



NEW DIETARY INGREDIENT NOTIFICATION FOR  
SAFFLORIN™

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# NEW DIETARY INGREDIENT NOTIFICATION FOR SAFFLORIN™

## Table of Contents

1.	Justification.....	3
2.	Name and Address of the Manufacturer .....	3
3.	Name of the New Dietary Ingredient .....	4
4.	Applications .....	4
5.	Description of the New Dietary Ingredient.....	4
5.1.	Chemistry .....	4
5.2.	Specifications .....	5
5.3.	Process description .....	5
5.3.1.	General .....	5
5.3.2.	Process principle.....	5
5.3.3.	Process outline .....	6
5.3.4.	Control of manufacture .....	6
5.3.5.	Control of the end-product Safflorin™ .....	7
6.	Occurrence in the Diet .....	7
6.1.	CLA in the diet .....	7
6.2.	Level of Consumption.....	7
7.	Safety determination of Safflorin™ .....	8
7.1.	Safety studies from literature .....	8
7.1.1.	Studies with animals .....	8
7.1.1.1.	Effects of CLA on blood glucose and insulin levels .....	8
7.1.1.2.	Effects of CLA on liver .....	9
7.1.1.3.	Additional toxicological data .....	9
7.1.2.	Studies with humans.....	12
7.1.2.1.	Effects of CLA on blood glucose and insulin .....	12
7.1.2.2.	Effects of CLA on liver .....	13
7.1.2.3.	Additional toxicological data on CLA .....	13
7.2.	Safety studies with Clarinol™ .....	14
7.2.1.	Genotoxicity.....	14
7.2.1.1.	Ames test .....	14
7.2.1.2.	In vitro chromosome aberration test .....	14
7.2.2.	Animal studies.....	15
7.2.2.1.	13 weeks sub-chronic oral rat study.....	15
7.2.2.2.	Effect of Cla on insulin and glucose in leptin deficient mice ....	17
7.2.3.	Human clinical trials.....	18
7.3.	Discussion and Conclusions .....	19
	References .....	21

## **1. Justification**

Pursuant to section 413(a)(2) of the Federal Food, Drug, and Cosmetic Act ("Act"), and FDA's implementing regulations at 21 C.F.R. § 190.6, Loders Croklaan B.V. ("Loders Croklaan") submits this New Dietary Ingredient Notification for Safflorin™ .

Based on the information described herein, including the history of use and other evidence of safety of octadecadienoic acid isomers and citations to published articles, Loders Croklaan concludes that dietary supplements containing Safflorin™, when used under the conditions recommended or suggested by Loders Croklaan, will reasonably be expected to be safe.

## **2. Name and Address of the Manufacturer**

Manufacturer:

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### 3. Name of the New Dietary Ingredient

The name of the new dietary ingredient is Safflorin™.

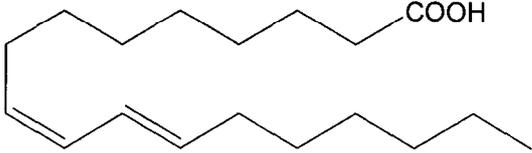
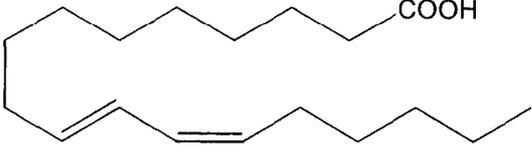
### 4. Applications

Safflorin™ is suitable as ingredient in dietary supplements in various forms, including softgels, capsules, and supplement bars and similar products. Loders Crokiaan recommends that dietary supplement manufacturers formulate products to provide a daily dosage of 2.0 g Safflorin™. The ingredient is intended for use by persons who wish to maintain their natural resistance in periods when increased susceptibility is at hand.

### 5. Description of the New Dietary Ingredient

Chemically seen Safflorin™ is a mixture of the *c9,t11*- and *t10,c12*- isomers of octadecadienoic acid in a 40:60 ratio. Depending on the application, Safflorin™ can be supplied as free fatty acids or esterified in glyceride form. The latter form is tasteless and therefore suitable for use in dietary supplement bars and similar products.

#### 5.1. Chemistry

octadecadienoic acid isomer:	CAS Number	Chemical Structure
<i>c9,t11</i> -octadecadienoic acid	872-23-1	
<i>t10,c12</i> -octadecadienoic acid	2420-44-2	

The main components of Safflorin™ are both present in the human diet. *c9,t11*-octadecadienoic acid, better known as conjugated linoleic acid (CLA), is found in dairy products and meat in considerable amounts. The *t10,c12*-isomer is found in vegetable oils merely, and together with an approximately equal amounts of the *c9,t11*-isomer.

In fact FDA has already concluded that the synthetically produced CLA's Safflorin™ contains are components of certain foods, such as the CLA's in processed vegetable oils. (FDA letter to I.S. Bass, Mar. 12, 2003.)

## 5.2. Specifications

Safflorin™ is a clear, colorless to pale yellow liquid at ambient temperature, free from foreign odors or off flavors. Both the free fatty acids and the glycerides<sup>1</sup> products do comply with the following specifications

### Specifications:

CLA total (all isomers)	≥ 78 %
CLA (c9,t11 + t10,c12 isomer)	≥ 74 %
CLA t10,c12 isomer	≥ 40 %
Monounsaturated fatty acids	≤ 15 %
Saturated Fatty Acids	≤ 8 %
Water	≤ 0.1 %
Peroxide Value	≤ 1 meq/kg
Iron (Fe)	≤ 0.5 ppm
Nickel (Ni)	≤ 0.1 ppm
Lead (Pb)	≤ 0.1 ppm
Copper (Cu)	≤ 0.05 ppm
Color yellow (Lovibond, cell 5¼")	≤ 10
Color red (Lovibond, cell 5¼")	≤ 2
Additives:	
Tocopherols	≥ 250 ppm
Microbiology:	
Total viable count	≤ 1000 /g
Yeast	≤ 10 /g
Moulds	≤ 10 /g
Enterobacteriaceae	≤ 10 /g
Salmonellae	absent in 25 g
Escherichia coli	absent in 1 g

## 5.3. Process description

### 5.3.1. General

Safflorin™ is the 40:60 mixture of c9,t11 and t10,c12 CLA. It is produced by partial hydrolysis, and molecular distillation of a 50:50 mixture of c9,t11 and t10,c12 CLA glycerides produced of Safflower oil. The raw material applied has been notified to the FDA on 4/23/03 (NDI notification Conjugated Linoleic Acid (CLA); filed as docket RPT 189).

### 5.3.2. Process principle

The raw material is partially hydrolyzed using a specific food grade enzyme. The partially hydrolyzed mixture is then purified by short-path molecular distillation. The distillate is Safflorin™ (glycerides).

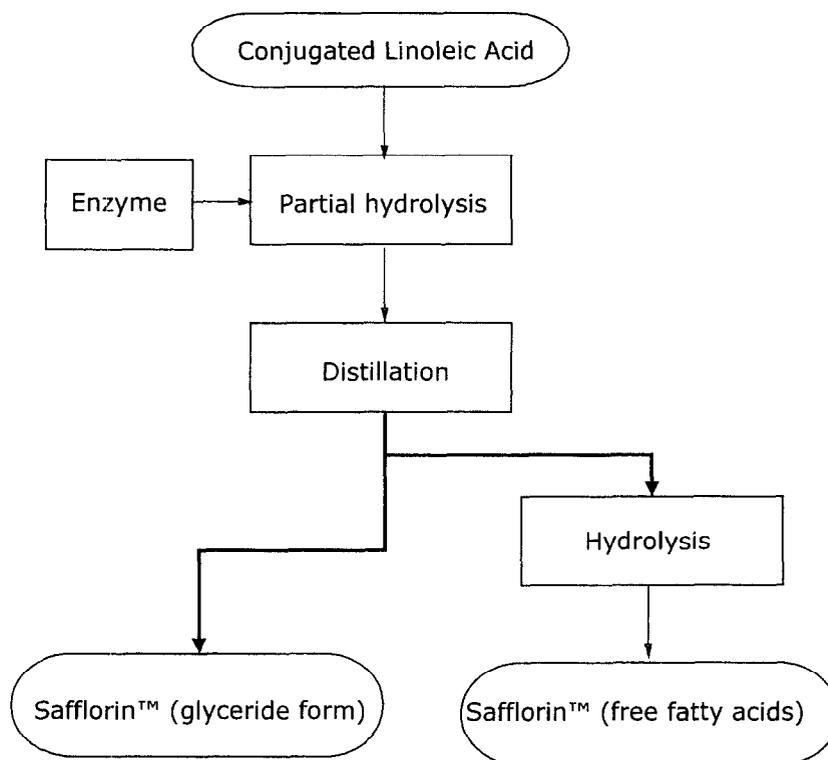
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<sup>1</sup> Whilst the glyceride product is the result of esterification of the fatty acid product with glycerol the fatty acid contents stated for the fatty acid product does also reflect the fatty acids distribution in the glycerides product.

To produce Safflorin™ fatty acids, the Safflorin™ glycerides are fully hydrolyzed using specific food grade enzymes.

This process is consistent with generally applied food industry practices.

### 5.3.3. Process outline



### 5.3.4. Control of manufacture

Good Manufacturing Practice (GMP), which includes HACCP risk analysis as well as a hygiene and transport standard, is applied throughout the manufacturing process. As a consequence, control takes place within continuous monitoring. Sampling schemes and analysis schedules are applied to raw materials and end-products as well as products in all stages of processing.

Highly modern production facilities and electronic monitoring systems allow precise process control at all stages. Contact with air is excluded as the whole production of Safflorin™ as well as tapping in drums is carried out under nitrogen.

For the production of Safflorin™ a computer assisted sampling routine is used. All samples are logged in the system under a specific sample code for each process step. The necessary analyses, including the specifications the sample has to comply with, are defined within this code. It becomes immediately obvious whether the results are inside specifications or not. So, if need be, an instant intervention in the process is possible.

### 5.3.5. Control of the end-product Safflorin™

The finished product is routinely tested to assure it meets specifications. These are set to ensure a high degree of purity consistent with its use as dietary supplement.

## 6. Occurrence in the Diet

### 6.1. CLA in the diet

Many different octadecadienoic acid isomers are present in fats and oils which are consumed in the normal diet.

Sehat *et al* (1999) analyzed cheese, cow milk and beef for the presence of octadecadienoic acid isomers. They detected a range of isomers including *t7,c9-*, *t8,c10-*, *t9,c11-*, *c9,t11-*, *t10,c12-*, *c11,t13-*, *t11,c13*-octadecadienoic acid in these three products. The predominant isomer was *c9,t11*-octadecadienoic acid. Vegetable oils were found to contain equal amounts of *t10,c12-* and *c9,t11*-octadecadienoic acid (Chin *et al.* 1992). Further, used frying fats were proven to contain a mixture of octadecadienoic acid isomers octadecadienoic acid classified as *trans,trans-* and *cis,trans + trans,cis*-isomers (Sebedio *et al.*, 1988). Finally, partially hydrogenated vegetable oils have been demonstrated to contain octadecadienoic acid isomers as well (Dutton 1979, Banni *et al.* 1994). A more detailed investigation on octadecadienoic acid isomer formation during partial hydrogenation of soybean oil was done by Jung *et al.* (2002). They demonstrated the formation of many octadecadienoic acid isomers with *c9,t11-* and *t10,c12-* octadecadienoic acid among the main isomers during hardening.

In conclusion several different octadecadienoic acid isomers, including *c9,t11-* and *t10,c12-* octadecadienoic acid, are present in the daily diet through various food sources. FDA has concluded that synthetically produced octadecadienoic acid is a component of certain foods, such as the octadecadienoic acid in processed vegetable oils. (FDA letter to I.S. Bass, Mar. 12, 2003.)

### 6.2. Level of Consumption

For the general US population, Ritzenthaler *et al.* (2001) estimated the total dietary octadecadienoic acid intake to be 212 and 151 mg/day for men and women, respectively. This study, with 51 men and 51 women between ages of 18 and 60, compared 3-day food duplicates with dietary records and food-frequency questionnaires.

Earlier surveys, performed in US sub-populations, estimated the intake to be 137 and 52 mg/d in college-aged males and females, respectively (Ritzenthaler *et al.* 1998), or 291 and 15 mg/day in lactating women with high and low dairy diet (Park *et al.* 1999). Herbel *et al.* (1998) measured 127mg/day as average daily octadecadienoic acid intake in a study with healthy young men and women.

In conclusion the daily dietary octadecadienoic acid intake can be estimated at 210 and 150 mg/day for men and women in the general population with subgroups up to 300 mg/day.

## **7. Safety determination of Safflorin™**

The safety determination of Safflorin™ is based on numerous published animal and human studies performed with CLA (table 1 and 2).

CLA is applied as a generic term to mixtures of isomers of conjugated linoleic acid. However, the isomer composition of the CLA used varies among the different studies. Where possible, the predominate isomers in the CLA mixtures used in the studies are specified in table 1 and 2. As discussed below, the results of these studies show that Safflorin™ is safe for use as a dietary supplement.

### **7.1. Safety studies from literature**

#### **7.1.1. Studies with animals**

##### **7.1.1.1. Effects of CLA on blood glucose and insulin levels**

In mice, a significant increase in insulin levels was observed in male AKR/J mice fed 1% CLA in the diet for a period of 39 days. However, there was no effect on plasma glucose (DeLany *et al.*, 1999). In a similar study, male AKR/J mice were fed 1% CLA for a period of 5 weeks. In this study plasma insulin was increased, but not significantly compared to controls. There was no effect on plasma glucose (West *et al.*, 2000). In female C57BL/6J mice fed 1% CLA in the diet an oral glucose tolerance test 17 weeks after dosing found no effect on glucose tolerance. However, insulin resistance measured after 9 weeks of CLA supplementation was increased (Tsuboyama-Kasaoka *et al.*, 2000). Both the *cis9,trans11* and *trans10,cis12* CLA isomers have been tested individually in female C57BL/6J mice. The isomers were administered for 4 weeks at a level of 0.4% in the diet. Only in mice receiving the *trans10,cis12* isomer, non-fasting insulin levels were increased but plasma glucose remained within normal range (Clément *et al.*, 2002).

The results of these four studies with mice are consistent: dietary CLA treatment, in particular the *trans10,cis12* isomer, significantly elevated insulin levels, but no effects were found on plasma glucose levels.

The effects of CLA on insulin and glucose levels in studies with rats are less consistent. In the leptin deficient rat model, Zucker ZDF *fa/fa*, 1.5% CLA in the diet for a period of 14 days was found to decrease plasma insulin and improve glucose tolerance in male fatty rats compared to fatty rats receiving control diet (Houseknecht *et al.*, 1998). However, these results may be due in part to reduced feed intake. In a similar study, 1.5% CLA and 1.5% of the *cis9,trans11* isomer were fed to male Zucker ZDF *fa/fa* rats for a period of 15 days. This study included a pair-fed group for the 1.5% CLA group. Fatty rats fed CLA and pair-fed rats maintained normal glycemia during the study period whereas rats fed control and *cis9,trans11* isomer were hyperglycemic. The improved glucose tolerance by CLA cannot be explained completely by reduced food intake, as pair feeding was not as efficacious as the CLA diet. The CLA diet and pair feeding were equally as efficient in lowering plasma insulin levels (Ryder *et al.*, 2001). In another study with Zucker rats, 0.5% CLA in the diet for a period of 5 weeks caused a numerical reduction in insulin levels in male obese rats but had no effect on females or on the lean genotype (Sisk *et al.*, 2001).

In a rat model of non-insulin dependant diabetes mellitus (NIDDM), 1% CLA in the diet for a period of 4 weeks decreased serum leptin, but had no effect on serum insulin levels (Rahman *et al.*, 2001). In Sprague Dawley rats fed 1%, 3%

or 5% CLA in the diet for a period of 5 weeks, a slight increase in serum glucose was observed, but only at the level of 5% CLA (Stangl, 2000).

In hamsters, fed either the *cis9,trans11* isomer (1.6%) or CLA mix (3.2%) for a period of 6 weeks plasma glucose was significantly higher in the CLA mix group only. In addition, insulin resistance showed an increased trend in the CLA mix group only (Bouthegourd *et al.*, 2002). In a study in pigs, a 6-week CLA supplemental diet increased plasma concentration of insulin. Glucose remained unaffected (Stangl *et al.*, 1999). However, 2.0% CLA in the diet for a period of 47-49 days had no effect on either serum glucose or insulin levels in crossbred pigs (Ramsay *et al.*, 2001).

In summary, the results of studies on the effects of CLA on the glucose and insulin levels are highly variable and may depend on animal species, genotype and even sex. Insulin levels in mice and hamsters seem to be most sensitive to CLA.

#### **7.1.1.2. Effects of CLA on liver**

A number of animal studies have examined the effects of CLA on different organs, in particular the liver. Male AKR/J mice fed CLA (0.25-1.0%) for 12 weeks tended to have enlarged spleens and livers due to fat accumulation (DeLany *et al.*, 1999). An enlargement of the liver was found in female C57B1/6J mice, fed a low level of purified CLA *trans10,cis12* isomer (0.4%) for 4 weeks, but not in mice fed with 0.4% of the purified CLA *cis9,trans11* isomer (Clément *et al.*, 2002). A 5 month study with female C57B1/6J mice fed 1% CLA also showed an increase in liver weight, in combination with signs of fat deposition and vacuolization. In this study, the spleen was also enlarged (Tsuboyama-Kasaoka *et al.*, 2000).

On the contrary, male obese Zucker rats fed 0.5% CLA for 35 days showed reduced liver weight (Sisk *et al.*, 2001). However, the effects of CLA on liver of rats are less consistent. The same investigators found no effect on liver weight in female obese Zucker rats fed 0.5% CLA for 8 weeks. In addition, a toxicological evaluation of CLA published by Scimeca (1998) reported no effects on liver in male Fischer 344 rats fed 1.5% CLA for 36 weeks. Similarly, in a study with male and female grower pigs, dietary CLA (0.25-2.0%) had no effect on liver weight (Ramsay *et al.*, 2001).

In summary, similar to effects on insulin levels, mice consistently seem to be most sensitive to effects of CLA on liver. The study by Clément *et al.*, (2002) suggests that the effect may be due to the CLA *trans10,cis12* isomer specifically, as no enlargement was seen in mice fed 0.4% of the purified CLA *cis9,trans11* isomer. Studies that report an increase in livers of other animal species due to dietary CLA are scarce.

#### **7.1.1.3. Additional toxicological data**

Some general toxicological effects of CLA, such as increased body triglyceride content and increased body protein, were found in hamsters (Bouthegourd *et al.*, 2002) and mice (DeLany *et al.*, 1999). In rats, treatment with CLA caused an elevation in serum albumin and creatinin, but only at the highest dose level of 5% CLA (Stangl, 2000). The effects of CLA on cholesterol levels are contradictory. In pigs, 1% CLA in the diet caused an increase in cholesterol and in LDL:HDL cholesterol ratio (Stangl *et al.*, 1999). However, in male Sprague Dawley rats, a diet of CLA caused a reduction of serum LDL (3%) and HDL (1%) cholesterol (Stangl, 2000).

A toxicological evaluation of CLA was published by Scimeca (1998). Male Fischer 344 rats received a diet with 1.5% CLA for a period of 36 weeks. Intake of CLA ranged from  $1970 \pm 11\text{mg/kg bw/day}$  at week 1 to  $467 \pm 52\text{mg/kg bw/day}$  at week 36. There was no effect on body weight or food disappearance in rats receiving CLA. Similarly there was no treatment-related effect on haematological parameters. Absolute and relative thymus weight was significantly reduced compared to controls, and adrenal weight increased compared with controls. Histopathological evaluation of these and other organs selected from 10 animals in each group did not indicate any changes related to administration of CLA. The author suggested that the changes in weight of thymus and adrenal tissue might be due to the gross trimming of adipose tissue. The study concluded that the administration of CLA at 1.5% in the diet for a period of 9 months did not produce any evident toxicity in male F344 rats.

Reference	Subjects	9cis,11trans-isomer (I) or 10trans,12cis-isomer (II): dose; length	Effect on glucose (GI) and insulin (In) levels	Effect on liver (enzymes)	Other effects
Bouthevoud <i>et al.</i> (2002)	male Syrian hamsters	I: 1.6% in energy; 6-8 weeks I+II: 3.2% in energy, 6-8 weeks	I: GI: no effect In: no effect I+II: GI: increased In: non-significant increase	I and I+II: CPT-I <sup>1</sup> activity increased	I: body triglyceride content increased
Clément <i>et al.</i> (2002)	female C57Bl/6J mice	I+II: 0.4% by weight; 4 weeks II: 0.4% by weight; 4 weeks	I: GI: no effect In: no effect II: GI: no effect In: increased	I: no effect II: liver enlarged, fat deposit	II: leptin increased
DeLany <i>et al.</i> (1999)	Male AKR/J mice	undefined octadecadienoic acid, 0.0, 0.25, 0.50, 0.75, 1.0% by weight, 39 days	GI: no effect In: increased	liver enlarged	spleen enlarged, body protein increased
Houseknecht <i>et al.</i> (1998)	male ZDF rats, fatty and lean littermates	undefined octadecadienoic acid, 1.5% by weight, 2 weeks	GI: normalized In: reduced		
Rahman <i>et al.</i> (2000)	Male OLETF rats	I+II, as free fatty acid or triglyceride: 1.0% by weight, 4 weeks	GI: increased (FFA-octadecadienoic acid only) In: no effect		CPT-I and serum triglycerides increased, leptin decreased
Ramsay <i>et al.</i> (2001)	male and female grower pigs	I+II, 0.0, 0.25, 0.5, 1.0, 2.0% by weight, from body weight 20 to 55 kg.	GI: no effect In: no effect	no effect on liver weight	
Ryder <i>et al.</i> (2001)	Male ZDF rats	I and I+II, 1.5% octadecadienoic acid or pair fed, 2 weeks	I: GI: increased In: increased I+II, GI: normalized and pair: In: decreased		
Scimeca (1998)	Male Fischer 344 rats	I+II, 1.5% by weight, 36 weeks	GI: N/A In: N/A	no effect on liver weight	unrelated decrease in thymus and adrenal weights
Sisk <i>et al.</i> (2001)	male and female, lean and obese Zucker rats	I+II, 0.5% by weight, 7-8 weeks	GI: no effect In: non-significant reduction (male obese only)	males: reduced liver weight	lean: reduced fat pad weights obese: increased fat pad weights
Stangl (2000)	male SPF Sprague-Dawley rats	I+II, 0, 1, 3, 5% by weight, 5 weeks	GI: increased (5% level) In: N/A		reduction of serum LDL (3%) and HDL (1%) cholesterol, increased serum albumin and creatinine (5%)
Stangl <i>et al.</i> (1999)	female pigs	I+II, 1% by weight, 6 weeks	GI: no effect In: increased		increase in cholesterol and LDL:HDL ratio.
Tsuboyama-Kasaoka <i>et al.</i> (2000)	female C57Bl/6J mice	I+II, 1% by weight, 4 days to 8 months	GI: no effect In: increased (normalized by leptin infusion)	enlarged liver, with vacuolization and fat deposition	enlarged spleen, reduced leptin
West <i>et al.</i> (2000)	Male AKR/J mice	I+II, 1% by weight, 5 weeks	GI: no effect In: non-significant increase		

**Table 1 Studies on the effects of octadecadienoic acid on glucose and insulin levels, liver and other parameters in hamsters, mice, rats and pigs.**

CPT-I: Carnitine palmitoyltransferase I; UCP: uncoupling protein; LDL cholesterol: low density lipoprotein cholesterol; HDL cholesterol: high density lipoprotein cholesterol

### 7.1.2. Studies with humans

Several studies have examined the effects of CLA on humans (table 2). The doses of CLA ranged from 3-7g CLA/day and the treatment periods ranged from 4-52 weeks. The trials were carried out in a range of population groups: body builders, healthy individuals, overweight and obese individuals and individuals with metabolic syndrome.

**Table 2 Studies on the effects of CLA on plasma glucose and insulin and other parameters in humans.**

Reference	Subjects	9cis,11trans-isomer (I) or 10trans,12cis-isomer (II): dose; length	Effect on glucose (GI) and insulin (In) levels	Other effects
Belury <i>et al.</i> (2003)	subjects with type 2 diabetes mellitus	I+II	GI: decreased In: N.A.	decrease in serum leptin
Blankson <i>et al.</i> (2000)	healthy overweight and obese men and women	I+II, 1.7, 3.4, 5.1, 6.8 g/day, 12 weeks	GI: no effect In: no effect	decrease in blood lipids and in creatinine
(Gauillier <i>et al.</i> , 2004)	180 overweight men and women	I+II, 4,5 g/day, 52 weeks	GI: no effect In: no effect	
Kreider <i>et al.</i> (2002)	resistance-trained men	I+II, 6 g/day, 28 days	GI: no effect In: N/A	
Lowery <i>et al.</i> (1998)	healthy male body builders	I+II, 7.2 g/day, 6 weeks	GI: no effect In: no effect	
Medina <i>et al.</i> (2000)	Healthy women	I+II, 3 g/day, 64 days	GI: no effect In: non-significant increase	
Risérus <i>et al.</i> (2001)	obese middle-aged men	I+II, 4.2g/day, 4 weeks	GI: increased In: no effect	II and I+II: decrease in HDL cholesterol
Risérus <i>et al.</i> (2002a,b)	obese middle-aged men	II, 3.4 g/day, 12 weeks	GI: increased In: increased	
		I+II 3.4 g/day, 12 weeks	GI: no effect In: no effect	II: increase in lipid peroxidation and C-reactive Protein (CRP)
Smedman <i>et al.</i> (2001)	healthy men and women	I+II, 4.2 g/day, 12 weeks	GI: non-significant increase In: no effect	Increase in Apo B
(Whigham <i>et al.</i> , 2004)	60 obese men and women	I+II, 6.0 g/day, 52 weeks	GI: no effect In: no effect Liver enzymes: no effect	

#### 7.1.2.1. Effects of CLA on blood glucose and insulin

In a study by Blankson *et al.* (2000) overweight and obese subjects received 1.7-6.8g CLA/day for 12 weeks. No significant changes were found in glucose or insulin levels. Similarly, in a study by Kreider *et al.* (2002), supplementation with 6g CLA/day for 4 weeks had no effect on glucose in resistance-trained men. In a study by Medina *et al.* (2000) no significant effect was observed on insulin and glucose levels in healthy female subjects consuming 3g/day CLA for a period of 64 days. In a study of bodybuilders who consumed 7.2g/day CLA for a period of 6 weeks no effects on serum glucose levels were found (Lowery *et al.*, 1998). In the first of a series of trials conducted at the University of Uppsala examining the effect of CLA on body composition, Smedman and Vessby (2001) found no treatment-related effect on insulin and glucose levels in healthy volunteers receiving 4.2g/day CLA for a period of 12 weeks. A second study was performed in abdominally obese men receiving 4.2g/day CLA for a period of 4 weeks.

Risérus *et al.* (2001) reported an increase in plasma glucose levels in both treatment and control groups. There was no effect on insulin.

In a recent published study, Risérus *et al.* (2002a,b) looked at the effect of CLA and the individual CLA isomer, *trans*10,*cis*12 CLA, in overweight men with signs of metabolic syndrome. Three groups received either 3.4g control oil (olive oil), 3.4g CLA, or 3.4g purified *trans*10,*cis*12 CLA isomer for a period of 12 weeks. CLA had no effect on insulin and glucose levels but a decrease in insulin sensitivity, an increase in fasting insulin levels and an increase in fasting glucose levels were observed in those receiving the purified *trans*10,*cis*12 isomer. In agreement with this study, Belury *et al.* (2003) reported that there was a stronger correlative of the *trans*10,*cis*12 CLA isomer with decreased leptin levels than the *cis*9,*trans*11 CLA isomer, suggesting that the *trans*10,*cis*12 CLA isomer is more bioactive.

The latest studies of importance are those of Gaullier *et al.*, 2004, and Whigham *et al.*, 2004 in which effects on glucose, and insulin were not reported during a study of 52 weeks of CLA supplementation.

In summary, none of the two-isomer preparations seem to alter the glucose level in healthy subjects. There are indications that the few reported effects of CLA on insulin levels are due to the *trans*10,*cis*12 CLA isomer.

#### **7.1.2.2. Effects of CLA on liver**

In order to determine the effects of CLA on liver in humans, several studies included liver parameters such as aspartate transaminase (AST) and alanine transaminase (ALT) levels in the blood. None of the studies reported elevated levels of these liver enzymes (Blankson *et al.*, 2000; Smedman and Vessby, 2001; Lowery *et al.*, 1998), indicating no toxicological effects of CLA on liver in humans.

#### **7.1.2.3. Additional toxicological data on CLA**

Overweight and obese subjects exhibited a decrease in blood lipids (total cholesterol, HDL and LDL cholesterol) and in creatinine (Blankson *et al.*, 2000). In a study with overweight men with signs of metabolic syndrome, an increase in markers of lipid peroxidation, such as C-reactive protein, urinary 8-iso-prostaglandin factor<sub>2α</sub> (8-iso-PGF<sub>2α</sub>) and 15-keto-dihydro-PGF<sub>2α</sub> (15-K-DH-PGF<sub>2α</sub>), was reported in individuals treated with 3.4 g/day of the purified isomer *trans*10,*cis*12 CLA, but not in individuals treated with a CLA mix (Risérus *et al.* 2002b). In the same study, HDL cholesterol levels were lowered both by treatment with the *trans*10,*cis*12 CLA isomer and with the CLA mix (Risérus *et al.* 2002a). In a study of Smedman and Vessby (2001) on healthy men and women, no effects of CLA on blood lipids were found.

Although an increased lipid peroxidation is often associated with an increased coronary risk, this effect was found only after treatment with the *trans*10,*cis*12 CLA isomer in overweight and obese subjects and not after treatment with the CLA mix. A general trend in studies in which subjects are treated with CLA mix, is a decrease in cholesterol .

## **7.2. Safety studies with Clarinol™**

A program of safety studies was conducted on Clarinol™ that included *in vitro* studies, animal toxicology studies, and human clinical trials.

### **7.2.1. Genotoxicity**

Two *in vitro* mutagenicity studies were conducted on Clarinol™ glycerides; a reverse mutation assay in five histidine-requiring strains of *Salmonella typhimurium* (an Ames test) and an *in vitro* cytogenetics assay in human lymphocytes. Both studies were conducted in accordance with GLP and in accordance with OECD guideline 471 and 473 respectively.

#### **7.2.1.1. Ames test**

In the Ames test, Clarinol™ G80 was tested in five strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) in the presence and absence of metabolic activation (S9). Each concentration was tested in triplicate with and without S9. Positive and negative controls were included.

In the first experiment, Clarinol™ G80 was tested in strain TA100 at concentrations of 1.26, 6.29, 31.44, 157.2, 786 and 3930 µg/plate. Precipitation of the test article was observed on plates treated at 786 µg/plate and above, but there was no evidence of toxicity at any of the dose levels. In the remaining *Salmonella* strains, Clarinol™ G80 was tested at concentrations of 1.6, 8, 40, 200, 1000 and 5000 µg/plate. Evidence of toxicity was observed in strain TA102 at the 1000 and 5000 µg/plate +/-S9. The test article was found to precipitate at concentrations of 1000µg/plate and above.

The second experiment included a pre-incubation step (1 hour at 37 ± 1°C) before plating. In this experiment the following concentrations were tested: 51, 128, 320, 800, 2000 and 5000µg/plate. Signs of toxicity were observed in TA102 at 5000µg/plate in the presence of S9. Precipitation of the test article was observed on all plates treated at 2000µg/plate and above.

Statistically significant increase in revertant numbers was observed in strain TA100 (+S9), TA1535 (+S9), and TA102 (-S9). In all cases the observed increases were not dose related, occurring solely at either the lowest or an intermediate test dose and were not reproducible in comparable experiments. They were therefore not considered evidence of mutagenicity.

In conclusion, Clarinol™ did not induce mutation in five histidine-requiring strains of *Salmonella typhimurium* in the presence and absence of S9.

#### **7.2.1.2. In vitro chromosome aberration test**

Clarinol™ G80 was tested in an *in vitro* cytogenetics assay using human blood lymphocyte cultures. The material was tested in the presence and absence of S9. In the first experiment treatment was 3 hours followed by a 17-hour recovery period prior to harvest. The following dose levels were tested 128, 160 and 200µg/ml. The highest dose level chosen was reported to induce a 3% and 4% reduction in mitotic index in presence and absence of S9 respectively.

In experiment 2, treatment in the absence of S9 was 20 hours. In the presence of S9 it was 3 hours followed by a 17-hour recovery period prior to harvest. In this experiment dose levels of 192 (-S9) and 153.6 (+S9), 240 and 300 µg/ml were tested. The top dose level, 300 µg/ml, induced a 0% and 4% reduction in mitotic index in the absence and presence of S9 respectively. Positive and negative

controls were included in both experiments. Each dose of test material was tested in quadruplicate cultures; controls were tested in duplicate.

In both experiments Clarinol™ G80 +/-S9 produced frequencies of cells with structural aberrations which were similar to the concurrent negative controls. The numbers of cells with aberrations (excluding gaps) in all treated cultures fell within the historical negative control ranges.

In conclusion, Clarinol™ G80 did not induce chromosome aberrations when tested in the presence and absence of S9.

## **7.2.2. Animal studies**

### **7.2.2.1. 13 weeks sub-chronic oral rat study**

A 13-week sub-chronic oral toxicity study in rats was conducted on Clarinol™ G80 (O'Hagan and Menzel, 2003). The study was conducted in accordance with GLP. Clarinol™ G80 was administered at a dose level of 0%, 1%, 5% and 15% w/w in the diet to male and female Wistar rats (20 rats/sex/group) for a period of 13 weeks. Two control groups were included in the study; one group was maintained on the standard AIN-93 diet (7% fat), whilst the other group was maintained on an AIN-93G diet containing high level of control oil – 15% w/w safflower oil. In the treatment groups receiving Clarinol™ G80, the fat content of the diet was standardized to 15% w/w, with the control oil being used to make the total fat content up to 15% w/w.

The study included a recovery phase at the end of the 13-week period in order to assess the reversibility of any adverse effects that occurred during the treatment period. Rats (10 rats/sex/group) in the two control groups and those receiving high dose Clarinol™ G80 were maintained on study for a further 4 weeks. However, rats in the high dose Clarinol™ G80 group were switched to control diet containing 15% safflower oil for the duration of the recovery period.

Haematology and clinical chemistry parameters were measured at week 4, 8 and 13 and at the end of the recovery period. Neurobehavioral testing (arena testing, FOB and motor activity) was performed on 10 rats/sex/group prior to exposure and on a weekly basis during the 13 weeks of study.

#### Clinical signs, body weight and food consumption

No treatment related clinical signs were observed during the study. One control animal in the recovery group was killed *in extremis* on day 107. A reduction in body weight was observed in the first week of study in high dose animals. This effect was linked to a reduction in food consumption. On day 7 body weights were lower in high dose males and females compared to both control groups. On day 14 body weight in the high dose females was lower compared with the high fat control group (HF). The reduction in body weight at the start of the study meant that animals in 15% Clarinol™ G80 group had consistently lower body weights throughout the study.

During the study, food consumption by both sexes was significantly lower in all groups fed high fat diets in comparison to control rats receiving the low fat diet. This effect was due to the difference in caloric value between the low fat and high fat diets. In high dose male rats food reduction was significantly reduced on days 3 and 7. In females it was lower on days 3, 7, 10 and 14. An intermittent reduction was also observed on day 31, 35, 42 and 66 in females. The lower food consumption at the start of the study was caused by reduced palatability of the high dose diet. Food conversion efficiency among the groups was similar throughout the study.

The mean intake of Clarinol™ G80 in male rats was 490, 2433 and 7591mg/kg bw/day in the low mid and high dose levels respectively. In females it was 551, 2728 and 8140 mg/kg bw/day. A slight reduction in water consumption was observed in the high dose rats towards the end of the study on days 78, 79, 80 and 81 compared with both control groups.

#### Ophthalmoscopic and neurobehavioral examination

No treatment related effects were observed in ophthalmoscopic examination of high dose and control rats. The results of neurobehavioral testing did not indicate any neurotoxic potential.

#### Haematology

Haematology measurements showed a reduction in mean corpuscular volume (MCV) in high dose females at week 13 and a decrease in absolute and relative white blood cells at week 13 in high dose males. The reduction in MCV value was not considered toxicologically relevant, as it was not accompanied by changes in red blood cells and packed cell volume. There were no haematological findings at the end of the recovery period.

#### Clinical chemistry

During the treatment period there was a marked elevation in liver enzymes e.g. alkaline phosphatase, aspartate transaminase (AST) and alanine transaminase (ALT), in the high dose animals. Sorbitol dehydrogenase was also increased in high dose females at week 13. Total cholesterol was decreased in high dose males throughout the 13-week treatment period. There was no effect in females. Plasma triglyceride levels were decreased in all groups of male rats receiving the high fat diet compared to the low fat control group. However, triglyceride levels were increased in high dose females during the study. Albumin was increased in high dose male and female rats at week 8 and 13 compared to both control groups. The albumin/globulin ratio was increased in high dose animals throughout the study compared to both control groups. Plasma insulin levels were increased in high dose females at week 8 and 13, in males it was increased at week 4 and 8. Glucose was significantly decreased in high dose males at week 13 compared to both control groups.

At the end of the recovery period AST and ALT remained increased in high dose females but only compared to the low fat control (LF) group. Glucose was significantly decreased in high dose males compared to the LF controls. There was no significant difference in cholesterol and triglyceride levels at the end of the recovery period.

There were no significant changes in urinary parameters. Examination of urinary sediment revealed a significant increase in urinary crystals in high dose males. The crystals had disappeared at the end of the recovery period.

#### Organ weights

There were a number of changes in organ weight recorded at the end of the 13-week treatment period. Relative liver weights were increased in mid-dose males and high dose males and females. Relative spleen and kidney weights were also increased in the high dose group. Relative adrenal weights were increased in high dose males only.

At necropsy an increase in hepatocellular vacuolation was significantly increased in high fat control animals and in males of the 1% Clarinol™ G80 group. This lesion remained at the end of the recovery period and was now observed in males of the high dose group that had switched to the HF control diet for the 4-week recovery period. The hepatocellular vacuolation was considered to be related to the feeding of the control oil, safflower oil.

In high dose females there was a significant increase in multifocal hepatocellular hypertrophy. The enlargement of the hepatocytes was mainly seen in the periportal region of the liver. After the 4-week recovery period the hypertrophy had almost completely disappeared. Only two females of the high dose group demonstrated this condition to a very slight degree.

At the end of the 13-week treatment period, the amount of brown adipose tissue was significantly reduced in mid and high dose male rats and in all Clarinol™ G80 treated female groups compared to both control groups. At the end of the recovery period the amount of brown fat remained significantly less in the high dose group.

#### Summary

A number of general toxicological effects of Clarinol™ G80 have been found in rats with respect to clinical chemistry and organ weights, but only at the high dose level of 15%. Relative liver weights were also increased in male rats treated with 5% Clarinol™. In the absence of any clinical chemistry changes and histopathology this small increase in relative liver weight is not considered adverse. As discussed in section IV.C.1(a)(2), studies on the effects of CLA on rat liver report variable results. The effects of Clarinol™ G80 on human liver are discussed in section IV.C.2(c).

A No Observed Adverse Effect Level (NOAEL) of 5% Clarinol™ G80 was identified in the study. This is equivalent to an overall mean intake of 2433 and 2728 mg/kg bw/day in male and female rats respectively.

#### **7.2.2.2. Effect of Cla on insulin and glucose in leptin deficient mice**

In a study on the effect of Clarinol™ G80 on plasma and insulin levels, female C57B1/6 lep/lep mice received either Clarinol™ in the form of a free fatty acid (Clarinol™ A-80) or in the form of a triglyceride (Clarinol™ G80) at dose levels of 1% and 2.5% in the diet for a period of 11 weeks. Control animals were given a supplement of 2.5% sunflower oil, and 1.5% sunflower oil was added to the diet of mice receiving 1% Clarinol™. At day 14, 35 and 70 days mice were fasted for 5 hours and blood samples were taken for the analysis of insulin, NEFA and triglycerides. Glucose tolerance was also measured. At the end of the study mice were killed and liver perigenital fat pad, brown adipose tissue, gastrocnemius muscle and pancreas were collected.

Clarinol™ caused a dose-related decrease in body weight gain, reaching a plateau after 2 weeks. At the top dose level mice failed to grow on diets with FFA and TG Clarinol™.

After 14 days of treatment, mice receiving Clarinol™ showed a dose-related decrease in glucose tolerance. At 1% in the diet the area under the curve was raised in mice fed 1% TG Clarinol™ and significantly so in mice fed 1.0% FFA-Clarinol™. In both groups receiving 2.5% Clarinol™ there was a significant increase in the area under the glucose curve. After 35 days the decrease in glucose tolerance remained. However, it was no longer dose-related and indeed an inverse dose relationship was observed, the decrease being significant in the lower dose groups only. After 70-days of supplementation with Clarinol™ a dose related improvement in glucose tolerance was observed. The reduction in glucose tolerance compared to control values remained significant at the top dose level only. A similar pattern was observed for blood glucose levels. After 14-days, fasted blood glucose concentrations showed a dose related increase with Clarinol™, after 35 days the fasting blood glucose levels remained elevated but the highest levels were found in mice receiving 1% Clarinol™. After 70-days there were no significant differences in blood glucose levels between the Clarinol™

treated groups and controls. Blood glucose was also measured at day 75 and in the animals treated with 2% Clarinol™, there was a trend in reduction of blood glucose levels.

After 14-days fasted plasma insulin concentrations showed a dose related increase and after 35-days fasted plasma insulin concentrations remained significantly elevated but as with the glucose measurements there was a trend towards an inverse dose-relationship. After 70-days plasma Clarinol™ significantly reduced insulin concentrations in comparison to control values. At day 75 there were no significant differences between any of the groups.

No significant differences were found in plasma free fatty acids between mice fed Clarinol™ and controls at day 14 or 35. After 70-days, plasma NEFAs concentrations were reduced by 2.5% Clarinol™ supplementation but not by 1% supplementation. No difference was seen between the TG and FFA formulations.

Intra-scapular brown adipose tissue was significantly decreased by Clarinol™ feeding. Similarly epididymal fat-pad weight was also reduced. There was no significant effect on pancreas weight in mice fed Clarinol™. Liver weights were increased in mice fed Clarinol™. The increase was statistically significant at the high dose level with both Clarinol™ G80 and A80.

In summary, the ratio of blood glucose to plasma insulin, a measure of insulin sensitivity, revealed that after 14 and 36 days, insulin sensitivity in leptin deficient mice treated with Clarinol™ was significantly reduced. This is in concurrence with the published studies discussed in section IV.C.1.(a). However, after 70-days there was a tendency for improved sensitivity, although this was not significant. Liver weights were increased in mice treated with 2.5% Clarinol™. This is in agreement with previously discussed studies as well.

### **7.2.3. Human clinical trials**

A clinical trial has been completed on Clarinol™ A60, a product with a lower level of CLA isomers than Clarinol™ A80. The study examined the effect of Clarinol™ A60 administration on substrate utilization, glucose and lipid metabolism, metabolic rate and body composition in regularly exercising individuals. The double blind, placebo controlled trial was conducted at the University of Cape Town. Sixty-four regularly exercising men and women aged 21-45 years were randomized into two groups receiving either 3.9g/day Clarinol™ A60 or 3.9g of high oleic sunflower oil for a period of 12 weeks. Two participants subsequently withdrew from the trial. The Clarinol™ A60 product tested in this study had a lower total CLA isomer content, 65.9% total CLA isomers, compared to the Clarinol™ G80 being tested in the clinical trial underway at the University of Wisconsin (see below) and tested in the toxicology studies.

Treatment with Clarinol™ A60 was associated with a small but statistically significant decrease in body fat levels in women but there was no change in body mass. Mean plasma insulin concentrations were also significantly lower in women taking Clarinol™ A60. Plasma glucose did not differ between the two groups. Insulin sensitivity, as measured by the increment in glucose concentrations versus the increment in insulin concentrations and by the ISI and QUICKI models, insulin resistance as measured using the HOMA model and fasting glucose/insulin ratio did not differ significantly between treatment and placebo group. Similarly, muscle carnitine palmitoyl transferase activity did not differ between the groups. No treatment related effects were found on total cholesterol, LDL-cholesterol, and HDL-cholesterol.

A second, 52-week clinical trial on Clarinol™ G80 is currently underway at the University of Wisconsin Clinical Nutrition Clinic. The trial is double blind placebo controlled study of the effects of Clarinol™ on the prevention of weight regain.

The study is being conducted in overweight and obese individuals (BMI in range 27-35kg/m<sup>2</sup>) and is divided into three phases:

- a. a weight loss phase on very low calorie diet
- b. a maintenance (of weight loss) phase
- c. open label phase.

In the first two phases participants receive either Clarinol™ G80 or placebo capsules in addition to a weight loss/management and exercise program. The daily intake of Clarinol™ G80 is 7.5g, which is equivalent to 6g/day of the CLA isomers. In the third phase of the study all subjects will be moved to the Clarinol™ G80 group and continue with this treatment until the end of the study.

A total of 62 subjects were randomized in the first phase of the study. Fifteen subjects have dropped out of the study due mainly to lack of time or commitment (10 subjects) and other non-treatment related reasons e.g. pregnancy, general intolerance to taking capsules, hair loss. Safety parameters including clinical chemistry parameters, insulin and glucose levels are being measured throughout the study.

Preliminary data from the first 24 weeks of the trial indicate that Clarinol™ G80 had no effect on liver function as there was no evidence of a treatment related effect on liver enzymes (AST and ALT). Similarly there was no effect observed on plasma insulin and glucose levels. More detailed analysis of the data is expected shortly.

In summary, studies with Clarinol™ G80 in human trials have not revealed any toxicological effects on blood lipids, plasma glucose and insulin levels, liver enzymes and other parameters

### **7.3. Discussion and Conclusions**

CLA was found to be non-genotoxic in *in vitro* assays for gene mutation and chromosome damage.

In a 13-week sub-chronic oral toxicity study Clarinol™ was found to cause liver enlargement in male and female rats fed 15% Clarinol™. The effects in males are not considered adverse due to the fact that no histopathology or changes in clinical chemistry were found. In female rats the liver enlargement was accompanied by hepatocellular hypertrophy, but this was reversible upon withdrawal of the test material. Thus the hypertrophy appears to be an adaptive effect, most probably due to enzyme induction, in response to very high levels of Clarinol™ in the diet.

Insulin sensitivity in leptin deficient mice treated with Clarinol™ was significantly reduced, and liver weights were increased. This is in concurrence with several studies on CLA discussed in section IV.C.1.(a). Mice appear to be more sensitive to CLA treatment than rats, pigs or humans, which is possibly related to their extremely high rate of metabolism.

Several human clinical trials on CLA have not found any evidence of an adverse effect on liver function. The preliminary results from the study at the University of Wisconsin are of particular note. Doses of CLA of 7.5g/day, consumed over a period of 24 weeks had no effect on liver function, as measured by serum liver enzymes such as AST and ALT. These findings are in agreement with the published studies on CLA by Blankson *et al.* (2000), Lowery *et al.* (1998) and Smedman and Vessby (2001).

There are a number of both human and animal studies that have looked at the effect of CLA on insulin and glucose. The animal data have shown that CLA may have both a beneficial and a potentially adverse effect on insulin levels depending

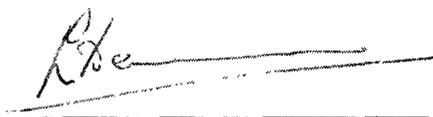
on the model that is used. In the 13-week rat study Clarinol™ G80 was found to increase plasma insulin levels in both males and females at a dose level of 15% in the diet. There was no effect at either 1% or 5%. However, the time course of the increase appeared to vary between the sexes. In males, insulin levels were significantly elevated compared to both control groups at week 4 and to the HF control only at week 8, after which they did not differ significantly. This was accompanied by a trend towards lower plasma glucose from week 8 onwards in high dose males but this effect was not significant compared to both control groups at all time points. In females plasma insulin levels were significantly increased at week 8 and 13 compared to both control groups. At the end of the recovery period this increase was reversed and plasma insulin levels were lower, though not significantly, in the former 15% CLA females compared to controls. No treatment-related effect on plasma glucose was observed in females.

Studies in humans with CLA have not found any adverse effect on insulin levels. There is some suggestion that the *t*10,*c*12 isomer solely consumed may have an adverse effect (Risérus *et al.*, 2002a). However, the human clinical trials on Clarinol™, which is a mix of isomers (mainly 9*c*,11*t* and 10*t*,12*c*) have found no evidence of an adverse effect on insulin and glucose. In fact, in the study conducted at the University of Cape town, Clarinol™ produced a significant reduction in plasma insulin levels in healthy regularly exercising women but plasma glucose and insulin sensitivity did not differ compared to controls in males and females. Similarly, preliminary results from the study at the University of Wisconsin in which volunteers receive 6g/day CLA have found no effect on plasma insulin and glucose.

In the 13-week toxicology study on CLA with rats, a NOAEL of 2433 and 2728mg/kg bw/day was identified in male and female rats respectively (average 2580mg/kg bw/day). Comparing this NOAEL with the recommended dose levels of Safflorin™ (1.6g or 27mg/kg bw/day for 60kg individual) gives a safety factor of 92. A safety factor of 100 is often expected when new ingredients, in particular food additives, are added to food. In this case a safety factor of less than 100 is acceptable because CLA isomers are found in food commodities such as meat and dairy products, so there is a long history of exposure to these compounds in the diet without any apparent adverse effect. In addition, data from the human clinical trials indicate that the endpoints on which the NOAEL is based, i.e. liver enlargement and increased insulin levels, are not seen in human exposure to CLA.

Based on the information described herein, including the history of use and other evidence of safety of CLA and citations to published articles, Loders Croklaan concludes that dietary supplements containing Safflorin™, when used under the conditions recommended or suggested by Loders Croklaan, will reasonably be expected to be safe.

## V. SIGNATURE OF DESIGNATED PERSON



Louis H.T. Dederen  
Safety & Regulatory Affairs Manager

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