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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD

ON

GLYCYRRHIZINIC ACID AND ITS AMMONIUM SALT

(opinion expressed on 4 April 2003)

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Opinion of the scientific committee on food on glycyrrhizinic acid and its ammonium salt

[Please note that more details on the use levels in food and on individual studies together with their reference citations can be found on the Annexes of this document]

Terms of Reference

The Committee is asked to consider if the opinion of the Committee expressed in 1991 on glycyrrhizin is still valid in the light of additional information resulting from toxicological and clinical studies published since then on both glycyrrhizinic acid and its salts. The Committee is asked to take into account dietary exposure from all known sources, including contributions due to its natural occurrence in liquorice and through the ingestion of food products to which it is added as a flavouring substance.

The Committee is also asked to evaluate ammonium glycyrrhizinate as a chemically defined flavouring substance for the possible acceptability of its inclusion in the Community Register.

Previous evaluations

Glycyrrhizinic acid has been given Generally Recognized as Safe (GRAS) status in the USA in 1985, however with upper limits for use levels in foods (Anonymous, 1985).

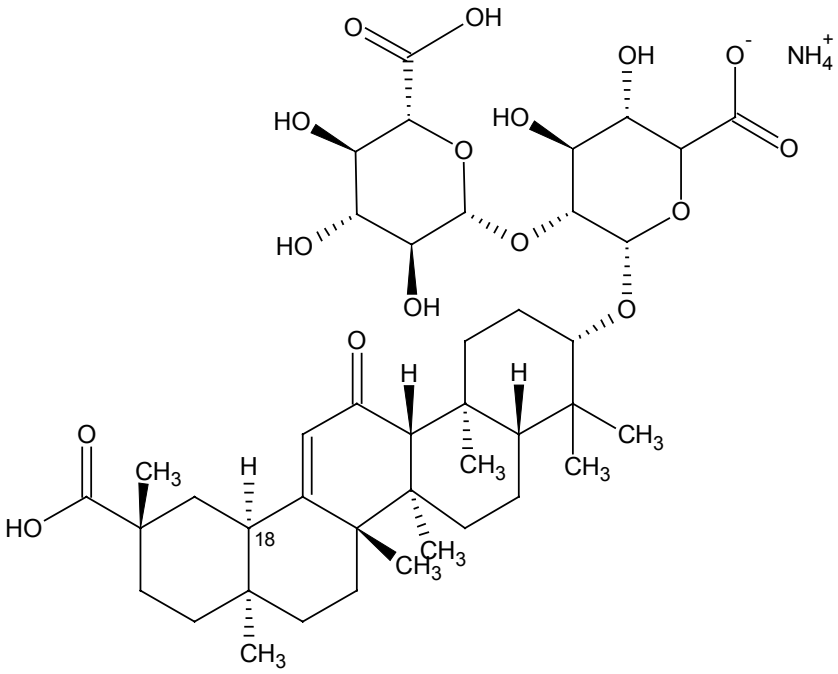
The Committee has considered the use of glycyrrhizinic acid as a sweetener in 1985. At that time the Committee was unable to endorse the use of the substance as a sweetener. In 1991 the Committee has evaluated the toxicological information obtained in human volunteer studies and the clinical information on glycyrrhizinic acid and concluded that the data were inadequate to derive an ADI. The data did indicate a concern for some sectors of the population, especially those suffering from hypertension. The Committee considered it prudent that regular ingestion should not exceed 100 mg/day, while it was explicitly mentioned that this was a provisional figure, which should be updated when new data

would become available. It was recognised that studies in human volunteers were in progress, the results of which were expected to be highly relevant (SCF, 1991).

In 1993 the Nordic Council of Ministers published an evaluation on glycyrrhizinic acid (Størmer *et al.*, 1993a). These authors concluded that: *"it appears that in the most sensitive individuals adverse effects occur at a regular daily intake of about 100 mg glycyrrhizinic acid. A regular daily intake of 100 mg/day was, established as a provisional LOAEL for adults."* In a subsequent publication, these authors advocated an ADI for glycyrrhizinic acid of 1-10 mg/person/day by applying an uncertainty factor of 10 to the above mentioned lowest-observed-adverse-effect level (LOAEL) (Størmer *et al.*, 1993b)

Chemical Identity

This document gives an evaluation of glycyrrhizinic acid and its monoammonium salt, which has the following chemical structure:



Substance Name	Glycyrrhizinic acid
Chemical name (IUPAC name not identified)	(3-beta, 20-beta)-20-carboxy-11-oxo-30-norolean-12-en-3-yl 2-O-beta-D-glucopyranuronosyl-alpha-D-glucopyranosiduronic acid
synonyms	Glycyrrhizin
CAS nr	1405-86-3
EINECS nr	215-785-7
FL nr	16.012
Molecular formula	$C_{42}H_{62}O_{16}$
Molecular weight	822.94 D*

* no information is available whether glycyrrhizinic acid may contain crystal water.

Substance Name	Ammonium glycyrrhizinate
Chemical name (IUPAC name not identified)	(3-beta, 20-beta)-20-carboxy-11-oxo-30-norolean-12-en-3-yl 2-O-beta-D-glucopyranuronosyl-alpha-D-glucopyranosiduronic acid, monoammonium salt
synonyms	Glycyrrhizinic acid, ammonium salt
CAS nr	53956-04-0
EINECS nr	258-887-7
FL nr	16.060
Molecular formula	$C_{42}H_{61}O_{16}\cdot NH_4$ (anhydrous)
Molecular weight	839.96 D (anhydrous)

Glycyrrhizinic acid is a naturally occurring triterpenoid saponin, which can be found in extracts of roots and rhizomes of the Liquorice plant *Glycyrrhiza glabra*, together with a number of other substances including other triterpenoids, polyphenols, polysaccharides essential oils and flavonoids. The crude dried aqueous extracts (also known as "block liquorice") may contain 4-25% glycyrrhizinic acid in the form of calcium, magnesium and potassium salts (Wang *et al.*, 2000; Størmer *et al.*, 1993a; EFFA, 2001). The ammoniated salt is manufactured by acid treatment of the aqueous extracts, followed by neutralisation

of the precipitated material with diluted ammonia. The monoammonium salt is then further purified by solvent extraction and other separation techniques (EFFA, 2001). Glycyrrhizinic acid can be considered as the di-glucuronic acid conjugate of glycyrrhetic acid. This aglycone part of the molecule may occur in two forms, (18-alpha- and 18-beta-). Although the 18-alpha form has been found in amounts up to 13% of the total glycyrrhetic acid present, it is not clear whether this form occurs naturally or whether it is only formed during processing due to isomerisation (Størmer *et al.*, 1993a).

Both glycyrrhizinic acid and ammonium glycyrrhizinate are in the Community register of chemically defined flavouring substances, adopted in Commission Decision 1999/217/EC (EU, 1999) and later amended. They are used because of their sweet taste (33-200 times sweeter than sucrose; Størmer *et al.*, 1993a; EFFA, 2001). As required by Commission Regulation (EC) No.1565/2000 (EU, 2000), specifications for marketed flavouring substances should be provided. Almost complete specifications were provided for ammonium glycyrrhizinate (for solubility in water only a qualitative statement was given; no data were provided for solubility in ethanol), but no specification data were provided for glycyrrhizinic acid.

Exposure Assessment

People may be exposed to glycyrrhizinic acid via food or other products that contain either the purified acid or its ammonium salt or via food in which the dried crude root extract of *Glycyrrhiza glabra* has been incorporated. Exposure to glycyrrhizinic acid may occur not only via the consumption of liquorice confectionery and sweets, but also through beverages, chewing gum, tooth paste and medicinal products. Other sources of intake may be sucking/chewing on dried *Glycyrrhiza* roots or chewing or smoking tobacco products.

<u>Exposure from the use as chemically defined flavouring substances</u>

The Upper Use Levels (UULs) of ammonium glycyrrhizinate and glycyrrhizinic acid in foods from various categories, as specified by EFFA (2001, 2003), are given in Annex I. According to EFFA (2001, 2003), the annual volumes of ammonium glycyrrhizinate and glycyrrhizinic acid for use as a chemically defined food flavouring substances are 1070

and 1965 kg, respectively. Based on these figures the Maximised Survey-Derived Intake (MSDI) [i] for ammonium glycyrrhizinate is (= 130 μg/person/day (~1.8 μg/kg bw/day) and the MSDI for glycyrrhizinic acid is 240 μg/person/day (~3.4 μg/kg bw/day).

When compared to the UULs for these substances, the MSDI calculations may result in unrealistically low intake figures for individuals who select to consume certain foods, flavoured at the UULs (see Annex I). For example, intake of 50 grams of candies, flavoured with ammonium glycyrrhizinate at the UUL would lead to an intake of ammonium glycyrrhizinate of 77.5 mg, while drinking of 1 liter of a glycyrrhizin-flavoured drink would provide an intake of 200 mg glycyrrhizinate. Both figures exceed the calculated MSDI by 3 orders of magnitude. Similarly, for glycyrrhizinic acid, consumption of 250 g of dairy products or edible ices would for both examples lead to an intake of 94 mg. For this substance, consumption of 20 g of fondant may already result in an intake of 100 mg.

Exposure via intake of liquorice confectionery

Glycyrrhizinic acid content

Størmer *et al.* (1993a) have given glycyrrhizinic acid levels which were determined in confectionery products marketed in Germany, Belgium and the United Kingdom. Glycyrrhizinic acid concentrations ranged from 0.29 to 7.9 mg/g liquorice confectionery; in the UK two products were above 3.5 mg/g and in Germany three products were above 4.2 mg/g. Certain "health" products such as liquorice-flavoured diet gum, and throat pearls may contain 15 and 47 mg/g, respectively**.**

In the Netherlands the mean glycyrrhizinic acid percentage in liquorice is 1.7 mg/g, based on an analysis of 19 samples, ranging from 0.3 to 5.1 mg/g. In 3 additional samples ("liquorice powder" and two brands of "bay liquorice") levels of 18 and 25-28 mg/g were found, respectively. "Bay liquorice" consists of 100% dried *Glycyrrhiza* root extract (Maas, 2000).

[i] Calculated as follows: [annual production volume * 10^9 μg]/ [population*survey correction factor*365 days]; with population = 10% (eaters only) of $375*10^6$ and survey correction factor = 0.6 (for under-reporting).

Consumption of liquorice confectionery and exposure to glycyrrhizinic acid

For Norway, Denmark, Iceland and Sweden average yearly consumption estimates of 1 to 2.5 kg of liquorice confectionery have been reported (Størmer *et al.*, 1993a). This average has been calculated for the entire population of consumers plus non-consumers.

The consumption of liquorice confectionery is not evenly distributed in the population. This is demonstrated by the intake data for liquorice confectionery in the Dutch population, based on the results from the 3rd Dutch food consumption survey (Kistemaker *et al.,* 1998). About 10% of the population that participated in this survey consumed liquorice confectionery at least once during the two days[ii]. The distribution of intake of liquorice confectionery within this group of regular consumers is shown in the table below:

percentile of regular consumers population	**Intake of liquorice confectionery (g/day)**	**intake of glycyrrhizinic acid* (mg/day)**
0	0,5	1
10	2	3
20	3	5
30	4	7
40	5	8
50	6	10
60	8	13
70	10	17
80	15	25
90	25	42
100	187.5	313

* calculation based on a mean glycyrrhizinic acid contents in liquorice confectionery of 0.17%

[ii] The survey method used is known to underreport consumption in occasional users. Hence the proportion of all consumers of liquorice (i.e. all eaters) in the entire Dutch population may be considerably larger. Additional data from the second survey (Hulshof and Kistemaker, 1995) indicated that about 60% of the population may eat liquorice at least once per month.

The average daily consumption among regular consumers was 11.5 g. Using the data of Maas (2000) that the mean content of glycyrrhizinic acid in liquorice confectionery in the Netherlands was 0.17%, this amount of would correspond to a daily intake of 19 mg glycyrrhizinic acid. About 3% of the regular consumers ate more than 50 g of liquorice confectionery per day. Sweet liquorice confectionery was the most popular type and the number of consumers of extra salted liquorice confectionery as well as the amounts consumed thereof were smaller. According to these data, about 2% of the regular consumers have a daily intake of glycyrrhizinic acid of over 100 mg/day (the maximum intake level, provisionally derived by the Committee in 1991).

Exposure via other sources

Herbal teas

Exposure to glycyrrhizinic acid via herbal teas may contribute significantly to the total daily intake of this substance. Maas (2000) described that of 33 brands of herbal teas, 13 contained < 10 mg/l, 16 contained 10 to 100 mg/l and 4 contained > 100 mg/l with a maximum of 450 mg/l (concentrations refer to the prepared beverage). Among these 33 brands, for 10 brands the label information stated liquorice plant material as ingredient, and for these teas the average glycyrrhizinic acid content was 126 mg/l (range 2 - 450 mg/l).

Beverages

Maas (2000) described that of the 18 brands of (herbal) alcoholic beverages studied, 6 contained < 10 mg/l, 2 contained 10 to 100 mg/l and 10 contained > 100 mg/l (range for all beverages: not detectable to 422 mg/l). Two non-alcoholic herbal drinks were also studied. One was a liquorice-syrup, which contained 411 mg glycyrrhizinic acid/l. In the other one no glycyrrhizinic acid could be detected (Maas, 2000).

Chewing gum

Based on the information provided in one case study, a brand of chewing gum is marketed which contains glycyrrhizinic acid in a concentration of 0.15% in a package size of 16 g. Consumption of the chewing gum in such a package in one day would result in a (maximum) intake of 24 mg of glycyrrhizinic acid/day (Rosseel and Schoors, 1993).

Tobacco products

Glycyrrhizinic acid in the form of liquorice extract (up to 4%) is also added to tobacco products, including chewing tobacco. Although exposure to glycyrrhizinic acid via chewing tobacco may elicit symptoms of pseudohyperaldosteronism (Blakey, 1998), for smoking tobaccos extensive exposure to glycyrrhizinic acid is not likely because of pyrolysis (Hoffmann and Hoffmann, 1977).

Hazard Assessment

The following text is a summary of the effects of glycyrrhizinic acid and its ammonium salt. More details on the individual studies, together with their reference citations, are contained in Annex II.

Glycyrrhizinic acid itself is hardly absorbed from the gastro-intestinal tract. Before absorption, glycyrrhizinic acid is hydrolysed to give glycyrrhetic acid, which is the ultimate biologically active metabolite. Bioavailability studies for glycyrrhetic acid have indicated that absorption from solutions (either as substance or as glycyrrhizinic acid) or from glycyrrhizinic acid in a liquorice matrix is equally effective and virtually complete. Due to excretion via the bile the substance is subject to entero-hepatic circulation, which may lead to prolonged maintenance of pharmacologically active plasma levels, especially in persons with slow gastrointestinal transit.

Glycyrrhetic acid is an inhibitor of the enzyme 11-beta-hydroxysteroid dehydrogenase-2 (11-BOHD-2), and via this inhibition it causes a cortisol-dependent increased sodium and water retention and an increased potassium excretion. When compensatory mechanisms (i.e. suppression of the renin-angiotensin-aldosterone axis) are surpassed, pseudohyper-aldosteronism may result, which is reflected in oedema, hypokalaemia and increased blood pressure. It can be concluded that not the acute but the repeated intake of glycyrrhizinic acid causes the effects and clinical symptoms may develop within exposure periods of a few days to weeks.

For the evaluation of the biological effects of glycyrrhizinic acid many studies in humans have been published, in addition to animal toxicity data. Based on all available data,

notwithstanding some *in vitro* and *in vivo* weak positive findings of questionable biological meaning, glycyrrhizinic acid and its hydrolysis product glycyrrhetic acid are considered to be non-genotoxic. In a limited chronic study with mice, no carcinogenic potential was detected. No carcinogenicity study in rats is available**.** In one developmental toxicity study with rats, indications were obtained for increased incidences of skeletal variants and ectopic kidneys at a dose level of 680 mg/kg bw/day (highest dose tested). Maternal effects were limited to increased water intake and a slight statistically insignificant decrease of plasma potassium. The authors of the study considered the findings of questionable relevance. However, in another study with rats at dose levels up to 1480 mg/kg bw/day the changes could not be confirmed, despite signs of (slight) maternal toxicity. An epidemiological study has indicated that while consumption of glycyrrhizinic acid at >499 mg/week is associated with a slight reduction in the length of the gestation period, teratogenicity was not associated with intake of glycyrrhizinic acid at levels up to 2464 mg/week.

The available information indicates that the pharmacological effects in humans and animals are similar (perturbation of sodium/potassium/water homeostasis) and in the past human dose-response data, mainly from case studies have been used to derive (provisional) recommendations with respect to maximum daily intake of glycyrrhizinic acid. Recently, two repeated-dose studies in human volunteers have been published from which two no-observed-adverse-effect levels (NOAELs) of 2 mg/kg bw/day (Bijlsma *et al.*, 1996) or 217 mg/person/day (Bernardi *et al*., 1994) can be derived. The NOAEL obtained in the study by Bijlsma *et al.* (1996) is considered the more appropriate because this study comprised larger groups of volunteers, included a placebo control group, and the exposure lasted for a longer period. At the next dose level above the NOAEL of 4 mg/kg bw/day (the highest dose tested), in 9 out of 11 volunteers, water retention, slight decreases in plasma potassium and suppression of the renin-angiotensin-aldosterone axis was observed. In addition in 1/11 volunteers clinical effects were observed as well.

Using a PBPK/PD model, Ploeger (2000a) predicted that at the intake level of 100 mg/day, 4 per 10^4 exposed persons (95% confidence limits: 4.6 per 10^6 - 3 per 10^2) might show signs of “pseudohyperaldosteronism”, but these should be considered as preliminary

results, because they were largely dependent on one fairly small study with human volunteers. 100 mg/day was the intake provisionally established by the Committee in 1991 that should not be exceeded on a regular basis. The model by Ploeger (2000a) also provides insight into the determinants of differences in sensitivity in humans. Gastrointestinal transit time, sensitivity of the target enzyme (11-BOHD-2) to glycyrrhetic acid and basal 11-BOHD-2 activity seem to be the most important determinants. Although there is no direct evidence, from the biomedical literature (Rose, 1994; Stewart, 2002) it is conceivable that the health of people with Cushing's syndrome, or other conditions related to hypertension, abnormal electrolyte or water homeostasis, may be adversely affected by exposure to glycyrrhizinic acid or its ammonium salt.

Conclusion

Previously, the Committee evaluated the toxicological information for glycyrrhizinic acid and concluded that the data were inadequate to derive an ADI (SCF, 1991). At that time, the Committee considered it prudent that regular ingestion should not exceed 100 mg/day, while it was explicitly mentioned that this was a provisional figure. Since then, new toxicological information, including data from human volunteer studies, has become available. Although these data provide a stronger basis for the upper limit for regular ingestion of glycyrrhizinic acid of 100 mg/day, the Committee still is of the opinion that an ADI for glycyrrhizinic acid and ammonium glycyrrhizinate cannot be derived, because the new human toxicity studies are too limited (small experimental groups, short duration). The Committee considers that this upper limit for regular ingestion of 100 mg/day provides a sufficient level of protection for the majority of the population. It is noted that this upper limit includes the intake of glycyrrhizinic acid via all products, liquorice confectionery as well as glycyrrhizinic acid- or ammonium glycyrrhizinate-flavoured products.

At the same time, the Committee realises that within the human population there are subgroups for which this upper limit might not offer sufficient protection. These subgroups comprise people with decreased 11-beta-hydroxysteroid dehydrogenase-2 activity (the target enzyme of glycyrrhizinic acid, for which genetic polymorphisms resulting in reduced basal activity have been described), people with prolonged gastrointestinal transit time, and people with hypertension or electrolyte-related or water homeostasis-related medical

conditions. A more extensive discussion on sensitive subgroups in the population can be found in Annex II (section on modeling "Pharmacokinetic-pharmacodynamic model").

The Committee notes that for ammonium glycyrrhizinate as well as for glycyrrhizinic acid, used as chemically defined flavouring substances, the Upper Use Levels in foods indicate that the Maximised Survey-Derived Intake (MSDI) exposure estimates (130 and 240 μg/person/day, respectively) may underestimate the intake for individuals who select to consume certain foods, e.g. foods flavoured at the UULs (see Annex I).

To complete the evaluation of glycyrrhizinic acid and ammonium glycyrrhizinate as chemically defined flavouring substances, the following information needs to be provided:

- for glycyrrhizinic acid: specifications as required by Commission Regulation (EC) No. 1565/2000.
- for ammonium glycyrrhizinate: adequate data on solubility, as required by Commission Regulation (EC) No. 1565/2000.
- data on occurrence of the 18-alpha isomer in the commercial product. The Committee notes that glycyrrhizinic acid and ammonium glycyrrhizinate are evaluated here irrespective of their chirality.
- for both glycyrrhizinic acid and ammonium glycyrrhizinate: more refined usage data (e.g market share data), as it seems these substances are used in many food categories, but within a given food category probably only in very few products.

References

Anonymous (1985). 21 CFR Parts 182 and 184 GRAS status of licorice, (Glycyrrhiza), Ammoniated glycyrrhizin and monoammonium glycyrrhizinate. Federal Register **50**, 21043-21044.

Bernardi, M., D'Intino, P.E., Trevisani, F., Cantelli-Forti, G., Raggi, M.A., Turchetto, E., Gasbarrini, G. (1994). Effects of prolonged ingestion of graded doses of liquorice by healthy volunteers. Life Sciences, **55**, 863-872.

Bijlsma, J.A., Van Vloten P., Van Gelderen C.E.M., Mensinga Tj.T., Mout, H.A., Elvers L.H., Van Leeuwen, F.X.R., Stolker, A.A.M., Van Ginkel, L.A., Looman, C.W.N., Van der Maas, P.J., Koomans, H.A. and Savelkoul, T.J.F. (1996). Onderzoek naar de effecten van verschillende doseringen glycyrrhizine bij gezonde vrouwelijke vrijwilligers. [Study into the effects of different dosages of glycyrrhizin in health female volunteers; in Dutch]. RIVM report 348801004, RIVM, Bilthoven, The Netherlands

Blakey, S.A. (1998). Hypokalemia in a tobacco chewer: licorice link. J. Pharm. Pract., **11**, 78-79.

EFFA (2001). Ammonium glycyrrhizinate. European Flavour and Fragrance Association (EFFA). Submission to SCOOP Working group on Chemically defined Flavouring Substances, document No: SCOOP/FLAV/8.4, SCF document No: SCF/CS/ADD/MsAd/203

EFFA (2003). European Flavour and Fragrance Association (EFFA) Submission to the European Commission, February 20th, 2003.

EU (1999). Commission Decision of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs drawn up in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council of 28 October 1996 (notified under number C(1999) 399) (text with EEA relevance) (1999/217/EC). Official Journal of the European Communities, **L084**, 27/03/1999, 1 - 137.

EU (2000). Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council (Text

with EEA relevance). Official Journal of the European Communities, **L180**, 19/07/2000, 8-16.

Hoffmann, D. and Hoffmann, I.(1997). The chancing cigarette, 1950-1995. Journal of Toxicology and Environmental Health, **50**, 307-364.

Hulshof, K.F.A.M. and Kistemaker, C. (1995). The consumption of licorice and the intake of ammonium chloride among Dutch population groups (second Dutch National Food Consumption Survey). TNO-report V 94-599. July, 1995.

Kistemaker, C., Bouman, M. and Hulshof, K.F.A.M. (1998) De consumptie van afzonderlijke producten door Nederlandse bevolkingsgroepen. Voedselconsumptie-peiling 1997-1998. [The consumption of separate products by Dutch population sub-groups. Food Consumption Survey 1997-1998; in Dutch]. TNO-report V98.812, TNO-Voeding, Zeist, The Netherlands.

Maas, P.(2000). Zoethout in levensmiddelen: onderzoek naar het glycyrrhizine gehalte van thee, kruidenmengsels, dranken en drop. [Liquorice root in food stuffs: survey of the glycyrrhizin content of tea, herbal mixtures, alcoholic drinks and liquorice.; in Dutch]. De Ware(n) Chemicus, **30**, 65-74.

Ploeger, B.A. (2000a). Development and use of a physiologically based pharmacokinetic-pharmacodynamic model for glycyrrhizinic acid in consumer products. PhD thesis University of Utrecht.

Rose, B.D. (1994). Clinical physiology of acid-base and electrolyte disorders. McGraw-Hill, Health Profession Division, New York.

Rosseel, M. and Schoors, D. (1993). Chewing gum and hypokalaemia. The Lancet, **341**, 175.

SCF (1991). Reports of the Scientific Committee for Food (29th series). Commission of the European Communities, Food Science and Techniques. Report No EUR 14482 EN, CEC, Luxembourg.

Stewart, P.M. (2002). Adrenal cortex: renin-angiotensin-aldosterone axis and hypertension. In: Besser, G.M. and Thorner M.O. (eds) Comprehensive clinical endocrinology. Elsevier Scientific Publ. Amsterdam.

Størmer, F.C., Reistad, R., Alexander J. (1993a). Adverse health effects of glycyrrhizinic acid in liquorice. A risk assessment. Nordiske seminar- og Arbejdsrapporter 1993:526.

Størmer, F.C., Reistad, R., Alexander J. (1993b). Glycyrrhizinic acid in liquorice - evaluation of health hazard. Food and Chemical Toxicology, **31**, 303-312.

Van Gelderen, C.E.M., Bijlsma, J.A., Van Dokkum, W. and Savelkoul, T.J.F (2000). Glycyrrhizinic acid: the assessment of a no effect level. Human and Experimental Toxicolology, **19,** 434- 439.

Wang, Z.Y., Athar, M. and Bickers, D.R. (2000). Licorice in foods and herbal drugs: chemistry, pharmacology, toxicology and uses. In: Mazza, G. and Oomah B.D. (2000) Herbs, Botanicals and Teas. Technomic Publ. Co. Inc. Lancaster, UK. pp. 321-353.

ANNEX I

Upper Use Levels (UULs) for glycyrrhizinic acid and ammonium glycyrrhizinateas chemically defined flavouring substances in food

FOOD CATEGORIES		UUL*	
		Glycyrrhizinic acid	Ammonium glycyrrhizinate
1	Dairy products, excluding products of category 2	375	40
2	Fats and oils, and fat emulsions (type water-in-oil)		
3	Edible ices including sherbet and sorbet	375	95
4	Processed fruits and vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes), and nuts and seeds		
1	Fruit		
2	Vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes), and nuts and seeds		
5	Confectionery Chewing gum Sweets (candy) Fondant	$ 400 - 1500 - 5000	150 1550
6	Cereals and cereal products, including flours and starches from roots and tubers, pulses and legumes, excluding bakery		45
7	Bakery wares	200	65
8	Meat and meat products, including poultry and game	25	
9	Fish and fish products, including molluscs, crustaceans and echinoderms	20	300
10	Eggs and egg products		
11	Sweeteners including honey		100
12	Salts, spices, soups, sauce, salads, protein products, etc.		50
13	Foodstuffs intended for particular nutritional uses		60
14	Beverages, excluding dairy products	50	100@
1	Non-alcoholic ("soft") beverages	50	200@
2	Alcoholic beverages	135 - 550#	200@
15	Ready-to-eat savouries		150
16	Composite foods (e.g. casseroles, meat pies, mincemeat), foods that could not be placed in categories 1 to 15	10	80

* UULs are given in mg/kg product (EFFA 2001, 2003).

$ For these products only a rather poorly defined range of UULs was given.

A range of UULs was given for products like liquors, aromatised wines, aromatised wine-based drinks, aromatised wine-product cocktails. Spirits < 15% alcohol: coolers, light alcoholic drinks.

@ In several alcoholic and non-alcoholic drinks and in teas, levels of glycyrrhizinic acid have been detected which are higher than the UULs specified in this table (see main text, section on exposure). This is probably explained by the fact that these beverages and teas are not flavoured with glycyrrhizinic acid or ammonium glycyrrhizinate as such, but are produced with *Glycyrrhiza* plant extracts or with dried plant material.

References to Annex I

EFFA (2001). Ammonium glycyrrhizinate. European Flavour and Fragrance Association (EFFA). Submission to SCOOP Working group on Chemically defined Flavouring Substances, document No: SCOOP/FLAV/8.4, SCF document No: SCF/CS/ADD/MsAd/203

EFFA (2003). European Flavour and Fragrance Association (EFFA) Submission to the European Commission, February 20th, 2003.

ANNEX II

Overview of toxicological background information for glycyrrhizin and glycyrrhetic acid

Introduction

Since the opinion of the Committee 1991, important new information has become available. Not only the evaluation by the Nordic Council of Ministers (Størmer *et al.*, 1993a), but also new studies in human volunteers and some new case studies have been published. In addition, extensive physiologically based toxicokinetic-dynamic models have been developed, which contribute to a better understanding of the phenomena observed in humans exposed to glycyrrhizinic acid. Furthermore, studies have been published in which the matrix effect of liquorice on the availability of glycyrrhizinic acid has been studied.

In contrast to many other substances, for glycyrrhizinic acid (and several salts) a considerable amount of human data are available. A wealth of animal data are available, some of which is rather old, which contributes to the overall understanding of the effects that are elicited by these substances. The data also show that the effects observed in humans and animals are similar. Much of this information has been reviewed by Størmer *et al* (1993a) and Ploeger (2000a) and therefore it is only briefly mentioned here, with reference to these two sources. The opinion of the Committee of 1991 was based on human data. In the present document, only relevant animal data for toxicological end-points for which there are no (extensive) human data are presented.

Toxicokinetic and toxicodynamic data

Absorption

Glycyrrhizinate as such, whether taken as free acid or as ammonium salt is not or hardly absorbed from the GI tract. Only at very high dose levels is glycyrrhizinic acid detectable in rat or human plasma after oral administration. However, the substance is hydrolysed in the GI tract most likely by bacterial activity, and the free aglycone, glycyrrhetic acid (the biologically active form; see below), is readily absorbed and can be detected in plasma, even after low doses. The time at which maximum plasma levels of glycyrrhetic acid are reached after oral intake of glycyrrhizinic acid, is similar in rats and humans (12-16 h and

8-12 h, respectively). At higher dose levels (> 25 mg/kg) the rate of hydrolysis of glycyrrhizinic acid may become saturated, thereby limiting the relative amount of glycyrrhetic acid that can be absorbed from the GI-tract. In human volunteers, after oral administration of an extract of *Glycyrrhiza* plant material, absorption of glycyrrhetic acid was *ca* 75% compared to the absorption from orally administered pure glycyrrhizinic acid (Cantelli-Forti *et al.*, 1994). Other human data have indicated that the relative bioavailability of glycyrrhetic acid is independent of the form in which it was administered (either as pure aglycone, or as pure glycoside or as glycoside present in a liquorice matrix; Mensinga *et al.*; 1998 see below). According to data presented by Størmer (1993a), absorption of glycyrrhetic acid from the GI tract is virtually complete. Combination of the data from Størmer (1993a) and from Mensinga *et al.*(1998) would indicate that the absorption of glycyrrhetic acid from the gut is virtually complete, irrespective of the form (viz. glycoside or aglycone) or the matrix (pure substance or solid liquorice matrix) in which it is administered.

Pharmacokinetics of glycyrrhizinic acid in rat bile have been determined after a single oral administration. Peak levels of glycyrrhizinic acid in bile samples from rats treated orally with the pure substance (480 mg/kg bw) were approximately 5 times higher than peak levels observed after administration of the same amount of pure glycyrrhizinic acid in the form of a liquorice extract. In case of glycyrrhetic acid, concentrations were very low, often below the detection limit (0.2 μg/ml bile). Based on comparison of total biliary excretion over 16 h post-dosing, the study indicates that biliary excretion and therefore gastrointestinal absorption of glycyrrhizinic acid administered as pure substance is about 7-8 times higher than when administered as liquorice extract. No such comparison could be made for glycyrrhetic acid, the biologically acitive form. In addition, glycyrrhetic conjugates were not determined and in particular these are subject to entero-hepatic circulation. (Cantelli-Forti *et al*., 1997).

The Committee noted that the study provides only information on the biliary kinetics of glycyrrhizinic acid, administered in only one dose level at which intra-intestinal hydrolysis is likely to be saturated. It does not provide information on the kinetics of glycyrrhetic acid. The amount of glycyrrhizinic acid that was retrieved in the bile was only *ca.* 1% of the dose administered. The Committee considers the study by Mensinga *et al.* (1998) in human

volunteers, of greater importance.

Distribution, metabolism and elimination

Neither glycyrrhizinic acid nor glycyrrhetic acid are taken up by tissues to any major extent. The main compartment for distribution is the plasma, where they are bound to serum albumin. Human lysosomal enzymes can only hydrolyse one of the glucuronide moieties of glycyrrhizinic acid, while rat lysosomal enzymes can split off both glucuronide groups. From studies with bile duct cannulated rats it can be concluded that an intravenous dose of glycyrrhizinic acid is excreted predominantly unmetabolised via the bile. Only at very high dose levels, up to 5% of an iv dose can be found in the urine. With iv doses of glycyrrhetic acid, in rats it has been demonstrated that sulphate and monoglucuronide conjugates but hardly any parent substance are excreted into the bile. Complete biliary excretion of an intraperitoneal dose of glycyrrhetic acid could be demonstrated within 5 h post dosing. All of the dose had been metabolised. Enterohepatic circulation of glycyrrhetic acid has been demonstrated in rats. Human data with respect to biliary excretion are not available, but since glycyrrhetic acid metabolites can be hydrolysed by bacteria in the human gastro-intestinal tract, in humans such a circulation can be expected (Ploeger, 2000a). Time curve data for plasma glycyrrhetic acid in humans have also provided evidence that in humans enterohepatic circulation does occur (Mensinga *et al.,* 1998; see below*).*

Toxicodynamic data

The most prominent effect of glycyrrhizinic acid is elicitation of a syndrome known as "pseudohyperaldosteronism". Originally it was thought that this was the result of a direct interaction of glycyrrhizinic acid and the aglycone glycyrrhetic acid with the mineralocorticoid receptor. However, at the end of the 1980's it became clear that glycyrrhizinic acid and glycyrrhetic acid produce "pseudohyperaldosteronism" because of interference with the cortisol/cortisone homeostasis. The 18-beta- form of glycyrrhetic acid is an inhibitor of the enzyme 11-beta-hydroxysteroid dehydrogenase-2 (11-BOHD-2), which is responsible for the conversion of cortisol to cortisone. Under normal circumstances this inactivation allows aldosterone to control mineral and water homeostasis. However, when the enzyme is inhibited, aldosterone is displaced from the

mineralocorticoid receptors by cortisol, thereby increasing mineralocorticoid activity. This results in sodium and water retention, in potassium excretion and in metabolic alkalosis. In addition, decreased plasma aldosterone and renin activity are observed, which can be considered a compensatory reaction to the over-stimulation of the mineralocorticoid receptors. Clinical signs are oedema and hypertension. In severe cases destruction of skeletal muscles (rhabdomyolysis and myopathy), ventricular tachycardia and renal failure may occur (Størmer *et al.*, 1993a; Bijlsma *et al.*, 1996; Ploeger, 2000a).

Animal studies

Acute toxicity

According to EFFA (2001), no standard oral LD50 studies are available. In the Hazardous Substances DataBase (2002) an oral LD50 in the rat for glycyrrhizinic acid of 14.2 g/kg bw has been mentioned.

Groups of 4 female adult rats were dosed with 0.5, 1, 1.5 or 2 g/kg bw of either 18-alpha or 18-beta glycyrrhetic acid ammonium salt, via intraperitoneal injection. Dose levels up to 1.5 g/kg bw were well tolerated and no changes in electrocardiograms were observed. However, at 2 g/kg bw of the 18-alpha isomer, but not the 18-beta isomer, all animals died due to atrio-ventricular block. Post mortem examination of these animals revealed brain, cerebellum and lung oedema and haematic stasis in kidneys, adrenals, spleen and liver. In two rats calcium salt calculi were observed in the kidneys. Focal changes in the papillary cardiac muscles were reported, showing oedema, myolysis and cell distortion with granular cardiomyocytes with pyknotic nuclei. Apoptosis of papillary muscle cells was observed (Rossi *et al.*, 1999).

Repeated dose toxicity study

Groups of 40 female adult rats were orally dosed with 30 mg/kg bw glycyrrhizinic acid, 15 mg/kg bw 18-beta-glycyrrhetic acid or 15 mg/kg bw 18-alpha glycyrrhetic acid, dissolved in water for up to 30 days. A fourth group received only water. Per group, 10 rats were sacrificed before treatment, 15 rats were sacrificed after 15 and 30 days of treatment, and 10 rats were sacrificed at d30 days after cessation of treatment.

Damage of the cardiac muscles (milder than observed in the acute studies, see above) was observed with glycyrrhizinic acid and 18-alpha glycyrrhetic acid after 15 and 30 days of treatment, and this damage appeared to be irreversible. Both glycyrrhizinic acid and 18-alpha glycyrrhetic acid caused renal tubular calculi and slight expansion of the bronchus-associated lymphoid tissue in the lungs after 15 days. These changes were not observed with the 18-beta isomer. Urinary electrolyte levels showed increased sodium, potassium and calcium excretion after 15 days of dosing with 18-alpha glycyrrhetic acid, but not with glycyrrhizinic acid or the 18-beta-isomer. After 30 days increased potassium and calcium excretion was also seen with the 18-beta isomer, but to a lesser extent. Thirty days after cessation of treatment, urinary electrolyte excretion with the 18-beta isomer returned to normal but in the 18-alpha -isomer-treated animals the situation was worse. In the blood only an increase in plasma sodium was observed, with all three treatments after both 15 and 30 days, which was completely reversed after 30 days of withdrawal. The authors concluded that the 18-alpha glycyrrhetic acid is considerably more toxic than the 18-beta-isomer, but a mechanistic explanation was not provided. It was also not clear whether the cardiac effects were a primary effect or secondary to changes in the renin-angiotensin system (Rossi *et al.*, 1999).

Genotoxicity

The genotoxicity of glycyrrhizinic acid has been studied in *in vitro* and in *in vivo* test systems. It should be noted that in *in vivo* studies glycyrrhetic acid rather than glycyrrhizinic acid is tested, because of presystemic hydrolysis of the latter. In i*n vitro* studies conversion of glycyrrhizinic acid to glycyrrhetic acid is probably not as effective as after oral administration *in vivo*.

Disodium and trisodium glycyrrhizinate did not induce gene mutations in *Salmonella typhimurium* strains TA 92, TA 1535, TA100, TA1537, TA94 and TA98 in dose levels up to 5 or 10 mg/plate, respectively, with and without metabolic activation (Ishidate *et al.*, 1984).

Liquorice powder and ammonium glycyrrhizinate were not genotoxic at concentrations ranging from 0.01 to 0.5 mg/ml in TA97, TA98 and TA100 *Salmonella* tester strains either with or without metabolic activation (Cooper and Berry, 1988; only abstract available).
Disodium but not trisodium glycyrrhizinate induced structural chromosomal aberrations in Chinese hamster fibroblast cultures (no specification of percentage of gaps). With trisodium only polyploid cells were induced (both substances tested only in absence of metabolic activation; Ishidate *et al*., 1984).

Disodium or trisodium glycyrrhizinate did not induce micronuclei in bone marrow cells of male ddY mice after single intraperitoneal injections with doses ranging from 0-140 mg/kg bw or 0-2000 mg/kg, respectively. Absence of micronuclei induction was also found after 4 daily intraperitoneal injections with 20 mg/kg (disodium salt) or 500 mg/kg bw (trisodium salt). With the trisodium salt, signs of bone marrow toxicity were observed after the single dose of 2000 mg/kg bw and after 4 daily injections with 500 mg/kg bw. (Hayashi *et al.*, 1988).

Ammonium glycyrrhizinate induced statistically significant dominant lethal effects (dead implants in offspring) in male rats at the maximum tolerated dose (40 g/kg in the diet). In mice no increases in dominant lethal events were observed at a dietary levels of 22.5 g/kg (maximum tolerated dose). It did not induce heritable translocations in the same mice also treated at 22.5 g/kg in the diet (Sheu *et al.*, 1986).

Based on all available data, notwithstanding some *in vitro* and *in vivo* weak positive findings of questionable biological meaning, glycyrrhizinic acid and its hydrolysis product glycyrrhetic acid are considered to be non-genotoxic.

Carcinogenicity

In a carcinogenicity study with B6C3F1 mice, groups of 50-70 males and females of 8 weeks of age were exposed to disodium glycyrrhizinate via the drinking water for 96 weeks. After the exposure period the animals were maintained for another 14 weeks withdrawal period.Concentrations in the drinking water were 0, 40, 80 and 150 mg/l for the males and 0, 80, 150 and 300 mg/l for the females, resulting for the males in dose levels of

0, 71, 166 and 229 mg/kg bw/day and for the females in dose levels of 0,117, 217 and 407 mg/kg bw/day (Kobuke *et al.*, 1985). For both sexes the respective top dose was equal to the MTD as determined in a 10 week pre-test. In this pre-test 100% mortality occurred at drinking water concentrations of 600 or 1250 mg/l in both sexes. At 300 mg/l, one male died within the first 4 weeks, but in the females no mortality was seen at this concentration. All animals exposed to 300 mg/l or higher showed marked atrophy in all visceral organs, but no changes were seen at lower drinking water concentrations.

A dose-related reduction in drinking water intake was observed in both males and females. At week 78 survival was 40, 40, 33 and 50% for the males and 90, 76, 90 and 70% for the females. In total 49 males and 18 females survived until the end of the study (week 110). Effects on body weights were not observed. No effect on tumour incidence or "time to death with tumours" was observed in either sex. Commonly observed tumours were liver cell tumours in the males and lymphoid leukaemia in females, but without any relationship to treatment. In male animals older than 52 weeks amyloidosis was observed at a high incidence, frequently affecting spleen, liver, kidney and/or adrenals. This condition was observed in 51% and in 33% of control and treated males, respectively, but not in any of the female treatment groups. It is noted that the mortality in his study was rather high. In the males mortality occured at a more or less constant rate thoughout the study, irrespective of the dose level. Mortality rates in all female groups increased sharply at about week 80. In the females, mortality in the top dose group was increased during the first 80 weeks of the study. Due to insufficient detail in the paper a more thorough (statistical) analysis of the mortality data is not possible.

No carcinogenicity data are available for the rat.

Reproductive and developmental toxicity studies

No standard reproductive toxicity studies with (salts of) glycyrrhizinic acid are available. However, ammonium glycyrrhizinate has been tested in dominant lethal tests in mice and rats and a heritable translocation test in mice at dietary exposure levels up to the maximum tolerated dose for exposure periods of 8 or 10 weeks for male mice and rats, respectively. No effects on male fertility were observed (Sheu *et al.*, 1986).

Groups of 14-17 female rats were administered 0, 0.08, 0.4 and 2% disodium glycyrrhizinate via the diet (average dose levels equal to 0, 60, 290 and 1480 mg/kg bw/day) from day 0 to day 20 of gestation. At day 20, per group 9-12 of the dams were killed and their litters examined for external and skeletal and visceral malformations. The remaining dams were allowed to deliver and rear their litters. These litters were allowed to grow up to 8 weeks post partum. No significant effects were observed during pregnancy or postnatally, apart from a reduction in maternal body weight gain *post-partum* in the mid- and high dose group. No indications for a teratogenic potential for disodium glycyrrhizinate were obtained (Itami *et al.*, 1985).

In a study by Mantovani *et al.* (1988) 16-20 pregnant rats were given ammonium glycyrrhizinate during day 7-18 of gestation via the drinking water in concentrations of 0, 100, 1000 and 2500 mg/l (equal to 0, 21, 239 and 680 mg/kg bw/day). At day 20, the dams were bled and blood samples were studied for aldosterone, sodium and potassium. Maternal adrenals were collected for histological examination. The uteri were examined for signs of embryo- and foetotoxicity and the offspring were studied for external, skeletal and visceral malformations. The only clinical sign of an effect in the dams was an increased water intake in the mid and high dose groups. No significant effects were observed except for a statistically significant increase in total skeletal variants at the high dose. Sternebral variants were significantly increased in both mid and high dose groups. At the low and high dose, a statistically significant increase in external haemorrhages and haematomas was found, but not in the mid dose, where an increase in internal haemorrhages was seen. Soft tissue examination revealed a higher incidence in ectopic kidneys in the low and high dose group litters and a decrease in other kidney variants in the low dose group. The authors indicated that the skeletal variants should be considered as a reversible effect of doubtful toxicological significance, while ectopic kidney is (partly) subjective. According to the study authors, the data indicate a slight adverse effect of ammonium glycyrrhizinate on foetal rats. The NOAEL in this study was 239 mg/kg bw/day.

Human studies

Many case reports noting adverse effects have been summarised by Størmer *et al*. (1993a). Five of these cases consumed very large amounts of liquorice confectionery (up to 1.8 kg/week, equivalent to doses of glycyrrhizinic acid of *ca.* 500 mg/day) for a long time. In another case a patient had consumed a laxative providing a dose of approximately 150 mg glycyrrhizinic acid for two to three times a week during 2-3 years. In a clinical study with this patient, daily administration of the laxative, resulting in exposure levels of 94 mg/day, resulted in profound hypokalaemia with sodium retention and depression of the renin-angiotensin-aldosterone system. The potsssium loss may have been aggravated by the laxative effect (Cummings *et al*., 1988). Other case studies refer to adverse effects in people who took glycyrrhzinic acid at daily dose levels up to 130 mg for only several days to weeks.

In a sequence of clinical trials, body weight gain, water retention, oedema and increased blood pressure were seen in 17 persons taking 1560 mg glycyrrhizinic acid/day during an undefined period of time. In 6 of these volunteers, intake of 780 mg/day resulted in less pronounced body weight increases and in 4 of these 6 also increased blood pressure was observed. One very sensitive person from this group of 17 showed an increase in blood pressure, when 130 mg/day was taken for a longer (unspecified) period of time. These clinical studies were performed with ”succus liquoritiae”, containing 26% of glycyrrhizinic acid (Smorenberg-Schoorl and Vree, 1963).

In another clinical study, effects related to pseudohyperaldosteronism were seen in groups of 8 male and 8 female volunteers exposed to pure glycyrrhizinic acid at doses of 400 mg and 800 mg/day during 2-4 weeks. It was concluded that females were more sensitive than males (Van Vloten *et al.,*1989).

<u>New case reports</u>

In the past decade several new cases of more or less severe intoxications with glycyrrhizinic acid have been reported. In the present opinion, only those studies are cited that show that occasionally quite serious adverse effects may occur at exposure levels at, or (only slightly) below the maximum level provisionally advised by the Committee (SCF,

1991), or because they show that adverse effects do not only occur after consumption of liquorice confectionery, but also after consumption of chewing gum and of (large) amounts of liquorice tea.

A 42 year-old man was referred to the hospital in a soporific state. He had experienced worsening headaches, nausea, vomiting and sensitive neuropathy on the left side of the body. An onset of slight hypertension had been diagnosed 6 months earlier. Clinical examination showed a blood pressure of 200/140 mm Hg and decreased plasma potassium (2.9 mmol/l, while 3.8-5 mmol/l is normal) and aldosterone levels (180 pmol/l instead of 320-2000 pmol/l). The patient's plasma potassium could not be easily restored by supplementation, and it was noted that he ate about 50 g of liquorice confectionery a day (estimated to correspond to about 100 mg glycyrrhizinic acid). He was advised to stop and after two weeks he was discharged from hospital without symptoms. His blood pressure had fallen to 120/85 mm Hg, while his plasma potassium concentration had risen to 4 mmol/l (Russo *et al.* (2000).

A second patient (46 year-old male) who consumed about 40 g of liquorice confectionery a day was hospitalised with deep asthenia, headaches and somnolence. Two weeks earlier hypokalaemia was observed (2.3 mmol/l) which did not respond to potassium supplementation. The patient was sluggish, did not respond to environmental stimuli and showed muscle weakness. His blood pressure was 215/125 mm Hg and an electrocardiogram showed sinus bradycardia and T-wave levelling. Plasma clinical chemistry was normal except for potassium, aldosterone and renin, which were all depressed. Despite treatment with potassium supplementation and anti-hypertensives, his condition only improved when he stopped eating liquorice confectionery. Upon discharge, his blood pressure had fallen to 150/80 mm Hg (Russo *et al.,* 2000). According to the study authors, similar cases of encephalopathy have been reported previously only after consumption of 500 g of liquorice confectionery which would correspond to 1000 mg glycyrrhizinic acid. This is far above the tentatively derived human LOAEL of 100 mg/day (Størmer *et al.*, 1993a), which is associated with hypertension without complications. According to the authors a significant inter-individual variation in sensitivity occurs within

the human population. It was further speculated that this variability might be associated with a partial 11-BOHD-2 deficiency.

In another case report a woman (41 years of age) was diagnosed with "essential hypertension" and treated with anti-hypertensives, to which her condition did not respond. Her blood pressure was 210/115 mm Hg. After adding a diuretic to the medication, she developed a hypokalaemia (1.9 mmol/l), with muscle cramps and muscle weakness. The plasma potassium level did not sufficiently respond to potassium supplementation. The woman also suffered from polyuria (4 l/day) but denied eating liquorice sweets. However, she drank 3 l of liquorice tea a day. After the consumption of this tea was stopped, blood pressure and potassium level gradually improved in the next two months. The study authors did not analyse the particular tea for glycyrrhizinic acid, but based on the average level of 126 mg/l in liquorice teas (range 2-450 mg/l), which can be derived from the data by Maas (2000), daily intake of glycyrrhizinic acid may have been 375 mg (Brouwers and Van der Meulen, 2001; range 6.5-1350 mg).

A 55-year old male patient was referred to hospital with atypical abdominal pain, which developed after he had quit smoking, but started to use chewing gum. He had an increased blood pressure, despite taking anti-hypertensives, a low plasma renin activity and a hypokalaemia, but the plasma aldosterone level was normal. Upon enquiry he told that he used two packs of a chewing gum (a brand not known to contain nicotine) , which according to the manufacturer contained 24 mg of glycyrrhizinic acid per pack of 16 g. Hence the patient was taking about 50 mg glycyrrhizinic acid/day. After quitting the use of chewing gum, the patient's blood pressure returned to normal (without the use of anti-hypertensives) with normal plasma potassium levels and no abdominal pains (Rosseel and Schoors; 1993).

New volunteer studies

The bioavailability of glycyrrhetic acid from liquorice was studied in 16 human volunteers (8 males, 8 females), who were exposed to equimolar amounts of A: 130 mg glycyrrhetic acid in an aqueous suspension, B: 225 mg glycyrrhizinic acid in an aqueous solution; C: 225 mg glycyrrhizinic acid in 150 g of sweet liquorice candy and D: 225 mg glycyrrhizinic

acid in 150 g of a salted liquorice candy. Every participant received all treatments, with a dose interval sufficiently long to avoid interference between the various treatments. Plasma levels of glycyrrhetic acid were observed for up to 56 hr post dosing and reached peak values after 3 hr (treatment A) or 8-10 hr (treatments B-D). No intra-individual differences were observed in the relative bioavailability of glycyrrhetic acid from the 4 different treatments. However, between the participants, Cmax and AUC values varied by factors of about 4 to 5, respectively: e.g. with treatment C: range Cmax 0.4 to 1.6 mg/l and range AUC: 4.8 tot 23.1 mg/l*hr. With respect to elimination of glycyrrhetic acid from the body, even within this small group of participants two sub-populations could be distinguished: one (9 persons) in which the plasma glycyrrhetic acid levels declined mono-exponentially with time and a second one in which elimination followed a biphasic pattern with very long terminal plasma half-lives (up to 35 hr) and occurrence of a secondary plasma peak at 18-32 hr post dosing, indicative of enterohepatic circulation. No differences in kinetics were observed between male and female participants (Mensinga *et al.*, 1998).

Sigurjónsdóttir *et al.* (2001) studied the relationship between intake of glycyrrhizinic acid and hypertension in human volunteers. These volunteers were divided into three different groups. A high dose group consisting of 9 women and 1 man (540 mg glycyrrhizinic acid/day), a mid dose group with 19 women and 11 men (270 mg glycyrrhizinic acid/day) and a low dose group with 12 women and 12 men (75 mg glycyrrhizinic acid/day). The glycyrrhizinic acid was administered in the form of sweet liquorice confectionery, which was obtained from two different producers (low dose from one supplier, and mid and high dose from another). A placebo control group was not studied, and the volunteers were fully aware of their treatment. The volunteers were studied in three separate experiments. Blood pressure was the only parameter studied.

The study authors reported that for intake of glycyrrhizinic acid and hypertension a linear dose-response relationship could be established. The regression co-efficient for this dose-response relationship amounted to 0.011 and 0.014 for 2 and 4 weeks of liquorice consumption, respectively. Based on the graphically presented data, the relationship depended heavily on 2 out of 64 volunteers; one with low and one with high glycyrrhizinic acid intake. No other parameters indicative for pseudohyperaldosteronism were studied and

no indication was provided as to the bioavailability of the glycyrrhetic acid from the different brands of liquorice. There are so many flaws in the study design that it can only be concluded that the claim for the weak linear dose-response relationship is scientifically insufficiently underpinned.

Four groups of 10 healthy female volunteers received orally 0, 1, 2 or 4 mg pure glycyrrhizinic acid/kg bw/day for 8 weeks after an acclimatisation period of two weeks (Bijlsma *et al*., 1996; Van Gelderen *et al*., 2000). The study was done according to a placebo-controlled, randomised, double blind design. The exposure period was followed by a wash-out period of two weeks. Criteria for study enrolment were age 18-40 years, body weight 50-90 kg, not taking oral contraceptives, not being pregnant or breast-feeding, plasma potassium > 3.5 mmol/l, and a positive outcome of a physical examination, including blood electrolytes and kidney function tests. During the study the volunteers were not allowed to smoke, to consume any glycyrrhizinic acid-containing food product or to take alcoholic drinks, medication or drugs. The study was performed with female volunteers, because in a pilot study with higher dose levels (Van Vloten *et al.,*1989) women appeared to be more sensitive to glycyrrhizinic acid than men. In the study the following parameters were investigated: physical examination, physical condition, body weight, blood pressure, oedema, plasma-potassium, sodium, chloride, calcium, bicarbonate, plasma renin, aldosterone and atrial natri-uretic peptide and urinary potassium, sodium and creatinine. In addition plasma glycyrrhetic acid levels were determined. The volunteers filled out a diary for physical complaints every day and a dietary questionnaire had to be filled out every other week.

During the study one volunteer from the 2 mg/kg group dropped out because of hypokalaemia (not cross-checked but reversed after one week) and one volunteer from the 4 mg group left the study because of loss of concentration and general discomfort, oedema, body weight gain and raise of blood pressure. Headache, nausea, change in defecation pattern, swollen face and tickling in arms and legs occurred somewhat more often in the 4 mg/kg group, but the overall number of complaints diminished in all groups during the experiment. Although at the beginning of the exposure period an increase in body weight of about 1 kg was observed in 23/28 participants in the 1, 2 and 4 mg/kg bw groups, statistical significance was only observed in the 1 and 4 mg/kg groups. No consistent

changes in body weight were found later in the study. Average plasma glycyrrhetic acid levels increased gradually during the first 7 days of the exposure period and remained about constant during the next 8 weeks at about 0, 0.16, 0.26 and 0.94 mg/l in the 0, 1, 2 and 4 mg/kg bw/day groups, respectively. After the two weeks post-exposure period, glycyrrhetic acid could no longer be detected in any of the volunteers.

The highest dose of 4 mg/kg bw/day caused a significant reduction of plasma renin activity and aldosterone concentration, while at the dose of 2 mg/kg bw/day only a non-significant decrease in aldosterone concentration could be observed. The atrial natri-uretic peptide was significantly increased at 4 mg/kg at the end of the exposure period, but decreased to normal values after two weeks of wash-out. Average plasma potassium levels decreased significantly during exposure in the 4 mg/kg bw/day group and non-significantly in the 2 mg/kg bw/day group, but were never outside the normal physiological range. In the 4 mg/kg bw group, the plasma bicarbonate concentration was significantly increased as compared to the control group. The mean systolic and diastolic blood pressures in the 4 mg/kg-group remained more or less constant but as the values decreased in the control group, a statistically significant difference with the control group was observed. Both systolic and diastolic blood pressures in all groups remained within the normally accepted range. Volume expansion was observed, which may lead to hypertension at a later stage. No relevant dose-related differences between control and exposed groups were observed in any of the other parameters studied.

The body weight changes in the 1 mg/kg group were not accompanied by changes in the renin-angiotensin-aldosterone system and are therefore considered not treatment-related. In this study the NOAEL for glycyrrhizinic acid can be established at 2 mg/kg bw/day (on average equal to *ca.* 130 mg/person/day (Bijlsma *et al.*, 1996; Van Gelderen *et al.*, 2000). At this dose level, the plasma glycyrrhetic acid level was never higher than 800 microg/l in any of the volunteers.

Bernardi *et al.* (1994) administered daily doses of 108, 217, 380 and 814 mg glycyrrhizinic acid, as 'liquorice pills' during 4 weeks to four groups of 3 male and 3 female healthy volunteers. The liquorice was a dried aqueous root extract. The glycyrrhizinic acid content,

as assayed by HPLC, was 7.64% w/w ratio. Different dose levels were achieved by giving different numbers of pills to the participants in the respective groups. A control group was not incorporated in the study, but changes were detected by comparison with baseline values. Note that in this study the statistical evaluation of the data was performed by comparison of group averages at baseline level with group averages after 1, 2, or 4 weeks of treatment. The study might have gained in sensitivity if the baseline data and post exposure data would have been compared on an individual basis. The following parameters were studied: body weights, wrist, ankle and mid-arm muscle circumference, triceps skin fold, heart rate, mean arterial pressure, creatinine clearance, daily diuresis, daily renal sodium and potassium excretion, serum sodium and potassium, plasma renin and aldosterone concentrations, blood glucose and haematocrit.

Three persons were withdrawn from the experiment at the end of week 2. One female from the group administered 380 mg experienced continuous headache. Another female from the group administered 814 mg complained of headache, increase in body weight and peripheral oedema. She also showed borderline arterial hypertension and hypokalaemia. It should be noted that she was taking an oestrogenic-progestagenic drug at the same time. The remaining drop-out was a man at 814 mg who developed arterial hypertension. His family history was positive for this effect. These adverse effects subsided within 24-48 hrs after treatment suspension. The two remaining females at 814 mg showed mild periorbital oedema and body weight increase through the second week which was their pre-menstrual period. Afterwards, oedema disappeared and body weights decreased again, though not completely to the baseline value. There were signs of sodium retention in the volunteers of the 380 and 814 mg dose groups, but the changes in renal sodium excretion reached statistical significance only in the 380 mg group after 3 weeks. It was noted that renal sodium excretion showed marked temporal variability among the subjects. Plasma renin-activity was significantly decreased in volunteers at 380 and 814 mg. A decline in plasma aldosterone concentration was also observed in these two groups, but the decrease reached statistical significance only at 814 mg. In the highest dose group the volunteers showed an increase in body weight and a reduction in serum potassium concentration which reached statistical significance at week 2 and week 1, respectively. However, the serum potassium concentration in the 814 mg dose group was consistently lower than in any of the other

groups at any time point (1, 2 or 4 weeks). The NOAEL based on the study report was 217 mg/person/day. At higher dose levels sodium retention and depression of plasma renin and aldosterone levels were observed. According to the study authors, the female participants were slightly more sensitive to glycyrrhizinic acid than the males. (Bernardi *et al.*, 1994).

Epidemiological studies

Reproductive toxicity

In an epidemiological study (Strandberg *et al.*, 2001), the influence of liquorice consumption on several gestation and offspring parameters was studied in 1049 pregnant women in Finland, who gave birth to singleton healthy babies between March and November 1998. Glycyrrhizinic acid intake was assessed from questionnaire information on liquorice consumption together with data on the course of the pregnancy and parturition. The cohort was split into three subgroups with respect to their glycyrrhizinic acid intake, with low (< 250 mg /wk; n=751), medium 250-499 mg/wk; n=145) and high (> 499 mg/wk; n=110) intakes, respectively. Of the entire cohort 2.3% reported not to use liquorice at all. Average glycyrrhizinic acid intake among the liquorice consumers amounted to 363 mg/wk (S.D. 348; range 1-2464 mg/wk). No correlation could be found between glycyrrhizinic acid intake and birth weight. When glycyrrhizinic acid intake was considered as a continuous independent variable, regression analysis showed that for every 500 mg increase in glycyrrhizinic acid intake, the length of gestation diminished by 1.26 days ($p = 0.009$, 95% confidence interval 0.31-2.24). When considered as three sub-cohorts (see above) a significant decrease of the length of the gestation period was only found in the high intake group. When the high intake group was compared to the low intake group, the odds ratio for delivery < 38 weeks was 2.5 (95% confidence interval 1.1-5.5; $p = 0.03$). No effects were observed on any of the other pregnancy parameters studied, on birth weight or on type of delivery, and no effects were observed on maternal blood pressure. Although the authors speculated about a causal relationship between glycyrrhizinic acid intake and reduced length of the gestational period, they could not definitely conclude that this relationship occurred. Confounding factors might have influenced the outcome of the study (e.g. a positive correlation was also observed between glycyrrhizinic acid intake and intake of chocolate, which was not corrected for). No comparison was made with a group of mothers who did not take liquorice during pregnancy.

Colley and Gibson (1982) studied the outcome of 6408 singleton pregnancies, all supervised by one hospital. 313 Mothers had taken cough mixtures during pregnancy, and 137 of these had used a medication ("S&A") which contained an unspecified amount of liquorice extract. No changes were observed in rates of congenital malformations or perinatal death. Non-significant increases in the rates of low birth weight, poor Apgar score and intrauterine growth retardation were observed for the users of "S&A", as compared to the control group, but it is impossible to attribute these changes to liquorice intake, because the intake of liquorice from other sources was not studied (neither for users nor for controls). No stratification for smoking, maternal age, premature birth (total incidence slightly decreased in the user group) or other confounding factors was applied. Because of these limitations, the study is not useful for the evaluation of the reproductive toxicity of glycyrrhizinic acid.

Pharmacokinetic-pharmacodynamic model

Ploeger and co-workers (2000b, 2001a, 2001b) have developed a physiologically based pharmacokinetic-pharmacodynamic (PBPK/PD) model for glycyrrhizinic acid, glycyrrhetic acid and occurrence of adverse clinical effects in humans. The model has been calibrated on the basis of various literature data. It can accurately predict plasma glycyrrhetic acid levels in human volunteers after oral intake of glycyrrhetic acid, glycyrrhizinic acid itself or two liquorice confectionery treatments (sweet and salted liquorice), which were determined in an independent clinical trial. Important features of the model are that it includes the presence of a gall bladder, it takes into account the rate of bowel movements and stomach emptying, hydrolysis of glycyrrhizinic acid in the GI-tract and enterohepatic circulation of glycyrrhetic acid. In addition, it can estimate the effects of intake of glycyrrhizinic acid (and subsequently glycyrrhetic acid) on the activity of 11-BOHD-2 by prediction of the ratio cortisol/cortisone in 24-h samples of human urine following repeated intake of glycyrrhetic acid, as proposed by Heilmann *et al.* (1999). The sub-model which predicts changes in urinary cortisol/cortisone ratios was also calibrated on the basis of literature data and validated against data that were obtained from independent experiments in human volunteers who received multiple oral administrations of either glycyrrhizinic acid or glycyrrhetic acid.

By using this PBPK/PD model Ploeger and co-workers (2000a, 2001b) demonstrated that the the most important factors to determine interindividual variability in the response to glycyrrhizinic acid with respect to perturbation of the cortisol/cortisone status were:

- motility of the gastrointestinal tract: slow bowel movements result in a prolonged residence time of glycyrrhizinic acid and glycyrrhetic acid in the enterohepatic circulation and therefore in a higher systemic exposure. Experimentally, this is confirmed by findings of Mensinga *et al.* (1998)
- variability of the absorption rate constant: a rapid absorption results in a more extensive enterohepatic circulation and in a higher internal exposure to glycyrrhetic acid.
- variability of the IC50 (plasma glycyrrhetic acid concentration at which 50% of the 11-BOHD-2 activity is lost). A lower IC50 results in an effect on the cortisol/cortisone status at lower plasma levels of glycyrrhetic acid.
- variability of the "base-line" activity of 11-BOHD-2. Indeed, several cases of congenital apparent mineralocorticoid excess, a condition which is similar to pseudo-hyperaldosteronism, have been identified, in which mutations in the gene encoding for the 11-BOHD-2 enzyme could be identified. These mutations cause partial or complete loss of enzyme activity (Mune *et al.,* 1995). In addition to this, "base-line" variability may also result from differences in the level of expression of the 11-BOHD-2 gene (Ferrari *et al.*, 2001).

These findings and the PBPK/PD model were further used in a Monte Carlo simulation to estimate the proportion of the population that might experience disturbances of the cortisol/cortisone status and, linked to this, biochemical or even clinical manifestations of intoxication at various intake levels of glycyrrhizinic acid. These calculations were based on the following starting points:

- at the NOAEL of 2 mg glycyrrhizinic acid /kg bw/day, the plasma level of glycyrrhetic acid is never higher than 800 μg/l, as observed in the study by Bijlsma *et al.* (1996).
- the population distribution of sensitivity to glycyrrhizinic acid with respect to biochemical and clinical manifestations at the LOAEL of 4 mg glycyrrhizinic acid/kg bw/day is adequately reflected by the distribution of responders vs. non-responders in

the Bijlsma study. In this study, at the LOAEL, 1/11 participants did not show any effect, while in 10/11 participants biochemical changes were observed, in one of whom also clinical signs were seen.

Ploeger (2000a) calculated that at the maximum intake level of 100 mg/day, provisionally established by the Committee in 1991 (SCF, 1991), about 18% (95% confidence limits: 1.6 - 73.5%) of the exposed population would experience plasma glycyrrhetic acid levels greater than 800 μg/l, while in 26% of the exposed population (95% confidence limits: 12 - 47%) disturbances of the cortisol/cortisone status would be detectable in the urine. Overt clinical adverse effects (hypertension) would occur in about 4 per 10^4 persons (95% confidence limits: 4.6 per 10^6 - 3 per 10^2). These estimates apply to intake of glycyrrhizinic acid from any source, hence to the entire population.

In addition to the sensitive groups that were identified by Ploeger (2000a) and co-workers, some other groups in the population can be expected to be adversely influenced exposure to glycyrrhizinic acid or its ammonium salt. Although there is no direct evidence showing this, from the biomedical literature (Rose, 1994; Stewart, 2002) it is conceivable that the health of people with Cushing's syndrome, or other conditions related to hypertension or abnormal electrolyte or water homeostasis, may be adversely affected by exposure to glycyrrhizinic acid or its ammonium salt.

References to Annex II

Bernardi, M., D'Intino, P.E., Trevisani, F., Cantelli-Forti, G., Raggi, M.A., Turchetto, E., Gasbarrini, G. (1994). Effects of prolonged ingestion of graded doses of liquorice by healthy volunteers. Life Sciences, **55**, 863-872.

Bijlsma, J.A., Van Vloten P., Van Gelderen C.E.M., Mensinga Tj.T., Mout, H.A., Elvers L.H., Van Leeuwen, F.X.R., Stolker, A.A.M., Van Ginkel, L.A., Looman, C.W.N., Van der Maas, P.J., Koomans, H.A. and Savelkoul, T.J.F. (1996). Onderzoek naar de effecten van verschillende doseringen glycyrrhizine bij gezonde vrouwelijke vrijwilligers. [Study into the effects of different dosages of glycyrrhizin in health female volunteers; in Dutch]. RIVM report 348801004, RIVM, Bilthoven, The Netherlands

Brouwers, A.J.B.W. and Van der Meulen, J. (2001). "Drophouthypertensie"; ook door zoethoutthee ["Liquorice hypertension"; also by liquorice tea; in Dutch]. Ned. Tijdschr. Geneeskd., 1**45**, 744-746.

Cantelli-Forti, G, Maffei, F., Hrelia, P., Bugamelli, F., Bernardi, M., D'Intino, P., Maranesi, P. and Raggi, M.A. (1994). Interaction of liquorice on glycyrrhizin pharmacokinetics. Environmental Health Perspectives, **102**, Suppl. 9, 65-68.

Cantelli-Forti, G., Raggi, M.A., Bugamelli, F., Maffei, F., Villari, A. and Trieff, N.M. (1997). Toxicological assessment of liquorice: biliary excretion in rats. Pharmacological Research, **35**, 463-470

Colley, D.P., Kay, J. and Gibson, G.T. (1982). Three common Australian cough mixtures: a study of their use in pregnancy. Australian Journal of Pharmacy (April 1982 issue), 213-217.

Cooper, E.P. and Berry, C.W. (1988). Mutagenic potential of Glycyrrhizin. Journal of Dental Research, **67**, abstract 339 (Special Issue, March).

Cummings, A.M.M., Boddy, K., Brown, J.J., Fraser, R., Lever, A.F., Padfield, P.L. and Robertson, J.I.S. (1980). Severe hypokalaemia with paralysis induced by small doses of liquorice. Postgraduate Medical Journal, **56**, 526-529.

EFFA (2001). Ammonium glycyrrhizate. European Flavour and Fragrance Association (EFFA). Submission to SCOOP Working group on Chemically defined Flavouring

Substances, document No: SCOOP/FLAV/8.4, SCF document No: SCF/CS/ADD/MsAd/203

Ferrari, P., Sansonnens, A., Dick, B. and Frey, F.J. (2001). In vivo 11-beta-HSD-2 activity; variability, salt-sensitivity, and effect of licorice. Hypertension, **38**, 1330-1336.

HSDB: Hazardous Substances Data Base (2002). Issued by the National Library of Medicine through Silver Platter International N.V., Ovid Technologies, Amsterdam.

Hayashi, M., Kishi, M., Sofuni, T. and Ishidate Jr., M. (1988). Micronucleus tests in mice on 39 food additives and eight miscellaneous chemicals. Food and Chemical Toxicology, **26**, 487-500.

Heilmann, P., Heide, J., Hundertmark, S. and Schoneshofer, M. (1999). Administration of glycyrrhetinic acid: significant correlation between serum levels and the cortisol/cortisone ratio in serum and urine. Experimental and Clinical Endocrinology and Diabetes, **107**, 370-378.

Ishidate, M., Sofuni, T., Yoshikawa, K., Hayashi, M., Nohmi, T., Sawada, M. and Matsuoka, A. (1984). Primary mutagenicity screening of food additives currently used in Japan. Food and Chemical Toxicology, **22**, 623-636.

Itami, T., Ema, M. and Kanoh, S. (1985). Effect of disodium glycyrrhizinate on pregnant rats and their offspring. Journal of the Food Hygiene Society of Japan, **26**, 460-464.

Kobuke, T., Inai, K., Nambu, S., Ohe, K., Takemoto, T., Matsuki, K., Nishina, H., Huang, I.-B. and Tokuoka, S. (1985). Tumorigenicity study of disodium glycyrrhizinate administered orally to mice. Food and Chemical Toxicology, **23**, 979-983.

Maas, P.(2000). Zoethout in levensmiddelen: onderzoek naar het glycyrrhizine gehalte van thee, kruidenmengsels, dranken en drop. [Liquorice root in food stuffs: survey of the glycyrrhizin content of tea, herbal mixtures, alcoholic drinks and liquorice.; in Dutch]. De Ware(n) Chemicus, **30**, 65-74.

Mantovani, A., Ricciardi, C., Stazi, V., Marci C., Piccioni, A., Badellino, E., De Vincenzi, M., Caiola, S. and Patriarca, M. (1988). Teratogenicity study of ammonium glycyrrhizinate in the Sprague-Dawley rat. Food and Chemical Toxicology, **26**, 435-440.

Mensinga, Tj.T, Sips, A.J.A.M., Van den Ham, W. and Meulenbelt, J. (1998) Gezondheidsrisico's veroorzaakt door het eten van drop. [Health risks caused by the consumption of liquorice confectionery; in Dutch]. RIVM report 236850003, RIVM, Bilthoven, The Netherlands

Mune, T.M, Rogerson, F.M., Nikkilä, H., Agarwal, A.K. and White, P.C (1995). Human hypertension caused by mutations in the kidney isozyme of 11-beta-hydroxysteroid dehydrogenase. Nat. Genet. **10**, 394-399.

Ploeger, B.A. (2000a). Development and use of a physiologically based pharmacokinetic-pharmacodynamic model for glycyrrhizinic acid in consumer products. PhD thesis University of Utrecht.

Ploeger, B.A., Meulenbelt, J. and De Jongh, J. (2000b). Physiologically based pharmacokinetic modelling of glycyrrhizic acid, a compound subject to presystemic metabolism and enterohepatic cycling. Toxicology and Applied Pharmacology, **162**, 177-188.

Ploeger, B.A., Mensinga, T., Sips, A., Seinen, W., Meulenbelt, J. and De Jongh, J. (2001a). The pharmacokinetics of glycyrrhizinic acid evaluated by physiologically based pharmacokinetic modelling. Drug Metabolism Reviews, **33**, 125-147.

Ploeger, B.A., Mensinga, T., Sips, A., Deerenberg, C., Meulenbelt, J. and De Jongh, J. (2001b). A population physiologically based pharmacokinetic/pharmacodynamic model for the inhibition of 11-beta-hydroxysteroid dehydrogenase activity by glycyrrhetic acid. Toxicology and Applied Pharmacology, **170**, 46-55.

Rose, B.D. (1994). Clinical physiology of acid-base and electrolyte disorders. McGraw-Hill, Health Profession Division, New York.

Rosseel, M. and Schoors, D. (1993). Chewing gum and hypokalaemia. The Lancet, **341**, 175.

Rossi, T., Fano, A., Castelli, M., Malagoli, M., Ruberto, A.I., Baggio, G., Zennaro, R., Migaldi, M. and Barbolino, G. (1999). Correlation between high intake of glycyrrhizin and myolisis of the papillary muscles: an experimental in vivo study. Pharmacology and Toxicology, **85**, 221-229.

Russo, S., Mastropasqua, M., Mosetti, M.A., Persegani, C. and Paggi, A. (2000). Low doses of liquorice can induce hypertension encephalopathy. American Journal of Nephrology, **20**, 145-148.

SCF (1991). Reports of the Scientific Committee for Food (29th series). Commission of the European Communities, Food Science and Techniques. Report No EUR 14482 EN, CEC, Luxembourg.

Sheu, C.W., Cain, K.T., Rushbrook, C.J., Jorgenson, T.A., Generoso, W.M. (1986). Tests for mutagenic effects of ammoniated glycyrrhizin, butylated hydroxytoluene, and gum arabic in rodent germ cells. Environmental Mutagenesis, **8**, 357-367.

Sigurjónsdóttir, H.Á., Franzon, L., Manhem, K., Ragnarsson, J., Sigurdsson, G. and Wallerstedt, S. (2001). Liquorice-induced rise in blood pressure: a linear dose-response relationship. Journal of Human Hypertension, **15**, 549-552.

Smorenberg-Schoorl, M.E. and Vree H.M. (1963). Het gebruik van dropjes en "essentiële" hypertensie [Consumption of liquorice candies and "essential" hypertension; in Dutch]. Ned. T. Geneesk., **107**, 2013.

Stewart, P.M. (2002). Adrenal cortex: renin-angiotensin-aldosterone axis and hypertension. In: Besser, G.M. and Thorner M.O. (eds) Comprehensive clinical endocrinology. Elsevier Scientific Publ. Amsterdam.

Størmer, F.C., Reistad, R., Alexander J. (1993a). Adverse health effects of glycyrrhizinic acid in liquorice. A risk assessment. Nordiske seminar- og Arbejdsrapporter 1993:526.

Strandberg, T.E, Järvenpää, A.-L., Vanhanen , H. and McKeigue, P.M. (2001). Birth outcome in relation to licorice consumption during pregnancy. American Journal of Epidemiology, **153**, 1085-1088.

Van Gelderen, C.E.M., Bijlsma, J.A., Van Dokkum, W. and Savelkoul, T.J.F (2000). Glycyrrhizinic acid: the assessment of a no effect level. Human and Experimental. Toxicology, **19,** 434- 439.

Van Vloten, P., Savelkoul, T.J.F., De Groot, G., Koops, R., Loeber, J.G., Van Leeuwen, F.X.R., Looman, C.W.N. and Van der Maas, P.J. (1989). Onderzoek naar de biologische effecten van verschillende doseringen glycyrrhizine bij menselijke

vrijwilligers (pilot study). [Investigation into the biological effects of various dose levels of glycyrrhizin in human volunteers (pilot study); in Dutch]. RIVM report 348801 001, RIVM, Bilthoven, The Netherlands.