Substantial Equivalence Application for the Approval of an Astaxanthin – Rich Carotenoid Oleoresin, Zanthin® derived from *Haematococcus pluvialis* for use in dietary supplement tablets and capsules. **Non - Confidential Version**

Application written and submitted on behalf of US Nutra LLC, USA by the natural products consulting company:

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Introduction

Zanthin® is the registered trade mark for a super critical carbon dioxide extract of the dried algae *Haematococccus pluvialis*, which is manufactured by US Nutra in Eustis, Florida, USA. *Haematococcus pluvialis* algae contains astaxanthin based carotenoids which are known to have powerful antioxidant effects and are also responsible for the pink colouration in Salmonoids. This algae has been marketed in the EU prior to 1997 in the form of capsules containing the dried algal meal of *Haematococcus pluvialis* by a Swedish company called Astacarotene.* US Nutra intends to market Zanthin®, for use in capsules by dietary supplement companies within the EU as an alternative form of extracted astaxanthin/*Haematococcus* algae. This will offer a standardized astaxanthin product that is free from algal cellular debris, has a long shelf life and offers consumers a wider choice in their selection of astaxanthin containing products. Approval of this product is sought under the EC regulation No. 258/97 which is concerned with the introduction of novel foods and ingredients into the EU and ensures that the novel food in question is assessed for its safety prior to its introduction to the general public. For plant derived foods that have a safe history of use a simplified procedure for pre market approval can be utilized providing that the food product can be demonstrated to be substantially equivalent with regard to its composition, nutritional effects, and potential toxicity as outlined in article 3.4 of the regulation 258/97. According to the procedures laid down in article 3.4, this submission will put forward the case that Zanthin®, a CO_2 extract of *Haematococcus pluvialis*, when used in capsules, is substantially equivalent to existing *Haematococcus* derived astaxanthin encapsulated containing products that are currently on sale and were on sale prior to 1997 within the EU. Since the product is derived from plant material obtained from non - GM sources, the classification under section 4, "Scientific Classification of Novel Foods for the Assessment of Wholesomeness" which facilitates the nutritional and safety of the novel food, is applicable. This product is classified as class 2 "Complex NF from non –GM sources" and is also applicable to sub heading 2.1 - "The source of the NF has a history of food

* Astacarotene AB, Sweden is now owned by Fuji Chemical Industries, Inc Japan. However in this submission Astacarotene will be used as the name of the company rather than Fuji, as it has more relevance to the historical use of the product in the EU.

Contents

1.0 Specification of astaxanthin rich oleoresin derived from *Haematococcus pluvialis*
1.1 Origin and occurrence of astaxanthin in nature.
1.2 Chemistry of astaxanthin
1.3 Description and composition of *Haematococcus pluvialis* dried algal meal.
1.3.1 Introduction
1.3.2 Dried algal meal
1.4 Description and composition of astaxanthin rich oleoresin.
1.5 Substantial equivalence of the dried algal meal and astaxanthin rich oleoresin.
1.5.1 Equivalency Experiments
1.5.2 Results
1.5.3 Conclusion
2.0 Effect of the production process applied to astaxanthin - rich carotenoid oleoresin.
2.1 Production of the dried algal meal of *Haematococcus pluvialis.*
2.2 Extraction of the dried algal meal of *Haematococcus pluvialis* to produce an astaxanthin rich oleoresin.
2.2.1 Stability of the algae and oleoresin.
2.3 Substantial equivalence of the dried algal meal compared to the dried algae used to make astaxanthin rich oleoresin.
2.3.1 Introduction
2.3.2 Production, source and strain of *Haematocccus pluvialis* algae used in Astaxin.
3.0 History of use of the organism used as a sources of astaxanthin rich oleoresin
3.1 Natural occurrence of astaxanthin in the food chain
3.2 Natural occurrence of *Haematococcus* in the food chain
3.3 History of use as a supplement for humans
4.0 Anticipated intake/extent of use of astaxanthin rich oleoresin
4.1 Use level for astaxanthin rich oleoresin.
4.2 Previous use and levels of the dried algal meal and astaxanthin rich oleoresin.
4.3 Substantial equivalence.
5.0 Information from previous human exposure to astaxanthin rich oleoresin and its source
5.1 Ingestion of astaxanthin and *Haematococcus pluvialis* via the food chain.
5.2 Clinical studies on *Haematococcus pluvialis* and astaxanthin rich oleoresin.
6.0 Nutritional information
6.1 Nutritional equivalence of astaxanthin rich oleoresin compared to existing foods.
6.2 Nutritional benefits of astaxanthin rich oleoresin.
7.0 Microbial information of astaxanthin rich oleoresin
8.0 Toxicity and Safety Studies
8.1 Toxicity studies.
8.1.1 Toxic components of *Haematococcus pluvialis*
8.2 Bioavailability studies.
8.3 Safety studies – human clinical trials.
8.4 Conclusion
9.0 Appendices

10.0 References

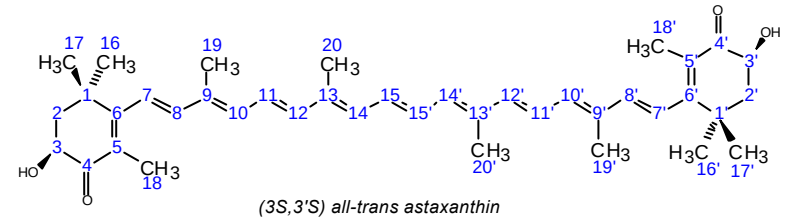
1.0 Specification of astaxanthin rich oleoresin derived from *Haematococcus pluvialis*

1.1 Origin and occurrence of astaxanthin in nature.

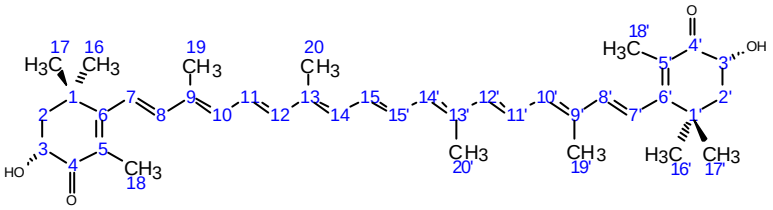
Astaxanthin (*β,3'-dihydroxy-β,β-carotene-4,4'-dione,* CAS 471-53-4) is a naturally occurring carotenoid that is found in various organisms such as salmon, trout, lobsters and shrimps (Maher 2000). Astaxanthin is responsible for the pink colouration in the flesh of these species (Turujman *et al.,* 1997) and is not synthesised de novo in salmonoids, but is entirely obtained from their diet (Foss *et al.,* 1984). Astaxanthin is also found in the yeast (*Phaffia rhodozyma*) and the micro algae *Haematococcus pluvialis* and salmonoids obtain astaxanthin principally from ingestion of krill and micro algae. (Grung 1992).

1.2 Chemistry of astaxanthin

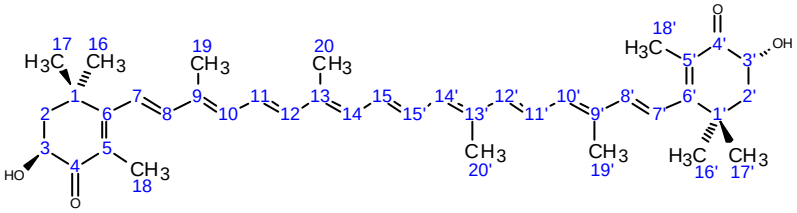
Astaxanthin is a member of the carotenoid family and exists in several stereochemical forms:



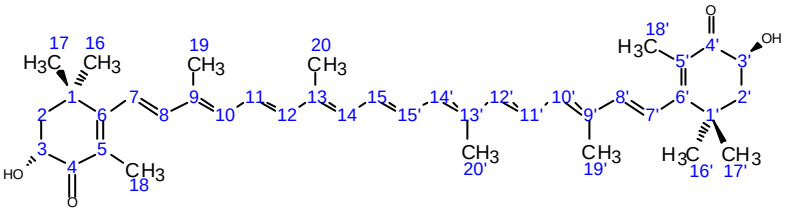
(3S,3'S) all-trans astaxanthin



(3R,3'R) all-trans astaxanthin



(3S,3'R) all-trans astaxanthin



(3R,3'S) all-trans astxanthin

These different optical forms are distributed in nature to varying degrees, as the following table indicates:

Table 1 Distribution of Astaxanthin stereoisomers in selected organisms (Bjerkeng 1997)

SPECIES	(3S,3'S)	(3R,3'R) and (3R,3'S)	(3R,3'R)
Yeast (*Phaffia sp*.)	---	<2%	>98%
Micro algae (*Haematococcus*)	100%	---	---
Synthetic Astaxanthin (Carophyll Pink La Roche)	25%	50%	25%
Atlantic Salmon	78-85%	2-6%	12-17% (Schiedt et al 1981) cited in Foss 1984

It can be seen that the algae sources of astaxanthin, biosynthesize this carotenoid with virtual optical purity in the 3S, 3'S form. In *Haematococcus pluvialis* the 3S, 3'S form also matches the predominant isomer found in salmon species (Schiedt *et al.,* 1981). Free astaxanthin is only found in nature in small quantities, with the fatty acid mono and diesters being the predominant forms together with both varying amounts of trans and cis isomeric forms of astaxanthin (Bjerkeng *et al.,* 1997, Renstrom *et al.,* 1981).

1.3 Description and composition of *Haematococcus pluvialis* dried algal meal.

1.3.1 Introduction

Haematococcus pluvialis is a unicellular green algae that is commonly found in freshwater bodies. Its taxonomic classification is as follows:

Family: Haematococcaceae
Order: Volvocales
Class: Chlorophyceae
Division: Chlorophyta

When the green cells of *Haematococcus pluvialis* experience environmental stress, such as high light intensity or phosphate deprivation, they differentiate from a vegetative stage to form aplanospores in a resting stage. At this point the cell volume increases, producing a hard cellular wall that accumulates the red coloured xanthophyll, astaxanthin and its fatty ester derivatives. (Boussiba *et al.,* 1992, Cohen *et al.,* 2002). *Haematococcus* is thought to biosynthesize astaxanthin to protect itself against UV induced cellular damage (Kobayashi *et al.* 1992). Interestingly, astaxanthin has recently been shown to have potential in reducing skin DNA damage and also to reduce time dependant sun burn in humans (Cyanotech 2002). It has also been shown to be a powerful anti oxidant (Naguib 2000). *Haematococcus* has been approved as a food supplement for use in fish farmed salmon, and the presence of this algae in the food chain, via salmon, is therefore clearly established. Consequently, since the mid 1990's, many companies have begun to market, worldwide the use of *naturally derived* astaxanthin as a dietary supplement for human use. Such companies include Mera Pharmaceuticals, Soft Gel Technologies, Inc., La Haye Laboratories and Cyanotech in the USA, Itano in Japan and Britannia Health in the UK. Since *Haematococcus pluvialis* is known to contain high amounts of astaxanthin, many of these companies are selling the dried form, or an extract of the algae to consumers who are interested in ingesting this natural product for their potential long term health benefits, rather than obtaining astaxanthin by consuming salmon meals on a daily basis.

1.3.2 Dried algal meal

To produce a consistent, stable and bio available form of *Haematococcus pluvialis* most companies, culture the algae using open ponds or bioreactors (Wiener *et al.,* 2003). Once the material is in the "red" cyst stage of maximal astaxanthin production, it is then cracked, to enable the secondary metabolites to be bio available and then dried and vacuum packed for encapsulation, tablet production or transported to manufacturers who extract the algae to produce standardized extracts of *Haematococcus* derived astaxanthin. It is this dried form of *Haematococcus pluvialis* that is formulated together with other materials such as preservatives, to produce a "dried algal meal" that companies such as Astacarotene, market as a dietary supplement.

The composition of cultured *Haematococcus pluvialis* algae has been shown to contain the following components: Carotenoids: Astaxanthin (1% of total carotenoids), the monoester (76%) and diester (7%) of astaxanthin, â,â – carotene (1%), an adonirubin ester (3%), Lutein (7%), violaxanthin (2%) and neoxanthin (1%), (Renstrom *et al.,* 1981). This carotenoid composition is in close agreement with earlier published work on *Haematococcus pluvialis* (Renstrom *et al.,* 1981 and references cited therein). Carotenoid analysis of commercially produced *Haematococcus pluvialis* also shows a similar pattern in the range and amounts of carotenoid production, indicating that despite different culturing methodologies and strains, the overall effect on the phytochemical profile of this material, is very similar. Typical ranges for the amounts of individual carotenoid pigments found from different sources of *Haematococcus pluvialis* have been shown to have the following % variation:

Astaxanthin (total) 81-99; free astaxanthin 1-5; astaxanthin monoesters 46-79; astaxanthin diesters 10- 39; *â* –carotene 0-5; lutein 1-11; canthaxanthin 0 -5.5; and other carotenoids 1-9; (Aquasearch 2000i, page 12 and references cited therein).

The ratio of E/Z isomers of astaxanthin have also been shown to have the following similar distribution despite the fact that they are from different algae sources:

Table 2. E/Z ratios of Astaxanthin isomers in various sources of *Haematococcus* algae

Source of algae	All – E Astaxanthin	9-Z Astaxanthin	13-Z Astaxanthin
Aquasearch[1]	1.30	0.10	0.20
Aquasearch[1]	1.90	0.30	0.30
Aquasearch[1]	2.10	0.40	0.40
US Nutra[2]	2.88	0.48	0.68

Figures expressed as % of Total Astaxanthin. References: 1. Aquasearch 2000i, page 89 ; 2. See appendix 2

1.4 Description and composition of astaxanthin rich oleoresin.

Astaxanthin rich carotenoid oleoresin is a supercritical carbon dioxide extract of the dried algae *Haematococcus pluvialis.* This solvent is widely used in the food industry to extract among other things, caffeine from coffee beans and flavours from hops. The extraction process uses supercritical CO_2 without the use of any other organic solvent or entrainers. This recovers the fat soluble components found in *Haematococcus pluvialis* algae to produce an oleoresin that consists of lipid soluble fatty acids, and carotenoids (appendix 8 - CONFIDENTIAL). Typically 10kg of the dried algae is

extracted to produce 3kg of the oleoresin, which contains 10.2% of total carotenoids with the other 89.8% consisting of lipids and water (appendix 1). Analysis of this extract has shown it to contain the following components:

Table 3. Fatty acid composition of CO_2 extract[1]

Fatty Acid		CO_2 Extract[1]
		% of total FA
12:0	Lauric	0.07
13:0	Tridecanoic	0.10
14:0	Myristic	0.51
15:0	Pentadecanoic	0.03
16:0	Palmitic	12.21
16:1 n-7	*cis*-9-Palmitoleic	0.32
17:0	Heptadecanoic	0.11
17:1	*cis*-10-Heptadecenoic	1.87
18:0	Stearic	0.79
18:1 n-9 *c/t*	*cis*-9-Oleic and/or *trans*-9-Elaidic	24.14
18:2 n-6 *c/t*	*cis*-9,12-Linoleic and/or *trans*-9,12-Linolelaidic	30.68
20:0	Arachidic	1.77
18:3 n-6	-Linolenic	14.83
20:1	*cis*-11-Eicosenoic	0.25
18:3 n-3	Linolenic	0.18
21:0	Heneicosanoic	1.65
20:2	*cis*-11,14-Eicosadienoic	0.48
22:0	Behenic	0.06
20:3 n-6	*cis*-8,11,14-Eicosatrienoic	1.34
22:1 n-9	*cis*-13-Erucic	0.06
20:3 n-3	*cis*-11,14,17-Eicosatrienoic	8.37
20:4 n-6	*cis*-5,8,11,14-Arachidonic	0.12
20:5 n-3	*cis*-5,8,11,14,17-Eicosapentaenoic	0.06

Table 4. Carotenoid composition of CO_2 extract[1]

Carotenoid	CO_2 Extract[1]	
	% w/w	% of total
E-Astaxanthin	5.92	59
9Z-Astaxanthin	1.48	15
13Z-Astaxanthin	2.58	26
Total Astaxanthins	9.98	99
â-Carotene	0.03	0.3
Lutein	0.07	0.7
Canthaxanthin	0.03	0.3
Total Other Carotenoids	0.13	1.3
Total Carotenoids	10.1	

1. Data derived from appendix 2

1.5 Substantial equivalence of the dried algal meal and astaxanthin rich oleoresin.

U.S. Nutra has performed a set of experiments to demonstrate the relative similarities between its Zanthin® Astaxanthin oleoresin extract and the raw algae material from which it is derived.

1.5.1 Equivalency Experiments

In order to demonstrate the relative equivalency of *Haematococcus* algae and the extract from which it is derived, U.S. Nutra compared the lipophilic constituent fatty acid and carotenoid compounds. For raw, unprocessed *Haematococcus* algae, a sample from ALGAtechnologies, Lot AST10203 was analyzed. U.S. Nutra's commercial CO_2 extract lot 031029HAOR was used for comparison. No official analytical procedure for the measurement of carotenoids in *Haematococcus* are available at present. Therefore the methods have been developed "in house" by US spectrophotometric and HPLC analytical methods and using analytical methods gleaned from the scientific literature and from emerging industry standards. Analyses were performed in duplicate at U.S. Nutra by in-house analytical chemists. The fatty acids were analysed by GC using the US Pharmacopoeia method and the analytical protocols including supporting references from the scientific literature are included in appendix 3. To compare the carotenoid content in the oleoresin and the algae, the oleoresin was taken for analysis as is, while the algae was first extracted with an acetone (algae/acetone – 1:500 w/v) in a centrifuge tube, then separated from the algae cells by centrifugation. To test for astaxanthin content the extracts were enzymaticaly hydrolyzed with cholesterol esterase, then separated via HPLC using a normal phase silica column. To test for other carotenoids, the solutions of extracts were injected into the HPLC column without hydrolysis. A C_{30} column was used for lutein and canthaxanthin analysis, and a C_{18} column - for â-carotene.

Fatty acid profile samples were first hydrolyzed and methylated with 5% sulfuric acid solution in methanol, then the resultant methyl esters of fatty acids were extracted with hexane and separated using GC WAX column. The initial hydrolysis was performed both with oleoresin and *Haematococcus* algae (without any additional extraction).

1.5.2 Results

Comparative fatty acid and carotenoid results for both the dried algae and the CO_2 extract are presented in appendix 2. Twenty-three identifiable fatty acids in the algae and the extract were quantified and reported on a relative w/w basis. As can be seen in appendix 2, all fatty acids were found in similar proportions. Total carotenoids were found at a 4.1% w/w level in the algae which increased to 10.1% w/w in the extract due to the exclusion of the remaining algal biomass (carbohydrates, proteins, ash). Relative proportions of total astaxanthins and other carotenoids remained virtually constant. However, there was a small change in the astaxanthin isomeric proportions. E- to Z- transformation has been documented for astaxanthin under solvation and heat treatment, (Chen et al., 1999), so this minor change in the isomeric ratios is expected even under the mild conditions of supercritical carbon dioxide extraction. The toxicological issues relating to this slight change in the geometric isomeric ratios are discussed in section 8.1.1.

1.5.3 Conclusion

These results demonstrate that the relative ratios of the phytochemicals found in the dried algae and the extract are substantially equivalent. However the extract, has a much higher concentration of lipids and carotenoids than the dried algae. Since the CO_2 extract will be diluted down to give a 5mg concentration of astaxanthin in the

final encapsulated product (see section 4.0), it is only of importance here to show that the relative proportions of these compounds have not changed.

2.0 Effect of the production process applied to Astaxanthin - rich carotenoid oleoresin.

The production of the astaxanthin – rich carotenoid oleoresin derived from *Haematococcus pluvialis* is carried out in a number of stages. Essentially the algae is cultured in bioreactors, harvested at maximum astaxanthin content and then dried. The dried algae is then extracted with supercritical carbon dioxide to produce the final oleoresin. These production methods are outlined in more detail in the relevant sections below.

2.1 Production of the dried algal meal of *Haematococcus pluvialis.*

The commercial production of cultured *Haematococcus pluvialis* is undertaken by several companies round the world. Cyanotech and Mera Pharmaceuticals in North America cultivate the algae using an "open pond" system, the Japanese firm Fuji Chemical Industries Ltd. Japan has an indoor facility in Sweden and dome shaped bioreactors in Hawaii, while the Israeli company, ALGAtechnologies uses solar powered photobioreactors in a closed, strictly controlled system (Weiner et al., 2003). All these companies harvest the algae at maximal astaxanthin concentration, the so called "red stage" of cultivation, before crushing (to increase bioavailability) and drying the material for production or extraction.

ALGAtechnologies supplies the dried form of *Haematococcus* algae to US Nutra for extraction at their plant in Florida, USA. The exact production of the algae by ALGAtechnologies is explained in the flow chart in appendix 4 (CONFIDENTIAL), and is also described in a US Patent (Cohen et al., 2000). The exact source and strain that ALGAtechnologies employ is described in appendix 5 (CONFIDENTIAL), but most companies use the following strains of *Haematococcus*:

***Haematococcus* Strains & Species** (From Cohen 2000)
Strains & Species Source
H. pluvialis flotow CCAG, Gottingen
H. pluvialis flotow NIES Tsukuba, Japan
H. lacustris UTEX 16 CCAT, USA
H. pluvialis flotow ETTL 1958/3 Ceska republika
H. pluvialis Flo-TAKAOOVA 1983/1 Same as above
H. Droeabicensis CCAP 43/2G CCAP, UK
H. pluvialis Flo. 1844 em. Willie K-0084 SCCAP, Denmark

The certificate of analysis and the material safety data sheets for ALGAtechnologies *Haematococcus pluvialis* algae are included in appendices 6 and 7.

2.2 Extraction of the dried algal meal of *Haematococcus pluvialis* to produce an astaxanthin rich oleoresin.

The algae is supplied to US Nutra in a dried, cracked form for optimum extraction. Typically the algae prior to extraction contains around 4 (+/-) 1 % w/w of total astaxanthin (appendix 7), i.e., around 40g of total astaxanthin is present in a 1Kg batch of dried algae. This is then extracted at US Nutra's plant in Florida using supercritical carbon dioxide. This process extracts the lipid soluble material from the algae and which after solvent removal, produces a 30% w/w yield of a dark red

oleoresin. This oleoresin contains 10%w/w total carotenoids (consisting of 83% total Astaxanthin, i.e a combination of free astaxanthin, esterified astaxanthin, cis/trans astaxanthin isomers). The full details of the production method and the extraction process are included in appendix 8 (CONFIDENTIAL). The resulting oleoresin is combined with other previous batches of extracted *Haematococcus* to produce a consistent 10% total astaxanthin content and this is defined as "astaxanthin – rich is registered as a trade mark under the name of Zanthin®.
It is this oleoresin, free from proteins, carbohydrates and minerals that will be diluted in a suitable carrier oil to produce capsules containing 5mg of total astaxanthin by dietary supplement manufacturers. The exact composition of Zanthin® is included in the following relevant appendices:

Appendix 1 - Certificate of Analysis of Zanthin®;
Appendix 2 - Fatty acid and carotenoid analysis of Zanthin®;
Appendix 3, - Analytical protocols;
Appendix 9 - Heavy metal and mineral analysis, pesticide content.

Batch to batch consistency is demonstrated with previous certificate of analyses and these are included in appendix 10. As can be seen from appendix 9, Zanthin® contains no significant amounts of pesticide residues or evidence of inorganic or heavy metal contamination.
With regard to the manufacturing facility at US Nutra, this facility currently falls under food grade regulations in the USA.

2.2.1 Stability of the algae, oleoresin and capsules.
Stability studies of Zanthin® have been conducted over a 14 month period and as can be seen from this data, no significant change occurred during that time in the level of total carotenoids (see appendix 11).The stability of the dried algae supplied by ALGAtechnologies is tightly controlled by storing and delivering the material in vacuum sealed, oxygen - free aluminum foil bags, which are stored at less than 5°C, without exposure to light and air (See appendix 6 and 7). Even though US Nutra only plan to sell Zanthin® to dietary supplement manufacturers, US Nutra has also provided evidence of the suitability and stability of their oleoresin once it has been encapsulated (appendix 11).This data indicates that the stability of capsules containing Zanthin® showed no degradation of astaxanthin over an 8 month period.
Similar data is enclosed for the beadlet form of the oleoresin (Appendices 11a -11c).

2.3 Substantial equivalence of the dried algal meal compared to the dried algae used to make astaxanthin rich oleoresin.
This substantial equivalence submission is also based on the similarity between the use of dried *Haematococcus* pluvialis algae in Astacrotene's products which have been sold in a capsular form, in the EU prior to 1997 (and up to the present day), and the dried algae that is extracted by US Nutra, to produce Zanthin®

2.3.1 Introduction
The description and background to the history of use of *Haematococcus* derived astaxanthin is detailed in section 3.0, but briefly summarizing that section, Astacrotene a Swedish company has been selling capsules containing dried

Haematococcus pluvialis algae (called "Astaxin") in the EU prior to 1997. The algae is produced in Sweden. ALGAtechnologies based in Israel are also a manufacturer of *Haematococcus* dried algae. The following section details the similarity of each company's production methods.

2.3.2 Production, source and strain of *Haematocccus pluvialis* algae used in Astaxin.

The full production details, the source and the strain of Astocarotene's dried algae meal remains confidential to the company. However, Astacarotene have had a full scale production plant for producing the algae since at least 1996 (Astacarotene 1999i). Astacarotene's US Patent also mentions that a suitable source of the alga is *Haematocccus pluvialis*, which is obtainable from NIVA, Norway. The patent also describes the production of the algae through culturing techniques and that the cells are harvested at the cysts stage of maximal astaxanthin production. These are then homogenized in order to break the cell walls and then dried to form a powder or extracted into suitable oil (Lignell et al., 1998). A later patent also confirms *Haematococcus* species as the source of the dried algae used in Astacarotene's clinical trial work (Lignell et al., 2001). A product sheet for Astaxin states that the product contains *Haematococcus pluvialis* as the source of algae (Astaxin 2000i,ii), and this is corroborated by its inclusion on the labeling of the product (Astaxin 2000iii). A specification sheet for Astacarotene's dried algae states that the material contains 3.4% astaxanthin (appendix 12), which is of the same order of astaxanthin content as that produced by ALGALtechnologies (appendices 6 and 7)

While the exact strain of *Haematococcus* used by Astacarotene is a commercial secret, it is well known that the phytochemical profile of the same species of plants including algae, are often very similar, although this may vary depending on cultivation and climatic changes among others (Evans 2001). Expert opinion has also supported that different strains of *Hamatococcus pluvialis* algae are likely to be substantially equivalent in their phytochemical content, if the manufacturing processes are similar (see appendix 17 and 17a). Further evidence to support the equivalence of Astacarotenes and Algatechnologies *Haematococcus* algae is provided in appendix 18 (CONFIDENTIAL). Therefore due to the fact that the same species are used, and that both companies prospective production methods are very similar (see appendix 19 – (CONFIDENTIAL) for summary comparison and appendix 20 – (CONFIDENTIAL) for confirmation by an expert in *Haematococcus* production methods), for both sources of algae (i.e., ALGALtechnologies summarized in section 2.1 and Astacarotene's Astaxin) and that their total astaxanthin content is the same, it can be concluded that both sources of algae are substantially equivalent.

3.0 History of use of the organism used as a source of Astaxanthin rich oleoresin

3.1 Natural occurrence of astaxanthin in the food chain

Astaxanthin is a common component in the food chain, being found in various fish and crustaceans that are consumed as foods, and in particular salmon which contains from 1-37mg/kg of astaxanthin.

Table 5. Amount of Astaxanthin present in different species (Maher 2000)

Species	Amount of Astaxanthin present per Kg
Atlantic Salmon	3-11mg
Sockeye Salmon	26-37mg
Rainbow Trout	1-13mg
Yeast (*Phaffia sp.*)	30-800mg
Algae (*Haematococcus pluvialis*)	10,000-30,000mg

3.2 Natural occurrence of *Haematococcus* in the food chain

Wild salmonoids obtain astaxanthin principally from ingestion of krill. In recent years fish farmed salmon have had their diets artificially supplemented with astaxanthin to increase the pink colouration of their flesh. The source of supplemented astaxanthin is usually obtained from synthetic sources or by using meal or extracts of the algae *H. pluvialis* (White et al., 2002). Salmon is considered a healthy and safe food product and is highly recommended by nutritionists (Davidson 1993). *Haematococcus* algae has been widely used as colour additive for fish farmed salmon globally. For example, Cyanotechs NatuRose TM, which contains *Haematococcus pluvialis* has been on sale since 1997 and its use has been given approval in the USA (AquaNIC 2000), aswell as in Japan and Canada, with European approval currently in process (Cyanotech 1999). Astaxanthin has also been approved as a permitted feed additive in salmonoids (E161j) in Europe. Under council directive 70/524 a mixture of canthaxanthin with astaxanthin is allowed providing the total concentration of the mixture does not exceed 100mg/kg in the complete food stuff (FSA 2000). This data shows that astaxanthin and *Haematococcus pluvialis* algae are already an established part of the food chain, albeit indirectly due to the consumption of salmon.

3.3 History of use as a supplement for humans

Several companies have also been marketing the use of *Haematococcus pluvialis* as a dietary supplement for use in humans. Astacarotene has been extensively marketing Astaxin (dried *Haematococcus pluvialis* capsules) in Europe since at least 1995 within the EU. This is supported by documentation at the Swedish Patent and Registration Office in Sweden and the Ministry of Food and Fisheries in Denmark and which are included in the reference section (FSA letter 2003). In the USA, companies such as Cyanotech have been selling *Haematococcus pluvialis* for human use since at least 1999 with no reported adverse reactions. For example, the following New Dietary Ingredient Notifications for astaxanthin-related products were filed in Docket No. 95S-0316 and were not objected to by FDA:

- Neptune Technologies & Bioressources – premarket notification for Krill-based ingredients filed on May 15, 2002 (RPTs 131, 132, 133 and 145);
- Micro Gaia Inc. – premarket notification for astaxanthin extract of *Haematococcus pluvialis* algae filed on March 7, 2002 (RPT 119);
- Igene Biotechnology, Inc. – premarket notification for *Phaffia rhodozyma* filed on May 4, 2000 (RPT 74);
- Aquasearch, Inc. – premarket notification for *Haematococcus pluvialis* algae filed on February 22, 2000 (RPT 65);and
- Cyanotech Corp. – premarket notification for *Haematococcus pluvialis* algae filed on March 22, 1999 and May 25, 1999 (RPTs 45 and 50).

It can be seen from the above data that *Haematococcus* and astaxanthin have a history of use in humans, in the USA and significant use in the EU, prior to 1997.

4.0 Anticipated intake/extent of use of astaxanthin rich oleoresin

Astaxanthin occurs in the food chain primarily through the consumption of salmon, lobster, trout and also dietary supplementation. In the UK, a salmon meal approximately equates to the consumption of a tin of 418g of Atlantic salmon (John West Food products 2002). Based on the data summarised in table 5 above, the average amount of astaxanthin found in Atlantic salmon is around 7mg per kg. Therefore, a 418g meal would equate to an approximate consumption of around 3mg of astaxanthin per meal. In other parts of the world where Sockeye salmon is consumed, an average meal of 418g of salmon containing an average 30mg/kg would contain much higher amounts of astaxanthin and would equate to around 12mg of astaxanthin per meal.

It can be assumed therefore that an ingestion of around 3-12mg per day of astaxanthin, offers a reasonable guideline on the amount of astaxanthin one could consume on a daily basis that would be expected to be reasonably safe.

4.1 Use level for astaxanthin rich oleoresin.

US Nutra plan to market Zanthin® for use in tablets, and in hard and soft gel capsules containing up to a maximum daily intake of 5mgs of astaxanthin. This is well within the levels of astaxanthin found in a single salmon meal. Dietary supplements containing *Haematococcus pluvialis* marketed by Cyanothech and Astacarotene also contain similar levels of astaxanthin (see section 4.2 below). These companies have been marketing these products for human consumption on a daily basis. Clinical trials performed with this intake of *Haematococcus* derived astaxanthin and consumed on a daily basis have not reported any adverse effects (see section 5.2 below).

4.2 Previous use and levels of the dried algal meal and astaxanthin rich oleoresin.

A number of companies sell astaxanthin containing at least 2 – 5mgs in a capsular form. In the USA Aquasearch Inc, market Astafactor soft gels containing 5mg of astaxanthin derived from *Haematococcus* algal meal (Aquasearch 2000i). Micro Gaia market AstraReal capsules that contain 2mg of astaxanthin based on an extract of *Haematococcus*. In the UK, Life Long Products, Devon, United Kingdom sells BioAstin in the form of capsules each of which contains 4mg of astaxanthin, in the form of *Haematococcus* algae manufactured by Cyanotech Inc, USA and Britannia Health Products Ltd, markets Astaxin in which each capsule contains 4mg of astaxanthin in the form of an *Haematococcus* algae meal (manufactured by Astacarotene, Sweden). Most of the clinical trials that have been conducted by various companies have used *Haematococcus* derived astaxanthin in levels from 2 -8mgs (see section 5.2)

4.3 Substantial equivalence.

Zanthin® will be marketed to dietary supplement companies for use in tablets and capsules containing up to 5 mgs of astaxanthin. This is in line with other companies astaxanthin levels and is also based on the clinical and toxicological data that has been published for *Haematococcus*. It has been demonstrated that US Nutra's carbon dioxide extracted oleoresin is phytochemically substantially equivalent to

Astacrotenes dried algae. US Nutra plans to dilute their concentrated 10% astaxanthin oleoresin, such that each capsule will provide no more than 5mgs of astaxanthin per capsule. These capsules, in terms of their intended intake and use are substantially equivalent to those astaxanthin products that are currently on the market.

5.0 Information from previous human exposure to astaxanthin rich oleoresin and its source

This section will detail the previous exposure of *Haematococcus* derived astaxanthin in humans.

5.1 Ingestion of astaxanthin and *Haematococcus pluvialis* via the food chain.

As detailed in section 3 and 4, astaxanthin is found in the food chain primarily through the ingestion of salmanoids and more recently through the human consumption of *Haematococcus* containing dietary supplements. This also includes a carbon dioxide extract of Haematococcus that Cyanotech have recently begun to market. This contains up to 7% Astaxanthin (Cyanotech 2003).

5.2 Clinical studies on *Haematococcus pluvialis* and astaxanthin rich oleoresin.

A number of clinical trials have been conducted on *Haematococcus pluvialis*:

* Lignell et al., (2001) conducted a double blind study on 40 young, healthy male students. Capsules containing 4mg of astaxanthin in the form of *Haematococcus* algal meal (manufactured by Astacarotene AB, Sweden), were given daily over a six month period.

* Another study by Chew, an academic based at Washington State University, USA, using 0, 2, or 8mg astaxanthin (109g astaxanthin/kg as the oleoresin concentrate from *Haematococcus pulvialis*) in a double blind placebo control study with fourteen subjects (Chew 2003).

* A twenty one patient trial investigating the effects of a carbon dioxide extract of *Haematococcous* algae on preventing sunburn, took capsules containing 4mg of astaxanthin over a two week period (Cyanotech 2000).

* A recent 35 patient double blind placebo controlled trial of 8 weeks duration, consumed an astaxanthin rich extract of *Haematococcus pluvialis*, at an intake of 6mgs per day (Spiller 2003).

No adverse effects have been reported in these clinical trials or by companies currently marketing astaxanthin as a dietary supplement in the USA or Europe. These findings demonstrate that *Haematococcus* derived astaxanthin, including CO_2 extracts of astaxanthin, have an extensive history of previous exposure in humans.

6.0 Nutritional information

6.1 Nutritional equivalence of astaxanthin rich oleoresin compared to existing foods.

The presence of astaxanthin in the diet has been discussed previously in section 3. US Nutra's oleoresin will in many cases be used to supplement the human diet with astaxanthin carotenoids which would have been provided by the ingestion of salmon meals. Concern has been raised on the depleting stock of wild salmon available to consumers and fish farmed salmon has received bad press principally due to the infestations of lice in aquaculture farms. To obtain astaxanthin from the ingestion of regular portions of astaxanthin containing fish and crustaceans is also unappealing. The use of a dietary supplement containing astaxanthin would therefore offer a real alternative to those consumers who would like to obtain the benefits of this carotenoid without consuming species such as salmon or trout on a regular basis.

6.2 Nutritional benefits of astaxanthin rich oleoresin.

Carotenoids have received a lot of attention for their potential health benefits in recent years. These compounds have been shown to be powerful anti oxidants and are implicated in the prevention of a variety of disease states such as, cancer, cardiovascular health, immune functioning and visual health (Maher 2000).
Astaxanthin has been studied for its anti oxidant potential and has recently been shown to be one of the most powerful antioxidants, *in vitro* compared to other known anti oxidants such as lutein and Vitamin E (Naguib 2000).

Astaxanthin has also shown potential in the following disease areas:
* Eye health – Macular degeneration has been shown to be linked with light induced oxidative processes within the eye. Reduced risk from increased intake of carotenoids especially lutein has been shown recently (Lyle et al 1999). Astaxanthin is thought to offer potential in this area due to its high anti oxidant properties and its ability to cross the blood brain barrier. (Tso *et al.,* 1996)

*Cardiovascular health – High levels of LDL – cholesterol are linked to the development of atherosclerosis. Anti oxidant supplementation may reduce this risk by increasing the amounts of HDL, which is inversely correlated with coronary heart disease (Guerin et al., 2003). In animal models, astaxanthin has been shown to increase the amount of HDL found in the blood (Murillo 1992). Regular supplementation with astaxanthin could therefore be beneficial for maintaining cardiovascular health.

*Astaxanthin has also been shown to reduce swelling in rat's paw, (Kurashige et al., 1990) to have potential in the maintenance of a healthy prostate (Anderson 2001) and also as an immune modulator (Chew et al, 2003). New potential applications of astaxanthin will continue to appear as researchers around the world become more aware of the health promoting effects of this carotenoid.

7.0 Microbial information of astaxanthin rich oleoresin

US Nutra has conducted studies on the microbiological load of the oleoresin (appendix 14). A combination of CO_2 extraction of the dried algae, manufacture under U.S. food regulations and the use of preservatives contribute to the inhibition of food borne microbes in this product.

8.0 Toxicity and Safety Studies

This section reviews the safety and toxicity studies conducted on *Haematococcus* derived astaxanthin. Although some of this data has been published in peer reviewed journals, many have also been derived from company sponsored research provided by astaxanthin supplement manufacturers.

8.1 Toxicity studies.

A number of toxicity studies have been undertaken on *Haematococcus pluvialis* and these are summarised below in table 6.

Table 6. Toxicity studies

SOURCE OF ASTAXANTHIN	AMOUNT OF MATERIAL TESTED	AMOUNT OF ASTAXANTHIN PRESENT	TOXICITY STUDY	REFERENCE
Haematococcus pluvialis dry algal meal contains 2% of total Astaxanthin	50mg/kg of algal meal which corresponds to 3.5g algal meal per 70-kg body weight of an adult man	1mg of total Astaxanthin, or equivalent to a dose of 70mg of total Astaxanthin to an adult man	28 day rat study. Post mortem observation failed to detect any sign of toxicity.	Astafactor Technical Report 1
Haematococcus pluvialis dry algal meal contains 2% of total Astaxanthin	10,400 – 18,000 mg/kg of the algal meal which equates to single doses of 720g to 1.2kg in humans		Single dose acute toxicity study with male and female mice. No abnormalities were observed in post mortem examination. All mice served the trial period.	Koyo Mercantile Company Limited refile, cited in Astafactor Technical Report 1
Haematococcus pluvialis dry algal meal contains 2% of total Astaxanthin	5g/kg Algal meal		13 day LD_{50} acute toxicity study in 3 Co operative groups in rats, were fed 5g/kg of algal meal. No differences in body weight or abnormalities in post mortem examination suggesting an LD_{50} greater than 5000mg/kg	Astafactor Technical Report 1
NatuRose™ *H. pluvialis* spray dried dark red powder contains 1.5% total Astaxanthin content (70% monoesters, 10% diesters, 5% free Astaxanthin)	5g of algae/kg of rat, corresponds to 350g single dose of algal meal to an adult man of 70kg	Total Astaxanthin equates to 75mg/kg or equivalent to 5.25g dose to an adult man. Implies an LD_{50} in man is higher than 5.25g single dose of Total Astaxanthin	13 day Acute oral toxicity study. No visible or post mortem abnormalities were observed.	Cyanotech Technical Report 1.
Haematococcus colour (food additive in Japan)	0 – 0.25% *Haematococcus* colour.		80 F344 rats were split into 4 groups of 20 and were fed a powder containing 0%, 0.025%, 0.075%, and 0.25% *Haematococcus* colour, for 13 weeks none of the animals died and there were no exposure related changes in body weight gain or food consumption slight increase in the levels of cholesterol were observed but the differences were slight and not defined as an adverse effect.	Ono et al. (1999).

It can be seen from the above data that no toxic effects were reported for *Haematococcus* from the dosage levels studied. When this data is extrapolated to an

equivalent dosage form in humans, it strongly suggests that up to 5g of astaxanthin (present in the form of *Haematococcus* algae), could be potentially safely ingested by humans. These studies further suggest that an intake of 5mg of *Haematococcus* derived astaxanthin is unlikely to produce any toxic effects in humans.

8.1.1 Toxic components of *Haematococcus pluvialis*

A thorough literature search has not identified any toxic components present in *Haematococcus pluvialis*, although the algae does contain small amounts of canthaxanthin. This compound has been shown in some individuals to form crystalline deposits in the retina, when taking around 90mg of canthaxanthin per day for tanning purposes. However these deposits have been shown to be reversible (Cited in Cyanotech 2000, page 33). The amounts of this material in a capsule containing US Nutra's astaxanthin rich oleoresin are well below this concentration, as there is approximately $1/100^{th}$ the amount of canthaxanthin compared to total astaxanthin in each capsule equating to around 0.005mg of canthaxanthin, per capsule per day. In conclusion there does not appear to be a toxicity issue with regard to the amounts of canthaxanthin present in this product. The slight changes in the ratio of trans/cis isomers of astaxanthin in US Nutra's product compared to the dried algae (appendix 2) also do not appear to have any toxicological significance (appendix 16).

8.2 Bioavailability studies.

A small number of bio availability studies have been conducted on astaxanthin and *Haematococcus* and these are summarised in table 7, below:

Table 7. BIOVALIABILITY STUDIES – SAFETY STUDIES

SOURCE OF ASTAXANTHIN	AMOUNT OF MATERIAL TESTED	AMOUNT OF ASTAXANTHIN PRESENT	TOXICOLOGY STUDY	REFERENCE
Not stated		100mg	Transport in the plasma by lipoproteins in a similar way to other carotenoids max levels (1.3+/- 0.1mg/L) were reached after 6.7 hours after administration elimination half life was 21 +/- 11hr.	Osterlie et al. (1999a) cited in Guerin 2002.
Carophyll Pink (Hoffman – La Roche) containing all E-, 92 and 132 – Astaxanthin (3R, 3'R: 3R, 3'S:35, 3'S ratio 1:2:1)	100mg	100mg	3 human volunteers were given a single meal containing 100mg of Astaxanthin max plasma concentrations of Astaxanthin (1.24 mg/ml) were observed after 6 hours.	Osterlie (1999b).
Algal meal from *Haematococcus* (Astacarotene)	100mg Astaxanthin per kg feed in the form of an algal meal	100mg	24 rats were divided into 2 groups. 12 were given feed without algal meal, the other group received the feed containing algal meal. Astaxanthin was found in particular in thigh and heart muscle, with no adverse effects reported but endurance tests were increased in those rats taking the algal meal.	Lignell (2001) US Patent 6, 245, 818

These studies suggest that astaxanthin is rapidly metabolised over a 24 hour period and that a daily intake of up to 5mg astaxanthin in a capsular form would not result in any residual build up of this material in the body.

8.3 Safety studies – human clinical trials.

The following clinical trials have been conducted mainly on the *Haematococcus pluvialis* meal, although two studies have been reported specifically on the carbon dioxide extract of *Haematococcus* algae. In particular, the study conducted by Chew at Washington State University, specifically used US Nutra's carbon dioxide extracted oleoresin.

Table 8. SAFETY STUDIES – HUMAN CLINICAL TRIALS

SOURCE OF ASTAXANTHIN	AMOUNT OF MATERIAL TESTED	AMOUNT OF ASTAXANTHIN PRESENT	STUDY WOULD BE EQUIVALENT TO CO_2 EXTRACT	REFERENCE
Mera Pharmaceuticals *H. pluvialis* algal meal (contains up to 2% total Astaxanthin) (around 5mg per 250mg of algal meal)	228mg of algal meal	3.85mg	33 human volunteer's daily ingestion for 29 days. Medical examination (urine & blood analysis) did not result in any safety concerns.	Astafactor Technical Report 1.
Mera Pharmaceuticals *H. pluvialis* algal meal (contains up to 2% total Astaxanthin) (around 5mg per 250mg of algal meal)	1.14g of algal meal	19.25mg of Astaxanthin	33 human volunteer's daily ingestion for 29 days. Medical examination (urine & blood analysis) did not result in any safety concerns.	Astafactor Technical Report 1.
		14.4mg	13 healthy patients were divided into 3 groups and given 3 levels of Astaxanthin daily for 2 weeks. Maximum dose being 14.4mg/day. No ill effects were reported.	Miki (1998) cited in Astafactor Technical Report 1.
Astacarotene *Haematococcus* algal meal	100mg Astaxanthin per kg feed in the form of an algal meal	4mg	20 healthy volunteers were given a capsule containing 4mg of Astaxanthin, against 20 healthy volunteers receiving a placebo. No adverse effects were reported from the study but improvements in endurance test were increased in the Astaxanthin group.	Lignell (2001) US Patent 6, 245, 818
CO_2 extract of *Haematococcus* Algae	109g Astaxanthin/kg of oleoresin concentrate	2 or 8mgs	An 8 week double blind placebo control trial to investigate the immune boosting effects of Astaxanthin was carried out, with subjects taking 0, 2 or 8mg Astaxanthin capsules, once a day. No adverse effects were reported from the study.	Chew et al 2003
CO_2 extract of *Haematococcus* Algae		2mgs of Astaxanthin in each capsule	35 adults, randomized, double blind, placebo controlled trial of 8 weeks duration. Subjects ingested 3, 2mg capsule per day. No adverse effects were reported.	Spiller et al., 2003

As can be seen from the data presented, none of the trials conducted reported any adverse effects resulting from the ingestion of *Haematococcus* derived astaxanthin. In particular, Chews study of US Nutra's oleoresin found no adverse effects and Chew has stated that physical examination of the subjects who participated in the trial of US Nutra's oleoresin (Chew *et al.,*2003) confirmed that laboratory immune function tests showed no deleterious effects with astaxanthin supplementation in human subjects (appendix 15).

8.4 Conclusion

From the above safety, bio availability and toxicology studies and considering the history of use data presented in section 3, it can be concluded that no adverse effects would be expected for consumers who regularly ingest up to 5mgs per day of astaxanthin derived from US Nutra's *Haematococcus* astaxanthin oleoresin, in the short or long term.

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9.0 Appendices

Appendix_1. Certificate of analysis for Zanthin® extract complex.

Appendix_2. Analytical comparison of *Hamatococcus* algae raw material and extract.

Appendix_3. Official analytical protocol for carotenoid and fatty acid analysis.

Appendix_4. CONFIDENTIAL

Appendix_5. CONFIDENTIAL

Appendix_6i -6vi. Certificate of analysis of *Haematococcus* from Algatechnologies.

Appendix_7. Material Safety Data Sheet for Algatechnolgies *Haematococcus* algae.

Appendix_8. CONFIDENTIAL

Appendix_9. Analytical data on US Nutras' product – pesticide, mineral content.

Appendix_10i -10iii. Certificate of analysis – examples from different batches of US Nutra's CO_2 *Haematococcus* extract (Zanthin®).

Appendix_11. Stability data of Zanthin 5mg astaxanthin complex vegetarian soft gel and stability data on US Nutra's Astaxanthin-rich carotenoid oleoresin extracted from *Haematococcus pluvialis* (Zanthin®).

Appendix_11a. Stability data on the beadlet form of US Nutra's astaxanthin-rich carotenoid oleoresin (Zanthin®).

Appendix_11b. Product specification sheet on Zanthin® 2.5% astaxanthin complex beadlet, including ingredient list for the beadlet form.

Appendix_11c. Certificate of product compliance for Zanthin® beadlet form stating that the product contains non bovine gelatine type A and that the product is GMO – free.

Appendix_12. Astacarotene's specification sheet for dried *Haematococcus* algae.

Appendix_13. Supporting evidence for the sale of *Haematococcus pluvialis* prior to 1997 in the EU.

Appendix_14. Microbiological load for US Nutra's astaxanthin-rich carotenoid oleoresin.

Appendix_15. Statement from Dr Boon Chew on the safety of US Nutra's Astaxanthin-rich carotenoid oleoresin from a recent clinical trial with Zanthin®.

Appendix_16. Explanation and discussion on the changes for cis –trans in *Haematococcus* dried algae and US Nutra's Astaxanthin-rich carotenoid oleoresin(Zanthin®).

Appendix_17. Expert opinion on the phytochemical similarity of *Haematococcus pluvialis* algae obtained from different sources.

Appendix_17a. Background and biography of the expert used in appendix 17.

Appendix_18. CONFIDENTIAL

Appendix_19. CONFIDENTIAL

Appendix_20 CONFIDENTIAL

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