Steviol glycosides
as
Food Additive

SUMMARY of New Application by

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Stevia glycosides – New submission

Summary Document.

- **Nature of proposed additive:** Steviol glycosides, that are extracted from *Stevia rebaudiana* (Bertoni) Bertoni. Number given to steviol glycosides by the Codex Alimentarius is (E-) 960. The proposed additive has to be in accordance with the EUSTAS Quality Label and is therefore named "**EUSTAS® Stevia glycosides**". It has at least 95 % purity and all the glycosides of the mixture are well specified. The quality of stevia glycosides on the European market will be under the control of the European Stevia Association (www.eustas.org). Bad qualities found will immediately be reported to the authorities of the country where they were found.

- Source:

Steviol glycosides are purified extracts of *Stevia rebaudiana* Bertoni. There are four main glycosides: stevioside, rebaudioside A, rebaudioside C and dulcoside A. Stevioside and rebaudioside A are the dominant components and the ratio of these two is the main determinant of taste 'quality'. Where stevioside is more than 50% of the total glycosides the taste is 'common/traditional', with a 'liquorice' after-taste. Where rebaudioside A makes up more than 50%, the taste is 'improved' with a reduced after-taste. Other minor glycosides present generally make up less than 5% of the total and include rebaudioside B, D, E and F, steviolbioside and dulcoside B.

- Method of manufacture

Most steviol glycosides are now produced in extraction factories in China, Japan or South America (Paraguay and Brazil). There is none yet produced in Europe. Most imports to Europe, now and in the short term, are likely to come from Japan, Paraguay, Brazil and China. All manufacturers use the same basic steps to extract steviol glycosides from the leaves of the stevia plant, although there is some variation in the later stages of purification and separation of glycosides. The process generally involves:

- Extraction from the leaves by dissolving the steviol glycosides in warm/hot water in a batch system 3 – 5 times or by a continuous reverse flow system,
- Flocculation and precipitation of suspended matter,
- Filtration,
- Concentration by vacuum assisted evaporation,
- Adsorption (and release by ethyl alcohol) in a resin exchange process,

- Ion-exchange purification,
- Further filtration and concentration,
- Spray drying or crystallisation.

The overseas products that will be imported in Europe will have to meet the EUSTAS Quality Label given in Table 8 and will have at least 95% purity with identification of all the sweeteners present.

- Proposed use:

The steviol glycosides will be used primarily as a non-calorie sweetener and/or flavour enhancer. They can be used in the same wide range of products that can contain sugar.

In Europe, they are expected to be used primarily in:
- soft drinks and cordials,
- milk, soy and mineral drinks,
- canned fruit, jams and juices,
- ice creams, yoghurts and other dairy products,
- cakes, biscuits, pastries and desserts,
- sugar free beers and alcoholic beverages,
- toppings, sauces, chutneys, spreads etc,
- cereals, muesli bars and confectionaries.

As steviol glycosides (crystals or powder) are 250 to 300 times sweeter than sucrose, they can be used at rates of up to 0.004 times the rate of sugar used. They can be used in conjunction with sugar or other sweeteners and will replace some (or all) of the sweetener now used. For example, a soft drink, instead of containing 10% sugar, may contain 5% sugar together with 0.008% of steviol glycosides expressed as steviol (or equivalent to 0.02% of stevioside), making a reduced calorie product. Alternatively, it may contain 0.016% of steviol glycosides expressed as steviol (or equivalent to 0.04% of stevioside) and no sugar.

Previously, the quantum of the extract was expressed as a weight (usually mg) of stevioside, although it was actually a mixture of very similar glycosides. As the molecular weights of the various glycosides are different, JECFA has suggested that the concentrations/amounts of steviol glycosides should be expressed as steviol content, which is equivalent to approximately 40% of the stevioside content. To obtain the steviol equivalents of the different steviol glycosides, their amounts should be multiplied by the factor given in Table 1. To obtain the amounts of steviol glycosides out of the

steviol equivalent, multiply the amount of steviol equivalent by the factors in the lower half of Table 1. Quantities quoted in this document are now also expressed as steviol equivalents, unless otherwise indicated. In the part on Toxicology, quantities are still expressed as stevioside, to be consistent with the reports of all the trials undertaken, which have been written on the basis of stevioside content. However, in Table 19 of proposed ADI's, both quantities as steviol glycoside and as steviol equivalents are given.

Table 1: Conversion of steviol glycosides to steviol equivalents and vice-versa

To obtain the steviol equivalent of:	**Molecular weigths**	**Multiply the amount by:**
Stevioside	804.38	0.395
Rebaudioside A	966.43	0.329
Rebaudioside C	950.44	0.334
Dulcoside A:	788.38	0.400
Rubusoside	642.33	0.496
Steviolbioside	642.33	0.496
Rebaudioside B	804.38	0.395
Rebaudioside D	1128.48	0.282
Rebaudioside E	966.43	0.329
Rebaudioside F	936.42	0.340
To obtain the steviol glycoside	**Molecular weigths**	**Multiply steviol equivalent by**
Stevioside	804.38	2.532
Rebaudioside A	966.43	3.039
Rebaudioside C	950.44	2.994
Dulcoside A:	788.38	2.500
Rubusoside	642.33	2.016
Steviolbioside	642.33	2.016
Rebaudioside B	804.38	2.532
Rebaudioside D	1128.48	3.546
Rebaudioside E	966.43	3.039
Rebaudioside F	936.42	2.941

- Summary of toxicological tests

Core studies on toxicology:

a) Absorption/Metabolism/Toxicokinetics.

- Absorption studies have been performed in *in vitro* assays (everted intestines, Caco-2 cell layers) as well as in *in vivo* studies. From the different studies it is obvious that the absorption of steviol glycosides is extremely low, but the absorption of steviol, the aglycone of steviol glycosides, is good.

- Metabolism studies have been performed in different animals, in bacteria and in *in vitro* assays as well as in human volunteers. Whole animal studies using single and repeat doses were done.
None of the digestive enzymes from the gastro-intestinal tract of different animals and man are able to degrade stevioside into steviol, the aglycone of stevioside. Moreover, incubation of stevioside (97% purity) in 3 freshly taken samples (intubation) of stomach juice at 37°C for 6 h (1 mg/ml) revealed that there was no degradation of stevioside. This means that the stevioside ingested, reaches the intestines intact.
In human metabolism studies, no free steviol was found in the blood or urine, but steviol glucuronide was present as typical excretion product. Whereas Geuns *et al*. (2004, 2006, 2007) did not find stevioside in blood or urine samples (below the detection limit of the UV detector used), Simonetti *et al*. (2004), by use of LC-MS, found small amounts of stevioside in plasma of 7 volunteers (maximum concentration 0.1 µg/mL plasma) with a large inter-individual variation. In urine only very small traces of stevioside were detected in amounts that were too low be quantified (Pietta, personal communication).

The lack of stevioside in the feces proves that the bacterial flora degraded all the stevioside into steviol, which itself was not further metabolized, as also shown in pigs *in vivo* and in pig and human feces *in vitro* under anaerobic conditions. These results are opposite to those obtained with chickens in *in vivo* and *in vitro* experiments, which demonstrated that not all of the stevioside was degraded into steviol. In the human colon, only the group of bacteroidaceae were efficient in hydrolyzing stevioside into steviol. However, in human metabolism experiments, no free steviol could be detected in the blood or the urine of the volunteers.

After enzymatic hydrolysis by β-glucuronidase/sulfatase, steviol could be set free out of the steviol glucuronide that was the only conjugate detected in urine. The amounts of steviol obtained after enzymatic hydrolysis of plasma varied between the different subjects showing values between 0.7 and 21.3 µg/ml plasma in the period between 0 and 7 h after the first dose on the third day.

The absence of free steviol in the peripheral blood of the volunteers and the presence of conjugated forms, suggests that all free steviol occurring in the portal blood was converted into steviol glucuronide by the liver.

No free steviol was detected in urine. After enzymatic hydrolysis of urine extracts by β-glucuronidase/sulfatase, steviol was found as the only aglycone present. There was no indication for the occurrence of, e.g., steviol sulfates even after large scale extractions of urine. As no other metabolites were found, the following excretion route is suggested (Figure 1).

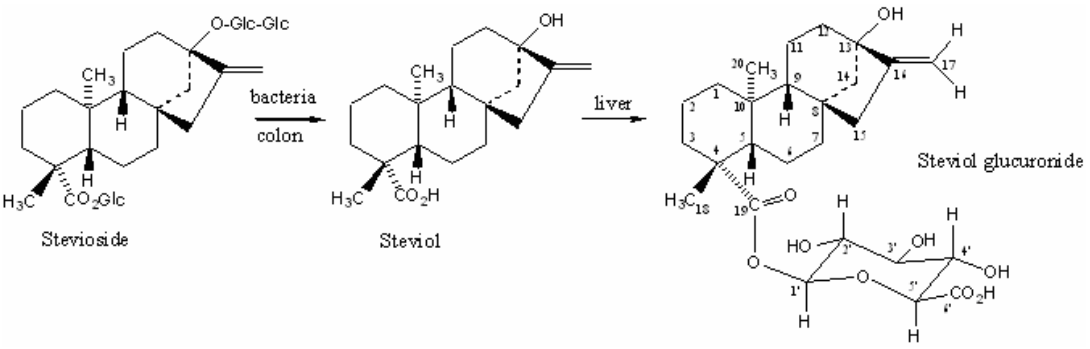

Figure 1. Hypothetical route from dietary stevioside to steviol glucuronide in human urine.

After degradation of stevioside to steviol by bacteria of the colon, part of the steviol is absorbed by the colon and transported to the liver by portal blood. In the liver, the steviol glucuronide is formed, which is released into the blood and filtered out by the kidneys into the urine. The high levels of steviol glucuronide in the urine suggest that there is no accumulation of steviol derivatives in the human body. The steviol glucuronide still present in the blood is expected to be excreted in the urine during the next 24 h.

Besides steviol glucuronide, no free steviol or any other of the possible steviol metabolites could be detected in blood or urine. Hepatic metabolism of steviol is extremely low, if existing at all which is in agreement with results of Koyama et al. (2003b) who demonstrated in *in vitro* experiments that the steviol metabolism by human microsomes was 4 times lower than that of rat microsomes, and this last one was already very low.

b) Toxicity: acute and subchronic

Results of acute and subchronic toxicity studies proved that oral steviol glycosides had no harmful effects in animal models or man.

c) Genotoxicity

Genotoxicity tests were performed with steviol glycosides, as well as with free steviol.

i. Induction of gene mutations in bacteria

a) Steviol glycosides (stevioside, rebaudioside A)

Steviol glycosides did not show mutagenic effects in gene mutation essays in bacteria with or without S9 activation mix.

b) Steviol

After metabolic activation, it was shown that so far unknown steviol metabolites caused mutations in *Salmonella typhimurium* TM677, i.e. transitions, transversions, duplications and deletions at the guanine phosphoribosyltransferase (*gpt*) gene (Matsui, 1996b). However, stevioside and even steviol were inactive in various other TA strains of *Salmonella typhimurium* with or without S9 mix at doses up to 5 mg/plate (TA 97, 98, 100, 102 and 104), in *Escherichia coli* WP2 *uvrA*/pKM101 and in the *rec*-assay using *Bacillus subtilis* even when activation S9 mix was present (99% purity, Matsui *et al*., 1996a; 96% purity, Klongpanichpak *et al*., 1997). The activity of steviol in *Salmonella typhimurium* TM677 was very low and was only about 1/3000 that of 3,4-benzopyrene and that of steviol methyl ester 8,13 lactone was 1/24500 that of furylfuramide (Terai *et al*., 2002). Although a weak activity of steviol and some of its derivatives was found in the very sensitive *S. typhimurium* TM677 strain, the authors concluded that the daily use of stevioside as a sweetener is safe. Moreover, the presence in the blood of the chemically synthesized steviol derivatives after feeding stevioside is not proven at all.

ii. Induction of gene mutations in mammalian cells *in vitro.*

a) Steviol glycosides (stevioside, rebaudioside A)

The conclusion from the experiments with mammalian cells *in vitro* is that steviol glycosides do not induce gene mutations. They may be chemopreventive agents of natural origin. It is not known if they have similar effects when orally administered, as the amounts of stevioside consumed as a sweetener (maximal amount estimated at about 200 mg/day) will be far below the concentration of 3.2 mM (≈ 2.57 g/l), the lowest concentration having a significant effect.

b) Steviol

After metabolic activation of steviol (99 % purity), some gene mutation and chromosomal aberration was found in Chinese hamster lung fibroblasts (Matsui *et al*., 1996a). CHL cells in logarithmic phase of growth were exposed to steviol dissolved in DMSO for 3h at 37°C in the presence of S9 mix. At the doses below 300 µg/ml, no effect was observed. At 350 µg/ml the increase was 47%, at 400 µg/ml about 236%. The positive control N,N-dimethylnitrosamine at 1000 µg/ml induced a 447 % increase of mutations. The mutagenicity of steviol without the metabolic activation was not studied.

Hye-Young et al. (1999) tested steviol (98.4% purity) in the mouse lymphoma L5178Y $tk^{+/-}$-3.7.2C gene mutation assay (MOLY). Steviol was tested at 42.66, 85.31, 170.63 and 340 µg/ml both in the presence and the absence of the metabolic S9 activation system. No sign of enhancement of mutation frequency by steviol was observed, whereas the positive control showed its expected stimulation.

iii. Induction of chromosomal aberrations in mammelian cells.

a) Steviol glycosides (stevioside, rebaudioside A)

Steviol glycosides were inactive in the chromosome aberration test using CHL cells with or without the metabolic activation (S9), in the micronuclei assay in regenerating hepatocytes and bone marrow cells of ddY mice (stevioside was administered orally at 62.5, 125 and 250 mg/kg BW), in the *in vitro* chromosome aberration assay using a Chinese hamster lung fibroblast cell line, in a micronucleus assay in male BDF1 mice to study the induction of micronuclei in erythrocytes, in the *in vivo* 'comet' assay in mice (doses used were 500, 1000 and 2000 mg/kg BW).

b) Steviol

Steviol did not induce an enhancement of micronuclei in hepatocytes and bone marrow cells of ddY mice (administered orally at 10, 50, 100 and 200 mg/kg BW).

No statistically significant increases in DNA damage were observed at concentrations of 500 µg/mL or below, either with or without metabolic activation using the 'comet' assay, *in vitro* (human lymphoblastoid cell lines TK6 and WTK-1) and *in vivo.*

d) Chronic toxicity and carcinogenicity

a) Steviol glycosides (stevioside, rebaudioside A)

Chronic toxicity and carcinogenicity studies by 3 research groups using rats demonstrated the safety of stevioside used as a sweetener. No chronic toxicity or carcinogenicity of steviol glycosides was reported. Steviol glycosides had no effects on rat urinary bladder carcinogenesis.

In addition to the above, a chronic study with hamsters over 3 generations did not show the appearance of abnormal structures in the reproductive tissues. Moreover, no preneoplastic or neoplastic lesions of the urinary bladder of rats were observed after feeding stevioside. Although use of F344 rats in some studies might not be the best choice for chronic carcinogenicity studies, the decrease of the incidence of adenomas of the mammary gland is relevant, as well as the delay and decrease of the number of papillomas in the two-stage mice skin assays, both by topical and oral administration. However, due to the extremely low absorption by the intestines and the low amounts needed for sweetening purposes, the advantages of oral steviol glycosides as chemopreventive agent seem to be less relevant.

b) Steviol

No chronic toxicity and carcinogenicity studies of steviol glycosides using mice seem to exist. However, various *in vivo* studies with mice, hamsters and rats were done in which large doses of steviol were administered. As oral steviol is easily absorbed by the colon after degradation of stevioside, these studies seem very relevant.
Four different research groups could not detect genotoxic effects of steviol although the maximum doses orally administered were 2, 4 and 8 g/kg BW depending on the research group and the animals tested. These values would suggest an ADI (safety factor 100) of steviol equivalents between 10 and 80 mg/kg BW or between 25 and 200 mg stevioside/kg BW, far above the values calculated in Part III (Toxicology).

e) Reproduction and developmental toxicity

- Reproduction

Studies with purified extracts

- One study was done over several generations (0, 0.5, 1 or 2.5 g/kg BW respectively) with hamsters as these animals are most sensitive towards stevioside and steviol. No abnormalities were found in growth and fertility in both sexes. It was concluded that a daily stevioside dose as great as 2.5 g/kg body weight does not affect growth or reproduction in hamsters. Gametogenesis and functions of reproductive tracts were normal in both sexes as judged from their histologies. Both the duration of pregnancy and the number of implanted foetuses were unaffected by the stevioside consumed.
- In a study with male and female Wistar rats receiving feed containing 0.15 %, 0.75% and 3% stevioside, the authors concluded that the addition of stevioside to feed up to a concentration of 3%, i.e. 3.16 g/kg BW, has no effect on mating, fertility or the development and the state of foetuses in rats given the diet prior to and during the early stage of pregnancy.
- In a study with rats fed 525 mg stevioside/kg for 21 days, the authors could not observe any difference in the copulation and conception averages. In the group of dissected animals, the number of foetuses, absorption of foetus, number of death, foetus measurements and weight did not differ with the control group. In the group of natural birth, the number of young delivered, their weight, growth and development did not differ from the control group.
- No significant effect was found on spermatogenesis, or on the interstitial cell proliferation and tumour formation in the testes of F344 rats fed a ration containing up to 1% stevioside (95.2% purity) for 22 months.

Results obtained with crude *Stevia* extracts

- The study by Planas and Kuć (1968) should be discussed, as, in the past, this has led to some confusion and controversy on fertility. These authors administered crude *Stevia* extracts to female albino rats (10 ml of a 5% decoction daily). The decoction was never administered to the male rats. The authors reported a 57–79% reduction of fertility. However, the results of Planas and Kuć (1968) were refuted by Shiotsu (1996) who did more reliable experiments with many more animals (Wistar rats) using methods as similar as possible to the methods used by Planas and Kuć. In addition, also the male rats were administered the *Stevia* decoction in Shiotsu's experiments. Every day, the animals were observed for general condition. No effect on general condition, body weight, water consumption, live birth rate or litter size was found.

- In 2 studies with Wistar rats receiving crude extracts for 31 or 60 days (up to 260 mg stevioside or 5.3 g stevioside/ kg body weight) *Stevia* extracts did not show any adverse effects on reproduction. The authors observed a decreased seminal vesicle weight by about 60 % (at 5.3 g stevioside/kg body weight) but they concluded that if *Stevia* extract does have some potential to decrease rat fertility at all, this effect is almost certainly not exerted on the male. Unfortunately, only one very high concentration was tested and no dose-effect studies were undertaken.
- In contrast to the above results, it was reported that very concentrated *Stevia* extracts similar to those of the authors above (5.3 g stevioside/kg BW) fed for 60 days to prepubertal male rats produced a decrease in final weight of testes, seminal vesicle and cauda epididymidis (Melis, 1999). In addition, the fructose content of the accessory sex glands and the epididymal sperm concentration were decreased. *Stevia* extract treatment tended to decrease the testosterone level. No alteration occurred in luteinizing hormone level. Melis (1999) suggested a possible decrease of the fertility of male rats. His results contradict those of Oliveira-Filho *et al.* (1989) who applied extracts with similar stevioside content, and these authors stated that there was certainly no effect on male fertility. The difference between the extracts of Oliveira-Filho *et al.* (1989) and Melis (1999) might be that the last author completely dried the *Stevia* water extracts, and it is known that various chemical alterations may occur in the extract during drying that probably were responsible for the observed effects. So it is not certain that the observed effects were indeed due to the stevioside present in the extract. It should also be mentioned that the extract concentrations were extremely high, at the start of the experiments around 5.34 % of the body weight (or around 5.3 g stevioside/kg BW). For an adult person of 65 kg this means 3.47 kg of dry *Stevia* leaves (± 347 g stevioside per day) or about 34.7 kg fresh leaves/day, i.e. more than 50% of his body weight. The significance of such experiments in which only one extremely large concentration was tested, should be questioned. Melis' results are also contrary to the other cited studies that did not reveal any effect on fertility of male or female animals.

After all, the results obtained with crude extracts are not comparable to those obtained with purified steviol glycosides which had no harmful effects. These results were given for the completeness of the dossier.

- Prenatal developmental toxicity

a) Steviol glycosides

- In several studies with rats, steviol glycosides, even very high doses up to 2100 (males) or 2400 (females) mg/kg body weight, did not provoke abnormalities found in the growth, general appearance and behaviour, viscera or skeleton of rat foetus.
- Chicken embryos are an isolated system and react very sensitively to administered toxicants, eg. LD_{50} values of 0.6, 8.8 and 5592 µg/kg egg were published for polychlorinated biphenyls. Fertile broiler eggs (Ross) were injected on day 7 with stevioside (> 97% purity, impurities being rebaudioside A, 2.7% and steviolbioside, 0.3%, 0.08, 0.8 or 4 mg/egg). From the results it was clear that injection of stevioside at day 7 of incubation i.e. during the critical developmental stages of the embryo (organogenesis) had no detrimental effects on hatchability and did not affect body weight or organ weights of the one-day old chick. The hatchlings developed and behaved normally. It was concluded that prenatal exposure to stevioside was not toxic to the chicken embryo.

b) Steviol

Administered stevioside has no effect on fertility, mating performance, pregnancy, number of foetuses, or on the growth and fertility of the offspring. However, when steviol (the aglucone of stevioside) was given to hamsters, the most sensitive animals, on day 6-10 of pregnancy at doses of 500-1000 mg/kg body weight/day, it induced toxicity. The number of live foetuses per litter and mean foetal weight decreased. The maternal kidneys showed a dose-dependent increase in severity of convoluted tubules in the kidneys. The NOAEL for maternal and foetal toxicity was 250 mg/kg BW/day. This study with steviol has nothing to do with the use of stevioside as a sweetener. When stevioside is fed to hamsters, no toxic effects were found, not even in 3 successive generations. When steviol is given in the feed, it can be resorbed directly by the intestines, whereas stevioside is not.
In the assay with chicken embryos injection of steviol at day 7 (99% purity, 0.025, 0.25 or 1.25 mg/egg) of incubation, had no detrimental effects on hatchability and did not affect body weight or organ weights of the one-day old chick. The hatchlings developed and behaved normally. It was concluded that prenatal exposure to steviol was not toxic to the chicken embryo.

Postnatal developmental toxicity

Even the highest dose of stevioside (2.5 g/kg BW) had no effect on growth or reproduction in hamsters in 3 generations.

In experiments on the development of chicken embryos, there were no differences in growth or general appearance after birth and all the hatchlings behaved normally (Geuns et al., 2003c). Both stevioside and steviol were tested.

Additives for infant formula: a special case

Steviol glycosides are not intented for use in infant formulae, follow-on formulae or weaning foods.

3.2 Other studies

Bio-availability of nutrients from the diet.

Modern broiler chickens are intensively selected for growth rate, and their body weight increases with a factor of more than 50x, from 50 g to over 2.5 kg in a time span of 6 weeks, making these animals especially suited to study the influence of food additives on growth. However, they have become very susceptible to even slight deviations from optimal environmental and nutritional conditions. If such aberrations occur, this is readily reflected in feed intake and growth rate. An other advantage in using broiler chickens is that they do no taste sweetness. As the supplementation of stevioside in the feed did not affect the growth parameters or feed conversion, it can be inferred that stevioside did not influence the uptake of other essential nutrients such as amino acids, vitamins, minerals etc.

In a similar way, steviol glycosides did not influence the growth parameters of rats or hamsters. In all of the above cited experiments, no indications of any influence on the bio-availability of nutrients, or on physiological effects were found.

Stevioside and caries

Although rather high concentrations of stevioside and *Stevia* extracts were shown to reduce the growth of some bacteria, the concentrations used for sweetening purposes are rather low. Therefore, the beneficial effect of the use of stevioside might be due to the substitution of sucrose in the food by a non-cariogenic substance.

Pharmacological effects

Some pharmacological effects of very high doses (750 to 1500 mg/day) were observed as decreased blood pressure in hypertension, and lowering of blood glucose in hyperglycemia. However, these high doses to provoke pharmacological effects will never been reached by the steviol glycosides used as a sweetener as only low amounts will be used, estimated to be 5-10 x lower than the amounts producing the pharmacological effects. Even then, only beneficial effects have been described, without an indication for the possible occurrence of hypotension or hypoglycemia.

Nutritional, microbiological, toxicological, allergenicity problems?

Steviol glycosides are unlikely to give rise to nutritional, microbiological, toxicological and/or allergenicity problems. The daily use of *Stevia* by about 150 million people in eg. Paraguay, Japan, Brasil, USA, Korea, Thailand and other people has never led to the demonstration of problems of this kind. In the literature, no reports on detrimental effects of either the living plants, or the dried leaves or stevioside can be found.

- Own conclusions about safety-in-use

- Number given to steviol glycosides by the Codex Alimentarius is (E-) 960.

- Many toxicological studies with steviol glycosides have been performed by different independent laboratories in different countries. Nearly all the studies confirm the safety of steviol glycosides as food additive.

- Absorption and metabolism studies revealed that the uptake of steviol glycosides is extremely low. Enzymes of the digestive tract are not able to degrade stevioside into steviol, nor is the stomach juice. Only the group of bacteroides of the colon are able to degrade stevioside to steviol. Some of this steviol is excreted with the feces, the rest is absorbed by the colon and glucuronated in the liver. The kidneys filter the steviol glucuronide into the urine and it is excreted. No accumulation of derivatives occurs in the body. Besides steviol glucuronide, no other derivatives could be detected. In the blood only steviol glucuronide was detected, but no free steviol.

- Acute and chronic toxicity studies showed that steviol glycosides have a very low toxicity. No toxicity was observed in chronic tests.

- Genotoxicity studies revealed that steviol glycosides did not induce gene mutations in bacteria. However, after metabolic activation, it was shown that so far unknown steviol metabolites caused mutations in a very sensitive strain of *Salmonella typhimurium* TM677, but not in other strains or in other bacteria.

- Steviol glycosides did not induce gene mutations in mammalian cells *in vitro.* However, after metabolic activation of steviol (99 % purity), some gene mutation and chromosomal aberration was found in Chinese hamster lung fibroblasts at high concentrations above 300 µg/ml that, however, will never been reached in the plasma, as the administration of high doses up to 750 mg daily did not reveal traces of steviol in the blood. However, no sign of enhancement of mutation frequency by steviol was observed in the mouse lymphoma L5178Y $tk^{+/-}$-3.7.2C gene mutation assay (MOLY), whereas the positive control showed its expected stimulation.

- Steviol glycosides as well as steviol were inactive in all the different chromosome aberration tests used.

Because of the positive score in 2 genotoxicity tests at relatively high concentrations, free steviol was also tested *in vivo* in different animal systems. Never any signs of harmful effects were found. This corresponds well with the metabolism studies with volunteers and the lack of traces of steviol in the blood. Therefore, the general conclusion is that the use of steviol glycosides as a sweetener is safe. Anyhow, in animal models it was shown that high doses of steviol glycosides inhibited the tumour formation. However, due to the extremely low absorption by the intestines and the low amounts needed for sweetening purposes, the advantages of oral steviol glycosides as chemopreventive agent seem to be less relevant.

- In chronic toxicity and carcinogenicity studies with steviol glycosides and free steviol, and using different animal models, never harmful effects were observed. There were no observable effects of steviol glycosides or steviol on reproduction nor on developmental toxicity. The study by Planas and Kuć (1968) has, in the past, led to some confusion and controversy on fertility. However, these results were refuted by Shiotsu (1996) who did more reliable experiments with many more animals. Many other authors have described the lack of effects of steviol glycosides in male and female reproduction. The significance of the administration of only one extremely large dose of crude stevia extracts to young rats, should be questioned. In this case, extracts from an amount of fresh stevia leaves of more than 50% of the body weight were daily administered to the young rats.

- In various studies on prenatal developmental toxicity, steviol glycosides and steviol were without effect, even when very high doses were administered, nor was there any effect on postnatal developmental toxicity.

- Steviol glycosides are not intented for use in infant formulae, follow-on formulae or weaning foods.

- Other studies revealed that there was no effect on bio-availability of nutrients from the diet. There is a clear beneficial effect of the use of steviol glycosides on caries formation, most probably due to the substitution of sucrose in the food by a non-cariogenic substance.

- Although there are pharmacological effects of high doses of steviol glycosides on blood glucose lowering and the lowering of hypertension, these effects will not occur when steviol glycosides are used in low doses for sweetening purposes.

- Steviol glycosides are unlikely to give rise to nutritional, microbiological, toxicological and/or allergenicity problems.

- A dietary exposure assessment estimated that for the majority of consumers the ADI (0-4 steviol equivalents) is not exceeded when steviol glycosides were added to the range of foods requested in the application. The estimated exposure for high consumers (children aged 2-6 years) marginally exceeded the ADI. However, this estimate is based on very conservative assumptions and when a dietary exposure estimate was undertaken with concentrations of steviol glycosides that reflect a more realistic level of use, it was estimated that dietary exposure for high consumers (children aged 2-6 years) was only 50% of the ADI. Moreover, the ADI value used is rather low as much higher values might be considered as suggested by Table 19.

Conclusion:

- The toxicological studies reveal that steviol glycosides are safe and should be authorised as food additives in Europe. There are no public health and safety concerns for "EUSTAS stevia glycosides" when used as a food additive at the maximum levels proposed by the applicant. There will be a continuous follow-up of the quality on the European market. Moreover, the group of steviol glycosides is not a new food additive and is already approved in many countries. It can be estimated that **daily** probably about 150 million of people consume steviol glycosides as such or as components of dried *Stevia* leaves (consumption in Thailand taken as a reference, see Enclosure 1).

- The introduction of steviol glycosides on the European market would have many benefits for the consumer by a reduction of energy intake, the possibility to consume all natural products and even totally organic meals are possible. Moreover, steviol glycosides are very sweet, and only low amounts need to be used. The steviol glycosides are very stable and can be cooked and baked without breakdown. They are also safe for PKU patients.

- There will also be a beneficial impact in the whole EU, translated in social benefits, health benefits with reduction of costs of medical care, and of course there are large economic and industrial benefits. Besides the possibility of growing *Stevia* in regions where now tobacco is grown, there will be job creations in European agronomy, research institutes and the food industry. There is also a beneficial environmental impact as sustainable techniques can be used for growing and processing *Stevia*. Besides this, the European companies will gain competitiveness with extern companies in using and developing products derived from *Stevia*, certainly now that steviol glycosides are already approved in many countries, and approval being expected in Australia and New Zealand, and the intention of Coca-Cola to use rebaudioside A in their soft drinks with, in addition, many health claims.

- Already on May 23rd 2007, the Food Standards of Australia New Zealand decided to publish the draft of its assessment report including an authorisation on its website. In this document they explicitly write that they did not wait until after JECFA had evaluated additional studies on potential pharmacological effects of steviol glycosides, as FSANZ considered the safety of steviol glycosides used as a sweetener completely proven. The draft of this document is also attached. There is also a very good document by Dr. O'Calaghan (2006) who concluded that there were no risks in using steviol glycosides. He made a study of published literature on pharmacological effects. As a specialist in hyper- and hypotension, he concluded that there is no risk in using Stevia, even not in case of hypotension. FSANZ has concluded that steviol glycosides are well tolerated and unlikely to have adverse effects on blood pressure, blood glucose or other parameters in normal, hypotensive or diabetic subjects at doses up to 11 mg/kg bw/day. The adequacy of the existing database and a new study in humans provides a basis for revising the uncertainty factors that were used by JECFA to derive the temporary ADI for steviol glycosides in 2005. In particular, the evidence surrounding the pharmacological effects of steviol glycosides on blood pressure and blood glucose has been strengthened so that the additional 2-fold safety factor for uncertainty related to effects in normotensive or diabetic individuals is no longer required. Therefore, a full ADI of 4 mg steviol equivalents/kg bw/day, derived by applying a 100-fold safety factor to the NOEL of 970 mg/kg bw/day (equivalent to 383 mg/kg bw/day steviol) in a 2-year rat study, has been established.

- In 2003, *Stevia rebaudiana* was approved for use as an active and/or excipient ingredient in Listed medicines in Australia (FSANZ, 2007). Stevioside is permitted in Listed medicines only in conjunction with the use of *Stevia rebaudiana* (it is not approved as an ingredient in its own right). There have been no known adverse effects for stevioside reported to the Therapeutic Goods Administration to date. This is very important information coming from FSANZ itself.

Although during the last JECFA meeting (June 2007) it was decided to extend the **temporary** ADI of steviol glycosides to 2008, there is enough information available to approve steviol glycosides as a sweetener only. This implies that in advertising no mention should be made of pharmacological effects that do not occur when using low doses for sweetening purposes. The lack of information is compensated for by the world-wide use of steviol glycosides and the daily exposure of about 150 million of consumers in the countries where this food additive is approved.

From an extended review of the scientific literature, an ADI value between 0 and 10 mg steviol equivalents/kg BW can be suggested. JECFA suggested a temporary ADI of 0-2 steviol equivalents (safety factor 200x). FSANZ fixed a value of 0-4 mg steviol equivalents/kg BW.

Because of the strictly described specification and the quality control by EUSTAS, the lack of risks for the consumers and their general interests in the natural sweetener in different countries, because of the safe use in many other countries outside the EU (about 150 million people daily consume Stevia or stevioside), and the huge possibilities for European agronomy and food industry, we propose to approve “EUSTAS® stevia glycosides” as food additive in Europe and fix an ADI value of 0-4 mg steviol equivalents/kg BW.

PART I ADMINISTRATIVE DATA

1. Name of petitioner

Since a few years, the European Stevia Association (EUSTAS, www.eustas.org) has been founded.

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Maladeta, nº 20,
22300 BARBASTRO, Huesca,
SPAIN
Tel: +34 974 311 478
Fax +34 974 311 359
Email: info@eustas.org

2. Name of manufacturers/importers:

As there are not yet manufacturers in Europe, the high quality Stevia glycosides will be imported by:
1) Granular AB; Carl Horn af Rantzien; Stora Ängsholmen; S-178 93 Drottningholm; Sweden
Tel. +468 759 0808
Fax: +468 759 0809
Email: carl@kambrium.se

2) Medherbs; Peter Grosser; Aunelstrasse 70, D-65199 Wiesbaden; Germany
Tel: +49-611-8460015; Fax: 0049-611-2046900
Email: info@medherbs.de

3) Anagalide SA; José-Cruz Cavero Buera; Calle Maladeta, 20; 22300 Barbastro (Huesca); Spain
Tel: +34 974 311 478
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4) Reisenberger GmbH; Ing. Franz Reisenberger; Stuttgarterstrasse 2; 2380 Perchtoldsdorf; Austria
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5) Stepaja bvba
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3. Name of person responsible for the dossier:

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