOLLOWING:

- (a) It is the responsibility of the user to ensure that the proposed use does not result in any statutory UK Maximum Residue Levels (MRLs) being exceeded. MRLs are set out in statutory instrument No. 1985 of 1994: 'The Pesticides (Maximum Residue Levels in Crops, Food and Feeding Stuffs) Regulations 1994' (The Stationery Office, ISBN 0-11-044985-1) and any subsequent updates.
- (b) These extrapolations **DO NOT APPLY** in the following situations:

<u>Column 1: Minor use</u>	<u>Column 2: Crops on which use is approved</u>	<u>Additional special conditions</u>
B. FRUIT CROPS		
Almond, Chestnut, Walnut, Hazelnut	Apple or cherry or plum	For herbicides used on the orchard floor ONLY
Almond, Chestnut, Walnut, Hazelnut	Products approved for use on two of the following: almond, chestnut, hazelnut and walnut	
Quince, Crab apple	Apple or pear	
Nectarine, Apricot	Peach	
Blackberry, Dewberry Rubus species (e.g. tayberry, loganberry)	Raspberry	
Whitecurrant, Bilberry, Cranberry	Blackcurrant or redcurrant	
Redcurrant	Blackcurrant	
C. VEGETABLE CROPS		
Parsley root	Carrot or radish	
Fodder beet, Mangel	Sugar beet	
Horseradish	Carrot or radish	
Parsnip	Carrot	
Salsify	Carrot or celeriac	
Swede	Turnip	
Turnip	Swede	
Garlic, Shallot	Half onion	
Aubergine	Tomato	
Squash, Pumpkin, Marrow, Watermelon	Melon	

Column 1: Minor Use

Mustard, Sunflower, Honesty, Sesame,
Linseed, Evening primrose,
Poppy (grown for oilseed),
Borage (grown for oilseed)
Canary flower e.g. *Echinus vulgare/Echinus
plantaginifolius* (grown for oilseed)

Column 2: Crops on which use is
approved.

Oilseed rape

VI. CLARIFICATIONS:

Under these arrangements the following crops are considered to be synonymous or equivalent and as such, uses on crops in Column 1 can be read across to uses in Column 2.

Column 1:

Hazelnut

French bean

Vining pea

Linseed

Wheat

Column 2: equivalent

Cobnuts, Filberts

Navy bean

Picking pea, Shelling pea, Non-edible podded pea

Linola, Flax

Durum wheat

Appendix 9: Comparison of lipid profile of Crossential SA14 with borage oil & blackcurrant seed oil

The anti-inflammatory activity of Crossential SA14 has been investigated along with a number of other oils rich in essential fatty acids [see Appendix 11]. Lipid profiles were determined for each of the oils prior to testing. For comparison the lipid profiles of borage oil, blackcurrant oil and Crossential SA14 are reproduced below.

Fatty Acid	Borage Oil	Blackcurrant Oil	Crossential SA14
C16:0	9.98	6.92	7.01
C16:1	0.39	0.20	0.19
C18:0	3.39	1.40	3.61
C18:1	16.37	11.76	16.41
C18:2 (LA)	38.79	44.68	14.96
C18:3 (ALA)	0.49	11.44	28.98
C18:3 (GLA)	20.68	16.27	11.83
C18:4 (SA)	0.13	3.02	12.99
C20:0	0.23	-	0.39
C20:1	3.83	0.86	0.68
C22:1	2.46	-	0.13
C24:1	1.23	-	0.14
Others	2.03	3.45	2.68

Appendix 10: Biochemical pathways for omega-3 & omega-6 fatty acids

OMEGA-6:

9,12-Octadecadienoic acid (linoleic acid)

↓ ⁶ Desaturase

6,9,12-Octadecatrienoic acid (*gamma*-linolenic acid)

↓ Elongase

8,11,14-Eicosatrienoic acid (dihomo-*gamma*-linolenic acid) → Prostaglandin E₁

↓ ⁵ Desaturase

5,8,11,14-Eicosatetraenoic acid (arachidonic acid) → Prostaglandin E₂
+ Leukotriene B₄

↓ Elongase

Docosatetraenoic acid

↓ ⁴ Desaturase

Docosapentaenoic acid

OMEGA-3:

9,12,15-Octadecatrienoic acid (*alpha*-linolenic acid / ALA)

↓ ⁶ Desaturase

6,9,12,15-Octadecatetraenoic acid (stearidonic acid / SA)

↓ Elongase

8,11,14,17-Eicosatetraenoic acid

↓ ⁵ Desaturase

5,8,11,14,17-Eicosapentaenoic acid → Prostaglandin E₃
+ Leukotriene B₅

↓ Elongase

7,10,13,16,19-Docosapentaenoic acid

↓ ⁴ Desaturase

4,7,10,13,16,19-Docosahexaenoic acid → Prostaglandin E₃
+ Leukotriene B₅

Appendix 11: Effect on PGE₂ and IL-1 α

APPENDIX 11



PROTOCOL NO. K0563
January 25, 1996

Anti-inflammatory Activity-UVB
MTT, PGE₂, IL-1 α
ZK1301

Croda Chemicals Ltd.
Cowick Hall
Snaith Goole
North Humberside, DN14 9AA
ENGLAND

TEST MATERIALS

Untreated Control		CN419
CN421	⇒ CROSSENTIAL	CN422
CN423	SALY	CN424

REPORT BY: Ronnda Bartel, Ph.D.

PERFORMED BY: Laurie K. Thomas



Final Report Addendum for Study No. K0563 Anti-inflammatory Activity - UVB

OBJECTIVE OF THE STUDY

The objective of this study is to determine the potential anti-inflammatory activity of topically applied oils using the skin²⁶ ZK1301 model with MTT, prostaglandin E₂ (PGE₂) and interleukin 1-alpha (IL-1 α) as the endpoints. The inflammatory response is generated by irradiation of the tissues with ultraviolet light, 295-400 nm (UVB). MTT, a substrate for mitochondrial succinate dehydrogenase, is an indicator of cellular viability. PGE₂ release is indicative of membrane perturbation events that activate phospholipase A₂. IL-1 α is a cellular cytokine released in response to inflammatory stimuli or chemical insult.

SUMMARY RESULTS

MTT Assay: None of the test materials produced any cytotoxicity (no UVB tissues) or phototoxicity (UVB treated tissues).

Prostaglandin E₂ Release Assay: Two materials, CN424 and CN421, showed potential anti inflammatory activity with PGE₂ release as the endpoint.

Interleukin 1 Alpha Release Assay: Two materials, CN422 and CN424 showed slight anti inflammatory activity with IL-1 α as the endpoint. Only CN424 caused a decrease in both PGE₂ and IL-1 α release induced by UVB irradiation

SUMMARY TABLE

<u>Test Material</u>	<u>MTT</u>	<u>PGE₂</u>	<u>IL-1α</u>
CN419	No effect	No effect	No effect
CN422	No effect	No effect	Decrease
CN423	No effect	No effect	No effect
CN424	No effect	Decrease	Decrease
CN421	No effect	Decrease	No effect

MATERIALS AND EQUIPMENT

Refer to the current directions for use:

ZS1301 DFU, FRD0033 (rev. 01) (see Attachment D)
 MTT Assay Kit, ZA0022 DFU, FRD0028 (rev. 02) (see Attachment E)
 PGE₂ Assay Kit, ZA0050 DFU, FRD0019 (rev. 02) (see Attachment F)
 IL-1 α Assay Kit, DLA50 (R&D Systems, Minneapolis MN) (see Attachment G)
 Dr. Honle Solar Simulator
 UVB H2 filter (295-400nm)

PROTOCOL

The attached testing protocol was followed as written (NB ref: 95-0248-42, 107, 108, 118, 120). The calibration of all equipment, (i.e., UV lamp, incubator, pipetters, etc.) was verified prior to beginning the study. In brief, the test materials were applied to the stratum corneum and allowed to incubate for 24 hours. Half of the tissues were then irradiated with 4 Joules of UVB light. The tissues were incubated another 24 hours and evaluated for cell viability using the MTT dye reduction assay. The media samples were collected and stored at -20°C prior to assay for inflammatory mediators. The samples were thawed to room temperature and assayed for PGE₂ and IL-1 α according to the assay kit directions for use.

DATA ANALYSIS

MTT Assay: The cytotoxicity of each test agent in the presence and absence of UVB treatment is expressed as the percent of the untreated

control MTT value. Less than 100 percent indicates that cell viability has been lost.

$$\frac{(\text{Test Material O.D.})}{(\text{Untreated Control O.D.})} \times 100 = \text{Percent Untreated Control}$$

PGE₂ Assay: The pg of PGE₂ released into the medium in each of the samples is calculated from optical density using a standard curve generated with each experiment (see the PGE₂ Assay Directions for Use).

Interleukin 1-alpha Assay. The pg of IL-1 α released into the medium in each of the samples is calculated from the optical density using a standard curve generated with each experiment. (see the IL-1 α Assay Directions for Use).

Factorial Analysis: To demonstrate the effects of test materials with and without UVB irradiation, the data was placed into a full factorial design (see Figure 4). The ratio of the values from each of the runs yields the fold change in the endpoint due to each of the variables, namely, the test agent and/or UVB.

RESULTS/CONCLUSIONS

MTT Assay: The cell viability data is summarized in Tables 1 and 2 in Attachment A. None of the test materials produced any cytotoxicity (no UVB tissues) or phototoxicity (UVB treated tissues).

PGE₂ Assay: The PGE₂ release raw data is summarized in Table 3 in Attachment A. The factorial analysis is shown in Table 5 in Attachment A. Each of the five test materials showed a slight increase in PGE₂ release without UVB (Run Ratio 3:1). This could be due to the physical manipulation of the tissues during the dosing regimen or to a slight irritation of the tissue in response to the test agent. A distilled water or other innocuous material would have to be used as a sham dosing control to determine which of these options is the case.

The test agent induced PGE₂ release is exacerbated by UVB irradiation by 3-6 fold with four of the five test materials (Run Ratio 4:3). The exception is material CN422 which showed a 11 fold increase. This suggests that this material may show some photoirritation.

UVB irradiation of the tissue causes ~13 fold increase in the release of PGE₂ (Run Ratio 2:1). This UVB induced increase is blocked ~50% by

preincubation with two of the test materials, CN424 and CN421 (Run Ratio 4:2). This suggests that these two materials may be blocking either the synthesis or release of PGE₂ from the tissues and may show some anti inflammatory activity.

IL-1 α Assay: The IL-1 α release data is summarized in Table 6 in Attachment A. The factorial analysis is shown in Table 7 in Attachment A. As with the PGE₂ endpoint, each of the test materials produced a moderate increase, ~2-5 fold, in IL-1 α (Run Ratio 3:1). A sham dosing control would be required to determine if this is due to the test material or the physical manipulation associated with dosing the tissues. In contrast to the PGE₂ endpoint, this test material induced increase in IL-1 α is not noticeably altered by the irradiation of the tissues with UVB.

Irradiation of the tissues with UVB alone caused an 8 fold increase in IL-1 α release. None of the test materials showed substantial inhibition of this UVB induced inflammatory response. Materials CN422 and CN424 inhibited the IL-1 α release by ~30% but only the CN424 showed inhibitory activity in both the inflammatory mediator endpoints (see Table below). These data suggest that three of the five materials (CN422, CN424 and CN421) may show some anti inflammatory activity but also, that they are working via different mechanisms in the tissue.

NOTE: One replicate in both the PGE₂ and IL-1 α release data for the UVB irradiated control was unusually low (see circled values in Tables 3 and 6). No assignable cause was found. However, since these two values were generated from the same cell culture supernatant, it is not likely to be an assay error. Both replicates were used in the data calculations since there were only duplicate samples. If the higher replicate were used alone, the trends in the data would remain the same but the magnitude of the inhibitory response would be larger.

ATTACHMENTS

- A - Data, Tables and Graphs
- B - ZK1301 Quality Control Release Form
- C - K0563 Study Protocol
- D - ZK 1301 Directions for Use
- E - ZA0022 MTT Assay Kit Directions for Use
- F - ZA0050 PGE₂ Assay Kit Directions for Use
- G - R&D Systems IL-1 α Assay Kit Directions for Use

ATTACHMENTS D TO
G HAVE NOT BEEN
INCLUDED IN THIS
COPY

TEST ARTICLE DISPOSAL PROCEDURE

Unless the Sponsor requests otherwise, it is the Advanced Tissue Science practice to retain test materials for one (1) month after the completion of the study. The test materials will automatically be returned to the Sponsor after one (1) month.

DATA RETENTION

All study documents and the original final report will be on file in the Advance Tissue Science archives for a period of no less than two years, unless indicated otherwise in writing.

DISCLAIMER

Advanced Tissue Sciences submits this report with the understanding that the data contained herein may be used as an important component of the sponsor's Product Safety assessment strategy. However, the data contained herein may not provide sufficient evidence by itself of the toxicity or irritation potential (or lack thereof) of a product or raw material. These data should be evaluated along with other sources of information about the test material, including, but not limited to, physicochemical data and clinical safety studies. Accordingly, the final assessment of any test material's safety and/or hazard potential is the sole responsibility of the sponsor.

Pages 70 - 74 containing
CONFIDENTIAL COMMERCIAL INFORMATION
have been redacted in their entirety



Advanced Tissue Sciences Customer Testing

Customer: Croda
 Cowick Hall
 Snaith Goole
 North Humberside, England DN149AA
 Phone:
 Fax:
 Contact person: Clair Packer

Protocol No.: K0563

Written by: Ronnda Bartel

Initial Protocol Approval:

<u>Department</u>	<u>Print Name</u>	<u>Signature</u>	<u>Date</u>
Quality Assurance	<u>Dadine Gonzales</u>	<u>[Signature]</u>	<u>11/1/95</u>
IVLT Research & Development	<u>Tracy Denny</u>	<u>[Signature]</u>	<u>12/14/95</u>
Customer			

Please read carefully! Protocol approval signifies concurrence with protocol design as written. The study will be billed per this protocol at the time the final report is completed.

Final Review of Data Gathered and Approval:

<u>Department</u>	<u>Print Name</u>	<u>Signature</u>	<u>Date</u>
Quality Assurance	<u>Dadine Gonzales</u>	<u>[Signature]</u>	<u>11/29/96</u>
IVLT Research & Development	<u>Ronnda Bartel</u>	<u>[Signature]</u>	<u>1/25/96</u>



Advanced Tissue Sciences Customer Testing

Customer: Croda
Cowick Hall
Snaith Goole
North Humberside, England DN149AA
Phone:
Fax:
Contact person: Clair Packer

Protocol No.: K0563

Written by: Ronnda Bartel

Initial Protocol Approval:

Department	Print Name	Signature	Date
Quality Assurance	<u>[Signature]</u>	<u>[Signature]</u>	<u>12/14/95</u>
IVLT Research & Development	<u>Tracy Donnelly</u>	<u>[Signature]</u>	<u>12/14/95</u>
Customer	<u>JULIE NICHOLS</u>	<u>[Signature]</u>	<u>12.12.95</u>

Please read carefully! Protocol approval signifies concurrence with protocol design as written. The study will be billed per this protocol at the time the final report is completed.

Final Review of Data Gathered and Approval:

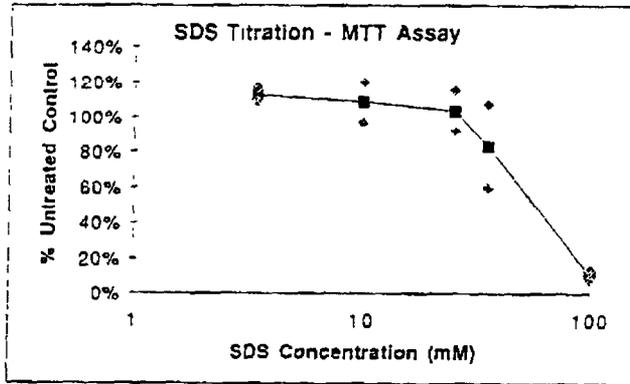
Department	Print Name	Signature	Date
Quality Assurance			
IVLT Research & Development			

ADVANCED TISSUE SCIENCES
 Skin²™
 Topical Testing Model
 Part Number ZK1300 Product Line
 Quality Control Report Form

Date Tested: 12/14/95
 ZS1300 Lot Number: 8090-3-128-09
 Skin-2 Assay Kit Lot Number: 13075

Quality control testing results

Test Method	Specification	Result
<i>Cell Line Testing</i>		
HIV-1 by PCR	No detectable levels	Negative
Heparans B by hybridizanor	< 1.0 ng/ml	Negative
Mycoplasma	None detected	Negative
Bioburden Screen	Sterile Bioburden	Negative
Replicate samples meet established specifications		PASS
MTT-50	20-65 mM	56.673 mM
Mean O.D. 540:	>= 0.6	1.2215
Average % CV:	<= 20%	4.657 %
		PASS



Note: Curve represents the average of two samples assayed from the same lot using the standard protocol from the Assay Kit Directions For Use.

Quality Control Approval: [Signature] Date: 12-14-95

10933 North Torrey Pines Road
 La Jolla, CA 92037-1005
 Fax (USA)

(619) 450-5730
 (800) 252-7273
 (619) 450-5703



December 15, 1995

Dear Valued Customer,

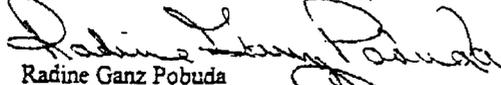
Advanced Tissue Sciences, Inc. has a dedicated Bioprocess Team that works with the company's manufacturing group. One of the responsibilities of the Bioprocess Team is to evaluate the dermal and keratinocyte cell lines used in our manufacturing process to assure continued optimal product performance. We would like to inform you that this lot of product contains the next approved dermal cell line. This cell line has completed an internal review and approval process prior to being incorporated into our manufacturing process. The product continues to function as intended for use.

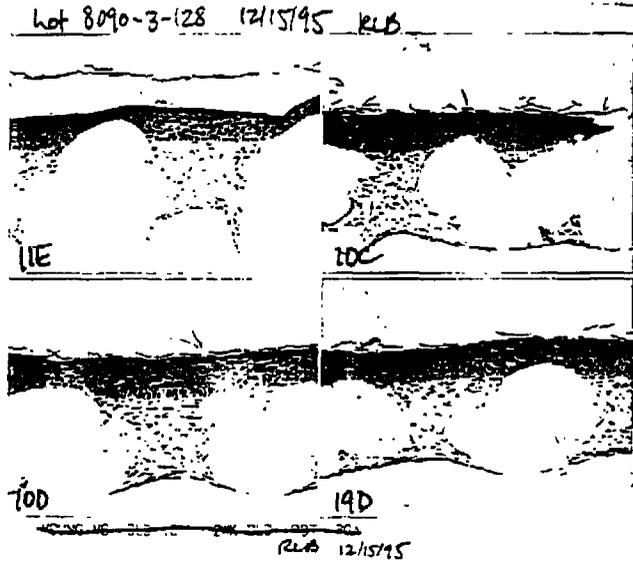
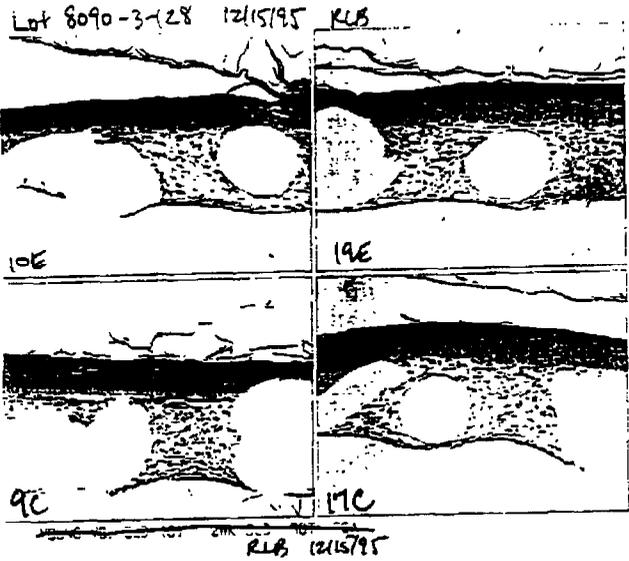
We have enclosed a copy of the histological sample taken of the lot released today with the Quality Control Release Form for your reference. All other aspects of the kit remain the same.

If you have any questions, please contact me at (619) 450-5845. We appreciate your support and comments.

Sincerely,

ADVANCED TISSUE SCIENCES, INC.


Radine Ganz Pobuda
Director of Quality Assurance



Testing Protocol for Study No. K0563 Anti-inflammatory Activity - UVB

1. DESCRIPTION OF PRODUCT

Skin² Model ZK 1301 will be used for this testing protocol.

2. BACKGROUND INFORMATION

This study is being conducted at the request of Croda Chemicals, LTD

3. OBJECTIVE OF THE STUDY

The objective of this study is to determine the potential anti-inflammatory activity of topically applied oils using MTT, PGE₂ and IL-1 α as the endpoints. MTT, a substrate for mitochondrial succinate dehydrogenase, is an indicator of cellular viability. PGE₂ release is indicative of membrane perturbation events that activate phospholipase A₂. IL-1 α is a cellular cytokine released in response to inflammatory stimuli or chemical insult.

4. TYPE OF STUDY

Customer request for testing. Date of request: November 16, 1995

5. STUDY SCHEDULE

Final report to be released 3 weeks from initiation of the protocol.

6. RESPONSIBILITIES

- 6.1 In Vitro Kit Manufacturing personnel are responsible for the production of the skin² product.
- 6.2 IVLT Applications Laboratory personnel are responsible for the protocol implementation.
- 6.3 Quality Control Department is responsible for final product release testing prior to protocol implementation.
- 6.4 Quality Assurance Department is responsible for reviewing the protocol and final report.

7. IMPACT ON PRODUCTION

There will be no impact on current product production.

8. MATERIAL UTILIZATION AND REPLICATE SAMPLING SIZE PER CUSTOMER REQUEST

- 8.1 This study will utilize (1) ZK 1301 Kit, (1) MTT Assay Kit, (1) PGE₂ Kit, (1) IL-1 α Kit.
- 8.2 This study will be conducted using 2 replicate samples per test condition.
- 8.3 Samples are supplied in septum capped vials under nitrogen and will be coded prior to testing. They must be stored in a light protective container at 2-8°C.

9. MATERIALS AND EQUIPMENT

Refer to the current directions for use.

- 9.1 ZS1301 DFU, FRD0033 (rev. 01) (see Attachment A)
- 9.2 MTT Assay Kit, ZA0022 DFU, FRD0028 (rev. 02) (see Attachment B)
- 9.3 PGE₂ Assay Kit, ZA0050 DFU, FRD0019 (rev. 92) (see Attachment C)
- 9.4 IL-1 α Assay Kit, DLA50 (R&D Systems, Minneapolis MN) (see Attachment D)
- 9.5 Dr. Honle Solar Simulator
- 9.6 UVB H2 filter (295-400nm)
- 9.7 Test materials: 1V2T # 1075, 1076, 1077, 1078, 1080

10. LIST OF RELEVANT SOPs

Not applicable

11. ANALYSIS OF DATA AND ACCEPTABILITY CRITERIA

- 11.1 All materials used will meet established quality control specifications.
- 11.2 Data analysis will be done by Advanced Tissue Sciences prior to decoding the samples. The final report will be prepared with decoded data.

12. PROTOCOL

12/14/05

Pages 82 and 83 containing
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have been redacted in their entirety

Appendix 12 – Effect on PGE₂ and TNF α

APPENDIX 12



PROTOCOL NO. K0590

UVB Anti Inflammatory Activity

ZC13111-03 skin²® Model ZK1301

**Croda Chemicals, Ltd.
Cowick Hall
Saith Goole
North Huberside, UK DN149AA**

TEST MATERIALS

CN506
CN480 ⇒ CROSSENTIAL
SA14
CN507

August 27, 1996

REPORT BY: Ronnda L. Bartel, Ph.D.
PERFORMED BY: Lynda Addington



In Vitro Laboratory Technology Contract Testing

Customer: Claire Packer
 Croda Chemicals Ltd.
 Cowick Hall
 Saith Goole
 North Humberside, DN149AA
 Phone: 011-1405-860551
 FAX 011-1405-860205

Study No.: KO590

Written by: Ronnda L. Bartel, Ph.D

Initial Protocol Approval

Department	Print Name	Signature	Date
Quality Assurance	Radine Ganz Pobuda	<i>Radine Ganz Pobuda</i>	7/10/96
IVLT Research & Development	Ronnda L. Bartel, Ph.D	<i>Ronnda Bartel</i>	7/10/96
Customer	Claire Packer	<i>Claire Packer</i>	15/7/96

Please read carefully! Protocol approval signifies concurrence with protocol design as written. The study will be billed per this protocol at the time the final report is completed.

Final Review of Data Gathered and Approval:

Department	Print Name	Signature	Date
Quality Assurance	Radine Ganz Pobuda	<i>Radine Ganz Pobuda</i>	8/26/96
IVLT Research & Development	Ronnda L. Bartel, Ph.D	<i>Ronnda Bartel</i>	8/26/96



ADVANCED TISSUE
S C I E N C E S

Final Report for Study No. K0590
UVB Anti Inflammatory Activity -- ZC13111-03

OBJECTIVE OF THE STUDY

The objective of this study is to determine the anti inflammatory potential of 3 test materials using the skin²⁸ ZK1301 tissue. Ultraviolet light (UVB) is used as the stimulus to induce an inflammatory reaction in the tissue. Cell viability is determined using the MTT dye reduction assay. MTT is a substrate for mitochondrial succinate dehydrogenase and is converted to an insoluble formazan by the activity of this enzyme. The amount of formazan produced is proportional to the number of viable cells in the tissue. The cytotoxicity of the test materials is estimated by the reduction in the cell viability of treated tissues relative to the untreated controls. PGE₂ release is indicative of membrane perturbation events that activate phospholipase A₂. Tumor Necrosis Factor alpha (TNF α) is a cellular cytokine released in response to inflammatory stimuli or chemical insults.

SUMMARY RESULTS

MTT Assay: None of the materials were cytotoxic. CN507 and CN480 may show some UVB protective effects.

PGE₂ Assay: None of the materials produced a significant increase in PGE₂ release in the non-irradiated control tissues. Two of the materials, CN507 and CN480, produced a slight decrease in the UVB induced PGE₂ release.

Tumor Necrosis Factor alpha Assay: The amount of TNF α produced by the tissues was at or near the detection limit of the assay. Longer incubation time would be required to use this cytokine as an endpoint in this type of study.

MATERIALS AND EQUIPMENT

- (1) ZK1301 skin^{2®} kit
 - (1) ZA0022 MTT Assay Kit
 - (1) ZA0050 PGE₂ Assay Kit
 - (1) DTA50 TNF α Assay Kit
 - Dr. Honle Solar Simulator
 - UVB H2 filter (295-400nm)
-
- Refer to the directions for use for standard materials and equipment requirements

ZK1301 DFU, FRD0033 (rev. 001) (see Attachment D)

ZA0022 DFU, FRD0028 (rev. 002) (see Attachment E)

ZA0050 DFU, FRD0019 (rev. 005) (see Attachment F)

DTA50 DFU (see Attachment G)

EXPERIMENTAL DESIGN

Tissue Model:	skin ^{2®} Model ZK 1301
Exposure Mode:	Topical application - 3 μ l
Concentration	Neat (oils)
Dosing Time:	24 hours
Sampling:	Triplicate per test condition
Assay Media:	Dulbecco's Modified Eagles Medium (DMEM)
Control:	Serum-Free Assay Media as Untreated Control
Test Materials:	(3) CN506, CN507, CN480
UVB Dose:	4 Joules
Incubation Time:	24 hours

SAMPLE HANDLING INSTRUCTIONS

Samples were supplied in septum capped vials under nitrogen and were stored in a light protective container at -20°C. To preserve the nitrogen atmosphere in the vials, an aliquot of the samples was withdrawn with a needle and syringe (~100 μ l) Following the testing, the test samples were archived at -20°C in a light protective box.

ASSAY PROCEDURE

The calibration and operation of all equipment was checked and documented prior to beginning the study. The assay procedure is outlined in detail in the Directions for Use (see Attachment D). In brief, the tissues were removed from the agarose shipping tray and placed in MILLICELL[®] plates

containing Serum-Free Assay Media under each MILLICELL³. Tissues were stored overnight in a 37°C, 5% CO₂, ≥90% humidity incubator prior to testing. The test materials were applied directly to the stratum corneum of the tissues. Once all tissues have been dosed the plates were placed in a 37°C, 5% CO₂, ≥90% humidity incubator for the exposure time. After 24 hours, tissues were rinsed and exposed to 4 Joules UVB. Tissues were then placed on new MILLICELLS and incubated overnight in a 37°C, 5% CO₂, ≥90% humidity incubator. The tissues were placed in a 6-well plate containing MTT and incubated for two hours. The formazan dye was extracted from the tissues with isopropanol and the optical density determined at 540 nm. Supernatants will be aliquoted into 2 tubes per tissue and frozen at -20°C for determination of PGE₂ and TNFα release. One set of tubes will be kept frozen for later IL-1α analysis, per sponsor request

DATA ANALYSIS

The percent untreated control values for each test material were calculated as follows:

$$\frac{(\text{Test Material O.D.})}{(\text{Untreated Control O.D.})} \times 100 = \text{Percent Untreated Control}$$

The MTT optical density values for the untreated control tissues were assumed to represent 100% viability

PGE₂ and TNFα release were measured with commercially available immunoassays. Concentrations of PGE₂ and TNF-α in the test samples were determined from a standard curve generated with each run. Data is expressed as total pg released per tissue.

RESULTS/CONCLUSIONS

The potential anti-inflammatory activity of 3 test materials was examined using the skin²⁶ ZK1301 tissues. Exposure of the tissues to ultraviolet irradiation was used to induce the inflammatory response. The results of this study are shown in Tables 1-3 and Figures 1 and 2 in Attachment A.

MTT Assay: None of the test materials produced any cytotoxicity in the presence or absence of UVB irradiation. Two of the materials, CN507 and CN480, showed a slight UV protective effect (i.e., higher MTT than the irradiated untreated control). The MTT values for these two treatment conditions was only slightly lower than the untreated, no UVB control

PGE₂ Assay: None of the test materials changed the amount of PGE₂ released in the non-irradiated tissues. The amount of PGE₂ released from CN507 treated was higher than the control but the difference is not significant. The UVB treatment caused an increase in PGE₂ release in all of the tissues. Two of the test materials, CN507 and CN480 produced a slight decrease in the UVB stimulated PGE₂ release.

Tumor Necrosis Factor alpha Assay: The release of TNF α for all of the tissues was at or near the detection limit of the assay. The non-irradiated tissues produced ~20 pg/tissue of TNF α . However, the irradiated tissues treated with CN507 and CN480 did not show detectable levels of TNF α suggesting that these materials may be decreasing the production of this cytokine. Longer incubation times (i.e., 48 hours) would be required to determine this effect.

ATTACHMENTS

- A - Data Tables and Graphs
- B - Quality Control Release Form
- C - K0590 Study Protocol
- D - ZK1301 Directions for Use
- E - ZA0022 MTT Assay Directions for Use
- F - ZA0050 PGE₂ Directions for Use
- G - DTA50 TNF α Directions for Use

ATTACHMENTS D TO G HAVE
NOT BEEN INCLUDED IN THIS
COPY

TEST ARTICLE DISPOSAL PROCEDURE

Unless the Sponsor requests otherwise, it is the Advanced Tissue Science practice to retain test materials for one (1) month after the completion of the study. The test materials will automatically be returned to the Sponsor after one (1) month.

DATA RETENTION

All study documents and the original final report will be on file in the Advance Tissue Science archives for a period of no less than two years, unless indicated otherwise in writing.

DISCLAIMER

The parties acknowledge and agree that the data contained in the final report may be used as a component of the client's product safety and efficacy assessment strategy. Client agrees and understands that the data contained therein may not provide sufficient evidence *by itself* of the safety, toxicity, irritation potential (or lack thereof) or efficacy of a product or raw material. Client agrees that the data contained in the final report will be evaluated along with other sources of information about the test material, including, but not limited to, physicochemical data and clinical studies. The parties agree that the final assessment of any test material's safety, efficacy and/or hazard potential is the sole responsibility of the client.

Pages 91 – 95 containing
CONFIDENTIAL COMMERCIAL INFORMATION
have been redacted in their entirety



Testing Protocol for Study No. K0590 Anti Inflammatory Assay - skin²® ZK1301

OBJECTIVE OF THE STUDY

The objective of this study is to determine the anti inflammatory potential of 2 test materials using the skin²® ZK1301 tissue. Ultraviolet light (UVB) is used as the stimulus to induce an inflammatory reaction in the tissue. Cell viability is determined using the MTT dye reduction assay. MTT is a substrate for mitochondrial succinate dehydrogenase and is converted to an insoluble formazan by the activity of this enzyme. The amount of formazan produced is proportional to the number of viable cells in the tissue. The cytotoxicity of the test materials is estimated by the reduction in the cell viability of treated tissues relative to the untreated controls. PGE₂ release is indicative of membrane perturbation events that activate phospholipase A₂. Tumor Necrosis Factor alpha (TNF α) is a cellular cytokine released in response to inflammatory stimuli or chemical insults.

MATERIALS AND EQUIPMENT

- (1) ZK1301 skin²® kit
- (1) ZA0022 MTT Assay Kit
- (1) ZA0050 PGE₂ Assay Kit
- (1) DTA50 TNF α Assay Kit
- Dr. Honle Solar Simulator
- UVB H2 filter (295-400nm)

Refer to the directions for use for standard materials and equipment requirements:

ZK1301 DFU, FRD0033 (rev. 001) (see Attachment A)
ZA0022 DFU, FRD0028 (rev. 002) (see Attachment B)
ZA0050 DFU, FRD0019 (rev. 002) (see Attachment C)
TNF α Protocol, FR0083 (rev. 001) (see Attachment D)

EXPERIMENTAL DESIGN

Tissue Model:	skin ²³ Model ZK 1301
Exposure Mode:	Topical application - 3 μ l
Concentration:	Neat (oils)
Dosing Time:	24 hours
Sampling:	Triplicate per test condition
Assay Media:	Dulbecco's Modified Eagles Medium (DMEM)
Control:	Serum-Free Assay Media as Untreated Control
Test Materials:	(2) CN467, CN463
Positive Control:	CN421
UVB Dose:	4 Joules
Incubation Time:	24 hours

SAMPLE HANDLING INSTRUCTIONS

Samples are supplied in septum capped vials under nitrogen and must be stored in a light protective container at 2-8°C. To preserve the nitrogen atmosphere in the vials, an aliquot of the samples must be withdrawn with a needle and syringe (~100 μ l). Following the testing, the test samples are to be archived at 2-8°C in a light protective box.

ASSAY PROCEDURE

The calibration and operation of all equipment is checked and documented prior to beginning the study. The assay procedure is outlined in detail in the Directions for Use (see Attachment A). In brief, the tissues are removed from the agarose shipping tray and placed in MILLICELL[®] plates containing Serum-Free Assay Media under each MILLICELL[®]. Tissues are stored overnight in a 37°C, 5% CO₂, \geq 90% humidity incubator prior to testing. The test materials are applied directly to the stratum corneum of the tissues. Once all tissues have been dosed the plates are placed in a 37°C, 5% CO₂, \geq 90% humidity incubator for the exposure time. After 24 hours, tissues are rinsed and exposed to 4 Joules UVB. Tissues are then placed on new MILLICELLS and incubated overnight in a 37°C, 5% CO₂, \geq 90% humidity incubator. The tissues are placed in a 6-well plate containing MTT and incubated for two hours. The formazan dye is extracted from the tissues with isopropanol and the optical density determined at 540 nm. Supernatants will be aliquoted into 2 tubes per tissue and frozen at -20°C for determination of PGE₂ and TNF α release. One set of tubes will be kept frozen for later IL-1 α analysis, per sponsor request.

- - - -

DATA ANALYSIS

The percent untreated control values for all of the dilutions of each test material will be calculated as follows:

$$\frac{(\text{Test Material O.D.})}{(\text{Untreated Control O.D.})} \times 100 = \text{Percent Untreated Control}$$

The MTT optical density values for the untreated control tissues are assumed to represent 100% viability.

PGE₂ and TNF α release are measured with commercially available immunoassays. Concentrations of PGE₂ and TNF- α in the test samples are determined from a standard curve generated with each run. Data is expressed as total pg released per tissue.

DISPOSITION OF MATERIALS

Retain samples per customer request and return after September 20, 1996.

ATTACHMENTS

- A: ZK1301 DFU, FRD0033 (rev. 001)
- B: ZA0022 DFU, FRD0028 (rev. 002)
- C: ZA0050 DFU, FRD0019 (rev. 002)
- D: TNF α Protocol, FR0083 (rev. 001)

STUDY SCHEDULE

Proposed Initiation Date:	July 22, 1996
Proposed Completion Date:	August 2, 1996
Proposed Report Date	August 23, 1996

**Appendix 13: Prophylactic effects on Experimental Allergic
Encephalomyelitis**

**Report on the Prophylactic Effects of DP2727
and SA14 on Experimental Allergic
Encephalomyelitis (EAE) in the Lewis rat**

for

Croda Universal Limited

by

Dr C. Bolton

Aim of the Study

The purpose of the investigation was to assess the prophylactic effects of DP2727 and SA14 on the emergence and development of neurological EAE in the Lewis rat.

Materials and Methods.

a) Induction of EAE

Male Lewis rats, weighing 200-250 g on the day of inoculation were injected, in each rear footpad, with 0.1 ml of an emulsion containing equal parts of guinea pig spinal cord, phosphate buffered saline (PBS) and incomplete Freund's adjuvant supplemented with 10mg/ml Mycobacterium tuberculosis H₃₇Ra. A minimum of 7 rats were used/treatment.

b) Assessment of EAE

Animals were weighed daily and assessed for neurological disease from day 7 post-inoculation (PI). Rats displaying symptoms of EAE were scored as follows:

0: 5; partial flaccid tail, 1; flaccid tail (FT), 2; hind limb hypotonia (HLH), 3; partial hind limb paralysis (PHLP), 4; complete hind limb paralysis (CHLP), 5: dead.

c) Dosing regime

DP2727 and SA14 diol were orally administered to individual treatments, 3 days prior to inoculation for EAE and continuing for 20 days PI, at a dose of 500 mg/kg body weight/day. Sensitised control animals received PBS vehicle or were undosed.

Results

a) Mean body weight changes

All animals in each treatment lost body weight immediately prior to the onset of EAE and beginning characteristically 9-11 days PI (Figure 1).

b) Onset and loss of symptoms

EAE was induced in all animals and the mean day of symptom onset was similar in each group (Table 1). The mean day on which the complete loss of neurological signs occurred was also comparable in dosed and untreated groups.

c) Incidence of EAE

The mean daily neurological scores for EAE-diseased rats receiving DP2727 or SA14 were not significantly different from values recorded for vehicle-treated or undosed

animals (Figure 2a and 2b). However, significantly fewer animals receiving DP2727 showed HLH compared to vehicle-dosed rats $P < 0.05$.

Conclusions

Treatment of EAE-inoculated Lewis rats with DP2727 or SA14 before and after sensitisation for disease did not alter the onset of neurological deficits or lessen the duration of symptoms. However, DP2727 did significantly reduce the number of animals experiencing HLH indicating the compound may have some ability to control the severity of disease.

Proposals for future studies

The results suggest DP2727 but not SA14 has limited activity in inhibiting the expression of neurological EAE. Further studies with DP2727 may be justified and could employ an extended pre-inoculation treatment schedule using higher doses of the compound in an effort to demonstrate conclusive effects on the development of Lewis rat EAE. Also, analogues of the parent compound could be assessed for activity in acute models of the disease.

Table 1 The effects of DP2727 and SA14 on the occurrence and development of EAE.

Treatment	No. diseased/total	Mean day of symptom onset (\pm SD)	Mean day of final symptom loss (\pm SD)	No. dead/total
Undosed	6/7	11.8 \pm 0.4	17.2 \pm 0.8	0/7
Vehicle	7/7	11.9 \pm 1.1	17.0 \pm 0.0	1/7
DP2727	6/7	12.7 \pm 1.5	17.7 \pm 0.8	0/7
SA14	7/7	11.0 \pm 0.6	18.3 \pm 2.0	0/7

Table 2 The effects of DP2727 and SA14 on the severity of EAE

Symptom	Treatment*			
	Undosed	Vehicle	DP2727	SA14
FT	6/7	7/7	6/7	7/7
HLH	6/7	7/7	3/7*	6/7
PHLP	2/7	3/7	2/7	2/7
CHLP	1/7	2/7	1/7	2/7

- * Number of animals showing symptoms/total
- * P<0.05 Fischer's exact probability test

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have been redacted in their entirety