European Food Safety Authority

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Theobromine as undesirable substances in animal feed[1]

Scientific Opinion of the Panel on Contaminants in the Food Chain

(Question N° EFSA-Q-2005-223)

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PANEL MEMBERS

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SUMMARY

Theobromine (3,7-dihydro-3,7-dimethyl-*H*-purine-2,6-dione) is a colourless and odourless 3,7-dimethylxanthine with a slightly bitter taste naturally present in the cacao tree (*Theobroma cacao* L.) and its seeds, and consequently in cocoa products and by-products. It is also a metabolite of caffeine in mammals. As a feed material, cocoa pod husk may be used in developing countries where cacao is grown. The cocoa bean shells, cocoa bean meal, cocoa germs and discarded confectionary are used for feed purposes in Europe.

[1] For citation purposes: Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on theobromine as undesirable substances in animal feed. *The EFSA Journal* (2008) 725, 1-66.

Theobromine shows moderate acute toxicity and the dog is more susceptible than rodents. In comparison with other methylxanthines, it has a weak action on the central nervous system and is a weak antagonist of adenosine receptors. Theobromine causes reproductive toxicity targeting the testes in rodents and dogs. It causes developmental effects with delayed ossification in mice exposed *in utero* and skeletal variations in off-spring of rabbits. When exposed to theobromine, dairy cows and calves showed reduction in milk yield/increase in fat (15 mg/kg b.w. per day) and adverse effects such as hyperexcitability, sweating and increased respiration and heart rates. In horses, which are particularly susceptible to theobromine, the liver and thyroid were affected, while pigs showed growth retardation, diarrhoea and lethargy. Theobromine exposure to laying hens caused liver and kidney toxicity, depressed weight gain and egg-production.

Data on theobromine levels in feed materials are lacking. Cocoa husk meal, cocoa bean shell and cocoa bean meal have been reported to contain 1.5-4.0, 8.0-16.9 and 20-33 g theobromine per kg material respectively. Current EU regulations on maximum levels (ML) of theobromine in feed material (300 mg/kg for complete feedingstuffs with the exception of 700 mg/kg for complete feedingstuffs for adult cattle) may not be fully protective for some target animal species, e.g. as effects on milk production in dairy cows and adverse effects in pigs may occur. Owing to the recognized susceptibility to theobromine toxicity, feed manufacturers do not include by-products of cocoa manufacture or confectionary by-products in feeds for dogs and horses.

Theobromine is well absorbed and widely distributed in the body. It is rapidly metabolised and unchanged theobromine and metabolites are mainly excreted in urine. There are no data indicating accumulation of theobromine. Data on carry over and and residues of theobromine in animal products derived from animals exposed to contaminated feed are not available for eggs, meat, offals and milk. Humans are exposed to theobromine mainly from chocolate confectioneries, cocoa drinks and bakery products containing cocoa or chocolate. In addition theobromine is a metabolite of caffeine. The Panel on Contaminants in the Food Chain (CONTAM Panel) concluded that theobromine exposure from animal products such as meat, milk and eggs is expected to be negligible in comparison with direct consumption of cocoa products.

KEYWORDS: Theobromine, 3,7-dihydro-3,7-dimethyl-*H*-purine-2,6-dione *Theobroma cacao* L, cocoa pod, toxicity, exposure, carry-over, animal health, human health.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

1. General background

Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed[2] has since 1 August 2003 replaced Council Directive 1999/29/EC of 22 April 1999 on the undesirable substances and products in animal nutrition[3].

The main modifications can be summarised as follows

- extension of the scope of the Directive to include the possibility of establishing maximum limits for undesirable substances in feed additives.
- deletion of the existing possibility to dilute contaminated feed materials instead of decontamination or destruction (introduction of the principle of non-dilution).
- deletion of the possibility for derogation of the maximum limits for particular local reasons.
- introduction the possibility of the establishment of an action threshold triggering an investigation to identify the source of contamination ("early warning system") and to take measures to reduce or eliminate the contamination ("pro-active approach").

In particular the introduction of the principle of non-dilution is an important and far-reaching measure. In order to protect public and animal health, it is important that the overall contamination of the food and feed chain is reduced to a level which is as low as reasonably achievable while providing a high level of public and animal health protection. The deletion of the possibility of dilution is a powerful means to stimulate all operators throughout the chain to apply the necessary prevention measures to avoid contamination as much as possible. The prohibition of dilution accompanied with the necessary control measures will effectively contribute to safer feed.

During discussions leading up to the adoption of Directive 2002/32/EC the Commission made the commitment to review the provisions laid down in Annex I on the basis of updated scientific risk assessments and taking into account the prohibition of any dilution of contaminated non-complying products intended for animal feed. The Commission therefore requested the Scientific Committee on Animal Nutrition (SCAN) in March 2001 to provide

[2] OJ L140, 30.5.2002, p. 10
[3] OJ L 115, 4.5.1999, p. 32

these updated scientific risk assessments in order to enable the Commission to finalise this review as soon as possible (Question 121 on undesirable substances in feed)[4].

The opinion on undesirable substances in feed, adopted by SCAN on 20 February 2003 and updated on 25 April 2003[5] provides a comprehensive overview on the possible risks for animal and public health as the consequence of the presence of undesirable substances in animal feed.

It was nevertheless acknowledged by SCAN itself and by the Standing Committee on the Food Chain and Animal Health that for several undesirable substances additional detailed risk assessments were necessary to enable a complete review of the provisions in the Annex.

2. Specific background

Theobromine (3,7-dihydro-3,7-dimethyl-1*H*-purine-2,6-dione) belongs to a class of alkaloid molecules known as methylxanthines. Methylxanthines occur naturally in as many as sixty different plant species and include caffeine (the primary methylxanthine in coffee) and theophylline (the primary methylxanthine in tea). Theobromine is the primary methylxanthine found in products of the cacao tree (*Theobroma cacao*), beans and shells.

Cocoa shells, beans and oilcake could serve as feedstuffs for livestock. However the presence of theobromine in feed can result in adverse effects[6].

Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed established maximum levels for theobromine in complete feedingstuffs.

SCAN concluded[7] that theobromine is a natural constituent of plants used for feed purposes. Above certain concentrations, they affect the health of domestic animals while they are without effects on the human consumer of products derived thereof. Any risk posed by feed ingredients containing these compounds is according to SCAN managed by modern techniques of feed formulation. SCAN therefore recommended[8] that theobromine should be

[4] Summary record of the 135th SCAN Plenary meeting, Brussels, 21-22 March 2001, point 8 – New questions (http://europa.eu.int/comm/food/fs/sc/scan/out61_en.pdf)
[5] Opinion of the Scientific Committee on Animal Nutrition on Undesirable Substances in Feed, adopted on 20 February 2003, updated on 25 April 2003 (http://europa.eu.int/comm/food/fs/sc/scan/out126_bis_en.pdf)
[6] Opinion of the Scientific Committee on Animal Nutrition on Undesirable Substances in Feed, point 9.4.3. *Theobromine*.
[7] Opinion of the Scientific Committee on Animal Nutrition on Undesirable Substances in Feed, point 9.5. Conclusions.
[8] Opinion of the Scientific Committee on Animal Nutrition on Undesirable Substances in Feed, point 9.6. Recommendations

excluded from the list of undesirable substances in annex to Directive 2002/32/EC as it concerns a natural constituent of feed ingredients which is not relevant to the control of contamination.

The requested detailed assessment of the risks for animal and public health related to the presence of theobromine in animal feed should provide information to judge if this conclusion and recommendation from SCAN is confirmed.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29 (1) of Regulation (EC) No 178/2002 the European Commission requests the European Food Safety Authority to provide a scientific opinion on the presence of theobromine in animal feed.

This scientific opinion should

- determine the toxic daily exposure levels of theobromine for the different animal species (difference in sensitivity between animal species) above which
    - signs of toxicity can be observed (impact on animal health) or
    - the level of transfer/carry over of theobromine from the feed to the products of animal origin results in unacceptable levels of theobromine or possibly toxic metabolites in the products of animal origin in view of providing a high level of public health protection.
- identify feed materials which could be considered as sources of contamination by theobromine and the characterisation, insofar as possible, of the distribution of levels of contamination for the different (groups of) feed materials.
- assess the contribution of the different identified feed materials as sources of contamination by theobromine
    - to the overall exposure of the different relevant animal species to theobromine,
    - to the impact on animal health
    - to the contamination of food of animal origin (the impact on public health), taking into account the dietary variations and variable carry over rates (bio-availability) depending on the nature of the different feed materials.
- identify eventual gaps in the available data which need to be filled in order to complete the evaluation.

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ASSESSMENT

1. Introduction

1.1. The cacao tree[9]

In most plants synthesizing methylxanthines, caffeine becomes the principal constituent. In some species, however, theobromine or methyluric acids accumulate rather than caffeine. This is the case for the cacao tree, *Theobroma cacao* L. (synonyms are *Cacao guianensis* Aubl., *Cacao minus* Gaertn., *Cacao sativa* Aubl., *Theobroma caribaea* Sweet, *Theobroma interregina* Stokes, *Theobroma kalagua* De Wild., *Theobroma leiocarpa* Bernoulli, *Theobroma pentagona* Bernoulli, *Theobroma saltzmanniana* Bernoulli, *Theobroma sapidum* Pittier, *Theobroma sativa* (Aubl) Lign. Et Le Bey, *Theobroma sphaerocarpa* Chevalier; subspecies, varieties and forms described include *T. cacao* ssp. *cacao* (L.) Cuatr. (criolla), *T. cacao* ssp. *sphaerocarpum* (Chevalier) Cuatr. (forastero, calabacillo, amelonado), *T. cacao* var. *catonga*, *T. cacao* f. *lacandonense* Cuatr. (balamte), *T. cacao* f. *leiocarpum* (Bernoulli) Ducke (porcelaine java criolla, cacao calabacillo), *T. cacao* f. *pentagonum* (Bernoulli) Cuatr. (alligator cacao, cacao lagarto) which accumulates theobromine. This plant is believed to have its origin in the forests of the Amazon and Orinoco areas of South America. It was first cultivated by Indians living in Mexico and Central America, but it was Linné (Linnaeus) who called the cacao tree *Theobroma*, which means 'food for the gods'. It is told in early mythical legends that the Mexican Indians used cacao in their religious rites. The cacao beverage used by the Aztecs consisted of ground cured beans whipped up in hot water and flavoured with pepper and other spices. Christopher Columbus brought the first specimen of cacao beans to Spain as a souvenir on his fourth voyage. By 1580, it was in common use in Spain. A century later, cocoa was well known in most *European* countries and with time became a product in every man's home.

The cacao tree requires a warm and humid climate. The major producers of cacao beans are generally African, South East Asian and Central and South American countries. The Ivory Coast dominates among global producers and produced 1,408,000 tonnes - about 40% of the

[9] Cacao refers to the plant and its parts while cocoa refers to the extracted material.

total output in the world world in 2005/2006. The Ivory Coast, Ghana and Indonesia produce nearly three quarters of cocoa in the world, which was 3,444,000 ton in 2005/2006 (Table 1). On commercial plantations, two cultivated forms dominate – forastero and criollo. The former is planted chiefly in Brazil and Africa, the latter in Central America.

Table 1. Leading cocoa bean producers in the world (1000 tonnes) in 2005/2006 (ICCO, 2007)

Producing countries	**2005/2006**
Ivory Coast	1408
Ghana	690
Indonesia	460
Cameroon	175
Nigeria	160
Brazil	150
Ecuador	118

The tree takes 7 years to mature and produces for 20 years, with an annual yield of around 50 to 60 pods. The ripe colourful, melon-like cocoa pod ripens in about 6 months with a pulp that is sweet and citrus-like. When the pods are ripe they are cut from the tree by hand, cut open and the fresh seeds (20 to 40 per pod) with adhering pulp are taken out and fermented in a heap or a box. The fermentation takes 4 to 6 days and it is followed by drying of the beans which is usually done in the sun for about one week (Feldman, 1998). These dried beans are the starting material for cocoa production. The left-over pods and pulp is a potential source of livestock feed or soil fertilization material becoming available at the site were cacao trees are grown. A simplified diagram illustrating the products associated with the manufacture of chocolate is given in Figure 1.

Cocoa liquor is used for production of chocolate, cocoa powder and cocoa butter. The left-over from the grinding and the shell are potential feed sources at the site of cocoa mass production. Pressing at ambient temperature of the cocoa liquor (cocoa mass) produced during crushing, removes the cocoa butter and produce a "pressing cake" with between 10 and 20% fat. Cocoa powder is produced by grinding the press cake. Thus, cocoa powder is a product comprised of 80-90% fat-free dry matter of cocoa and 10-20% cocoa butter.

1.2. Chemical composition of the cacao fruit, cacao bean and cacao products

The cacao tree contains several chemical constituents that could be classified as toxic or anti-nutritious. Examples of such compounds are the methylxanthines theobromine, caffeine, and

traces of theophylline, the biogenic amines β-phenethylamine and tyramine, oxalate, cyanogenic compounds, furfural, anandamides, tannin and trypsin inhibitor (Dodo *et al.*, 1992; Aremu *et al*., 1995; Rätsch, 1998). With the exception of the methylxanthines, the presence and levels of these compounds in the cacao pod is poorly investigated.

Theobromine was discovered in extracts from cacao beans (*Theobroma cacao*) by Woskresensky in 1842 and its chemical structure (Figure 2) determined by Emil Fischer at the end of the 19th century. Fischer was awarded the Nobel Prize in 1902 for working out the structural formulae of theobromine and the related methylxanthines caffeine and theophylline. Its chemical abstract name is 3,7-dihydro-3,7-dimethyl-1*H*-purine-2,6-dione and the CAS Registry Number 83-67-0. In this text the trivial names are used for the methylxanthines.

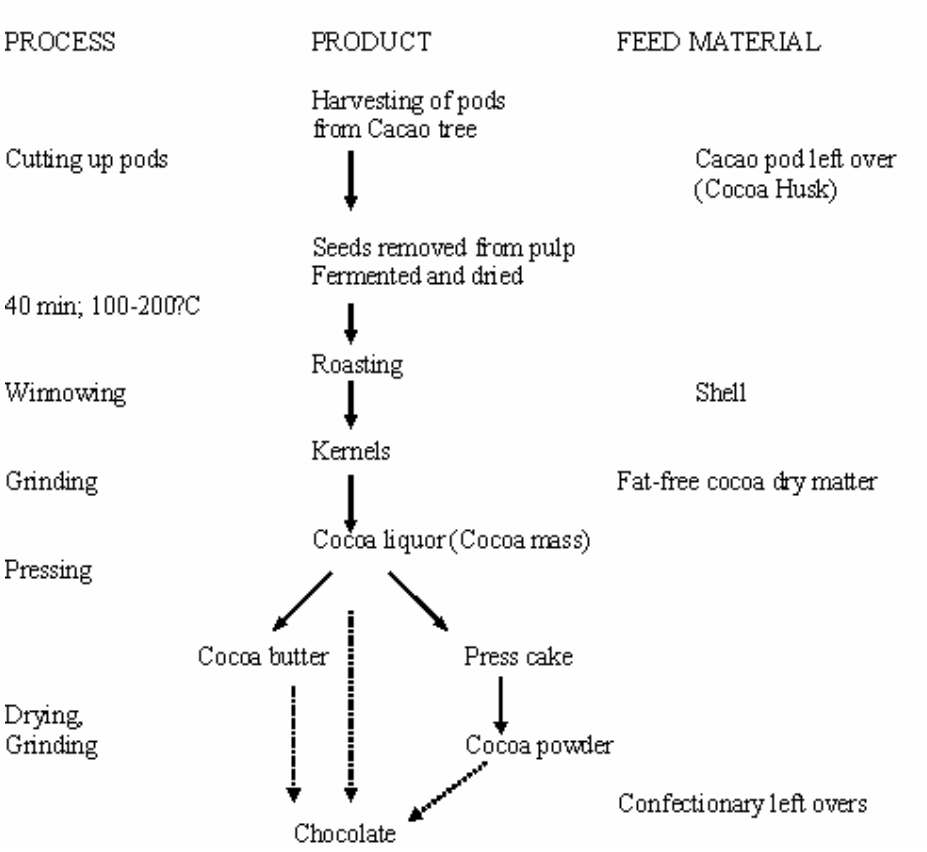

Figure 1. Processes in chocolate production and remains used as feed material.

Theobromine is a colourless and odourless substance (melting point 357°C) with a slightly bitter taste that is naturally present in all parts of the seed and in small quantities in the pod, most likely as a component of the chemical defence mechanism of the cocoa plant (IARC 1991; Windholz, 1983; Aneja and Gianfagna, 2001). It is therefore present in cocoa products and by-products of cocoa production. Theobromine, and to some extent caffeine, contributes

to the typical bitter taste of cocoa and chocolate. Within the European Union most of the import and processing of cocoa beans is in The Netherlands, Germany, Belgium, France and the UK. Whereas cocoa husk (the remains of the cacao pod when beans have been harvested) is the by-product of cocoa production, cocoa meal, cocoa shells, and discarded confectionary are the major by-products of cocoa manufacturing. The shells represent 8-10 percent of raw cocoa bean by weight. When excess cocoa is produced surpluses may be sold for livestock feeding under the name of cocoa meal.

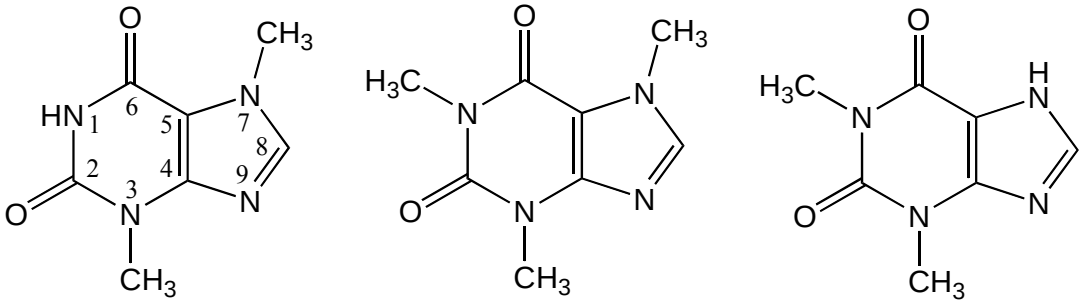

Figure 2. Molecular structure of theobromine (3,7-dimethylxanthine) (Mw 180.2), caffeine (1,3,7-trimethylxanthine) (Mw 194.2), and theophylline (1,2-dimethylxanthine) (Mw 180.2), and the numbering of the xanthine ring structure.

The fresh unfermented cacao beans contain 14-38 g theobromine and 1-8 g caffeine per kg seed material on a dry weight basis (Senanayake and Wijesekera, 1971; Chevalley, 1976; Fincke, 1989; Sotelo and Alvarez, 1991; Naik, 2001). Thus, the caffeine content is usually around 10-15% of the theobromine content. Traces of theophylline may be found. However, the amount of the individual methylxanthines is dependent on the genotype of the cacao tree. African cacaos contain less caffeine and more theobromine than cacaos from South America (Matissek, 1997). Sotelo and Alvarez (1991) also compared the theobromine content in various parts of the *T. cacao* fruit. Whereas the content in seed was high (see above), hull contained around 1 g/kg and shell around 0.2 g/kg. Senanayake and Wijesekera (1971) followed the theobromine and caffeine contents during a cycle of growth of the cocoa pod. They noted that the level of both compounds were negligible until the 3½-month stage when seeds were still mucilaginous. Subsequently, when the seeds started to become hard, both methylxanthines started to increase in amount but the increase was much stronger for theobromine than for caffeine. The increase was much stronger in seeds than in the hull.

During fermentation the methylxanthine concentrations decrease by around 25-40% due to exudation from the nibs to the shells and to the surrounding seatings, but there is no indication that theobromine is degraded (Ziegleder and Biehl, 1988). Hadorn (1980) measured the theobromine and caffeine content of well defined non-roasted, fermented, hand-shelled cocoa

beans and found average values of 29 g/kg theobromine and 2.3 g/kg caffeine on a dry weight basis in beans from Africa (n=7). Beans from Asia contained 23.3 g/kg theobromine and 6.2 g/kg caffeine (n=7), and beans from Latin and South America 25.6 g/kg theobromine and 6.5 g/kg caffeine (n=24).

Also subsequent steps in the cocoa production influence the methylxanthine content of the product. The fermented and dried cocoa beans are roasted to develop the flavour, often for about 40 minutes at 100°C to 220°C (Feldman, 1998). The roasted beans are broken up and the thin shell is removed by winnowing. The remaining kernels of the beans, the so-called nibs are crushed between grinding stones to produce cocoa liquor or cocoa mass. The nibs and the cocoa mass contain around 55% fat (cocoa butter), the rest being fat-free dry matter of cocoa. The water content is very low, less than 2%. Due to the heat evolved as a result of the grinding, the cocoa butter is melted, producing cocoa liquor (cocoa mass), a fluid. Cocoa liquors vary considerably in caffeine and theobromine content with levels between 8 and 17 g theobromine per kg (w/w), with an average of 12 g/kg, whereas the caffeine contents were between 0.6 and 4.2 g/kg (w/w), the average being 2 g/kg. Pressing of the cocoa liquor (cacao mass) produced during crushing, removes the cocoa butter and produce a "press cake" with between 10 and 20% fat. Cocoa powder is produced by grinding the press cake. Thus, cocoa powder is a product comprising 80-90% fat-free dry matter of cocoa and 10-20% cocoa butter. Cocoa powders contain higher amounts of theobromine and caffeine than cocoa liquors because these components are present in the non-lipid portion of the liquor. Since cocoa powders may be produced from different liquors, based on different cacao beans, the theobromine and caffeine content can vary considerably (Fincke, 1989). In a German study on 88 samples of cocoa powder the theobromine content varied from 18 to 38 g/kg, being highest in products from West Afrika and lowest in products from the Pacific. The caffeine content varied from negligible levels, predominantly in West African products, to 9.9 g/kg in products from the Pacific and South America (Fincke, 1989). Hadorn (1980), De Vries *et al*. (1981) and Shively and Tarka (1984) reported similar levels in analysis based on 5, 18 and 8 products, respectively. Slightly lower values were reported by Zoumas *et al.* (1980).

Relatives of *Theobroma cacao* L. may contain theobromine but are not used for cocoa production. Seeds of *Theobroma bicolor* and *Theobroma angustifolium* contain 1.7 and 0.4 g theobromine/kg seed, respectively (Sotelo and Alvarez, 1991). Other *Theobroma* species contain tetramethyluric acid instead of theobromine as the principal methylxanthine, as does 9 *Herrania* species with a similar morphology (Lima Vasconcelos *et al.,* 1975; Marx and Maia, 1991; Hammerstone *et al.,* 1994). *Herrania* was previously considered a section of *Theobroma* but is now a separate genus.

Among other plants, theobromine is the dominating methylxanthine only in two other species - *Camellia ptilophylla* (Ashihara *et al.,* 1998) and *Camellia irrawadiensis* (Nagata and Sakai, 1985; Ashihara and Kubota, 1987). Low quantities of theobromine can also be found in several plants belonging to different, unrelated families and growing in very different parts of

the world (Willaman and Schubert, 1961; Franzke *et al*., 1967), Table 2. The common feature of the majority of these plants is that they grow in tropical or subtropical countries.

With exception of *Butyrospermum parkii*, which is used to prepare sheanut butter and gives sheanut cake as a by-product, none of the other species in Table 2 and none of the *Herrania* species are contained in waste products from industrial processing that might be of interest as feed ingredients for livestock. Sheanut butter is a minor product. The cake contains 4.5 g theobromine per kg cake and in addition saponin anti-nutrients (Atuahene *et al.,* 1998). However, sheanut cake is not a major feed constituent.

Table 2. Occurrence of theobromine (g/kg) in other plant species than those of the genus Theobroma (*recalculated). The only plant species used (locally) as feed is *Butyrospermum parkii*.

Species	**Traditional use**	**Theobromine content**	**Reference**
Butyrospermum parkii	cake (used locally for feed)	4.5	Atuahene *et al.*, 1998
Butyrospermum parkii	cake (used locally for feed)	4.6 - 5.7	Rhule, 1999
Camellia ptilophylla	cocoa tea	49.7 – 68.4 (tree)	Ye *et al.,* 1997
Camellia ptilophylla	cocoa tea leaves	8.8 – 26.2*	Ashihara *et al.,* 1998
Coffea lancifolia	-	0 - 1.4	Rakotomalala *et al.,* 1992
Coffea kianjavatensis	-	0 - 1.4	Rakotomalala *et al.,* 1992
Enicostemma axillare	medicinal plant	not quantified	Retnam and de Britto, 2003a
Hybanthus enneaspermus	medicinal plant	not quantified	Retnam and de Britto, 2003b
Ilex brevicuspis	adulterant to mate	not measured	Filip and Ferraro, 2003
Ilex paraguariensis	maté	5.1 – 11.2	Scherer *et al.,* 2002
Ilex paraguariensis	yerba maté	0.8 – 17.6	Pomilio *et al.,* 2002
Ilex paraguariensis	different tissues	0.05 – 0.21	Mazzafera, 1994
Ilex paraguariensis	wax of leaves	0 – 9.5	Athayde *et al.,* 2000
Ilex perado	leaves	not quantified	Bohinc *et al.,* 1975
Paullinia pachycarpa	-	traces – 0.25	Weckerle *et al.,* 2003
Paullinia cupana	guarana	0.05 – 12.6	Weckerle *et al.*, 2003

Species	Traditional use	Theobromine content	Reference
Paullinia cupana	many different products	0.12 – 1.75	Meurer-Grimes *et al.,* 1998
Paullinia cupana	guarana	not quantified	Bempong *et al.*, 1993
Paullinia yoco	-	0.06 – 0.53	Weckerle *et al.*, 2003

1.3. Biosynthesis of theobromine and caffeine in *Theobroma cacao*

Several reviews on the biosynthesis and metabolism of caffeine and related purine alkaloids are available (Suzuki *et al.,* 1992; Baumann, 1996; Ashihara and Crozier, 1999; Andersson *et al.,* 2004), but none of these focus specifically on the biosynthesis of theobromine. One experimental study, however, focused on theobromine biosynthesis in *Theobroma cacao* fruits (Zheng *et al.*, 2004), and another on theobromine biosynthesis and degradation in *T. cacao leaves* (Koyama *et al.,* 2003).

It has long been agreed that the ring nucleus of methylxanthines is formed via the classical scheme of purine nucleotide biosynthesis (Ashihara and Crozier, 1999), but there have been diverging views regarding the route from xanthine to the methylxanthines. The dispute has recently been settled, however, as convincing data from studies on tea and coffee plant materials demonstrate that the route of synthesis from the simple purines to the methylxanthines goes over the corresponding nucleotides. Further information is available in Andersson *et al.* (2004).

Most of the purine alkaloids accumulating in seeds appear to be synthesised in the seeds (the cotyledons and the embryonic axis) during the final stages of fruit development (Timbie *et al.,* 1978). Zheng *et al.* (2004) were, however, unable to exclude the possibility that some purine alkaloids are translocated from the pericarp to the seed of *T. cacao*.

Although theobromine, and to some extent caffeine, may be regarded as the end product of a long biosynthetic pathway in *T. cacao*, both compounds can be catabolised by mature cacao fruits. In young small fruits (2 g), in medium size fruits (100 g), and in seed coats, cotyledons and placenta of large ripe fruits (500 g) nearly all radiolabelled theobromine remain unchanged after 18 hours of incubation. The pericarp of large ripe fruits, however, metabolised 30-35% of the theobromine during this period. Metabolites formed were carbon dioxide (29%), uric acids (2.7%) and 3-methylxanthine (1.7%) (Zheng *et al.,* 2004). Also 8.6% of the caffeine was metabolised in the pericarp of large ripe fruits, metabolites formed being carbon dioxide (5.7%), theophylline (2.0%), and theobromine (0.7%). Taken together, the data in *T. cacao* indicate that the degradation pathway is theobromine → 3-methylxanthine → xanthine → uric acid → allantoin → allantoic acid →→ carbon dioxide + NH_3 + glyoxylate (Zheng *et al.,* 2004). This route of theobromine degradation has been

demonstrated also in *T. cacao* leaves (Koyama *et al.*, 2003), and is analogous to the catabolic routes for caffeine in coffee and tea plants.

1.4. Toxicology in laboratory animals and hazard assessment for humans

The pharmacology and toxicology of theobromine and other methylxanthines have been reviewed by Tarka *et al.* (1982), Nehlig *et al.* (1992), Sawynok and Yaksh (1993), Nehlig and Debry (1994), Fredholm (1995), Sawynok (1995), Garrett and Griffiths (1997), Eteng *et al.* (1997) and Fredholm *et al.* (1999). Theobromine salts, at doses of 300 to 600 mg per day, were previously used in humans as dilators for coronary arteries (Moffat, 1986). There is no current therapeutic use of theobromine in human medicine. In some studies cocoa products have been used. It should be noted that these contain caffeine in addition to theobromine and that some of the biological effects observed may be ascribed to caffeine.

The biological effects of methylxanthines, including theobromine, which all have been used as pharmaceuticals, can be classified as pharmacological and when adverse as toxic. Usually the pharmacological effects occur at lower doses than the adverse effects. In this context where occurrence of theobromine in feed is not intended, but is naturally present in the feed ingredient also pharmacological effects may be considered as adverse.

In comparison with the methylxanthines caffeine (1,3,7-trimethylxanthine) and theophylline (1,3-dimethylxanthine), the action of theobromine (3,7-dimethylxanthine) on the central nervous system is weak. While the main molecular target of caffeine and theophylline is their antagonistic effect on adenosine receptors, in particular A_1, A_{2A} and A_{2B} subtypes, theobromine is a weak antagonist with a two- and threefold lower affinity to A_1 and A_{2A} receptors than caffeine (Snyder *et al.,* 1981; Carney, 1982; Carney *et al.,* 1985; Shi and Daly, 1999; Fredholm *et al.,* 1999; Fredholm, 2007). In mice, theobromine did not, as compared to caffeine, elicit changes in density of adenosine receptors or downstream alterations in other receptors (Shi and Daly, 1999). At doses higher than those associated with adenosine receptor antagonism methylxanthines such as caffeine and theophylline inhibit phosphodiesterase (Fredholm *et al.,* 1999). Theobromine is apparently a weak inhibitor of phosphodiesterase as it does not interfere with adenosine 3´,5´-phosphate cyclic AMP signalling as do other xanthines (Robinson *et al.,* 1967; Heim and Ammon, 1969). Theobromine and its derivatives act as smooth-muscle relaxants, diuretics, cardiac stimulants, and coronary vasodilators (Windholz, 1983). The diuretic action of theobromine, which is brought about by increased glomerular filtration rate and inhibited reabsorption of sodium and water, is more sustained than that of theophylline, but less pronounced (Fredholm, 1984). Theobromine in chocolate may cause heartburn upon relaxation of the lower oesophageal sphincter resulting in reflux of acid gastric contents (Babka and Castell, 1973).

Acute and subacute toxicity

The acute oral LD_{50} values were 950 and 1356 mg/kg b.w. in rats and mice, respectively (Tarka, 1982). The oral LD_{50} in dogs appears to be about 300 mg/kg b.w. (Gans *et al.,* 1980). A dog consuming 610 mg/kg b.w. in a cake (the absorbed dose being much less) died after 5 hours. Post-mortem examination revealed strongly dilated blood vessels in the intestine, systolic heart, bleeding in spleen and lungs (Beccari, 1936).

Adverse effects of large doses of theobromine in humans may include nausea and anorexia (Reynolds, 1982). Long-term consumption of large quantities of cocoa products, giving a methylxanthine intake of 1.5 g per day, may result in sweating, trembling and severe headaches (Czok, 1974). Some of these latter effects may be due to caffeine.

The target organs of theobromine toxicity in rodents are the thymus and the testes. Thymus atrophy is observed at doses of 250-300 mg/kg b.w. in rats, whereas 850 and 1840 mg/kg b.w. is required in hamsters and mice, respectively (Tarka *et al.*, 1979). A significant decrease in absolute and relative thymus weight, with loss of cortical lymphocytes was observed in rats receiving 2-10 g theobromine/kg feed mixed into the diet (corresponding to 90-140, 215-290 mg/kg b.w., in males and females, respectively) for 4 weeks in two studies and 7 weeks in a third (Tarka *et al.*, 1979; Gans, 1982, 1984). In hamsters and mice only the highest theobromine concentrations in the diet (10 g/kg) produced a decreased thymus weight (Tarka *et al.*, 1979). No abnormal histological changes were seen in any of the hamster tissues examined. Several of the mice receiving the higher doses of theobromine died before the end of the study.

The testes have been shown to be affected in both rodents and the dog (see section on reproductive and developmental toxicity).

Long-term studies of toxicity and carcinogenicity[10]

No studies on the carcinogenicity of theobromine in experimental animals were available.

A number of short-term carcinogenicity studies in model systems have been performed. In one such short-term carcinogenicity study of questionable quality intraperitoneally injected theobromine (18 mg/kg b.w. 6 times during 36 hrs.after ethylcarbamate treatment (single *s.c.* dose of 0.1 mg/g b.w.) significantly reduced both the incidence and number of lung tumours induced by ethylcarbamate in ICR/Jc1 mice (Nomura, 1983).

Slattery and West (1993) in a case-control study on newly diagnosed cases of prostate cancer (n=362) and age-matched controls (n=685) in Utah, the United States, observed an increased risk for prostate cancer in older men with theobromine intakes between 11 and 20, or over 20 mg per day. The odds ratios were 2.06 (95 percent confidence interval (CI)=1.33-3.20) and 1.47 (CI=0.99-2.19). The Utah population from which cases and controls were recruited

[10] For long-term studies in rats see section on reproductive and developmental toxicity

comprised predominantly Mormons or members of the Church of Jesus Christ of Latter-day Saints, whose religion does not allow consumption of coffee.

Genotoxicity

The potential genotoxic properties of methylxanthines including theobromine have been investigated in a variety of experimental systems. These studies have been reviewed by e.g. Kihlman (1977), Tarka (1982), Rosenkranz and Ennever (1987), Kihlman and Andersson (1987), and IARC (1991).

In lower eukaryotes (e.g. *Euglena gracilis, Physaarum pollycephalum, Schizosaccharomyces pombe*) and bacteria (e.g. *E. Coli*, phage T5-resistance, Klebsiella pneumoniae) theobromine induced mutations, but gave negative results in various *Salmonella typhimurium* strains with and without metabolic activation. In cultured rodent cells theobromine induced gene mutations (at cytotoxic concentrations), sister chromatid exchange (SCE), but not chromosomal aberrations or cell transformation. In human lymphocytes *in vitro*, SCE and chromosomal aberrations were seen in some experiments (IARC, 1991). In *in vivo* studies, theobromine induced SCE and micronuclei, but not chromosomal aberrations, in the bone marrow of Chinese hamsters. Theobromine did not induce dominant lethal effects in mice or rats (IARC, 1991).

Brusick *et al.* (1986) evaluated the genotoxic activity of cocoa powder in several *in vitro* assays and concluded that it is inactive in the Ames assay (mutations in *Salmonella typhimurium*), the mouse lymphoma assay, cytogenetic assays measuring chromosome breakage and SCE, and a cell transformation assay using Balb/c-3T3 cells.

Reproductive and developmental toxicity

In Osborne-Mendel rats given 1.0% theobromine mixed in the diet for 3 weeks and 0.5% theobromine (approximately 250 mg/kg b.w. per day) for another 61 weeks severe testicular atrophy was observed in 94% of the animals and aspermatogenesis in 82% (Weinberger *et al*., 1978). The rats had lower weight gains than the controls. Similar findings were obtained for caffeine. Haematological- or blood chemistry parameters and histopathology of organs were not significantly affected except for the sexual accessory organs which were atrophic. Seminiferous tubules were not distinctly delineated, spermatogonia were generally absent and when present degenerated with reduced mitotic activity. Leydig cells in many severely atrophied testes appeared to be hyperplastic. The authors confirmed their finding in a follow up study on Holtzman rats fed a diet with 0.5% theobromine for 19 weeks. In this experiment the testosterone levels were increased.

Subsequently, several studies have investigated the effects of dietary exposure to pure theobromine on the male reproductive tract (Tarka *et al.,* 1979, 1981; Gans, 1982, 1984). The exposures varied from 2 to 10 g/kg of the diet (corresponding to 90-140, 215-290, 310-380, 420, and 490-560 mg/kg b.w., respectively) for 4 to 8 weeks. The theobromine exposure led

to decreased food consumption and body weight gain possibly due to reduced palatability (Gans, 1982, 1984). Caffeine had similar or possibly stronger effects than theobromine (Gans, 1984). A considerable extent of damage to the testes was noted at doses above 300 mg/kg b.w. and exposure times of at least 4 weeks. The destructive effects of high theobromine doses on the rat testis to a large extent are irreversible (Tarka *et al.*, 1981).

In male dogs short- and longer-term (up to one year) theobromine administration at doses from 25 to 150 mg/kg b.w. per day failed to cause testicular atrophy (Gans *et al.*, 1980).

Rats treated 5 times daily by oral gavage with 500 mg theobromine/kg b.w. were examined after 1, 2, 4, 6 and 10 weeks. The most striking morphological observation was a retarded release of late spermatids into the tubular lumen 2 weeks post treatment indicating selective interference with germ cell kinetics possibly due to Sertoli cell toxicity (Russell, 1984; Ettlin *et al.* 1986). In addition theobromine exposure resulted in decreases in cauda epididymal sperm reserve, seminiferous tubule fluid volume, lactate concentration in seminiferous tubule fluid, binding activity of androgen binding protein, and reduced content of the androgen binding protein in seminiferous tubule fluid (Wang and Waller, 1994). Also more recent studies on male Sprague-Dawley rats gavaged daily with 0, 250 or 500 mg theobromine/kg b.w. for 2 or 4 weeks, resulted in reduced weight gain at the higher doses and similar effects on testicular and thymus tissue, and in addition relative prostate- and seminal vesicle weight were reduced at the highest dose (Funabashi *et al.*, 2000).

In later studies toxicity of theobromine in cocoa extracts or cocoa powder generally showed similar or less pronounced effects to those of pure theobromine, including Sertoli cell toxicity as a primary target and failed release of spermatides (Wang *et al.* 1992; Wang and Waller, 1994).

Effects on reproduction

The US National Toxicology Programme (NTP) assessed the reproductive effects of theobromine on Swiss CD-1 mice (NTP, 1984). The mice were fed 0, 1, 2.5 or 5 g/kg of theobromine in the feed (estimated intake: 0, 126, 335 and 630 mg/kg b.w. per day) for 18 weeks (1 week of premating, 14 weeks of cohabitation, and 3 weeks thereafter). The end-points studied were fertility, number of live and dead pups, average pup weight, and sex ratio. The number of litters per rat pair were reduced at the highest dose and the percentage of live pups per litter were reduced at all doses. Cross-over matings were performed to study whether the reproductive toxicity was limited to only one sex given 5 g theobromine/kg feed (630 mg/kg b.w.). The studies showed reproductive capacity to be severely impaired in female mice administered theobromine, as demonstrated by (reduced) number of live pups per litter, reduced proportion of pups born alive, and reduced pup body weights. In males ingesting a diet containing 5 g theobromine/kg, the incidence of abnormal sperm was significantly increased. The highest dose caused increased liver weights and reduced brain weights in both sexes and in males the testicular weight was reduced. Histopathological studies revealed no

morphological changes in the reproductive organs, and hormonal patterns were unchanged in both sexes. The F1 generation was not examined for reproductive toxicity. The studies have been summarised by Lamb *et al.* (1997).

When theobromine was administered in the diet of pregnant rats, at doses of 4 or 8 g/kg diet (corresponding to approximately 290 or 471 mg/kg b.w. per day) on day 15 to 20 of gestation, average daily food consumption decreased in the high dose group without any external signs of toxicity (Fujii and Nishimura, 1973). Upon sacrifice, on day 21 of gestation, no effect on number of resorptions was observed, but both doses reduced the average body weight of the foetuses.

Tarka and co-workers (1986b) performed studies to evaluate the effects of dietary cocoa powder (containing 24.6 g theobromine/kg and 1.9 g caffeine/kg) on gestation, parturition, lactation, and postnatal growth and viability, and to determine the effect of dietary cocoa powder and theobromine on teratogenesis in rats. In female CD-rats given diets containing 0, 25, 50 or 75 g cocoa powder/kg feed from day 0 of gestation to day 21 of lactation, no clinical signs were observed in the dams. The length of gestation was unaffected by the treatments. No gross abnormalities were observed in the dams and no effects were observed on the litter size at birth or sex ratio. Three weeks after delivery, the litter size was slightly reduced at the highest dose and pup survival slightly reduced at the two highest doses of cocoa powder (50 and 75 g/kg feed). Additional groups of CD rats were fed diets with 0.68 or 1.35 mg theobromine/kg diet (corresponding to 53 and 99 mg/kg b.w.) on day 6-19 of gestation. No treatment-related clinical signs were observed on the dams. The number or percentage of pregnant dams and the mean number of corpora lutea did not differ between the cocoa powder or theobromine treated and control rats. No differences between the treatment groups were observed for number of implantations, percentage of live foetuses, mean litter size, number of dead and resorbed foetuses, and mean sex ratio. The incidence of foetuses with neither external, visceral nor skeletal anomalies was not different between the cocoa powder treated, theobromine treated and control groups. The highest dose of cocoa powder (corresponding to 102 mg/kg b.w. of theobromine) and theobromine (99 mg/kg b.w.) induced a significant increase in incompletely ossified or absent sternebrae, indicating a delay in osteogenesis. The authors concluded that the variations observed could be attributed to non-specific maternal toxicity and not related to the specific test item given.

As investigations had indicated growth promoting effects of low doses of theobromine on rat pups and weanling rats (Hart and Grimble, 1990a; Zoumas and Tarka, 1976), Hart and Grimble (1990b) studied the influence of theobromine on lactational performance in albino Wistar rats. The daily dosage of theobromine from drinking water during pregnancy was 2 mg/kg b.w. This dose had no influence on food and fluid intake and did not influence body weight gain. The exposure increased litter weight, but had no influence on litter growth rate, milk volume produced and the general composition of the milk.

Effects in off-spring

In New Zealand rabbits, administered theobromine by gavage at doses of 25, 75, 125 and 200 mg/kg b.w. per day during gestation days 6-29, maternal deaths were seen in the highest dosed group (40%). Decreased foetal body weights were seen at the two highest doses and skeletal variations were increased with 75 mg/kg b.w. and above. Other groups fed diets with theobromine giving daily doses of 21, 41 and 63 mg/kg b.w. per day showed no maternal toxicity, but skeletal variations were increased at the doses of 41 and 63 mg/kg b.w. (Tarka *et al.,* 1986a).

Skopiński *et al.* (2003) studied the effect of daily dietary administration of 400 mg bitter chocolate to two month old mice (3.5 mg theobromine per mice, approximately 140 mg/kg b.w.) during pregnancy and lactation. Endpoints studied were organ weight, lengths of limbs, and bone vascular endothelial growth factor (VEGF) concentration in 4-week old offspring, due to the suspected inhibitory effect on angiogenesis and production of angiogenic growth factors (Skopińska-Różewska *et al.*, 1998). The exposure resulted in a decrease in the relative length of limbs and thigh bones and a decreased content of VEGF in femoral bones of offspring.

Evaluations

Since neither data on theobromine with respect to carcinogenicity in experimental animal, nor epidemiological data in humans of theobromine *per se* was available, IARC (1991) concluded that the evidence for theobromine being carcinogenic in humans and experimental animals was inadequate. Therefore the compound was not classified as to its carcinogenicity to humans (group 3). No ADI or equivalent health based guidance value for theobromine has been established by international bodies. Testicular toxicity appears to occur at about 300 mg/kg b.w. in rats, whereas doses up to 150 mg/kg b.w. did not induce testicular toxicity in dogs. The NOAEL for testicular toxicity is 150 mg/kg b.w. per day. A dose of about 126 mg/kg b.w. of theobromine induced reproductive toxicity in mice (reduced live pups per litter). In rabbits a dose of 41 mg/kg b.w. given during pregnancy caused variations in skeletal development in the off-spring, with a NOAEL of 21 mg/kg b.w.

2. Methods of analysis

Purine derivatives including methylxanthine derivatives (Figure 2) are heteroaromatic compounds with characteristic UV absorptions in the 260-280 nm area and molar extinction coefficients of 1.3×10^4 $M^{-1}cm^{-1}$. This gives basis for sensitive analytical detection of these compounds following various chromatographic separations. However, a great number of different biomolecules including a great number of ubiquitous purine derivatives, give UV absorption in the area of 260-280 nm, which means that efficient separation capacities of the

applied analytical procedures are required for reliable determination of the individual methylxanthines in feed materials.

Initial problems in the analytical procedure are the solubilities and extractabilities of the compounds in question. Several studies have been devoted to the feasibility of efficient extraction of theobromine and other methylxanthines from cocoa beans including use of CO_2 as supercritical fluid (SF) in extraction procedures (SFE). The lower extractability of theobromine in comparison with caffeine in aqueous solvents and by SFE (Salvadori *et al.*, 1994; Hatfull *et al.*, 1980) limits the use of the techniques, and further work is needed to optimize the extraction procedure including SFE (Li *et al.*, 1991; Li and Hartland, 1992, 1996; Johannsen and Brunner, 1995; Saldaňa *et al.*, 2002). Due to the above mentioned interference with other compounds, and the extraction problems also discussed, some older determinations might be unreliable.

Chromatographic procedures such as paper chromatography and thin-layer chromatography suffer from too low separation capacities (IARC, 1991; Matissek, 1997) and focus is therefore, directed at LC methods with higher separation capacities as high performance LC (HPLC) (Hurst *et al.*, 1985; Casoli and Verine, 1990; Delbecke and Debackere, 1991; Gurney *et al.*, 1991; Salvadori *et al.*, 1994; Hieda *et al.*, 1995). The low solubility of theobromine and caffeine in aqueous solutions and thus also in the mobile phase used in HPLC are closely related to the temperature. Furthermore, all HPLC methods with high separation capacities require suitable sample clean-up prior to the separation procedure (Taylor, 1982; Blanchard *et al.*, 1990; Naik, 2001). With the appropriate sample preparation including extraction and clean-up procedures, HPLC with diode array detection (DAD) presents an efficient procedure (Zambonin *et al.*, 2004) as the case is for micellar electrokinetic capillary chromatography (MECC) (Perez-Martinez *et al.*, 1995). In general, gas chromatography (GC) have high separation capacities but is also a technique, which require appropriate sample clean-up and derivatization procedures. This most often results in significant losses and experimental difficulties both for GC and GC-MS (mass spectroscopy).

More recently, LC-MS and LC-ESI-MS methods have been developed and validated for measurement of methylxanthines in beverages and capsules of dietary supplements (Zhu *et al.*, 2004; Marchei *et al.*, 2005). The main advantages of these systems are the rapid and simple extraction and sample preparation required and the specificity of the analysis. The limit of detection lies in the region 0.02-0.03 mg/kg, and the limit of quantification around three times higher at 0.06-0.09 mg/kg. The response of the LC-ESI-MS method was linear in the region 0.0049-1.96 mg/kg. A similar technique based on gradient capillary high-performance liquid chromatography frit-fast atom bombardment mass spectrometry (FAB-MS) (LC-frit-FAB-MS) to measure methylxanthines in human plasma and urine has been developed (Hieda *et al.*, 1995).

3. Statutory limits for theobromine in feed materials

The Council Directive 2002/32/EC prescribes that all products intended for animal feed must be of sound, genuine, and of merchantable quality and should pose neither a risk to animal health and productivity nor to human health or the environement. Annex 1 to Council Directive 2002/32/EC[11] lists at present a number of compounds that are considered undesirable in animal feeds and prescribes maximum limits in different feed commodities. The current EU maximum levels (ML) for certain feed materials containing theobromine are given in Table 3.

Table 3. EU legislation on theobromine in feed materials as listed currently in Annex 1 of Council Directive 2002/32/EC.

Undesirable substances	**Product intended for animal feed**	**Maximum content in mg/kg relative to a feedingstuff with a moisture content of 12%**
Theobromine	Complete feedingstuffs with the exception of: - complete feedingstuffs for adult cattle	300 700

4. Occurrence in feed materials[12]

The edible part of the cocoa bean, the cotyledon, is usually used by the cocoa production industry and does not go to the feed industry. The nutrient composition of the raw cocoa bean and the fermented product is well documented (e.g. Jinap *et al.*, 1993; Aremu *et al.,* 1995). The proximal composition of cocoa bean cotyledons is influenced by the month of harvest as well as by the genotype studied (Alvarado *et al.,* 1983). The principal components of the cotyledons are lipids (48%) of which around 57% are based on saturated fatty acids, the main ones being palmitic acid 25-30%, oleic acid 32-37%, and stearic acid 30-37%. Linoleic and arachidonic acids are present in smaller concentrations i.e. 2-4% and 0.7-1%, respectively (Horman and Bracco, 1986; Staphylakis and Gegiou, 1985; Bucheli *et al.,* 2001). The type of fatty acids present in the triglycerides and the way they arrange themselves during crystal formation determine the hardness of the cocoa butter. In relation to methylxanthines it has been shown that young beans do not contain these compounds. Theobromine appears during the eight week of maturation and reaches a maximum around week 20, after which the level is slightly reduced. Caffeine does not appear until at week 16 (Senanayake and Wijesekera, 1971; Jinap *et al.,* 1993; Bucheli *et al.,* 2001). The ripe unfermented bean usually contain 6-

[11] OJ L 140, 30.5.2002, p. 10–22

[12] Where the moisture content of materials has not been stated, a value of 12% has been assumed.

30.6 g theobromine and 0.2-5.3g caffeine per kg bean (Paiva *et al.,* 1982; Alvarado *et al.,* 1983; Ziegleder and Biehl, 1988).

Considerable efforts have been made to utilise the various parts of the harvested pod which are discarded en route to the finished chocolate. Potential uses as animal feed are summarised below but has also in the past been reviewed by Greenwood-Barton (1965) and Owusu-Domfeh (1972). In these reviews references to the composition of cocoa germ and mucilage (pulp) can be obtained.

To increase the utilisation of cocoa wastes several technologies to extract or reduce theobromine content have been explored (Adomako, 1985). Methods to reduce the methylxanthine content have been reviewed (Sebald *et al.,* 1996).

It is generally acknowledged that pigs, poultry and companion animals (including horses, cats and dogs) are particularly susceptible to theobromine poisoning and for this reason cocoa shells and meal are not used in the manufacture of feeds for these animals. FAO (2008) report that cocoa shells and oilcake are acceptable to ruminans in small amounts (up to 0.8 kg/day have been fed without adverse effect); however, Ewing (2002) has recommended a maximum inclusion rate for cocoa residues in ruminant diets of 2% (equivalent to a maximum of approximately 0.5 kg/day). In practice, because of their low digestibility (Aregheore, 2002) and palatability compound feed manufacturers limit the inclusion of cocoa by-products in ruminant feeds, which ensures that levels of theobromine are below those specified by legislation.

Small quantities of chocolate may also be available as feed ingredients from chocolate manufacturers in the form of rejected (non-standard) confectionary products. The majority is handled in solid form, and, following processing to remove packaging material, is blended with other feed materials for pigs. A proportion of the reject chocolate is processed with other forms of confectionary and marketed as liquid chocolate, mainly to pig farmers (Crawshaw, 2001).

4.1. Feed materials derived from the fruits of the cacao tree

Particularly in developing countries there is a scarcity of protein and energy rich food and feed plants. Those that are available are used as food by man, and will be too expensive for use as animal feed. As it is only the seeds of the cocoa fruit that are used for human food production, there are abundant amounts of agricultural by-products available for alternative use. In many places, what is left over when the cocoa beans have been removed from the cacao pod (cacao pod husk) is left to rot on the plantation or is used as fertilizer (Oladokun, 1986). However, by-products of cocoa manufacture, including cocoa husk (cocoa pod), cocoa bean shell (cocoa hull), cocoa seed meal and cocoa germ have been investigated for food use. Whereas the last three may become available in the country where cocoa beans are processed,

the cocoa husk is not usually exported from the country where the Cacao tree was grown. Within the European Union most of the processing takes place in The Netherlands, Germany, Belgium, France and the United Kingdom. The chemical composition of cocoa husks, shells and meals has been widely reported and are summarised in Table 4.

Table 4. The proximate composition (% dry matter) of by-products from the manufacture of cocoa

Proximates	**Cocoa husks**[13]	**Cocoa bean shell**[14]	**Cocoa bean meal**[15]
Moisture	5.4-15.3	4.9-12.0	2.1-16.9
Crude protein	6.3-10.4	13.2-20.1	17.8-28.6
Crude fibre	23.4-36.2	9.3-20.5	5.3-22.0
Ether extractable components	0.5-2.4	1.9-22.0	1.1-17.8
Nitrogen-free extract	31.8-61.4	40.2-52.5	25.9-51.1
Ash	7.0-15.3	6.0-10.8	3.0-15.1

The variation in proximate content could be due to year-to-year variation, different varieties being studied (Alvarado *et al.,* 1983), and differences in the preparations of the samples for analysis and the analytical method.

4.1.1. The cocoa husk

It takes between twenty and twenty-four weeks for cocoa pods to develop from the time the flowers are fertilized until the fruits are fully ripened, naturally depending on the climatic conditions and where the tree is growing. Growth of the pods is generally slow at the beginning and then speeds up. When the beans are removed from the pod after harvest, they are covered by the mucilage pulp. Both the initial colonisation and subsequent microbial activity is largely dependent upon the nature of the pulp surrounding the beans. Pulp of mature fruit generally contains 82-87% water, 10-13% sugar and 1% pectine (Hardy, 1960). The sugar constitutes mainly glucose and fructose which are metabolised by microorganisms such as yeast and lactic acid bacteria during fermentation, and traces of sucrose.

The parts of the cocoa pod left over, the cocoa husk, represents between 2/3 and 3/4 of the total weight of the fruit (average fruit weight about 400 g) and is usually discarded by local

[13] From Braude and Foot, 1942; Schneider, 1947; Haines and Echeveria, 1955; Morrison, 1957; Dittmar, 1958; Evans, 1960; Bateman and Fresnillo, 1967; Oyenuga, 1968; Devendra and Gohl, 1970; Owusu-Domfeh *et al.,* 1970; Ashun, 1973; Wong and Abu Hassan, 1988; Falaye, 1990; Abiola and Tewe, 1991; Donkoh *et al.,* 1991; Fleischer *et al.*, 1991; Fagbenro, 1995; Aregheore, 2002

[14] From Crowther, 1936; Knapp and Churchman, 1937; Black and Barron, 1943; Schneider, 1947; Pearman *et al.,* 1951; Morrison, 1957; Evans, 1960; Bonadonna *et al.,* 1963; Oyenuga, 1968; Owusu-Domfe *et al.,* 1970; Abiola and Tewe, 1991; Aregheore, 2002

[15] From Braude and Foot, 1942; Schneider, 1947; Pearman *et al.,* 1951; Morrison, 1957; Evans, 1960; Velloso *et al.,* 1965; Oyenuga, 1968; Fagbenro, 1988; Flachowsky *et al.,* 1990; Offem, 1990; Odunsi and Longe, 1995a; Odunsi *et al.*, 1999

farmers after harvest when the beans have been taken care of (Ashun, 1975). Ashun (1975) presents a calculation by Dittmar that cocoa plantations would produce around 4,650 kg of dry cocoa pod per hectare.

Typical values for the composition of cocoa husks are given in Table 4. More detailed information on protein quality is given by Oyenuga (1968), Ashun (1975), Okai *et al.* (1984), Falaye (1990) and Donkoh *et al.* (1991). The ash composition has been investigated in more detail by Ashun (1975), Agyeman and Oldham, 1986; Aregheore (2002) and Gowda *et al.* (2004), and the fatty acid composition by Ashun (1975). Vadiveloo and Fadel (1992) freeze dried and milled the cocoa husk and analysed the milled material for nutrient composition. These studies showed that cocoa husk in addition to the nutrients shown above contain high amounts of soluble phenolics and condensed tannins, and a high content of uronic acids (Vadiveloo and Fadel, 1992). The theobromine level has been reported to be around 1.5-4.0 g/kg dry weight (Barnes *et al.,* 1985; Abiola and Tewe, 1991; Falaye and Jauncey, 1999; Falaye *et al.,* 1999). These data indicate that cocoa husk is rich in fibre, and is poor in metabolisable energy and crude protein, in particular for non-ruminants.

Studies to assess the quality and safety of cocoa husk as livestock feed have been performed since 1944. Those that have some bearing on the safety of the feed material are presented in section 6.

4.1.2. Cocoa bean shell

After the beans have been isolated from the pods, usually with some associated fruit flesh, dried and fermented, the manufacturing of various cocoa products starts. During this process around 10-12% in weight of fibrous material surrounding the nib (cotyledons) is discarded. This material mainly consists of seed coat and embryo, often called cocoa shell, and is separated from the cotyledons (the edible part) by roasting. The shell is a dry, crisp, slightly fibrous brown husk with a pleasant odour resembling that of chocolate. The fibre content is equivalent to medium quality grass hay in feeding value. When the shell is removed, it may contain 2-3% of an unseparated cocoa nib. In Samoa and other Pacific Island countries where cocoa is produced, the shell that covers the cocoa bean is used as mulching material on the farm (Aregheore, 2002). Typical values for the composition of cocoa bean shells are given in Table 4. More detailed information on protein and fatty acid quality is given by Owusu-Domfeh *et al.* (1970). The phytase activity of cocoa shell has been reported to be low (Eeckhout and De Paepe, 1994).

The chemical composition of cocoa shell indicates it might be a useful ingredient for ruminant feeding. Ashun (1975) reviewed the feeding studies up to the 1960's. These studies proved cocoa shell to be a useful ingredient in cattle feeding (for meat or milk production). It may also be used as fuel or as manure. Studies on rumen dry matter degradability, and apparent digestibility of cacao bean shells, as well as the influence of the shells on the rumen

fermentation and performance of sheep and dairy cows allowed Flachowsky *et al.* (1990) to conclude that cacao bean shells may be used as roughages in ruminant diets up to 5% of dry matter intake. The factor limiting the use of cocoa bean shell in feed is the theobromine level which is dependent on the way the cacao bean is prepared for the market. Originally the shell contains a limited amount of theobromine. This is acquired from the nib during fermentation. The shell of most well-fermented commercial cacao beans contains over 1% theobromine – five samples contained between 0.80 and 1.69%. Abiola and Tewe (1991) reported a level of 1.9%. Knapp and Churchman (1937) presented a complete analysis of a commercial sample of roasted shells; the average theobromine content was 13 g/kg (8.0-16.9 g/kg), and the caffeine content around 1 g/kg. In another study Alvarado *et al.* (1983) measured the theobromine and caffeine content of five different shell fractions collected over the whole growing season and observed 14.0 g/kg (7.5-21.0 g/kg) theobromine and 1.4 g/kg (0.8-2.3 g/kg) caffeine, respectively.

4.1.3. Cocoa bean meal

When excess cocoa is produced surpluses may be sold for livestock feeding under the name of cocoa bean meal hereafter sometimes referred to as cocoa meal. Cocoa meal may also be prepared from discarded cocoa beans, press cake of cocoa beans, or residues from cocoa factories. The composition of the meal varies considerably depending on the amount of shell fragments incorporated in the meal and the degree of oil extraction. Reports of the proximate composition of the cocoa meal are summarised in Table 4. More detailed information on protein quality and mineral content is given by Owusu-Domfeh *et al*. (1970), Adegbola and Omole, 1973), and Offem (1990), and the fibre and carbohydrates by Flachowsky *et al.* (1990). The high fibre content and the content of the cell wall constituents (neutral and acidic detergent fibre and lignin) suggest that cacao bean shells are more suitable for ruminants than monogastrics (Flachowsky *et al.*, 1990). A drawback of the cocoa meal is its high theobromine content, typically 20-33 g/kg. The caffeine content is lower, around 1-4 g/kg.

Adegbola and Omole (1973) studied the influence of treating ground cocoa meal with various concentrations of sodium hydroxide or warm water of various temperatures to improve the usefulness of cocoa meal as a grower-fattener ration for swine. Water treatments for a few hours above 60^{o}C efficiently extracted theobromine. The hot water treatment retained nutritional quality of the product better than an alkali treatment.

4.1.4. Occurrence of theobromine in European feed materials

Data on the concentrations of theobromine in feed materials in the EU are scarce. In preparation for this Opinion, EFSA issued a call for occurrence data within the Member States. Data were received from Belgium and the Czech Republic (Table 5). As part of routine surveillance in Belgium, 26 samples of feed materials were analysed for theobromine

in feed for different animal species during the period 2000-2005. One complementary feed for pigs contained 229 mg/kg theobromine, while levels in two complementary feeds for cattle were found to have theobromine levels of 321 and 385 mg/kg. For five complementary or complete feeds, levels of theobromine were between 10 and 70 mg/kg, while in 20 samples levels were <10 mg/kg (i.e. below the level of quantification (LOQ)). One sample of cocoa hulls contained 6,100 mg theobromine/kg. Thus in none of the complete feedingstuffs tested from Belgium did theobromine content exceed the MLs. Both samples of complete feedingstuffs (for rabbits) from the Czech Republic exceeded maximum permitted levels of theobromine. Also samples of cocoa meal and hulls contained high levels of theobromine. In addition, a private company from France (INZO) submitted data on analysis of cocoa hulls, 16 analyses were performed and the concentration of theobromine was between 160 and 10,800 mg theobromine/kg cocoa hulls with an average of 6150 mg/kg.

Table 5. Levels of theobromine (mg/kg) in feed materials in the EU reported to EFSA

Feeding material	**Provider**	**Theobromine content (mg/kg)**
Complete feed, rabbits (n=2)	Czech Republic	554; 774
Complete feed, rabbit (n=2)	Belgium	<LOQ*
Complete feed, dog (n=3)	Belgium	3x<10
Complete feed, pig (n=4)	Belgium	2x<LOQ; 1x<10; 229
Complete feed, poultry (n=2)	Belgium	17.6; 63
Complementary feed, bovine (n=3)	Belgium	47.4; 321; 385
Complementary feed, horse (n=10)	Belgium	7x<LOQ; 1x<10; 25.8; 27.1
Complementary feed, dairy cows (n=1)	Belgium	<LOQ
Cocoa husk (n=1)	Belgium	6,100
Cocoa husk (n=15)	INZO, industry data	160-10,800 (mean 6150)
Cocoa hulls (n=1)	Czech Republic	6,004
Cocoa meal (n=1)	Czech Republic	21,968

The LOQ of the Belgian data was 5 mg/kg and the LOQ of the Czech Republic data was approximately 100 mg/kg, n is number of samples.

5. Estimating the intake by farm livestock in the EU

Cocoa pods are generally not imported into the EU, and therefore the cocoa husks are not normally available. Cocoa meal is only exceptionally used as feeding material, while the availability of chocolate confectionary for use as livestock feeds is highly variable. The latter is most commonly available in those EU countries in which cocoa processing takes place (predominantly Germany, the UK, The Nederlands, France and Italy). In addition, there is a seasonal effect, with peaks of chocolate production at Christmas and Easter leading to

increased feed availability. More recently, there has been considerable interest in the use of rejected chocolate confectionary as an energy source in power generators.

Chocolate factories produce waste, equivalent to about 5-10% of their total output. Liquid chocolate may occasionally be available, and it is usually used in pig diets. However, most of the chocolate entering the animal feed chain does so as finished products which have been rejected by the factory because they have not met quality control standards, e.g. material produced at the start or end of production runs, or as a result of factory breakdowns (Crawshaw, 2001).

Given this degree of variability, it is difficult to estimate intake by livestock. Crawshaw (2001) estimated that approximately 20,000 tonnes of chocolate enters the animal feed chain in the UK each year. The majority is handled in a solid form, and processed before inclusion in blended feeds for ruminants and pigs. About 25% is blended with other confectionary waste and marketed to pig farmers as liquid chocolate.

Estimates of intake by pigs, poultry, fish and ruminants are given in Table 6. These are based on typical diet formulations as used in previous feed Opinions of this Panel, and assume that the complete feedingstuffs contain the maximum permitted level of theobromine[16]. The data on intakes are summarised in the table below, and show that under these conditions intakes (mg/kg liveweight/day) range from 6.4 to 21.4 (growing lambs, suckler cows) to 21.4 (broilers).

It should be noted that these levels of exposure assume the ML of theobromine in complete feedingstuffs. Although there are relatively few data on the theobromine contents of feeds in the EU, the data that are available would suggest that intake or dietary concentrations will be lower than indicated in Table 3.

[16] 300 mg/kg in a complete feedinstuff with a moisture content of 12% (700 mg/kg for mature cattle)

Table 6. The estimated intake of theobromine by livestock consuming complete feedingstuffs containing the ML of theobromine[17].

Pigs, poultry and fish					
Livestock	Body weight	Total complete feed, kg/day	Theobromine intake		
	kg	kg/day	mg/day	mg/kg of diet	mg/kg b.w. per day
Finishing pigs	100	3.7	1110	300	11.1
Sows	250	6.5	1950	300	7.8
Poultry (broilers)	2.1	0.15	45	300	21.4
Poultry (laying hens)	1.9	0.115	34.5	300	18.2
Fish[18]	4.5	0.09	27.0	300	6.0

Ruminants							
Livestock	Assumed body weight	Total DM intake	Concentrate intake	ML	Theobromine intake		
	kg	kg/day	kg/day	mg/kg	mg/day	mg/kg of diet	mg/kg b.w. per day
Dairy cow	650	22.00	14.00	700	9800	445	15.1
Suckler cow	550	16.00	5.00	700	3500	219	6.4
Growing cattle	300	8.00	7.00	300	2100	263	7.0
Lactating ewe	70	1.8	1.5	300	450	250	6.4
Growing lamb	20	0.6	0.45	300	135	225	6.8
Dairy goats	65	2.2	1.5	300	450	205	6.9

Amongst feed manufacturers, the toxicity of theobromine to dogs and horses is well-recognised and consequently they do not include by-products of cocoa manufacture or confectionary by-products in feeds for these animals. It is recognised that owners of horses and dogs may give chocolates to their animals, but the risks associated with doing so are outside the scope of this Opinion.

6. Adverse effects on livestock, pets and fish

There are many published reports of feeding studies with animals fed on cocoa products. The main endpoints studied are production parameters as weight gain, milk yield, and general

[17] 300 mg/kg, with the exception of mature cattle (700 mg/kg)
[18] Mature Salmonids

health. Not all of the reports discuss theobromine as a potential toxin in the cocoa products and quite few studies have quantified theobromine. The present risk assessment is mainly based on the studies where theobromine was measured and discussed to be involved in effects. Several feeding studies with cocoa pods have been conducted on livestock, the pods have a low concentration of theobromine and in general it is the restricted nutritive value that restricts its usability.

6.1. Ruminants

6.1.1. Cattle

Knapp and Churchman (1937) reported on experiments where dairy cows were fed 0.9 kg (2 pounds) of dried cocoa shell per day for up to more than seven months. The theobromine concentration was 10.3 g/kg cacao shell. Thus, each cow ingested 9.3 g theobromine per day. The body weights of the cows were not reported. Assuming that the cows weighed around 400 kg, the cows ingested 23 mg theobromine/kg b.w. per day. The general health of the cows was good, milk yield was normal but there was a tendency to increased butter fat yield. No change in taste of the milk was noted. There was a very slight stiffening effect in the stools, which was considered an advantage. The authors concluded that cocoa shells can be safely used as an accessory fodder for cattle when fed at a level of 0.9 kg per day.

Aplin and Ellenberger (1927) reported that the fat content of milk was increased but the milk yield decreased in dairy cows when 20% of a grain ration of 1.4-4.5 kg/day was substituted with cocoa meal (with approximately 2% theobromine) for one week. Thus, the daily amount of cocoa meal ingested was 0.28-0.90 kg, corresponding to approximately 5.6-18 g theobromine/cow per day. If we expect the cow to weigh around 400 kg, the daily dose of theobromine was 14-45 mg/kg. Another group of cows received a grain ration with pure theobromine added at 0.4% (giving the same dose of theobromine) and obtained similar results. Thus, an effect on milk production was observed in cows fed theobromine at about 14-45 mg/kg b.w. per day for a period of one week.

Weniger and co-workers performed a series of feeding studies with cocoa shell (7.0 g/kg theobromine), cocoa bean meal (29.6 g/kg theobromine) and pure theobromine in cows. Feeding lactating cows for over a year with cocoa shell meal, or for over one month 250-750 g cocoa bean meal per day, or 12-31 days 7.4-22.2 g pure theobromine daily, resulted in significant reduction in milk yield but increased fat content of the milk (Weniger *et al.,* 1955, 1956). The studies identified theobromine as the likely cause of the observed effects. A dose-response was noted. Assuming a body weight of around 450 kg the lowest dose corresponded to 15 mg/kg b.w. per day. Twentyfive g pure theobromine/day (corresponding to approximately 55 mg/kg b.w.) had no toxicological effects on the cows.

Curtis and Griffiths (1972) reported on six calves (3-5 months old) reared on a dairy farm, which for some weeks were given a feed with 5-10% waste chocolate. They got a stiff gait,

and when let out into the orchard they became hyper-excited, sweating and had greatly increased respiration rates and very rapid pulse; there was no evidence of blindness or nystagmus. One of the calves died. Post-mortem examination revealed no major abnormalities. The investigators concluded that the caffeine content of the cocoa material might have resulted in the hyper-excitability, whereas the theobromine may have been responsible for the death. When the chocolate waste was withdrawn from the other five calves they recovered in ten days. The concentrations of theobromine and caffeine were expected by the authors to be 0.3 and 0.1%, respectively. The estimated doses of theobromine and caffeine may have been 45-90 and 15-30 mg/kg b.w., respectively.

To conclude on cattle, effects on milk yield and fat content seem to occur in dairy cows fed theobromine at approximately 15 mg/kg b.w. Adverse effects were found in calves fed theobromine around 45-90 mg/kg b.w.

6.1.2. Goats

Aregheore (2002) evaluated the inclusion of cocao shell or cocao dust (a waste byproduct in the manufacture of chocolate) into goat feed. Goats of 18-20 month (20.5-21.3 kg b.w.) were fed a diet containing up to 50% of a cocoa product (unknown theobromine content) for 56 days. In the control diet this portion instead was dried brewers´ grain. Goats fed cocoa dust (from cocoa powder production) in particular, but also those fed cocoa shell had significantly reduced voluntary dry matter intake which resulted in correspondingly reduced body weight gain. The author indicated that the effect could be due to occurrence of theobromine in the cocoa material. If we estimate the theobromine concentration in the cocao shell to be 13 g/kg and that of cocao dust to be similar to that of cocoa bean meal, 20 g/kg, the theobromine intake was 6.9 and 9.7 g/animal per day, corresponding to 290 and 420 mg/kg b.w. per day.

Thus, as a conclusion for goats, reduced dry matter intake and body weight gain were found at the lowest theobromine level tested, assumed to be approximately 300 mg/kg b.w per day for 56 days.

6.1.3. Sheep

Theobromine was supplied to diets of lambs with an initial weight around 28 kg in the form of cocoa shell (9.9 g theobromine and 1.5 g caffeine per kg cocoa shell) in rations at 0, 4.63, 9.25, 13.87 and 18.5% of the diet, or corresponding levels in the form of theobromine in rations at 0, 0.05, 0.10, 0.15 and 0.20% of the diet for a period of 98 days (Tarka *et al.,* 1978). Cocoa shell levels at 4.63 and 9.25% and theobromine at 0.05 and 0.10%, were found to stimulate feed intake and growth. Feeding higher rations of cocoa shell or amounts of theobromine led to a depression in feed intake and weight gain. Histopathological studies of

the liver, thymus, kidney and adrenals revealed no abnormalities in the animals. Thus, the NOAEL of theobromine was 1 g/kg diet, corresponding to approximately 35 mg/kg b.w.

Aly (1981) administered sheep a single dose of 40 mg theobromine per kg body weight in the form of the pure compound. No adverse effects appeared during the twelve-day follow-up period. In a second experiment the same author fed a single dose of 3 g cocoa shells per kg body weight (approximately 50 mg theobromine per kg b.w.), and followed the sheep for 12 days. Also in this case, no effect was observed. In the third and last experiment, sheep were fed the same daily ration (3 g cocoa shells/kg b.w. with approximately 50 mg theobromine/kg b.w.) for five days. The feed intake was reduced day by day and also a significant reduction in body weight which was normalised within some days on normal feedingstuff.

As a conclusion, the NOAEL in lambs exposed for 98 days was approximately 35 mg/kg b.w In adult sheep reduced feed intake was observed when fed theobromine at approximately 50 mg/kg b.w per day for 5 days. No effects were observed when this level was given as a single dose.

6.2. Horses

Five horses received individually different quantities of cocoa bean meal containing 5.8 g theobromine/kg (Kelly and Lambert, 1978). An 11-year-old thoroughbred mare was given 1.4 kg cocoa bean meal waste (8 g theobromine) by stomach tube. The horse became completely anorexic within 24 hours, and developed diarrhoea. The pulse increased the second hour after the intake but temperature and respiratory rate changed only marginally. The second horse was a five-year-old thoroughbred mare given only 50 g cocoa bean meal corresponding to 0.29 g theobromine. The horse appeared normal after the intake and faeces was not influenced. The third horse was a 10-year-old light draught mare given 200 g cocoa bean meal containing 1.16 g of theobromine. The blood picture was normal. Elevated serum levels of glutamic oxalacetic transaminase was found on days 4 and 6 without change in creatine phosphokinase, which was changed days 1 and 2, and the plasma triiodothyronine level which was changed days 6 and 8, the out-of-phase occurrence of the markers makes no explanation facile. The fourth horse was a six-year-old thoroughbred mare given 250g cocoa bean meal containing 1.45g theobromine on two occasions, separated by 24 hours. The mare was anorectic the second day with insignificant fluctuations in temperature, pulse and respiratory rates. The packed cell volume, total leucocytes and plasma protein were elevated on day 1 and 5, the first day probably due to reduced water intake, the fifth day probably due to the diuretic effect of theobromine. The increase in creatine phosphokinase on day 5 was not accompanied by an increase in plasma triiodothyronine level. The last horse was a 10-year-old thoroughbred mare given 250g cocoa bean meal containing 1.45 g theobromine at three occasions, each dose separated by 24 hours. Appetite was somewhat reduced on day 2 and 3. Temperature, pulse and respiratory rates showed no significant changes. No explanation could

be offered for a sustained leucopenia. Blood glucose levels were increased on day 6 only but sodium values were consistently high throughout the study. Although not fully authenticated, the evidence obtained from the studies on pulse rates, blood glucose, creatine phosphokinase and triiodothyronine values suggests that theobromine exerts similar pharmacokinetic effects in horses as in laboratory animal species and in man. The weights of the horses were not given, but if assumed to be around 600 kg, the single dose of 0.29 g theobromine which produced no effects corresponds to 0.5 mg/kg b.w. The single dose of 1.16 g theobromine, which produced some biochemical effects, corresponds to 2 mg/kg b.w.

6.3. Pigs

Braude and Foot (1942) studied pigs given a feed with 8% cocoa meal (theobromine 25-27 g/kg) or 8% or 16% detheobromined cocoa meal (4.5 g theobromine/kg cocoa meal) for 126 days. Thus, the dietary theobromine content was around 2.1 g/kg for those fed the untreated cocoa meal feed, and 0.36 and 0.72 g per kg feed for those fed the detheobrominated feed. Estimated average daily doses of theobromine in these three groups were 70, 12 and 24 mg/kg b.w. The pigs had a weight of around 28-30 kg at the start of the experiment and reached slaughter weight of around 85-92 kg before it was over. Test diets resulted in growth retardation, particularly strong during the first 4-5 weeks and strongest in the 8% cocoa meal group but were less pronounced later in the study. Pigs that received 8% cocoa meal lost their appetite, appeared lethargic, and became dirty (caused by soft faeces). One of the animals in this group died and two other were severely affected but recovered. Veterinary examination of the dead pig revealed pneumonia, fluid in the pericardial sac, patchy inflammation in the small intestine and liver enlargement. Pigs receiving 8 or 16% detheobrominated cocoa meal appeared a little lethargic during the first weeks and gained weight more slowly than controls. A follow-up study on pigs with initial weights around 52-60 kg were fed 10% cocoa meal in the diet for 50 days (Braude and Foot, 1942). The feed was accepted with proper weight gain and no influence on liver weight. The theobromine concentration in the feed was approximately 2.6 g/kg. The estimated daily dose of theobromine was around 87 mg/kg b.w.

In another study, Braude (1943) gave 14-15 week old pigs a diet with 5 or 7.5% cocoa meal containing 28 g theobromine and 14 g caffeine per kg cocoa meal (an exceptionally high caffeine content) over 24 weeks. Estimated daily doses of theobromine and caffeine in the 5% cocoa meal diet were about 50 and 25 mg/kg b.w., and in the 7.5% cocao meal diet 80 and 40 mg/kg b.w. Both average daily weight gain and food utilization figures were below normal. At the higher dose the pigs (initial weight 54 kg) became lethargic and produced dark diarrhoea. Three of the five pigs receiving this dose died; the days before dying body temperatures were increased and behaviour abnormal. On post-mortem examination lungs showed pneumonic lesions, hearts looked normal but the pericardial fluid was excessive, livers were congested and deep magenta coloured, and stomach and large intestine were also congested. Kidney, bladder and spleen were normal. The 5% cocoa meal diet was not

sufficient to cause signs of toxicity but did not allow the pigs to grow at a normal rate. Follow up studies with older pigs showed that dietary inclusion of 10% cocoa meal produced signs of toxicity after nine weeks also in these animals.

Yang *et al.* (1997) performed experiments with starter pigs (4.9-9.6 kg) to investigate the effect of substituting dried whey in feed for a by-product of milk chocolate production. The chocolate product was a dried by-product of milk chocolate, candy and food industries, and consisted of approximately one-third whole milk, one-third cocoa and one-third sucrose. The methylxanthine content of the feed was not stated. The feeding periods were 2, 4 or 5 weeks in different experiments. The endpoint studied was growth performance. The studies, using two races of pigs, showed that the milk chocolate product could replace dried whey at a dietary level of 5% without reducing pig performance, but at 10% or more the dried by-product reduced performance. The authors indicated that this could be due to theobromine. Nonetheless, pigs strongly preferred the milk chocolate product over dried whey. Assuming the theobromine concentration in the milk chocolate product to be 1.35 g/kg (Andersson *et al.*, 2004), 5% of the product in the ration means a theobromine concentration in the ration of 68 mg/kg, indicating the NOAEL was approximately 7 mg/kg b.w. per day. .

To conclude, for young growing pigs a NOAEL of 7 mg/kg b.w. may be derived. Older growing pigs may tolerate higher doses.

6.4. Birds

Chickens

Day and Dilworth (1984) fed broiler chickens starter diets with 0, 1, 2, 4, or 6% cocoa shell meal (at the expense of maize) from day 1 to 21 of age. By analysis, cocoa shell meal contained 13g theobromine per kg. The cocoa shell meal did not significantly affect 3-week body weights, but feed conversion at 3 weeks was significantly affected by feeding 6% cocoa shell meal. The investigators claimed that performance tended to be depressed over 1% cocoa shell meal. The addition of pure theobromine to four additional diets to levels identical to those provided by 1, 2, 4 and 6% cocoa shell meal depressed performance somewhat more than did cocoa shell meal and reached significance at the two highest doses. The highest dietary theobromine concentration without significant adverse effects (NOAEL) was 260 mg/kg (corresponding to 2% cocoa shell meal), estimated to correspond to a theobromine dose of 26-39 mg/kg b.w. per day.

Odunsi and Longe (1995a) fed six groups of day-old chickens (Isa Brown pullet type) isonitrogenous (but not isocaloric) diets with 0, 5, 10, 20, 30 or 40% cocoa bean cake for 9 weeks. As the theobromine content of the cocoa bean cake was 22.4 g/kg, the diets contained 1.1, 2.2. 4.5, 6.7, and 9.0 g theobromine per kg feed, respectively. At 4 weeks of age, 2 chickens were randomly selected for blood collection, and at 8 weeks of age, 2 chickens were sacrificed to evaluate the influence of the feed on the relative weights of liver, kidney and

heart to the body weight. The experiment was ended after 9 weeks. Feed intake and weight gain were depressed at 20% inclusion of cocoa bean cake and above. As the metabolisable energy of the cocoa bean cake-containing feed was reduced, an increased feed consumption was expected. Feed intake was however reduced at 20% cocoa bean cake and above. Clearly, the reduced weight gain was correlated to the reduced feed consumption (and reduced protein intake). Inclusion rates of 10% or more cocoa bean cake resulted in reduced kidney and heart weight and increased liver weight. No effects were observed at the 5% cocoa bean cake level, estimated to correspond to a theobromine dose of 110 mg/kg b.w. per day after the first week. Due to the relatively higher feed intake in the first week of life, the estimated theobromine dose during their first days was 165 mg/kg b.w. per day. Mortality was low and not related to treatment. Haematological parameters such as haemoglobin concentration, packed cell volume and red blood cell count were reduced with increase in cocoa bean cake. The authors interpreted these findings as possibly a consequence of the reduced feed intake.

Odunsi and Longe (1998) fed 28-day old broiler chickens a standard maize-groundnut based diet, or diets with 15 or 30% cocoa bean meal (22.4 g theobromine/kg) for 14 days. Broiler chickens that received the cocoa meal had reduced feed intake and weight gain, and increased mortality with dose. The lowest inclusion of cocoa bean meal, 15%, corresponds to 3.4 g theobromine/kg diet (estimated to be 340 mg/kg b.w. per day). The experiment also included diets with cocoa bean meal that had been pretreated to reduce the content of theobromine. Hot water-extracted cocoa bean meal contained 9.8 and cocoa pod ash-treated cocoa bean meal 3.3-17 g theobromine/kg. However, the pretreatment also changed the nutritional composition of the meal. The theobromine reduced cocoa bean meals were mixed in diets at 15, 30 or 45% levels and fed to the 28-day old broilers for 14 days. The pretreatment of the feeding material reduced the adverse effects but also reduced feed intake and weight gain seemed to be found also at the lowest theobromine concentration; 15% cocoa pod ash treated cocoa bean meal with a theobromine concentration 0.95 g/kg diet, estimated to be 95 mg/kg b.w. per day.

In another experiment, Odunsi *et al.* (1999) fed hot water pretreated cocoa pod ash, alkali treated and untreated cocoa bean meal to 28-days old Anak 180 broiler chickens for four weeks. The theobromine concentration in the hot water extracted cocoa bean meal, the alkali-treated cocoa bean meal, and the non-treated cocoa bean meal were 9.8, 6.3 and 22.4 g/kg. The three types of cocoa bean meal were included in separate diets at levels of 15% and 30%, respectively. Chickens receiving 15% of the untreated cocoa bean meal or more, corresponding to an intake of 3.4 g theobromine or more per kg diet, performed less well than chickens on the control diet. The most pronounced effects were reduced feed intake, reduced daily weight gain, reduced haemoglobin levels and increased creatinine levels. These negative effects were not observed in chickens given the hot-water or alkali-treated cocoa bean meal feeds at an inclusion rate of 15% of the diet, reducing theobromine exposure to 1.5 and 0.95 g theobromine per kg feed (estimated to be 150 and 95 mg/kg b.w. per day). However, the higher inclusion rate of pretreated cocoa bean meal, 30%, resulted in those adverse effects.

In conclusion, the NOAEL of theobromine in young chickens was found to vary between 260 and 1100 mg/kg diet (approximately 26-110 mg/kg b.w. per day). In older broiler chickens, a LOAEL of 950 mg/kg (approximately 95 mg/kg b.w. per day) was found.

Laying hens

Fangauf and Haenfel (1938) reported that substituting 20% of laying Leghorn hens feed with cacao shell meal for four months resulted in a decreased feed consumption, reduced weight gain, reduced egg production and lower egg weight than in fowls given normal hen diets. Assuming the theobromine concentration in cocoa shell meal to be 13 g/kg, the diet contained 2.6 g/kg, corresponding to 160 mg/kg b.w. per day.

Black and Barron (1943) reported on a poisoning episode in laying hens. Among 300 hens that were fed a diet including 15% cacao shell 80 birds died suddenly in convulsions, possibly after a short period of nervous excitability. The cocoa shell contained 17 g theobromine/kg. After death, hens were lieing on their backs with their legs tightly drawn against their bodies. The comb frequently showed a marked bluish tinge and the cloaca was violently everted as though in an effort to expel faeces. The only organ changes observed post mortem were a colour change of the liver, and mottled appearance of kidneys that, coupled to histological changes, indicated subacute glomerulo-nephritis. During the episode egg-production was reduced by around 80%. When the cacao shell ration in the feed was reduced to 7.5%, egg production rose again. The cause of the poisoning episode mentioned above was tested in a small feeding experiment in which groups of three fowls were fed for 200 days diets with 0, 7.5, 15 or 30% cacao shell (contained 17 g theobromine/kg). All hens in the highest dose group died. In the 15% dose group two hens died. Hens in the 7.5% dose group (1.3 g theobromine/kg diet, estimated to be around 80 mg/kg b.w. per day) survived and consumed a normal amount of feed but the droppings were looser than normal.

Black and Barron (1943) concluded that feeding cocoa meal containing 15 g of theobromine per kg may be lethal to hens. The authors concluded that feeding 15% and upwards of cocoa meal to laying birds is extremely harmful; it decreased appetite and egg production, and caused scouring and high mortality.

Four groups of 20-week old layers (Isa Brown pullet type) were supplied isonitrogenous (but not isocaloric) diets with 0, 5, 10 or 20% cocoa bean meal for 25 weeks (Odunsi and Longe, 1995b). Assuming the same feed was used as in the study of Odusi and Longe (1995a), the pullets were given diets containing 1.1, 2.2. 4.5, 6.7, and 9.0 g theobromine per kg feed. Egg production was followed carefully. Delayed start of egg production was observed in all groups fed cocoa bean meal. It was not known whether the effect was caused by theobromine. Otherwise, there were no adverse effects on layers and laying performance. During the second half of the laying period, no influence of the diets was observed. Thus, the diet with 5% cocoa bean meal assumed to contain 1.1 g theobromine/kg feed (estimated to correspond to 66 mg/kg b.w. per day), was the lowest observed effect level.

In conclusion, for laying hens the LOAEL is 1100 mg/kg diet (corresponding to 66 mg/kg b.w. per day). No NOAEL has been identified.

6.5. Rabbits

Tarka *et al.* (1986a) evaluated the teratogenic potential of theobromine and cocoa powder in New Zealand White rabbits by supplying the tested compounds from day 6 to day 29 of gestation. In these studies the animals were either gavaged doses at 25, 75, 125 and 200 mg theobromine per kg b.w. or supplied theobromine or cocoa powder via the feed. The highest gavaged dose was close to the LD_{50} value (40% of the animals died). The most frequent clinical signs of the theobromine exposure were anorexia and vaginal discharges. The primary finding at autopsy was gastric mucosal haemorrhage/congestion. Mean foetal weights were similar to those of the control animals at the doses 25 and 75 mg/kg b.w. but were reduced in animals receiving 125 or 200 mg/kg b.w. Also the percentage of dead and resorbed foetuses and the number of malformations and developmental variations were increased in the two higher dose groups. Malformations were increased at 75 mg theobromine/kg b.w. per day and above. Maternal toxicity prohibited a thorough study of the litters at the highest dose. In the dietary exposure studies to determine potential teratogenicity, theobromine at 0, 0.625, 1.25 or 1.875 g/kg diet (which resulted in approximate doses of 0, 21, 41 or 63 mg/kg b.w. per day) or cocoa powder (24.6 g theobromine and 1.9 g caffeine/kg) at 0, 2.5, 5.0 or 7.5% in the diet (approximately 0, 25, 50 or 75 mg methylxanthines/kg b.w.) were used. Some diarrhoea and soft stools were observed in all dose groups fed cocoa powder. Four deaths and a few abortions were registered in this study but not considered to be treatment related as not related to dosage. Maternal weight gain and food consumption were unaffected by the dietary treatment, as were the mean number of corpora lutea. Neither foetotoxicity, nor teratogenicity was associated with the dietary exposure to cocoa powder or theobromine in these studies. At the two highest doses of theobromine an increased incidence of incompletely ossified or absent sternebrae were noted. Similarly the highest dose of theobromine and cocoa powder, respectively, resulted in corresponding effects on metacarpal bones, indicating a delay in osteogenesis. A NOAEL of 21 mg/kg b.w., based on variations in skeletal development, was identified from this study.

Soffietti *et al.* (1989) performed two studies to investigate the toxic effects of theobromine on mature and immature male rabbits. In the first of these studies, mature (3.2-3.6 kg) New Zealand rabbits were for 120 days exposed to a diet containing 0, 0.5, 1.0 or 1.5% theobromine. Neurological symptoms, which were common at the beginning of the trial and progressively disappeared during the remainder of the experiment were noted in the two highest dose groups. Animals in these dose groups also showed decreased weight gain; at the highest dose even weight loss. Seven of the eight animals in both of the two highest dose groups died, and four of the eight animals in the 0.5% theobromine dose group as well. Only one of the animals in the control group died. No effects on haematological values were observed and urine analyses displayed only minor changes (moderate proteinuria and slight

haematuria in all treated groups). The rabbits that died showed severe pulmonary congestion, slight to moderate hydropericardium and scattered foci of myocardial necrosis. The fact that necrotic lesions were found spread in the myocardium supports death from cardiac failure as suggested by Decker and Myers (1972). In the abdominal cavity, slight ascites, liver and kidney congestion and redness of the gastro-intestinal mucosa was noted. Animals dying early in the study showed severe thymic lesions but no influence on the testes. The histopathological studies consistently revealed lesions related to theobromine administration in the heart (degeneration and necrosis of myocardial fibres throughout the myocardium), thymus (severe haemorrhages) and testes (dose-dependent degeneration and necrosis of seminiferous tubules, including vacuolation of spermatids and spermatocytes to multinucleated cell formation and oligospermia or aspermia with extensive degeneration of tubule cells; Sertoli and Leydig cells were not influenced). In lungs, congestion of the capillaries and intra-alveolar oedema was noted.

In the second experiment, immature rabbits (1.3-2.3 kg) of the same strain were given the same types of diets for 10 or 20 days (Soffietti *et al.,* 1989). Half the number of animals was killed after 10 days, the other half after 20 days. Clinical signs and mortality were dose-dependent. The earliest thymic alterations in immature rabbits consisted of a blurring of the demarcation between cortex and medulla accompanied, in the more advanced stages, by a decreased lymphocyte density. The feed intake of the rabbits was not given. Assuming it to be 5% of the body weight, the lowest theobromine concentration in the feed of 5 g/kg diet would correspond to an exposure of approximately 250 mg/kg b.w,

6.6. Dogs and cats

There are several reports of dogs being intoxicated or suspected to be intoxicated by chocolate, cocoa by-products or cacao bean mulch (a fertilizer material) (Clough, 1942; Decker and Myers, 1972; Sutton, 1981; Strachan and Bennett, 1994; Hovda and Kingston, 1994; Stidworthy *et al.,* 1997).

Chocolate poisoning in dogs is seen in veterinary practices (Hornfeldt, 1987). The most severe toxicity signs are related to the CNS including nervousness, restlessness, excitement, tremors, seizures and coma. In addition, cardiac stimulation, panting, vomiting, thirst, diarrhea, urinary incontinence and sudden death have been reported (Hooser and Beasley, 1986). Fatally poisoned dogs have exhibited cyanotic mucous membranes, swollen red gastric and duodenal mucosal surfaces, scattered petechial and ecchymotic thymic hemorrhages, congestion and hyperemia, renal hyperemia with cytoplasmic hyaline droplets, pyknosis and karyorrhexis of the convoluted tubules, and degenerative and fibrotic changes in the right atrial appendage (Drolet *et al*., 1984; Hornfeldt, 1987). These effects could partly be caused by caffeine also present in chocolate.

An 8 month old Airedale Terrier (12.3 kg) died 6 hours after having consumed around 250 g chocolate. Theobromine intoxication was confirmed by thin-layer chromatography. Before death the dog vomited, and became comatose with periodic clonic muscle spasms or convulsions. The visible mucous membranes were cyanotic. Autopsy revealed that the mucosal surfaces of the stomach and the first 12½ cm of the duodenum were red and swollen (Decker and Myers, 1972).

Based on the fairly high bioavailability of theobromine from chocolate products (see section 7.3.4), Loeffler (2000) concluded that 160 g chocolate or cocoa powder would lead to theobromine intoxication in a dog of 20 kg, corresponding to about 60 mg/kg b.w., and that 400 g could be lethal, corresponding to about 150 mg/kg b.w.

In hitherto unpublished studies by Percy and Borys, mentioned by Hornfeldt (1987), one dog died in each of the following theobromine dosage groups (form unknown) in an experimental study: 1000 mg/kg b.w. (n=2), 500 mg/kg b.w. (n=8) and 300 mg/kg b.w. (n=4). Below 200 mg/kg b.w., no deaths were observed (n=19).

Stidworthy *et al.* (1997) noted that two dogs weighing 22.6 and 25.8 kg suddently died after an intake of 20-30 g chocolate each. Strachan and Bennett (1994) examined post mortem two dogs that died after having shared 225 g cocoa powder (around 4.4 g cocoa powder/kg b.w.), corresponding to a theobromine dose of approximately 80 mg/kg b.w. Post-mortem examination revealed cyanosis, cardiac arrest in diastole and severe congestion of stomach, intestines and liver. Unclotted haemorrhagic fluid was present in the peritoneal and thoracic cavities and stomach contents included cocoa and tin foil. In another case, a springer spaniel that had consumed an entire 250 g packet of common household cocoa, corresponding to 208 mg/kg b.w. of theobromine, died around 12 hours after cocoa intake (Sutton, 1981). The dog showed diffuse pulmonary congestion, foamy oedematous fluid in the airways, and patchy over-inflated areas of the diaphragmatic lobes. Thymus had scattered petechial and ecchymotic haemorrhages and the liver, kidneys and pancreas were congested. Histopathological examination revealed pycnotic nuclei in the distal renal tubular epithelium.

There are also reports from the United States that dogs have been intoxicated by consuming organic mulch (a fertilizer) composed of cocoa beans and shells, used for fertilising home gardens (Drolet *et al.,* 1984; Hovda and Kingston, 1994; Hansen *et al.,* 2003). The theobromine content in these products varies considerably; contents from 0.2 to 3.0% have been reported. Reported symptoms of poisoning include CNS depression, restlessness, diarrhoea, vomiting, ataxia, haematuria, tachycardia, hyperpnea, muscle tremor, seizures and death.

In acute toxicity studies in male mongrel dogs, a single theobromine dose of 500 or 1000 mg/kg b.w. resulted in panting, restlessness, and muscle tremors beginning approximately 4 to 5 hours after theobromine administration, and persisting for 6 to 8 hours. None of the dogs given 200 mg/kg b.w. or less died, whereas 3 of the 14 dogs given 300-1000 mg/kg b.w. died (Gans *et al.,* 1980). There were no gross cardiac lesions in the dogs which died.

Gans *et al.* (1980) also performed subacute toxicity studies with ten of the dogs surviving the acute toxicity study. They received an oral dose (generally 125-150 mg/kg b.w. per day) for the subsequent 21 or 28 days. Seven of these dogs died during the experiment although they seemed to be in excellent health before death. The cause of death was not thoroughly investigated. None had thymic or testicular atrophy, as has been reported in rats. Instead six of the ten dogs had a degenerative, fibrotic cardiomyopathy limited to the right atrial appendage of the heart. The appendage was discoloured and on microscopic examination revealed extensive coagulation-type necrosis of atrial muscle fibres with polymorphonuclear leucocyte infiltration that with time were replaced by connective tissue. There were no gross cardiac lesions in the control dogs.

In follow-up studies, two groups of dogs received a daily dose of theobromine for 1 year at 25 or 50 mg/kg b.w. (Gans *et al.*, 1980). Other dogs were given 25 or 50 mg theobromine/kg b.w. per day for the first four months and subsequently 100 or 150 mg/kg b.w. per day for the eight following months. Three of 17 theobromine-dosed dogs died during the study. One of these dogs showed the effects on the right atrial appendage. Dogs that survived the 1-year study, including the controls, were killed and subjected to complete necropsies. As in the subacute toxicity study, the only gross and microscopic change associated with the theobromine was a fibrotic lesion in the right atrium of the heart in three of the five dogs given 150 mg theobromine/kg b.w. per day and in two of the four dogs given 100 mg theobromine/kg b.w. per day. No pathological lesions were found in dogs dosed 25 or 50 mg/kg b.w. for one year. To sum up, administration of theobromine leads to a degenerative and fibrotic lesion in the right atrial appendage of the heart accompanied initially by an acute inflammatory process. It subsequently results in necrosis of the atrial appendage muscle fibres, the replacement of necrotic muscle fibres by collagen, and infiltration of mononuclear cells. It was hypothesised by Gans *et al.* (1980) that injury to small arteries and arterioles in the affected right atrial tissue produce tissue hypoxia and ischemic degeneration. No clear relationship between dose, plasma concentration, frequency, and severity of the lesion could be determined.

According to Merck Veterinary Manual (2006), mild signs of intoxication (vomiting, diarrhea, polydipsia) may be seen in dogs ingesting 20 mg methylxanthines (theobromine and caffeine) per kg body weight, cardiotoxic effects may be seen at 40-50 mg/kg, and seizures may occur at doses ≥60 mg/kg. However, no references to these numbers were given.

To conclude on dogs, acute fatal intoxication seems to occur after single ingestion of theobromine at 80-300 mg/kg b.w. In one study dogs tolerated 50 mg/kg b.w. per day of theobromine for one year. Toxicity at lower doses, down to 20 mg/kg b.w. was reported in a veterinarian manual. These limited studies show that the margin for theobromine/chocolate between the acute fatal dose and the no adverse effect levels in dogs is low.

Considering dark chocolate with a high level of cocoa and theobromine (max reported is 6300 mg theobromine/kg chocolate), and assuming a 20 kg body weight, the lowest fatal dose

would be approximately 250 g dark chocolate, corresponding to 13 g dark chocolate/kg b.w. A safe intake would theoretically be below 3 to 8 g dark chocolate/kg b.w.

No studies were found on intoxication of cats, which is probably because cats don't like chocolate.

6.7. Wild animals

In a series of small tests, Johnston (2005) evaluated the effect of cocoa-derived methylxanthines on coyotes. In the first of these studies two animals were given a ration of theobromine and caffeine (13:1) in lard/rendered bacon fat/soybean oil that the animals could consume within 3 hours. One of the animals vomited shortly after the acute dose and survived. The other animal that had ingested 413 mg theobromine and 31.6 mg caffeine died following 15 seconds of relatively minor symptoms, which included recumbent posture and laboured breathing. In a second experiment coyotes were gavaged with a single dose of a theobromine/caffeine mixture (13:1) at 400, 450, 650 or 850 mg/kg body weight, diluted in 1:24 in water (4 animals/dose), and given 60 mL extra water. Before it was decided to administer the methylxanthines in water, two coyotes received 450 mg of the mixture/kg body weight in water suspension, and two other animals in soybean oil suspension. The two animals that received the latter suspension regurgitated the suspension shortly after dosing and survived. The two animals receiving the water-based suspension retained the methylxanthines and showed relatively mild signs of toxicosis: increased salivation and slight trembling for several minutes. Premortality symptoms were relatively mild in this study, but percent lethal toxicity ranged from 0 to 75% and was dose-dependent. The LD_{50} was calculated to be 516 mg/kg b.w. and the LD_{99} 619 mg/kg b.w. Another group of coyotes was treated with 600 mg methylxanthines/kg b.w. but the ratio of theobromine to caffeine differed: 1:1, 1:2, 2:1, 4:1, 5:1 or 6:1. Animals given the dose in the ratio 1:1 and 1:2 exhibited vigorous symptoms of toxicosis and were euthanized. Animals given the other rations died during the post-dosing observation and duration and magnitude of premortality symptoms generally decreased with increasing proportion of theobromine. From these data an additional acute toxicity study was designed with a ratio of theobromine to caffeine of 5:1 at the doses 250, 350, 450 and 650 mg/kg b.w. Percent lethal toxicity was dose-dependent, ranged from 0 to 100% and resulted in LD_{50} and LD_{99} values of 336 and 385 mg/kg body weight, respectively. Premortality symptoms were minimal.

In a Swedish case study, a red fox (*Vulpes vulpes*) and a European badger (*Meles meles*) were found dead on a golf-course close to a farm using chocolate waste as pig feed. Theobromine and caffeine were identified in gastric contents and theobromine in samples of liver tissue analysed by reversed phase HPLC (Jansson *et al.* , 2001). The gastric content of theobromine of the red fox was 420 mg/kg, whereas samples of the gastric content of the badger contained 270 mg/kg theobromine. Caffeine occurred at lower levels, 10 and 110 mg/kg, respectively. Liver samples contained 64 and 105 mg theobromine per kg, respectively. At necropsy both animals had acute circulatory collapses. The histological examination also showed acute non-

reactive oedema in liver, kidney, lung, lymph nodes, heart and meninges. Both animals had mild mononuclear corneal cell infiltration and corneal oedema, as well as multifocal hemorrages.

A case report describes that a male kea (wild parrot) died by consuming chocolate (Gartrell and Reid, 2007). The bird had previously been involved in behavioural tests of problem-solving ability. It was found dead and the crop contained 20 g of what appeared to be dark chocolate. A conservative estimate of the dose of ingested methylxanthines was 250 mg/kg b.w. of theobromine and 20 mg/kg b.w. of caffeine. Histopathological examination revealed acute degenerative changes to hepatocytes, renal tubules, and cerebrocortical neurons.

6.8. Fish

A study by Fagbenro (1995) explored the possibility of using heat-processed cocoa pod husk meal as an energy feedstuff to clariid catfish (*Claris isheriensis*). Isonitrogenous and isoenergetic diets containing 0, 15, 30 or 45% heat-processed cocoa husk meal were fed to the fish (mean 16.8 g) for 180 days. As the theobromine content of the cocoa husk meal was approximately 1.5 g per kg, diets contained 0, 0.23, 0.45 and 0.68 g theobromine/kg feed. Growth rates of the fish on the different diets were similar, and differences in feed:weight gain ratio or protein efficiency were non-significant. No pathological effects were noted in fish livers.

Pouomogne *et al.* (1997) fed juvenile Nile tilapia (*Oreochromis niloticus*) diets with 0, 10 or 20% cocoa husk (replacing maize flour, wheat bran and rice bran) for 13 weeks. Fish (mean b.w. 1.35 g) fed the three diets had similar growth rates. Although feed:weight gain ratio and protein efficiency ratio tended to be different at the various dietary levels of cocoa husk, there was no statistical significance. Carcass analysis of the whole fish revealed a slight decrease in protein content. No overt toxicity signs were seen in the tilapia fish. The authors recommended cocoa husk as a viable dietary supplement in juvenile tilapia diets. Although the study was not controlled for theobromine content, the authors concluded it did not have significant influence on the test result.

Also Falaye and Jauncey (1999) studied the acceptability and digestibility of cocoa husk in feed for Nile tilapia (*Oreochromis niloticus*). The cocoa husk contained 3 g theobromine per kg. Three semipurified isonitrogenous diets containing 0, 10 or 20% cocoa husk, corresponding to 0, 0.3, and 0.6 g theobromine per kg diet, were fed to satiation three times a day. The doses achieved were 40 and 80 mg per kg body weight. Although the feed with cocoa husk were acceptable to the fish (0.95-0.98 kg), the cocoa diets induced significant reductions in gross feed conversion efficiency as indicated by feed consumption and weight gain. Low digestibility and not theobromine was suggested to be the cause. The cocoa husk diet induced in a dose dependent manner significant reductions in both apparent protein and dry matter digestibility. The growth rates on cocoa husk were lower than the growth rate on

traditional fish feed. Apparent net protein utilization was not significantly affected at the lower cocoa husk level. It was concluded that the cocoa husk feed was accepted by the fish and increased consumption could compensate for their low digestibility and result in acceptable fish yields and returns.

During a 49-day long feeding trial, Nile tilapia (*Oreocromis niloticus*) fingerlings were fed isonitrogenous diets containing 0, 5, 10, 15 or 20% cocoa husks (Falaye *et al.,* 1999). As the theobromine content of the cocoa husk was only 1.5 g per kg, the diets contained 0, 0.075, 0.15, 0.23, and 0.30 g theobromine per kg feed. The cocoa husk diets were accepted by the fish (0.91-1.01 g) and resulted in positive feed consumption, growth and nutrient utilization. Feed conversion efficiency and fish weight gain were significantly reduced in fish fed cocoa husk diets and their specific growth rates were also depressed over the control fish, particularly beyond 10% cocoa husk incorporation. The reduced fish growth was caused by the high fibre content of cocoa husk which resulted in low apparent protein and dry matter digestibilities during the study. However, protein efficiency ratio was significantly enhanced, particularly at 20% cocoa husk. The comparatively low theobromine content of the diet used reduces the likelihood that it was theobromine that was the cause of the adverse effects observed.

Cocoa meal was evaluated as feed for 120 days to *Tilapia guineensis* (Fagbenro, 1988). There was no difference in survival as compared to fish fed a normal fish diet and fish given no feed. Mean daily weight gain and mean final weight were lower than in fish receiving traditional fish diet, but better than in fish not receiving any fish diet. Feed conversion was less favourable with the cocoa meal than with traditional fish diet. Fish production was not optimal without the traditional fish diet. It was speculated that the theobromine content of the cocoa meal feed might be the cause of its reduced value as feed, as the feed had a quite acceptable dietary composition. There were no indications that the cocoa meal reduced the quality of the pond water. The authors expected the theobromine content of the cocoa meal to be 10-15 g/kg. As the average daily feed intake was 6.9 g in fish weighing on average 133 g, the daily average theobromine dose was calculated to be 500-750 mg/kg body weight.

As a conclusion on fish, there are few data, but reduced weight gain was found in tilapia fed defatted cocoa cake with a theobromine content corresponding to 500-750 mg/kg b.w. In studies with cocoa husk, the high fiber content and not the relatively low theobromine content was regarded to be the cause of reduced growth.

7. Toxicokinetics

The absorption, distribution, metabolism and excretion of theobromine have been investigated in test species (rat, rabbit, mice, dog), horses and humans. Most studies were performed with the pure compound.

7.1. Absorption and distribution

Theobromine is well absorbed (>90%) from the gastrointestinal tract in humans and test species (mice, rat, rabbit, dog) with bioavailability close to unity (Miller *et al.*, 1984; Walton *et al.,* 2001; Dorne *et al.*, 2001). Theobromine is distributed throughout the total body water with a volume of distribution of <1 L/kg b.w. In humans, the bound and unbound volumes of distribution are 0.68 and 0.8 L/kg b.w., respectively (Lelo *et al.,* 1986).

7.2. Biotransformation

Biotransformation of theobromine takes place in the liver with minimal first pass elimination. (Yesair *et al.,* 1984). The major routes of theobromine metabolism, in humans and test species (rat, mice, rabbit, dog) are 3- and 7-*N*-demethylation. The demethylation generates 3- and 7-methylxanthine, compounds which are further oxidised to their respective methyluric acids. Theobromine may also be C8-oxidised to 3,7-dimethyluric and 6-amino-5-(*N*-methylformyl-amino)-1-methyluracil (6-AMMU), respectively (Figure 3)

In humans after oral uptake of methylxanthines, the 3-*N*-demethylation reaction predominates (50 to 60 %) over the 7-*N*-demethylation (about 20%) and C-8 oxidation (<15%) of an oral dose. The cytochrome P-450 (CYP) isoforms involved in these reactions are CYP1A2 for the 3-*N*-demethylation, CYP1A2 and CYP2E1 for the 7-*N*-demethylation and CYP2E1 for the C-8 oxidation (Gates and Miners., 1999; Dorne *et al.*, 2001; Walton *et al.,* 2001). Overall, CYP1A2 play a major role in the human metabolism of theobromine (Walton *et al.,* 2001).

In test species, evidence is generally lacking for which CYP isoforms are involved in theobromine metabolism. However, when induced CYP1A is involved in theobromine's 3-N-demethylation in the rat (Walton *et al.,* 2001) whereas no information is available for the dog, rabbit and mouse. For caffeine and theophylline, however, the CYP isoforms that are responsible for the 3- and 7-N-demethylation reactions in these species have been characterised (Walton *et al*., 2001).

Although, the general routes for demethylation and C-8 oxidation are mostly common to humans and test species, there are considerable quantitative species differences in the metabolism and excretion of theobromine.

In the rat, theobromine is excreted in the urine mainly as the parent compound (~30-50%) (Miller *et al.*, 1984). The major biotransformation route is C8-oxidation, whereas *N*-demethylation is a minor route (~10% of the dose). The limited data available suggests that C8-oxidation of theobromine in the rat is catalysed by a cytochrome P450, although it is unclear if this is CYP1A2 (Miller *et al.*, 1984).

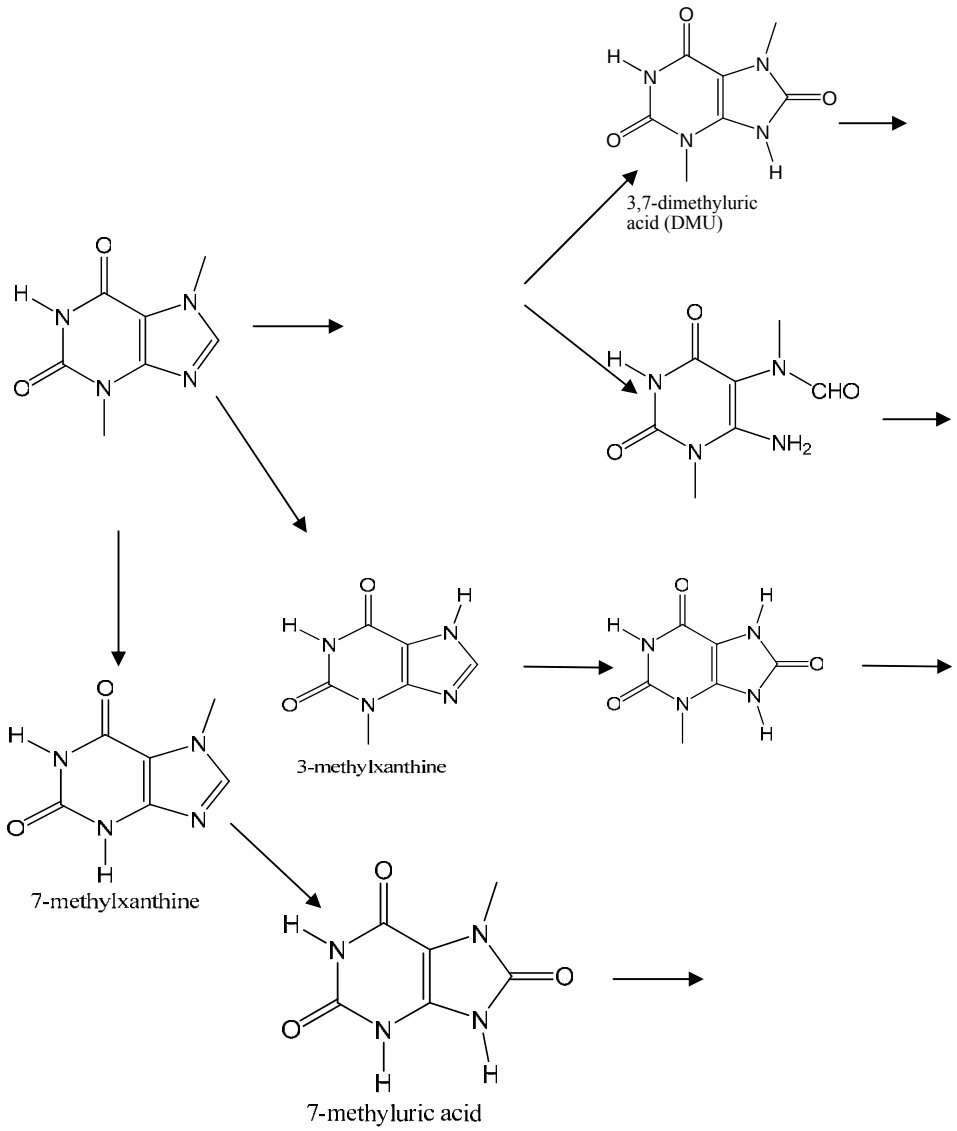

Figure 3. Biotransformation of theobromine in mammals

The dog excretes a large proportion of an oral dose of theobromine unchanged in the urine (~37%) and predominantly 7-*N*-demethylates the rest, generating 3-methylxanthine (Miller *et al*., 1984). Small quantities of an apparently unique and unidentified biotransformation product was found in the dog but not in the other four animal species (rats, mice, hamsters, and rabbits) compared in this pharmacokinetic study. Recovery of radioactivity ranged from 60-89% of the dose in urine, and from 2-38% of the dose in feces, with most material being excreted during the first 48 hr after dosing. About 4-5 hours after dosing around 25% of the

supplied radiolabelled theobromine had been excreted in urine. Excretion of unchanged theobromine ranged from 14 to 37% (37% in dog, 32% in rat, 16-22% in mouse, and 14% in rabbits).The amount of theobromine excreted in faeces varies substantially between species and range from low in rabbits and dogs (1.6% and 4.5% respectively) to intermediate in mouse (9-12%) and high in rats, where also a sex difference is noted (16% in females and 38%. in males).

Demethylation coupled to ring opening and formation of 6-amino-5-(N-methylformylamino)-1-methyluracil also constituted an important route of metabolism ranging from 7.5 to 28% depending on species (14-28% in mouse, 17-18% in rat, 10% in rabbit and 7.5% in dog). Additional major metabolites were 7-methylxanthine in the rabbit (36%), and 3-methylxanthine in the dog (20%). Ring N-demethylation at position 3 predominated over N-7-demethylation in all species except the rat and dog. Mono- and dimethyluric acids constituted 3.4-4.7% in rat, 6.6-8.2% in mouse, 3.7% in rabbit, and 5.7% in dog. Thus, oxidation of methylated xanthines to the corresponding uric acids was a relatively minor metabolic pathway in all species, but had greatest activity in mice (Miller *et al.*, 1984).

It should be noted that genetic polymorphisms of cytochrome P450s, particularly CYP1A2, have been reported in humans and in beagle dogs (Ghotbi *et al.*, 2007; Tenmizu *et al.*, 2004; Kamimura, 2006). Around 10-15% of beagle dogs have been estimated to be CYP1A2-deficient (Fleischer *et al*., 2008). Such metabolic polymorphisms might play a role for the sensitivity to theobromine in particular dog breeds as the CYP1A subfamily is known to catalyse 3-N-demethylations also in the dog, at least when using caffeine as a test substrate. Demethylation of the 7-position of caffeine is perfomed by phenobarbital inducible CYP isoforms (CYP2B11, 2C11 and 3A12) (Walton *et al.*, 2001).

In conclusion, excretion patterns of theobromine and its metabolites were qualitatively comparable among species, indicating that theobromine is metabolised via similar pathways. Except for the excretion of small quantities of an unidentified but apparently unique metabolite by dogs, only quantitative species- and sex-related differences have been observed in metabolic disposition of theobromine.

7.3. Elimination

7.3.1. Humans

There are several studies on the pharmacokinetics (absorption, distribution, metabolism, and excretion) of theobromine in man (Cornish and Christman, 1957; Drouillard *et al.,* 1978; Miners *et al.,* 1982; Tarka *et al.,* 1983; Lelo *et al.,* 1986). Following oral absorption, theobromine has a relatively low renal clearance in healthy adults with 1.0 mL/min/kg similar to other methylxanthines, i.e. caffeine 1.2 mL/min/kg and theophylline 0.9 mL/min/kg (Dorne *et al.,* 2001). Peak concentrations are usually reached within 2-3 hours, plasma protein

binding is low (15-25%) with an unbound plasma clearance of 1.4 mL/min/kg (Resman *et al.,* 1977; Lelo *et al.*, 1986). Theobromine's half life is longer than that of caffeine and theophylline and ranges from 7 to 12 hours (Tarka *et al.,* 1983, Shively *et al.,* 1985, Lelo *et al.,* 1986). Mumford and co-workers (1996) compared the oral absorption of theobromine after administration of capsules, cola beverages and chocolate candy. From capsules, plasma peak concentrations were at 6.72 mg/L approximately 3 hours after capsule administration in contrast to chocolate or cola for which they were higher (8.05 mg/L) and more rapid (2 hours). The authors concluded that an ordinary dietary portion of cola or chocolate may result in plasma levels of biological significance (Mumford *et al.,* 1996, Andersson *et al*., 2004) and the psychopharmacological effects associated with chocolate have been shown to depend on the combination of both theobromine and caffeine (Smit *et al.,* 2004).

There are no data reporting the toxicokinetics of theobromine in children and neonates. The oral clearance rates of caffeine and theophylline are faster in children (1.5-fold) but much lower in neonates (5-7-fold) compared to healthy adults due to the immaturity of CYP1A2 metabolism in neonates. Hence it is likely that theobromine clearance will also be affected in neonates (Cresteil, 1998; Renwick *et al.*, 2000, Dorne *et al.,* 2005). Breast milk distribution of theobromine has been investigated in six breast-feeding mothers following ingestion of 113 g of Hershey's milk chocolate (corresponding to 240 mg theobromine) and the compound has been shown to pass freely into milk. Peak concentrations were reached after 2-3 hours with a half life of 7.1 hours, a plasma clearance of 1 mL/min/kg. Milk protein binding was around 20% with mean concentration ratios of 0.82 for milk/plasma and 0.92 for saliva/plasma. In four nursing mothers, the consumption of 240 mg theobromine every six hours together with an average breast feeding volume of milk of 1 litre per day was predicted to result in 10 mg or 1-2 mg/kg per day theobromine exposure in the neonate. Such doses are expected to be pharmacologically active (stimulatory effects on the nervous system, diuresis and cardiac muscle, smooth muscle relaxant properties) in most neonates (Resman *et al.,* 1977).

Theobromine has also the capability to cross the placenta, and enter gonadal tissue. However no data are available on theobromine diffusion through the blood/brain barrier (Andersson *et al.,* 2004).

7.3.2. Rats

The toxicokinetics of oral theobromine in rats has been investigated in a number of studies (Arnaud and Welsch, 1979, Shively and Tarka, 1983, Bonati *et al.*, 1984, Shively *et al.*, 1986) and has been shown to be linear (clearance and metabolite profile) between 1 and 100 mg/kg per day after both acute and chronic exposures (Bonati *et al.*, 1984). The compound is almost equally distributed in plasma and in blood cells, and bound to plasma proteins only insignificantly. The plasma half life is 5.5 hours (Shively and Tarka, 1983; Walton *et al*., 2001). A meta-analysis revealed sex differences in theobromine kinetics so that oral clearance was 5.40 and 1.41 for the male and female rat respectively with an overall value of 3

ml/min/kg (Walton *et al.,* 2001). Shively and Tarka (1983) compared the toxicokinetics of theobromine in pregnant and nonpregnant rats dosed orally with 5, 10, 50 or 100 mg theobromine/kg body weight by comparing the urinary metabolites after a low (5 mg/kg) and a high (100 mg/kg) dose. In nonpregnant rats the t_{max} was reached after 15-36 minutes, elimination kinetics were independent of dose, with an average theobromine half-life of 5.5±1.5 hours. After 48 hours, analysis of theobromine's metabolic profile in the urine of animals dosed orally 5 or 100 mg/kg b.w revealed similar qualitative metabolic patterns in pregnant and non-pregnant rats and the authors concluded that pregnancy did not have an effect on theobromine elimination in female rats.

7.3.3. Rabbits

Theobromine toxicokinetics was investigated in male and female (non-pregnant and pregnant) rabbits after a single oral dose and two weeks daily oral dosing at 1, 5, 10, 50 and 100 mg/kg per day. No significant differences between groups were found for the toxicokinetic profile of theobromine and only at the highest doses (100 mg/kg for males and 50 mg/kg for pregnant rabbits) was there a tendency towards accumulation. The overall clearance was 1.8 mL/min/kg (Latini *et al.*, 1984).

Tarka and co-workers (1986a) dosed pregnant New Zealand white rabbits between 25 and 200 mg theobromine/kg b.w. on day 6-29 of gestation. In rabbits dosed with 75 mg theobromine per kg body weight this exposure led to serum levels between 24 and 86 mg/L, whereas the levels were between 14 and 203 mg/L in rabbits dosed 200 mg theobromine/kg b.w.

7.3.3. Mice

No data describing toxicokinetic parameters of theobromine in mice were available.

7.3.4. Dogs

In a single dose study on dogs, the theobromine plasma half-life was around 17.5 hours (Gans *et al.*, 1980). In a 1-year feeding study, the time to peak plasma concentration of theobromine was dose-dependent. At lower doses, 15-50 mg/kg b.w. per day, the peak appeared after around 3 hours and varied in magnitude between dogs. At higher doses, 150 mg/kg b.w. per day, peak plasma concentrations of theobromine were attained at around 15 hours and were considerably higher than concentrations observed following a single dose of that magnitude. In this case the plasma half-life was 14.5 hours.

The pharmacokinetics of 5 mg intravenously supplied theobromine and approximately 5 mg theobromine supplied orally in the form of chocolate (0.7 g per kg body weight), was

investigated in female beagle dogs (Loeffler *et al*., 2000a, 2000b). Plasma levels peaked directly after intravenous administration (42 mg/L at 5 min), and urinary levels at approximately 3 hours (Loeffler *et al*., 2000a). The half-life of theobromine in plasma was in this case 6.5 ±0.6 hours. When supplied in the form of chocolate, maximum plasma levels of theobromine (20.5 mg/L) were found around 2 hours after dosing. The plasma half-life was 6.8 ±2.8 hours, which allowed theobromine to be detected in plasma for up to 36 hours. Urinary levels peaked at (180 mg/L) at 12 hours after dosing and were mostly unchanged theobromine. The bioavailability of theobromine from chocolate was estimated to be 77% (Loeffler *et al*., 2000b). The toxicokinetic studies performed with theobromine in dogs does not give a totally coherent picture, but this could be due to different matrixes having been used to deliver the compound. When 12.3 mg theobromine per kg b.w. was given to dogs in the form of a chocolate bar, plasma levels peaked at 12 mg/L in about 4 hours, and decreased fairly slowly. It was unclear if this was due to delayed gut absorption from the chocolate bar, a long serum half-life or both (Glauberg and Blumenthal, 1983). When theobromine in another study was given as tablets at a dose of 15 mg/kg body weight, peak plasma levels were 16-33 mg/L at around three hours after dosing. This resulted in plasma half-lives between 13.5 and 19 hours (Percy and Borys, referred to by Hornfeldt, 1987).

In conclusion, theobromine is fairly bioavailable from cocoa feeds and chocolate products, and metabolized in the dog to a rather limited extent. Such limited metabolism may be partly due to the presence of a genetic polymorphism in the CYP1A2 isoform based on its apparent significance for 3-N-demethylation using caffeine as a test substrate. Even though such a genetic polymorphism of CYP1A2 has been shown in dogs, the molecular basis of the dog's susceptibility to theobromine is still unclear and the relationship between CYP1A2 and theobromine metabolism in the dog remains to be confirmed (see section 7.2).

7.3.5. Horses

Nine toxicokinetic studies describe the urinary excretion of theobromine in horses (mostly in thoroughbred mares) (Kelly and Lambert, 1978; Moss, 1980; Moss *et al.,* 1980; Lambert *et al.*, 1985; Haywood *et al.,* 1990; Aramald *et al.,* 1991; Delbeke and Debackere, 1991; Salvadori *et al.,* 1994; Dyke and Sams, 1998).

Five thoroughbred mare horses individually received different quantities of cocoa bean meal containing 5.8 g theobromine/kg (Kelly and Lambert, 1978). The horses were given between 50 g (1.7% of the daily ration) and 1.4 kg cocoa bean meal (0.29 to 8 g theobromine), corresponding to 1.7 to 28% of the daily feed, by stomach tube. No theobromine was found in the blood serum. Overall, theobromine was rapidly absorbed from the gastrointestinal tract and metabolised, its persistence in the body tissues was indicated by its presence in urine for up to 12 days with peak concentrations appearing from 22 hours to 5 days.

Nine thoroughbred male horses weighing 300-400 kg were given cocoa husk (5.8 g theobromine per kg feed) at a single feeding to produce exposures of 10, 20, 50 or 100 mg/kg

body weight theobromine (Lambert *et al.*, 1985). Theobromine was present in the urine for up to 8 days.

Haywood *et al.* (1990) fed racehorses 7 kg cocoa husks in the form of cocoa husk, spread over a morning ration and an evening ration, for four days. The doses were chosen after having determined the quantity of theobromine required in the feed to reach the limit of detection of urinary metabolites with the analytical method used (1 mg/kg feed). The various feed formulations contained 1.2, 2.0, 6.6 and 11.5 mg theobromine per kg husks. The maximum urinary levels were dose-dependent and appeared at around 80 hours after the initial feeding but with significant differences between horses. An intake of around 50 mg theobromine over four days resulted in peak urinary concentrations around 0.4-0.9 mg/L. A threshold level for theobromine in urine (2.0 mg/L) was determined as being of relevance to feed, and above which doping of the animal could be concluded.

In a similar study, Delbeke and Debackere (1991) gave five horses, weighing 365-517 kg, feed consisting of oats and a pelleted ingredient containing EC permitted theobromine levels (38.4 mg) twice daily to horses for 2½ days. Peak excretion rate varied from 2 to 12 hours after the last administration. The theobromine excretion rate was correlated to urinary flow.

Salvadori *et al.* (1994) determined the clearance time after administration of a guaraná powder under the tongue of a thoroughbred mare twice a day over 5 consecutive days. The ppwder contained 2.16 g theobromine per kg powder and theobromine could be detected in urine for up to 13 days.

Dyke and Sams (1998) determined the urinary excretion of methylxanthines in three horses (450-500 kg) following feeding of the mares with 20 chocolate-coated peanuts containing approximately 19.6 g chocolate (1.87 mg theobromine/g chocolate) for 8 days. Twenty-four hours after administration of the seventh dose and before administration of the last dose, theobromine concentrations in urine were between 3.3 and 3.7 mg/L and increased to a maximum of between 7.2 and 11.8 mg/L approximately 5-6 hours after ingestion of the last dose. At 5 days, theobromine in two horses was below the limit of quantification. After oral administration of radiolabelled theobromine to two ponies, 1.1 mg/kg and 0.79 mg/kg, theobromine was shown to have a plasma half-life of 12.8 and 27.2 hours respectively, and was excreted in urine as unchanged drug and 3,7-dimethyluric acid, 80% and 65% of the dose being accounted for. Excretion was complete after 100 hours (Moss *et al.*, 1980).

In horses, excretion of theobromine and its metabolites is very variable, dose dependent and related to variability in renal excretion and renal blood flow.

7.3.6. Livestock

Aly (1981) collected toxicokinetic parameters in single dose feeding studies on sheep with 40 mg theobromine/kg b.w. or 3 g cocoa shells/kg b.w. Whereas the half-life of theobromine in plasma was around 21 hours after ingestion of the pure compound, it was only 15.5 hours

after ingestion of the cocoa shell diet. These exposures gave no toxicological effects. Dosing 3 g cocoa shells/kg b.w. per day for five days resulted in reduced body weight. The urine from these animals contained the demethylated metabolites 3-methylxanthine, 7-methylxanthine and 7-methyluric acid.

8. Carry-over and residues

Data on carry over and residues of theobromine in animal products derived from animals exposed to contaminated feed are not available for milk, eggs, meat and offals. However, theobromine is completely absorbed, with an apparant low protein binding, and distributed into body tissues in approximate proportion to their water content. Such distribution would dilute down theobromine residues in meat and offals considerably so that low levels in these tissues can be expected.

9. Human dietary exposure

Consumption of chocolate on a daily basis results in human dietary exposure to theobromine of several milligrams per day. According to CAOBISCO (the Association of chocolate, biscuit and confectionery industries of the European Union), the average chocolate product consumption in the EU was 6.55 kg/person in 2006 (CAOBISCO/ICA, 2008). In milk chocolate, which contains between 10 and 15 % cocoa, Zoumas *et al.* (1980) reported an average of 1530 mg theobromine/kg milk chocolate (with a range of 1350-1860) whereas dark chocolate contained an average of 4600 mg theobromine/kg (with a range of 3600 to 6300). Dark chocolate contains between 30 and 80% cocoa, and the theobromine content varies depending on the percent of cocoa in the chocolate. A worst case scenario based on these data, and assuming that a 60 kg person eats 100 g of very dark chocolate (a max of 6300 mg theobromine/kg chocolate) per day, would result in a theobromine intake of 630 mg per day corresponding to 10.5 mg theobromine/kg b.w. Such exposure is very high as compared with possible exposure resulting from carry-over of theobromine from tissues of farmed animal species. Hence, it is evident that animal-derived products would contribute little to human dietary exposure of theobromine, even if accidentally present at high inclusion rates of cocoa meal or husks in the animal's diet.

A study examined the level of theobromine in mother's milk after ingestion of 113 g (4 ounces) milk chocolate (containing a total of 240 mg of theobromine). Peak theobromine concentrations of 3.7 to 8.2 mg/L were found in all fluids including breast milk at 2 to 3 hour after ingestion of the chocolate If this amount of chocolate were to be ingested four times a day, it would potentially lead to an exposure of the breast fed infant of about 10 mg theobromine/day (corresponding to 1-2 mg/kg b.w.) (Resman *et al.,* 1977). Such an exposure might result in pharmacologically active theobromine levels since newborn babies and infants

have very low CYP1A2 activity (Cresteil, 1998; Renwick *et al.* , 2000; Dorne *et al.* , 2001) and would metabolise theobromine much more slowly than adults. However, such consumption of chocolate is highly unlikely.

CONCLUSIONS

Chemistry, occurrence in plants and parts used as feed materials

- Theobromine (3,7-dihydro-3,7-dimethyl-*H*-purine-2,6-dione) is a colourless and odourless methylxanthine (3,7-dimethylxanthine) with a slightly bitter taste that is naturally present in all parts of the seed and at smaller quantities in the pod of the cacao tree (*Theobroma cacao* L.). Except for the cacao tree and a few other plant species caffeine or tetramethyluric acid are the principal methylxanthines synthesized by plants. Theobromine is also a metabolite of caffeine in mammals.
- Theobromine is present in cocoa products and by-products of cocoa production and manufacturing. While the cacao pod husk may be used as feed material in developing countries where cacao is grown, cocoa bean shells, cocoa bean meal and cocoa germ as well as discarded confectionary is used for feed purposes in Europe. Cocoa bean shells can be a suitable feed ingredient for ruminants.

General toxicological effects

- Theobromine shows moderate acute oral toxicity in experimental animals with LD_{50} values ranging from around 300 to 1350 mg/kg b.w. In comparison with other methylxanthines, theobromine has a weak action on the central nervous system and is a weak antagonist of adenosine receptors.
- Target organs of theobromine toxicity in rodents are the testes (Sertoli cells) and the thymus. Dogs showed also cardiomyopathy upon prolonged exposure. Theobromine causes reproductive toxicity and incomplete ossification in mice, and variation in skeletal development in off-spring of rabbits. In rats, the NOAEL for testicular toxicity was identified at 150 mg/kg b.w. and in rabbits the NOAEL for variation in skeletal development in the off-spring was 21 mg/kg b.w..
- The evidence for mutagenic and clastogenic effects of theobromine is equivocal. No long-term carcinogenicity studies are available.
- No ADI or equivalent has been established for theobromine.

Adverse effects of theobromine in target animals

- In dairy cows, reduction in milk yield and increase in fat content occurred when fed theobromine at approximately 15 mg/kg b.w. per day. Adverse effects (hyperexcitability, sweating and increased respiration and heart rates) were found in calves fed theobromine between 45 and 90 mg/kg b.w. for some weeks.
- In goats, reduced dry matter intake and body weight gain were found at the lowest theobromine dose tested, approximately 300 mg/kg b.w. per day for 56 days.
- In lambs exposed to theobromine for 3 months, the NOAEL was identified as approximately 35 mg theobromine/kg b.w. per day. At higher doses, depression of feed intake and weight gain was seen. In adult sheep reduced feed intake was observed at exposure of approximately 50 mg theobromine/kg b.w. per day for 5 days. No effects were observed when this level was given as a single dose.
- In horses a single dose of 0.29 g of theobromine (estimated to be about 0.5 mg/kg b.w.) did not cause any clinical or biochemical effects.
- In pigs, feeding studies with cocoa meal resulting in exposure to 24 mg/kg b.w. of theobromine caused growth retardation and diarrhoea, and made pigs lethargic. For young growing pigs a NOAEL of 7 mg/kg b.w. was identified. Older growing pigs appeared to tolerate somewhat higher doses.
- The NOAEL of theobromine in young chickens were found to vary between 260 and 1100 mg/kg diet (approximately 26-110 mg/kg b.w. per day), with depressed feed intake and weight gain at higher doses. In older broiler chickens, a LOAEL of 950 mg/kg (approximately 95 mg/kg b.w. per day) was found.
- For laying hens a LOAEL of 1100 mg theobromine/kg diet (corresponding to 66 mg/kg b.w. per day) may be identified, based on liver and kidney toxicity, depressed weight gain and egg-production.
- In rabbits, the NOAEL was 21 mg theobromine/kg b.w., based on variation in skeletal development observed at 41 mg/kg b.w.
- In dogs, acute fatal intoxication may occur after a single ingestion of theobromine at 80-300 mg/kg b.w. Dogs dosed up to 50 mg/kg b.w. for 1 year did not show adverse effects.
- In studies on fish, reduced weight gain was found in tilapia fed defatted cocoa meal with a theobromine content corresponding to 500-750 mg/kg b.w.

Theobromine in feed materials

- There is a lack of data on theobromine levels in feed materials. However, there are data on products and by-products produced in cocoa industry. Thus, cocoa husk meal has been reported to contain 1.5-4.0 g theobromine per kg material, cocoa bean shell 8.0-16.9 g/kg (and 1 g caffeine/kg), and cocoa bean meal 20-33 g/kg material (1-4 g caffeine/kg). Chocolate waste is expected to contain more variable contents of theobromine.

- Current EU regulation on maximum levels of theobromine in feed material may not be fully protective for some target animal species, e.g. as effects on milk production in dairy cows and adverse effects in pigs can occur at these levels.

Fate in animals and carry-over

- Theobromine is well absorbed and widely distributed. It is rapidly metabolised, and unchanged theobromine and metabolites are mainly excreted in urine,
- Data on carry over and and residues of theobromine in animal products derived from animals exposed to contaminated feed are not available for eggs, meat, offals and milk.

Human exposure

- Humans are exposed to theobromine mainly from chocolate confectioneries, cocoa drinks and bakery products containing cocoa or chocolate. In addition theobromine is a metabolite of caffeine. Theobromine exposure from animal products such as meat, milk and eggs is expected to be negligible in comparison with direct consumption of cocoa products.

RECOMMENDATIONS

- Occurrence data for theobromine in animal feed and further information on the use of various feed materials containing cocoa are needed.

- The rate of carry-over of theobromine to milk (and eggs) should be determined.

- In consideration that by-products of the production of chocolate confectionaries are used in feed materials particularly designated for pigs, it is recommended to conduct more dose-response studies on potential adverse effects in pigs.

DOCUMENTATION PROVIDED TO EFSA

Belgium. The Federal Agency for the Safety of the Food Chain.
Czech Republic. Central Institute for Supervising and Testing in Agriculture.

INZO, Château-Thierry Cedex, France.

REFERENCES

Abiola, S.S. and Tewe, O.O. 1991. Chemical evaluation of cocoa byprodukts. Trop. Gric. (Trinidad) 64: 335-336.

Adegbola, A.A., and Omole, T.A. 1973. A simple technique for preparing discsarded cocoa bern-meal foar use as livestock feed. The Nigerian Abricultural J. 10: 72-81.

Adomako, D. 1985. Some conversion technologies for the utilisation of cocoa wastes. 9th Internat. Cocoa Res. Conf.: 437-441.

Agyeman, K.O.G. and Oldham, J.H. 1986. Utilization of cocoa by-products as an alternative source of energy. Biomass 10: 311-318.

Alvarado, J. de D., Villacís, F.E. and Zamora, G.F. 1983. Efecto de la epoca de cosecha sobre la coomposicion de coddtiledoones ccrudos y fermentados de dos variedades de caca y fracciones de cascarilla. Archivos latiinamericanos de Nutericion 33: 340-355.

Aly, Z.H. 1981. Untersuchungenüber coffein und theobromin bei schafen III. Mitteilung: Verfütterung von Kakoschalen. Zbl. Vet. Mrd. A 28: 711-719.

Andersson, H.C., Hallström, H. and Kihlman, B.A. 2004. Intake of caffeine and other methylxanthines during pregnancy and risk for adverse effects in pregnant women and their foetuses. TemaNord 2004 564: 387.

Aneja, M. and Gianfagna, T. 2001. Induction and accululation of caffeine in young, actively growing leaves of cocoa (Theobroma cacao L.) by wounding or infection with Crinipellis perniciosa. Physiological and Molecular Plant Pathology 59: 13-16.

Aramald, T., Suzuki, E., Ishidaka, O., Momose, A. And Umemura, K. 1991. Pharmacokinetics of caffeine and its metabolites in horses after intravenous, intramuscular or oral administration. Chem. Pharm. Bull. 39: 2999-3002.

Aregheore, E.M. 2002. Chemical evaluation adn digestibilityt oc cocoa (Theobroma cacao) bypbroducts fed to goats. Tropical Animal. Health and Production 34: 339-348.

Aremu, C.Y., Agiang, M.A. and Ayatse, J.O.I. 1995. Nutrient and antinutrient profiles of raw fermented cocoa beans. Plant Food for Hyman Nutriton 48: 317-223.

Arnaud, M.J. and Welsch, C. 1979. Metabolic pathway of theobromine in the rat and identification of two new metabolites in human urine. J. Agric. Food Chem. 27: 524-527.

Ashihara, H. and Crozier, A. 1999. Biosynthesis and metabolism of caffeine and related purine alkaloids in plants. Advances in Botanical Research 30: 117-205.

Ashihara, H. and Kubota, H. 1987. Biosynthesis of purine alklaoids in Camellia plants. Plant Cell Physiol. 28: 535-539.

Ashihara, H., Kato, M., and Chuang.xing, Y. 1998. Biosynthesis and metabolism of purine alkaloids in leaves of cocoa tea (Camellia ptilophylla). J. Plant Res. 111: 599-604.

Ashun, P.K. 1973. Cocoa by-products for livestock feeding. Conspectus Agriculturae Scientificus 35: 119-129.

Aplin, R.D. and Ellenberger, H.B. 1927 . Effect of feeding cocoa meal to milking cows. University of Vermont and State Agricultural College, Vermont Agricultural Experiment Station, Burlington, Vermont. Bulletin 272: 1-20.

Athayde, M.L., Coelho, G.C. and Schenkel, E.P. 2000. Caffeine and theobromine in epicuticular wax of Iles paraguariensis A. St.-Hil. Phytochemistry 55: 853-857.

Atuahene, C.C., Donkoh, A, and Aante, F. 1998. Value of scheanut cake as a dietary ingredient for broiler chickens. Animal Feed Science Technology 72: 133-142.

Babka, J.C. and Castell, D.O. 1973. On the genesis of heartburn. The effects of specific foods on the lower esophageal sphincter. Digestive Diseases 18: 391-397.

Barnes, A.R., Amega, W.K., Manu, M. and Rhule, T. 1985. Utilization of cocoa husk meal by growing-finishing pigs. 9th International Cocoa Research Conference, pp 449-454.

Bateman, J. V. and Fresnillo, O. 1967. Digestibility of Theobroma cacao pods when fed to cattle. J Agric. Sci. Camb. 68: 23-25.

Baumann, T.W. 1996. Coffein. Bot. Helv. 106: 127-158.

Beccari, E. 1936. Massen-Vergiftung durch Theobromin-Derivate. Fühner-Wielands Sammlung von Vergiftungsfällen 7: 77.

Bempoong, D.K., Houghton, P.J. and Steadman, K. 1993. The xanthiine content of guarana and its preparations. Int. J. Pharmacog. 312: 175-181.

Black, D.J.G. and Barron, N.S. 1943. Observations on the feeding of a cacao waste product to poultry. The Veterinary Record 55: 166-167.

Blanchard, J., Weber, C.W. and Shearer, L.-E. 1990. HPLC analysis of methylxanthines in human breast milk. J. Chrom. Sci. 28: 640-642.

Bohinc, P., Korbar-Smid, J. and Bizilj-Kremžar, L. 1975. Theobromin in *Ilex perado* Blättern. Planta medica 28:374-378.

Bonadonna, T., Colanghis, S. and Curto, G. 1963. Tests of tolerance for meal of detheobrominised cocoa husk in diets of pigs and lactating cows. Zootec. Vet. 18: 2-16.

Bonati, M., Latini, R., Sadurska, B., Riva, E., Galletti, F., Borzelleca, J.F., Tarka, S.M., Arnaud, M.J. and Garattini, S. 1984. Kinetics and metabolism of theobromine in male rats. Toxicology 30: 327-341.

Braude, R. 1943. Toxic effects in the feeding of cocoa meal to pigs. The Veterinary J. 29: 302-307.

Braude, R. and Foot, A.S. 1942. Cacao by-products in pig feeding: cocoa-cake meal and de-theobrominized cocoa-cake meal as feeding-stuffs for pigs. Emp. J. Exp. Agric. 10: 182-188.

Brusick, D., Myhr, B., Galloway, S, Rundell, J., Jagannath, D.R., and Tarka, S. 1986. Genotoxicity of cocoa in a series of short-term assays. Mutation Research. 169: 115-121.

Bucheli, P., Rousseau, G., Alvarez, M., Laloi, M. and McCarthy, J. 2001. Developmental variation of sugars, carboxyllic acids, purine alkaloids, fatty acids, and endoproteinase activity during maturation of Theobroma cacao L. Seeds. Jagric. Food Chem. 49: 5046-5051.

CAOBISCO/ICA, 2008. Statistical Bulletin. Collected and Published by CAOBISCO in association with the CAOBISCO Statistics Network and International Confectionery Association. March 2008.

Carney, J.M. 1982. Effects of caffeine, theophylline and theobromine on scheduled controlled responding in rats. British Journal of Pharmacology 75:451-454.

Carney, J.M., Holloway, F.A. and Modrow, H.E. 1985. Discriminative stimulus properties of methylxanthines and their metabolites in rats. Life Science 36:913-920.

Casoli, P. and Verine, H. 1990. High performance liquid chromatographic determination of methylxanthines in canine serum, gastric and pancreatic juices. Biomed. Chromatogr. 4: 209-213.

Chevalley, J. 1976. Theobromine and caffeine content of cocoa beans from different botanical and geographical origins.

Clough, G.W. 1942. Theobromine poisoning in the dog. Veterinary J. 98: 196-197.

Cornish, H.H. and Christman, A.A. 1957. A study on the metabolism of theobromine, theophylline, and caffeine in man. Journal of Biological Chemistry 228: 315-323.

Crawshaw, R. 2001. Co-Product Feeds: Animal Feeds from the Food and Drinks Industries. Nottingham University Press, pp. 285.

Cresteil, T. 1998. Onset of xenobiotic metabolism in children: toxicological implications. Food Addit Contam.15 Suppl: 45-51.

Crowther, J. 1936. J. Minist. Agric. 43: 684.

Curtis, P.E. and Griffiths, J.E. 1972. Suspected chocolate poisoning of calves. The Veterinary Record 90: 313-314.

Czok, G. 1974. Zu Frage der bilolgische Werksamkeit von Methylxanthinen in kakaoprodukten. Zeischrift für Ernärungswissenschaft 13: 165-171.

Day, E.J. and Dilworth, B.C. 1984. Toxicity of jimson weed seed and cocoashell meal to broilers. Poultry Science 63: 466-468.

Decker, R.A. and Myers, G.H. 1972. Theobromine poisoning in a dog. JAVMA. 161: 198-199.

Delbeke, F.T. and Debackere, M. 1991. Urinary excretion of theobormine in horses given contaminated pelleted food. Veterinary Reasearch communications 15: 107-116.

Devendra, C. and Gohl, B.I. 1970. The chemical composition of caribbean feedingstuffs. Trop. Agriculture. 47: 335-342.

De Vries, J.W., Johnson, K.D. and Heroff, J.C. 1981. HPLC Determination of caffeine and theobromine content of various natural and red dutched cocoas. J. Food Sci. 46: 1968-1969.

Dittmar, H.F.K. 1958. Untersuchung and Kakao-Fruchtschalen. Gordian 58: 48-49.

Dodo, H.W., Fritz, P.J. and Furtek, D.B. 1992. A cocoa 21 kilodalton seed protein has trypsin inhibitory activity. Café Cacao Thé 36: 279-284.

Donkoh, A., Atuahene, C.C., Wilson, B.N., and Adomako, D. 1991. Chemical coomosition of cocoa pod husk and its effect on growth and food efficiency in broiler chicks. Animal Feed Science and Thechology 35: 161-169.

Dorne, J.L.C.M., Walton, K. and Renwick, A.G. 2001. Uncertainty factors for chemical risk assessment: human variability in the pharmacokinetics of CYP1A2 probe substrates. Food Chemical Toxicol. 39: 681-196.

Drolet, R. Arendt, T.D. and Stowe, C.M. 1984. Cacao bean shell poisoning in a dog. J. Am. Vet. Med. Assoc. 185: 902.

Drouillard, D.D., Vesell, E.S. and Dvorchik, B.H. 1978. Studies on theobromine disposition in normal subjects. Alterations by dietary abstention from or exposure to methylxanthines. Clinical Pharmacology and Therapy 23: 296-302.

Dyke, T.M. and Sams, R.A. 1998. Detection and Determionation of Theobromine and caffein in urin and administraiton of chocolate-choted peanuts to horses. J. Analytical Toxicology 22: 112-116.

Eeckhout, W. and De Paepe, M. 1994. Total phosphorus, phytate-phosphorus and phytase activity in plant feedstuffs. Animal Feed Science and Technology 47:19-29.

Etemg, M.U., Eyong, E.U., Akpanyung, E.O., Agiang, M.A: and Aremu, C.Y. 1997. Recent advances in caffeine and theobromine toxicities: a review. Plant Foods for Human Nutrition 51:231-243.

Ettlin, R.A., Armstrong, J.M., Buser, S. and Hennes, U. 1986. Retardation of spermiation following short-term treatment of rats with theobromine. Archives in Toxicology 9: 441-446.

Evans, R.E. 1960. Rations for livestock. London: Ministry of Agric. Her Majesty Sta. Office; Bull. 48.

Ewing, W.N. 2002. The Feeds Directory: Commodity products Guide. Context, Ashby de la Zouch, pp 118.

Fagbenro, O.A. 1988. Evaluation of defattted coca cake as a direct feed to the monosex culture of Tilapia guineensis (pisces: cichiladae). Aquaculture 73: 201-206.

Fagbenro, O.A. 1995. Evaluation of heat-processed cocoa pod husk meal as an energy feedstuff in produktion diets for the clariid catfish, Clarias isherensis (Sydenham). Aquaculture Nutrition 1: 221-225.

Falaye, A.A. 1990. Evaluation of the chemical and nutrient composition of cocoa husk (Theobroma cacao) and its potential as a fish feed ingredient. Nigerian Journal of Basic and Applied Sciences 4: 157-164.

Falaye, A.E., and Jauncey, K. 1999. Acceptability and digestibility by tilapia Oreochromis niloticus of feed containing coca husk. Aquaculture Nutririon. 5: 157-161.

Falaye, A. E., Jauncey, K., and Tewe, O.O. 1999. The growth performance of talapia (Oreochromis niloticus) fingerlings fed varying levels of cocoa husk diets. J. Aqua. Trop. 14:1-10.

Fangauf, R. and Haenfel, A. 1938. Bericht über einen Fütterungsversuch mit Kakaoschalenmehl bei Legehühnern. Archiv für Geflügelkunde 12:183-187.

FAO (Food and Agriculture Organization), 2008. Theobroma cacao. Animal Feed Resources Information System, FAO, Rome, Italy.

http://www.fao.org/ag/Aga/AGAP/FRG/AFRIS/index_en.htm

Feldman, E.B. 1998. Death by chocolate. Nutrition Today 33:106-112.

Filip, R., and Ferraro, G. E. 2003. Resarching on new species of ”Mate” Ilex brevicuspis. Phytochemical and pharmacology study. Eur. J. Nutr. 42: 50-54.

Fincke, A. 1989. Analytische und technologische Entwicklungen auf dem Gebiet der Kakaoerzeugnisse. Lebensmittelchem. Gerichtl. Chem., 43:49-55.

Flachowsky, G., Ivanowitsch Ochrimenko, W., Koch, H. and Leopold, G. 1990. Utilization of cacao bean shells as ruminants feeds. Beitr. Trop. Landwirtsch. Vet. Med., 28:93-99.

Fleischer, J. E., Barnes, A. R. and Adomako, D. 1991. Determination of the digestible crude protein, digestible energy and metabolizable energy of cocoa pod husk by pigs. Bull. Anim. Health Prod. Afr., 39:83-87.

Fleischer, S., Sharkey, M., Mealey, K., Ostrander, E.A. and Martinez, M. 2008. Pharmacogenetic and metabolic differences between dog breeds: Their impact on canince medicine and the use of the dog as a preclinical animal model. The AAPS Journal [How do you give a reference to an internet journal?]

Franzke, C., Grunert, S., Hildebrandt, U. and Griehl, H. 1967. Über das Vorkommen von Theophylline in Kaffee. Hoppe-Seyler´s Z. Physiol. Chem. 348, 1725-1726.

Fredholm, B. 1984. Cardiovascular and renal actions of methylxanthines. Prog Clin Biol Res., 158:303-30.

Fredholm, B. 1995. Adenosine, Adenosine Receptors and Actions of Caffeine. Pharmacol. Toxicol., 76:93-101.

Fredholm, B.B. 2007. Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ., 14:1315-1323.

Fredholm, B., Bättig, K., Holmén, J., Nehlig, A. and Zvartau, E.E. 1999. Actions of Caffeine in the Brain with Special Reference to Factors that Contribute to its Widespread Use. Pharmacol. Rev., 51:83-133.

Fujii, T. and Nishimura, H. 1973. Fetal hypoproteinemia associated with generalized edema caused by administration of methyl xanthines to the rat during late pregnancy. Japan J. Pharmacol., 23:894-896.

Funabashi, H., Fujioka, M., Kohchi, M., Tateishi, Y. and Matsuoka, N. 2000. Collaborative work to evaluate toxicity on male reproductive organs by repeated dose studies in rats. 22) Effects of 2- and 4-week administration of theobromine on the testis. J. Tox. Sci., 25:211-221.

Gans, J.H. 1982. Dietary influences on theobromine-induced toxicity in rats. Toxicology and Applied Pharmacology 63:312-320.

Gans, J.H. 1984. Comparative toxicities of dietary caffeine and theobromine in the rat. Food and Chemical Toxicology 22:365-369.

Gans, J.H., Korson, R., Cater, M.R. and Ackerly, C.C. 1980. Effects of short-term and long-term theobromine administration to male dogs. Toxicology and Applied Pharmacology 53:481-496.

Garrett, B.E. and Griffiths, R.R. 1997. The role of dopamine one the Behavioral effects of caffeine in Animals and Humans. Pharmacol. Biochem. Behav., 57:533-547.

Gartrell, B.D. and Reid, C. 2007. Death by chocolate: A fatal problem for an inquisitive wild parrot. New Zea. Vet. J. 55(3): 149-151.

Gates, S. and Miners, J.O. 1999. Cytochrome P450 isoform selectivity in human hepatic theobromine metabolism. Br.J.Clin.Pharmacol. 47: 299-305.

Ghotbi, R., Christensen, M., Roh, H.K., Ingelman-Sundberg, M., Aklillu, E. and Bertilsson, L. 2007. Comparisons of CYP1A2 genetic polymorphisms, enzyme activity and the genotype-phenotype relationship in Swedes and Koreans. Eur.J.Clin.Pharmacol. 63: 537-546.

Glauberg, A.,and Blumenthal, H.P. 1983. Chocolate poisoning in the dog. Journal of the American Animal Hospital Association 19:246-248.

Gowda, N. K. S., Ramana, J. V., Prasad, C. S., and Singh, K. 2004. Micronutrient, content of certant tropical conventional and unconventional feed resources of southern India. Tropical, Anim. Helth and Production 36:77-94.

Greenwood-Barton, L.H. 1965. Utilisation of cocoa by-products. Food Manufacture May 1965, p. 52-56.

Gurney, K.A., Evans, L.V. and Robinson, D.S. 1991. Extraction of purine alkaloids from cocoa tissues and determination by high-performance liquid chromatography. Phytochemical Analysis 2:15-19.

Hadorn, H. (1980) Theobromine-, caffeine- and total alkaloid contents of cocoa mass. CCB 5:26-28.

Haines, C. E. and Echieverria, A. J. 1955. Cacao pods as a substitute for corn in tropical dairy rations. Foreign Agricult., 19:99-101.

Hammertone J.F., Romanczyk, L. J., and Aitken, W. M. 1994. Purine alkaloid distributon within Herania and Theobroma. Phytochemistry 35:1237-1240.

Hansen, S., Trammell, H., Dunayer, E., Gwaltney, S., Farbman, D. and Khan, S. 2003. Cocoa Bean Mulch as a Cause of Methylxanthine Toxicosis in Dogs. Clin. Tox. 41.

Hardy, F. 1960. Cacao Manual. Turrialba, Costa Rica: Inter-Am. Inst. Agric. Sci. 395 pp. 42.

Hart, A. and Grimble, R.F. 1990a. The effects of methylxanthines on lactational performance. Annals of Natrition and Metbolism 34:297-302..

Hart, A. and Grimble, R.F. 1990b. The effects of methylxanthines on milk volume and composition, and growth of rat pups. Br. J. Nutr., 64:339-350.

Hatfull, R. S., Milner, I., and Stanway, V. 1980. Determination of theobromine in animal feeding stuffs. J Assoc. Publ. Analysts 18: 19-22.

Haywood, P. E., Teale, P., and Moss, M. S. 1990. The excretion of theobromine in thorughbred rachorses after feeding compunded cubes containing cocoa husk-stablishment of a threshold value in horse urine. Equine vet. J. 232: 244-246.

Heim, F. and Ammon, H.P.T. 1969. Caffeine and Other Methyl Xanthines, Schattauer-Verlag, Stuttgart.

Hieda, Y., Kashinura, S., Hara, K. and Kageura, M. 1995. Highly sensitive and rapid determination of theophylline, theobromine and caffeine in human plasma and urine by gradient capillary high-performance liquid chromatography-frit-fast atom bombardment mass spectrometry. Journal of Chromatography B 667:241-246.

Hooser, S.B. and Beasley, V.R. 1986. Methylxanthine poisoning (chocolate and caffeine toxicosis). In: Current Veterinary Therapy small animal practice IX, R.W: Kirk (Ed.), W.B. Saunders, Philadelphia, Pennsylvania, pp. 191-192.

Horman, I. and Bracco, U. 1986. Chocolate. In: Handbook of Food and Beverage Stability, Chemical, Biochemical, Microbiology and Nutritional Aspects, G. Chalambous (Ed.), Academic Press, Orlando, FL, pp. 397-401.

Hornfeldt, C.S. 1987. Chocolate toxicity in dogs. Modern Veterinary Practice 68:552-554.

Hovda, L. R., and Kingston, R. L. 1994. Cacao bean mulchpoisoning in dogs. Vet Muman Toicol. 36:3.

Hurst, W. J., Snyder, K. P., and Martin, R. A. 1985. Use of microbore high-performance liquid chromotography for the determination of caffein, theobromin and theopylline in cocoa. J Chromatography 318:408-411.

IARC 1991. International Agency for Research on Cancer (IARC) Monograph on the Evaluation of Carcinogenic Risks to Humans. Vol 51 Coffee, tea, mate, methylxanthines and methylglyoxal, IARC, Lyon, pp. 513.

ICCO (International Cocoa Organization, 2007. Quarterly Bulletin of Cocoa Statistics; Vol. XXXIII, No. 2, Cocoa Year 2006/2007. http://www.icco.org.

Jansson, D.S., Galgan, V., Schubert, B. and Hård af Segerstad, C. 2001. Theobromine intoxication in a red fox and a European Badger in Sweden. J. Wildlife Diseases 37:362-365.

Jinap S, S., Kamaruddin, S. W., and Radiah, O. 1993. Changes in some physico-chemico properties of cocoa beans during maturation. ASEAN Food J. 4: 134-138.

Johannsen, M. and Brunner, G. 1995. Measurements of solubilities of xanthines in supercritical carbon dioxide + methanol. J. Chem. Eng. Data 40:431-434.

Johnston, J.J. 2005. Evaluation of coca- and coffee-derived methylxanthines as toxicants for the control of pest coyotes. J Agric. Food. Chem. 53: 4069-4075.

Kamimura, H. 2006. Genetic polymorphism of cytochrome P450s in beagles; possible influence of CYP1A2 deficiency on toxicological evaluations. Arch. Toxicol., 80:732-738.

Kelly, W. R., and Lambert, M. B. 1978. The use of cocoa-bean mealin the diets of horses: Pharmacology and pharmacokinetics of theobromine. Br. Vet. J. 134: 171-180.

Kihlman, B.A. 1977. Caffeine and chromosomes. Elsevier Sci. Publ. Comp., Amsterdam, pp. 504.

Kihlman, B. and Andersson, H., C. 1987. Effects of caffeine on chromosomes in cells of higher eukaryotic organisms. Rev. Env. Health 7:279-382.

Knapp, A. W. and Churchman, A. 1937. Cacao shell and its use as an accessory fodder. Chemistry and Industry 9: 29-33.

Koyama, Y., Tomoda, Y., Kata, M. and Ashihara, H. 2003. Metabolism of purine bases, nucleosides and alkaloids in theobromine-forming Theobrom, cacao leaves. Plant Physiology and Biochemistry 41:977-984.

Lamb, J., Gulati, D., Choudhury, H., Chambers, R., Poonacha, K. and Sabharwal, P. 1997. Theobromine. Environmental Health Perspectives 105 (Suppl. 1):353-354.

Lambert, M.B.T., Miller, J., Kelly, R. and Evans, J.A. 1985. Urinary theobromine levels after adding cocoa husks to horses' feed. Proceedings of the 6th International Conference of Racing Analysis and Veterinarians (Hong Kong), pp. 137-141.

Latini, R., Bonati, M., Gaspari, F., Traina, G.L., Jirtano, L., Bortolotti, A., Borzelleca, J.F., Tarka, S.M.Jr., Arnaud, M.J. and Garattini, S. 1984. Kinetics and metabolism of theobromine in male and female non-pregnant and pregnant rabbits. Toxicology 30:343-354.

Lelo, A., Birkett, D.J., Robson, R.A., and Miners, J.O. 1986. Comparative pharmacokinetics of caffeine and its primariy demethylated metabolites paraxanthine, theobromine and theophylline in man. British Journal of clinical Pharmacology 22:177-182.

Li, S. and Hartland, S. 1992. Influence of co-solvents on solubility and selectivity on extraction of xanthines and cocoa butter from coca beans with supercritical CO 2. JThe Journal of Supercritical Fluids 5:7-12.

Li, S., Varadarajan, G. S. and Hartland, S. 1991. Solubilities of theobromine and caffeine in supercritical carbon dioxide: correlation with density-based models. Fluid Phase Equilibria 68:263-280.

Lima Vasconcelos, M. N, Leão da Silva, M., Soares Maia, J. G. and Gottlieb, O. R. 1975. Estudo químico das sements do copuaçu. Acta Amazonica 5:293-295.

Loeffler, B. 2000. Untersuchungen zur Pharmakokinetik von Coffein, Theophyllin und Theobromin beim Hund nach Aufnahme von Kaffee, Tee und Schokolade. Dissertation: Aus dem Institut für Pharmakologie, Pharmazie und Toxikologie der Veterinärmedizinischen Fakultät der Universität Leipzig. 104 pp.

Loeffler, B., Kluge, K., Ungemach, F.R. and Kietzmann, M. 2000a. Untersuchungen zur Pharmakokinetik von Coffein, Theophyllin und Theobromin beim Hund. Tierärztl. Prax., 28:71-78.

Loeffler, B., Kluge, K., Ungemach, F.R. and Kietzmann, M. 2000b. Plasma- und Urinkonzentrationen von Coffein, Theophyllin und Theobromin nach Applikation von Kaffee, Tee und Schokolade bei Hunden und ihre Dopingrelevanz bei Windhundrennen. Tierärztl. Prax., 28:79-85

Marchei, E., Pellegrini, M., Pacifici, R., Palmi, I. and Pichini, S. 2005. Development and validation of a high-performance liquid chromatography-mass spectrometry assay for methylxanthines and taurine in dietary supplements. Journal of Pharmaceutical and Biomedical Analysis 37:499-507.

Marx, F. and Maia, J. G. S. 1991. Purine alkaloids in seed of Theobroma species from the Amazon. Z Lebensm Unters Forsch., 193:460-461.

Matissek, R. 1997. Evaluation of xanthine derivatives in chocolate – nutritional and chemical aspects. Z. Lebensm. Unters. Forsch. A., 205:175-184.

Mazzafera, P. 1994. Caffein, theobromine and theophylline distributon in Ilex paraguaryensis. R.Bras. Fisiol. Veg. 6:149-151.

Merck Veterinary Manual 2006. (accessed 29 May 2008), http://www.merckvetmanual.com/mvm/index.jsp?cfile=htm/bc/211104.htm

Meurer-Grimes, B., Berkov, A. and Beck, H. 1998. Theobromine, theophylline, and caffein in 42 samples and products of guaraná (*Paullinia capuana*, sapindaceae). Economic Borany 52:293-301.

Miller, G.E., Radulovic, L.L., Dewit, R,H., Brabec, M.J., Tarka, S.M. and Cornich, H.H. 1984. Comparative theobromine metabolism in five mammalian species. Drug Metabolism and Disposition 12:154-160.

Miners, J.O., Attwood, J. and Birkett, D.J. 1982. Theobromine metabolism in man. Drug Metabolism and Disposition 10:672-675.

Moffat, A.C. 1986. Clarke's Isolation and Identification of Drugs, Second Edition, Pharmaceutical Press, London. 1223 p.

Morrison, F.B. 1957. Feeds and feeding, 22nd Ed., The Morrison Publ. Co., New York.

Moss, M.S. 1980. Methylxanthine Pharmocokinetics in the Horse. PhD Thesis. University of Bradford.

Moss, M.S., Hucklebridge, J.E., Jackson, P.J., Woodhead, S., Houghton, E. and Horner, M.W. 1980. The metabolism and excretion of caffeine in the horse. Proceed. 3rd Internat. Symp. Equine Medication Control, The University of Kentucky, Lexington, Kentucky, June 12-14, 1979, p. 347-350.

Mumford, G. K., Benowiz, N. L., Evans, S. M., Kaminski, B. J., Preston, K. L., Sannerud, C. A., Silverman, K. and Griffiths, R. R. 1996. Absorption rate of methylanthines following capsules, cola and chocolate. Eur. J Clin: Pharmacol. 51: 319-325.

Nagata, T. and Sakai, S. 1985. Purine base patterns of Camellia irrawadiensis. Phytochemistry 24:2271-2272..

Naik, J. P. 2001. Improved high-performance liquid chromatography method to determin theobromine and caffeine in cocoa and cocoa products. J. Agric. Food. Chem. 49: 3579-3583.

Nehlig, A. and Debry, G. 1994. Potential Teratogenic and Neurodevelopmental Consequences of Coffee and Caffeine Exposure: A Review on Human and Animal Data. Neurotoxicology and Teratology 16:531-543.

Nehlig, A., Daval, J.L. and Debry, G. 1992. Caffeine and the central nervous system: mechanism of actions; biochemical, metabolic and psychostimulant effects. Brain Res. Rev., 17:139-170.

Nomura, T. 1983. Comparative inhibiting effects of methylxanthines on urethan-induced tumors, malformations, and presumed somatic mutations in mice. Cancer Res., 43:1342-1346.

NTP 1984. Reproduction and fertility assessment in CD-1 mice when administered in feed. National Toxicology Program, National Institute of Environmental Health Sciences, NTP-84-265, 1984, 253 p.

Odunsi, A. A. and Longe, O. G. 1995a. Cocoa bean cake in poultry diets I. Chemical composition and nutritive value of cocoa bean cake in pullet chick diets. J. App. Anm. Res. 7: 91-97.

Odunsi, A. A. and Longe, O. G. 1995b. Cocoa bean cake in poultry diets. 2: Effects of feeding cocoa bean cake on growth and laying performance of pullets. Appl, Anim. Res. 7: 137-144.

Odunsi, A.A. and Longe, O.G. 1998. The nutritive value of hot water or cocoa pod ash solution treated cocoa bean cake for broiler chicks. Brit. Poult. Sci., 39:519-525.

Odunsi, A. A., Onifade, A. A. and Longe, O. G. 1999. Effect of alkali or hot water treatment of cocoa bean cake fed to broileer finishers as partial replacment for dietary groundnut cake. Arch. Zootec., 48: 337-342.

Offem, J.O. 1990. Individual variation in the amino acid and chemical composition of defatted cocoa bean meal of three varieties of cocoa (Theobroma cacao) from South-eastern Nigeria. J. Sci. Food Agric., 52:129-135.

Okai, D. B., Easter, R. A. and Frank, G. R. 1984. Nutritive value of some non-conventional feed ingredients. World Review of Animal Production 20:12-16.

Oladokun, M.A.O. 1986. Use of cocoa pod husk as fertilizer for maize production. Nigeria J. Agron., 1:103-109.

Owusu-Domfeh, K. 1972. The future of cocoa and its by-products in the feeding of livestock. Ghana J. agric. Sci., 5:57-64.

Owusu-Domfeh, K., Christensen, D.A. and Owen, B.D. 1970. Nutritive value of some Ghanaian feedstuffs. Canadian Jorunal of Animal Science 50:1-14.

Oyenuga, V.A. 1968. Nigeria's food and feeding-stuffs. Their chemistry and nutritive value. 3rd Ed. Ibadan Univ. Press. Ibadan, Nigeria, 99 p.

Paiva, M., Wright, D.C. and Janick, J. 1982. Alkaloid variation in *Theobroma cacao* L. Proceedings of the 8th International Cocoa Research Conference, p. 229-233.

Pearman, R.W., Raymond, W.D. and Squires, J.A.. 1951. Cocoa husks from Gold Coast as a source of furfural. Col. Plant. ANim. Production 2.

Perez-Martinez, I., Sagrado, S. and Medina-Hernandez, M.J. 1995. A rapid procedure for the determination of caffeine, theophylline and theobromine in urine by micellar liquid chromatography and direct sample injection. Analytica Chimica Acta 304:195-201.

Pomilio, A. B., Trajtemberg, S. and Vitale, A. A. 2002. High-performance capillary electrophoresis analysis of mate infusions prepared from stems and leaves of Iles paraguariensis using automated micellar electrokinetic capillary chromatography. Phytochem. Anal. 13: 235-2441.

Pouomogne, V., Takama, G. and Pouemegene, J.-B. 1997. A preliminary evaluation of cacao husk in practical diets for juvenile Nile tilapia (Oreochromis niloticus). Aquaculture. 156: 211-219.

Rakotomalala, J.-J. R., Cros, E., Clifford, M. N. and Charrier, a. 1992. Caffein and theobromine in green beans from Mascarocoffea. Phytochemistry 31: 1271-1272.

Renwick, A.G., Dorne, J.L. and Walton, K. 2000. An analysis of the need for an additional uncertainty factor for infants and children. Regul.Toxicol.Pharmacol. 31: 286-296.

Resman, B. H. Blumenthal, H. P. and Jusko, W. J. 1977. Breast milk distribution of theobromine from chocolate. J of Pediatrics. 91:477-480.

Retnam, K. R. and De Britto, A. J. 2003a. Phytochemical analysis of a medicinal plant Enicostemma axillare (Lam.) Raynal, by gas chromatography. J. Econ. Taxon. Bot. 27: 686-691.

Retnam, K. R. and De Britto, A. J. 2003b. Phytochemical analysis of a medicinal plant Hybanthus enneaspermus (L.) F.v. Muell., by gas chromatography. J. Econ. Taxon. Bot. 27: 701-706.

Reynolds, J.E.F. 1982. Martindale – The Extra Pharmacopeia, 28th edition, The Pharmaceutical Press, London.

Rhule, S. W. A. 1999. Performance of pigs on diets containing detoxigied sheanut cake. Tropical Animal Health and Production. 3:45-53.

Robison, G.A., Butcher, R.W. and Sutherland, E.W. 1967. Adenyl cyclase as an adrenergic receptor. Ann. N.Y. Acad. Sci., 139:703-723.

Rosenkranz, H.S. and Ennever, F.K. 1987. Evaluation of the genotoxicity of theobromine and caffeine. Fodd Chem. Toxicol., 25:247-251.

Russell, L.D. 1984. Spermiation – the sperm release process: ultra-structure observations and unresolved problems. In: Ultrastructure of Reproduction, J.P. Blerkom, P.M. Motta (Eds.), Hingham, Martinus Nijhoff, pp. 46-66.

Rätsch, C. 1998. The Encyclopedia of Psychoactive Plants. Ethnopharmacology and its Applications. Park Street Press, Rochester, Vermont, 942 p.

Saldaña, M.D.A., Zetzl, C., Mohamed, R. S. and Brunner, G. 2002. Extraction of methylxanthines from guaraná seed, maté leaves, and cocoa beans using supercritical carbon dioxide and ethanol. J Agric. Food. Chem. 50: 4820-4826.

Salvadori, M. C., Rieser, E. M., Riheiro Neto, L. M. and Nascimento, E. S. 1994. Determination of Xanthines by highperformance liquid chromatography and thin-layer chromatography in horse urin after ingestion of guaraná powder. Analyst. 119: 2701-2703.

Sawynok, J. 1995. Pharmacological Rationale for the Clinical Use of Caffeine. Drugs 49:37-50.

Sawynok, J. and Yaksh, T.L. 1993. Caffeine as an Analgesic Adjuvant: A Review of Pharmacology and Mechanisms of Action. Pharmacol. Rev., 45:43-85.

Scherer, R., Urfer, P., Mayol, M.R., Beliingheri, L. D., Marx, F. and Janssens, M. J. J. 2002. Inheritance studies of caffeine and theobromine content of mate (Ilex paraguariensis) in misiones, Argentina. Euphytica. 126:203-210.

Schneider, B.H. 1947. Feeds of the world. Agr. Exp. Sta., Charleston, W. Va.

Sebald, J., Schulmeyr, J. and Gehrig, M. 1996. Removal of xanthines from cacao. In: High Pressure Chemical Engineering, P.R. von Rohr and C, Trepp (Eds.), Elsevier Sci., Amsterdam, p. 333-338.

Senanayake, U.M. and Wijesekera, R.O.B. 1971. Theobromine and caffeine content of the cocoa bean during its growth. J. Sci. Food Agric., 22:260-261.

Shi, D. and Daly, J.W. 1999. Chronic effects of xanthines on levels of central receptors in mice. Cell Mol.Neurobiol. 19: 719-732.

Shively, C.A. and Tarka, S.M. Jr. 1983. Theobromine metabolism and pharmacokinetics in pregnant and nonpregnant Sprague-Dawley rats. Toxicology and Applied Pharmacology 67:376-382.

Shively, C.A. and Tarka, S.M. Jr.. 1984. Methylcanthine composition and consumption pattern of cocoa and chocolate products. Progress in Clinical and Biological Research, Methylcanthine containing Beverages and Foods 158.

Shively, C. A., Tarka, S. M., Arnaud, M. J., Dvorchik, B. H., Passananti, T. and Vesell, E. S. 1985. Heigh level of methylxanthines in chocolate do not alter theobromine disposition. Clin. Pharmacol. Ther. 37:415-424.

Shively, C.A., White, D.M. and Tarka, S.M. Jr. 1986. Diet-induced alterations in theobromine disposition and toxicity in the rat. Toxicology and Applied Pharmacology 84:593-598.

Skopińska-Różewska, E., Janik, P., Przybyszewska, M., Sommer, E. and Bialas-Chromiec, B. 1998. Inhibitory effect of theobromine on induction of angiogenesis and VEGF mRNA expression in v-raf transfectants of human urothelial cells HCV-29. Internat. J. Mol. Med., 2:649-652.

Skopinski. P., Skopińska-Różewska, E., Sommer, E., Chorostowska-Wynimko, J., Rogala, E.,Cendrowska, I.,Chrystowska, D., Filewska, M. Bialas-Chromiec, B. and Bany, J. 2003. Chocolate feeding of pregnant mice influences length of limbs of their progeny. Polish Journal of Veterinary Sciences 6: 57-59.

Slattery, M.L. and West, D.W. 1993. Smoking, alcohol, coffee, tea, caffeine, and theobromine: risk of prostate cancer in Utah (United States). Cancer Causes Control 4:559-563.

Smit, H. J., Gaffan, E. A. and Rogers, P. J. 2004. Methykxantines are the psycho-pharmacologically active constituent of chocolate. Psychopharmacology. 176: 412-419.

Snyder, S.H., Katims, J.J., Annau, Z., Bruns, R.F. and Daly, J.W. 1981. Adenosine receptors and behavioral actions of methylxanthines. Proc.Natl.Acad.Sci.U.S.A 78: 3260-3264.

Soffietti, M.G., Nebbia, C., Valenza, F., Amedeo, S. and Re, G. 1989. Toxic effects of theobromine on mature and immature male rabbits. J. Comp. Path., 100:47-58.

Sotelo, A. and Alvarez, R. G. 1991. Chemical composition of wild Theobroma species and their comparison to the cacao bean. J Agric. Food. Chem., 39: 1940-1943.

Staphylakis K. and Gegiou D. 1985. Free, esterified and glucosidic sterols in cocoa butter. Lipids 20:723-728.

Stidworthy, M. F., Bleakley, J. S., Cheeseman, M. T. and Kelly, D. F. 1997. Chocolate poisoning in dog. Veterinary Record 5: 28.

Strachan, E. R and Bennett, A. 1994. Theobomine poisoning in dogs. Veterinary, Record 12: 284.

Suzuki, T., Ashihara, H. and Waller, G.R. 1992. Purine and purine alkaloid metabolism in Camellia and Coffea plants. Phytochem., 31:2575-2584.

Sutton, R. H. 1981. Cocoa poisoning in a dog. Veterinary record 19/23: 563-564.

Tarka, S. M. 1982. The toxicology of cocoa and methylxanthines: A review of the literature. CRC critical Reviews in Toxicology : 275-312.

Tarka, S.M., Zoumas, B.L. and Trout, G.A. 1978. Examination of the effect of cocoa shells and theobromine in lambs. Nutr. Rep. Internat., 18:301-312.

Tarka, S.M., Zoumas, B.L. and Gans, J.H. 1979. Short-term effects of graded levels of theobromine in laboratory rodents. Toxicology and Applied Pharmacology 49:127-149.

Tarka, S.M., Zoumas, B.L. and Gans, J.H. 1981. Effects of continuous administration of dietary theobromine on rat testicular weight and morphology. Toxicology and Applied Pharmacology 58:76-82.

Tarka, S.M. Jr., Arnaud, M.J., Dvorchik, B.H. and Vesell, E.S. 1983. Theobromine kinetics and metabolic disposition. Clinical Pharmacology and Therapy 34:546-555.

Tarka, S. M., Applebaum, R. S. and Borzelleca, J. F. 1986a. Evaluation of the teratogenic potential of cocoa powder and theobromine in New Zealand white rabbis. Food Chemical Toxicology 24: 363-374.

Tarka, S. M., Applebaum, R. S. and Borzelleca, J. F. 1986b. Evaluation of the perinatal, postnatal and teratogenic effects of cocoa powder and theobromine in Sparague-Dawley/CD rats. Food Chemical Tox. 24: 375-382.

Taylor, O. W. 1982. A method for the detection and estimation of trace levels of theobromine in mixed horse feed. J Sci. Food Agric. 33:584-588.

Tenmizu, D., Endo, Y., Noguchi, K. and Kamimura, H. 2004. Identification of the novel canine CYP1A2 1117 C > T SNP causing protein deletion. Xenobiotica 34: 835-846.

Timbie, D.J., Sechrist, L. and Keeney, P.G. 1978. application of high pressure liquied chromatography to the study of variable affecting theobromine and coffeine concentrations in cocoa beans. Journal of Food Science 43:560-565.

Vadiveloo, J. and Fadel, J. G. 1992. Compositional analyses and rumen degradibility of selecterd tropical feeds. Animal Feed Science and Technology 37: 2656-279.

Velloso, L., A. J. Becker, M.E.B., Kalil, E-B.., Mellotti, L. and Sartini, H.J. 1965/1966. Bol. Indust. Animal, San Paulo 1965/1966, 23:147-152. Div. Zootec. Nutirc. Animal, Faz. Exp. Criacao, Sertaozinho, Brazil.

Walton, K., Dorne, J.L. and Renwick, A.G. 2001. Uncertainty factors for chemical risk assessment: interspecies differences in the in vivo pharmacokinetics and metabolism of human CYP1A2 substrates. Food Chemical Toxicol., 39:667-680.

Wang, Y. and Waller, D. P. 1994. Theobromine toxicity on Sertoli cells and comparison with cocoa extract in male rats. Toxicology Letters 70:155-164.

Wang, Y., Waller, D. P., Sinha Hikim, A. P. and Russell, L. D. 1992. Reproductive toxicity of theobromine and cocoa extract in male rats. Reproductive Toxicology 6:347-353.

Weckerle, C. S., Strutz, M. A. and Baumann, T. W. 2003. Purine alkaloids in Paullinia. Phytochemistry. 64:735-742.

Weinberger, M.A., Friedman, L., Farber, T.M., Moreland, F.M., Peters, E.L., Gilmore, C.E. and Khan, M.A. 1978. Testicular atrophy and impaired spermatogenesis in rats fed high levels of the methylxanthines caffeine, theobromine, or theophylline. Journal of Envirnomental Pathology and Toxicology 1:669-688.

Weniger, J.H., Funk, K. and Hartwig, W. 1955. Der Futterwert der Kakaoschalen und ihre Wirkung auf die Milchprodukten. Archiv für Tierernährung 4:337-348.

Weniger, J.H., Funk, K. and Grosse, F. 1956. Untersuchungen über die Vertütterung von Theobromin und Rückständen der Kakaofabrikation. Archiv für Tierernährung 6:206-223.

Willaman, J.J. and Schubert, B.G. 1961. Alkaloid-bearing plants and their contained alkaloids. USDA Tech. Bull. 1234, Documents Govt. Print Off, Washington DC.

Windholz, M., Ed. 1983. The Merck Index. M. Windholz, Ed., Rahway, NJ, Merck and Co.

Wong, H. K. and Abu Hassan, O. 1988. Nutritive value and rumen fermentation profile of sheep fed on fresh or dried cocoa pod husk-based diets. MARDI Res. J. 16:147-154.

Yang, H., Kerbger, J. A., Pettigrew, J. E., Johnston, L. J. and Walker, R. D. 1997. Evaluation of milk chocolate product as a substitute for whey in pig starter diets. J. Anim. Sci. 75: 423.429.

Ye, C., Lin, Y., Zhou, H., Cheng, F. and Li, X. 1997. Isolation and analysis of purine alkaloids from Camellia ptilophylla Chang. Acta Sci. Natur. Univ. Sunyatseni 36: 30–33.

Yesair, D.W., Branfman, A.R. and Callahan, M.M. 1984. Human disposition and some biochemical aspects of methylxanthines. In: The methylxanthine beverages and foods, G.A. Spiller (Ed.), A.R. Liss, Inc., New York, pp. 215-233

Zambonin, C.G., Aresta, A. and Palmisano, F. 2004. Determination of methylxanthines in urine by liquid chromatography with diode array UV detection. J. Pharmaceutical Biomed. Analysis 36:621-624.

Zheng, X.-Q., Koyama, Y., Nagai, C. and Ashihara, H. 2004. Biosynthesis, accumulation and degradation of theobromine in developing Theobroma cacao fruits. J. Plant Physiol. 161: 363-369.

Zhu, X., Chen, B., Ma, M., Luo, X., Zhang, F., Yao, S., Wan, Z., Yang, D. and Hang, H. 2004. Simultaneous analysis of theanine, chlorogenic acid, purine alkaloids and catechins in tea samples with the help of multi-dimension information of on-line high performance liquid chromatography/electrospray-mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 34:695-704.

Ziegleder, G. and Biehl, B. 1988. Analysis of Cocoa flavour components and flavour precursors. In: Analysis of Non-alcoholic Beverages, H.F. Linskens and J.F. Jackson (Eds.), Modern Methods of Plant Analysis, New Series Vol. 8, Springer Verlag, Berlin, pp. 321-393.

Zuomas, B.L. and Tarka, S.M. 1976. The effect of dietary theobromine on food intake and growth of rats. Federation Proceedings 35:341.

Zoumas, B.L., Wesley, R.K. and Martin, R.A. 1980. Theobromine and caffeine content of chocolate products. J. Food Science 45, 314-316.