European Food Safety Authority

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SCIENTIFIC OPINION

Gossypol as undesirable substance in animal feed [1]

Scientific Opinion of the Panel on Contaminants in the Food Chain

(Question No EFSA-Q-2005-222)

Adopted on 4 December 2008

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SUMMARY

Gossypol is a yellow compound produced by the cotton plant that confers resistance to pests. Gossypol exists in two enantiomeric forms, (+) and (-), and is experimentally often used as a racemate, (±)-gossypol, or complexed with acetic acid. (±)-Gossypol is found in cottonseed and cottonseed products in two forms: free gossypol, which is readily extractable with solvents, and bound gossypol. The latter form represents mostly covalent adducts of gossypol to proteins, from which free gossypol can be (partially) liberated by heating with acids. Cottonseeds are by-products of cotton fibre production, and are rich in oil and proteins and are therefore used for cottonseed oil production and as a feed supplement. Storage, steam and

[1] For citation purposes: Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on gossypol as undesirable substance in animal feed. *The EFSA Journal* (2008) 908, 1-56.

[2] In table 4 on page 22 the figures on gossypol intake for dairy cows, suckler cows and growing cattle have been revised from 2.8, 1.0 and 1.0 g/day respectively to 2864, 1023 and 1091 mg/day respectively in order to correspond to the unit measurement of the table. Theses changes do not change the overall conclusions of the opinion. The original version of the opinion is available on request.

heat, and extrusion of oil, reduce free gossypol concentrations and commercial production of cottonseed meals is now achieved routinely with only 0.1-0.2 % remaining as free gossypol.

Gossypol shows moderate acute toxicity in most species with oral LD_{50}s of 2400-3340 mg/kg for rats, 500-950 mg/kg for mice, 350-600 mg/kg for rabbits, 550 mg/kg for pigs and 280-300 mg/kg for guinea pigs. Signs of acute gossypol toxicity are similar in all animals and include dyspnoea and anorexia. Generally, (–)-gossypol is more biologically active than (+)-gossypol. However, (+)-gossypol is more slowly eliminated. The main target organ of gossypol toxicity following repeated exposure to lower doses in rats and humans is the testis with reduced sperm motility, inhibited spermatogenesis and depressed sperm counts. Suppressed spermatogenesis in humans is partly irreversible, particularly in males with varicocele. Gossypol also affects female reproductive organs and embryo development. Gossypol is not genotoxic and it did not induce tumours in a one year study in rat. No health-based guidance value (ADI, TDI) has been established for gossypol. The lowest oral doses inhibiting spermatogenesis in humans and monkeys were 0.1 and 0.35 mg/kg b.w., respectively. Gossypol is less toxic to ruminants, and inhibition of spermatogenesis, embryo development and increased erythrocyte fragility occur at doses of 6-18 mg/kg b.w. per day in cattle and cardiomyopathy in lambs at 2-3 mg/kg b.w. per day. Monogastric animals appear to be more susceptible to gossypol toxicity than ruminants.

Current legislation includes maximum limits for free gossypol in both cottonseed meal and complete feedingstuffs. Under normal feeding practices, the concentration in complete feedingstuffs will be less than half the maximum permitted level, even assuming the highest permitted concentrations in cottonseed meal and maximum recommended inclusion rates of the meal in livestock diets. The concentrations of free gossypol that theoretically could be reached according to the current legislation on maximum permitted concentrations in complete feedingstuffs would lead to an intake of gossypol that could result in adverse effects in livestock. The potential exposure to free gossypol, based on the maximum permitted concentration in cottonseed meal and recommended maximum inclusion rates in complete feed, would not be expected to result in adverse effects in ruminants, poultry and fish. However, not all monogastric livestock animals, e.g. pigs, have been fully investigated for potential reproductive effects occurring at low doses in some species.

There is a lack of data on gossypol content (free and bound) in feed materials used for livestock in the EU. However, information provided by the livestock feed industry indicates that amounts of cottonseed meal imported into the EU have declined significantly in recent years, and relatively little is now used as a feeding stuff for livestock in the EU. Industry sources confirm that it is not used as a feed for laying hens or fish. Gossypol is transferred to edible parts, muscle and offal of ruminants and poultry, and is probably transferred to cow's milk as it is transferred to breast milk in rats. There is very little quantitative information on transfer. At high experimental doses substantial amounts are transferred. No information was identified on the bioavailability of gossypol remaining in food products from animals fed gossypol containing feed. Human exposure to gossypol through the consumption of food products from animals fed gossypol seed derived products is probably low and would not result in adverse effects.

Key words: **Gossypol, cotton products, cottonseed, cottonseed meal, toxicity, exposure, carry-over, animal health, human health.**

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General background

Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed3 replaces since 1 August 2003 Council Directive 1999/29/EC of 22 April 1999 on the undesirable substances and products in animal nutrition[4].

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 red to a level as low as reasonably achievable 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 the discussions in view of 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 has therefore requested the Scientific Committee on Animal Nutrition (SCAN) in March 2001 to provide 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)[5].

The opinion on undesirable substances in feed, adopted by SCAN on 20 February 2003 and updated on 25 April 2003[6] 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.

[3] OJ L140, 30.5.2002, p. 10
[4] OJ L 115, 4.5.1999, p. 32
[5] 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)
[6] 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)

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 are necessary to enable a complete review of the provisions in the Annex.

Specific background

Gossypol is a polyphenolic compound that occurs naturally in the seed, foliage and roots of most cotton plants, and is relatively heat stable. Gossypol is a natural defence compound produced by plants against pests and diseases. Gossypol can be found in "free" or bound (attached to a protein) forms with the bound form being less toxic than the "free" form. In the seeds, almost all the gossypol is found in the free form. Heat and moisture processing converts the free form into the less toxic, bound form. Free gossypol is responsible for the toxicity of cotton products to non-ruminants and young ruminants. Adult ruminants are more tolerant of gossypol[7].

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

SCAN concluded[8] that free gossypol 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 contained by modern techniques of feed formulation. SCAN therefore recommended[9] that gossypol should be 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 gossypol 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 asks the European Food Safety Authority requests to provide a scientific opinion on the presence of (free) gossypol in animal feed.

This scientific opinion should:

- confirm that the level of free gossypol is relevant for the adverse effects of gossypol and not the level of total gossypol (free + bound)
- determine the toxic daily exposure levels of (free) gossypol for the different animal species (difference in sensitivity between animal species) above which

7 Opinion of the Scientific Committee on Animal Nutrition on Undesirable Substances in Feed, point 9.4.2. Free gossypol.
8 Opinion of the Scientific Committee on Animal Nutrition on Undesirable Substances in Feed, point 9.5. Conclusions.
9 Opinion of the Scientific Committee on Animal Nutrition on Undesirable Substances in Feed, point 9.6. Recommendations

  - signs of toxicity can be observed (animal health / impact on animal health) or
  - the level of transfer/carry over of (free) gossypol from the feed to the products of animal origin results in unacceptable levels of (free) gossypol 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 (free) gossypol 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 (free) gossypol
  - to the overall exposure of the different relevant animal species to (free) gossypol,
  - 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 possible gaps in the available data which need to be filled in order to complete the evaluation.

ACKNOWLEDGEMENTS

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ASSESSMENT

1. Introduction

Gossypol occurs in cotton plants, mostly in the seeds, which are grown for the production of fibre and oil. Cotton plants (*Gossypium* L.) are primarily grown in dry climates at temperatures between 11 and 25°C in North and South America, Africa and Asia, and are used for production of the raw material for cotton cloth and cottonseed oil. The worldwide annual production of cotton in 2007/08 was about 46 x 10^6 tonnes from about 35 million hectares, principally in the People's Republic of China and India (31 % and 23 % of the global production, respectively), but it is also a significant crop in the United States of America (USA), Pakistan, the Commonwealth of Independent States (in particular Uzbekistan), and Brazil. The largest cotton producers in Europe are Turkey and Greece; very small amounts of cotton are also produced in Spain and Bulgaria[10]. In 2007/08, the European Union (EU) imported 75,000 tonnes of cottonseed products, whereas earlier in this decade imports were in excess of 200,000 tonnes. For every kg of fibre, about 1.65 kg of cottonseed is produced (Sunilkumar *et al.*, 2006).

Following removal of the fibre, the seeds undergo further processing to remove the oil, either by crushing or solvent extraction. The remaining meal is used as a feed material because of the high protein content, particularly for ruminants that are less sensitive to gossypol than other species. Cotton plant derived feed material is not an important livestock feed in the EU.

Gossypol is a yellow pigment occurring in free or bound form in all parts of cotton plants, with the highest levels found in seeds (Figure 1; Adams *et al.*, 1960; Markman and Rzhekhin, 1969; Jaroszewski, 1998; Dodou, 2005). Non-processed whole cottonseeds, as well as processed cottonseed meal may therefore contain large amounts of free gossypol, which may cause adverse and toxic effects if used as a feeding stuff. Cotton also contains terpenoid phytoalexins other than gossypol, e.g. hemigossypol, desoxyhemigossypol, 2,7-dihydroxy cadalene, hemigossypolone and heliocides H1 and H2, but in low quantities (Bell *et al.*, 1975; Stipanovic *et al.*, 1978).

1.1. Chemistry

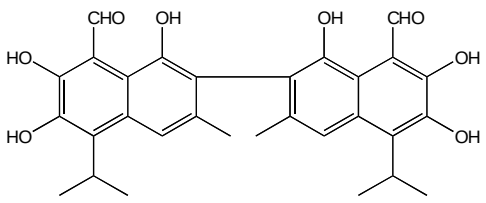


Figure 1. **Structure of gossypol**

[10] According to US Department of Agriculture: http://www.fas.usda.gov/cotton/circular/Current.asp (accessed August 15, 2008).

Gossypol (Chemical Abstracts Service CAS Registry Number 303-45-7), $C_{30}H_{30}O_8$ (MW 518.55) is crystalline, intensely yellow, insoluble in water and soluble in organic solvents and fats. It has an ultraviolet (UV) absorption maximum at about 385 nm (ε 18,000) (solvent dependent). It melts at temperatures around 200°C depending on its polymorphic form. Many polymorphic forms and crystalline solvates of gossypol exist depending on the solvent of crystallization. As racemic gossypol forms a 1:1 crystalline complex (solvate) with acetic acid that is less soluble in some organic solvents than gossypol itself, acetic acid is commonly used when isolating gossypol.

Because of the presence of the polyhydroxylated aromatic aldehyde moieties, gossypol exhibits complex tautomerism, which influences its chemical reactions. In addition to the symmetrical forms, the two halves of the molecule may adopt different tautomeric forms, giving rise to numerous molecular species. Moreover, water loss from the hemiacetal form, e.g., by heating in inert solvents above 90°C (Jaroszewski *et al.*, 1992a), results in reversible formation of anhydrogossypol (Figure 2). Oxidation of gossypol produces gossypolone (Figure 2).

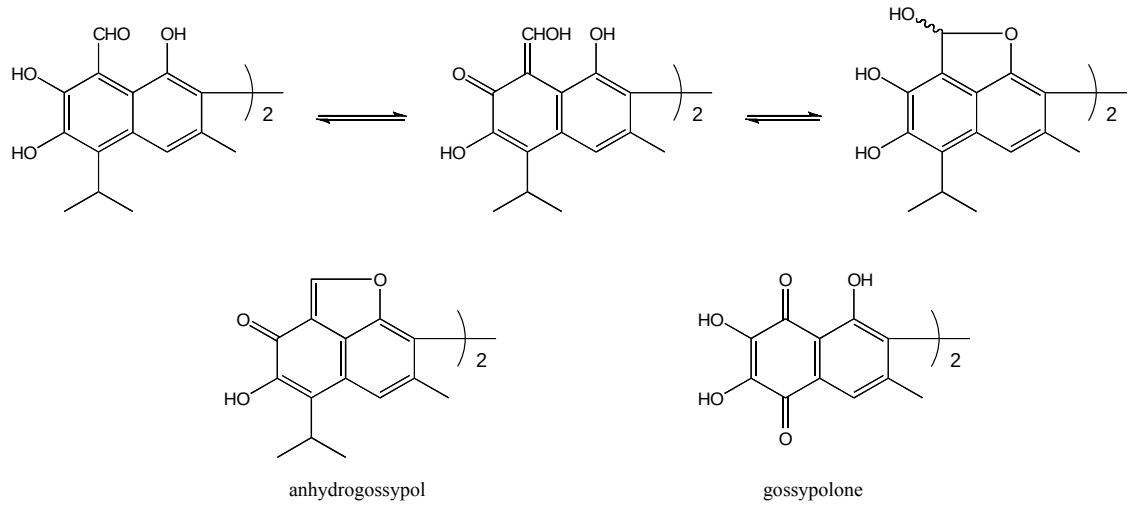


Figure 2**. Tautomeric forms of gossypol (upper structures) and common transformation products of gossypol (lower structures)**

Because rotation around the bond connecting the two halves of the gossypol molecule is hindered, the molecule is non-planar and has a two-fold symmetry axis (Figure 3). The gossypol molecule is therefore chiral due to axial dissymmetry. Hence, two enantiomers [(+) and (–)] of gossypol exist. (+)-Gossypol has the (*S*) configuration, and (–)-gossypol the (*R*)-configuration (Huang *et al.*, 1988; Freedman *et al.*, 2003).

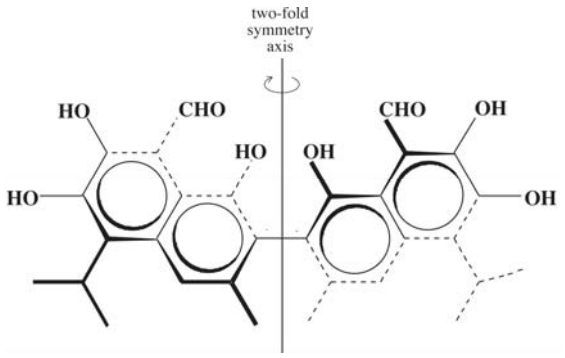


Figure 3. **Perspective view of the gossypol molecule showing the presence of a two-fold symmetry axis (C2 axis)**

The aldehyde groups present in the main tautomer of gossypol (the dialdehyde form shown in Figure 1) react readily with amines, including free amino groups in proteins (Strøm-Hansen *et al*., 1989) and form imines (Schiff bases), which can be further transformed to other products. Thus, reaction of gossypol with amino groups present in side-chains of amino acid residues and with other amino groups present in proteins (N-terminals of peptide chains) leads to formation of so-called bound gossypol, i.e., gossypol-protein conjugates. Such covalently protein-bound gossypol is not extractable with organic solvents, but can be liberated as free gossypol by treatment with acids. Protein-bound gossypol is formed upon storage of cottonseed products and its formation is accelerated by heat and moisture, the conditions encountered during processing of cottonseed. Although gossypol bound to proteins is considered less toxic or non-toxic, it may diminish the quality of proteins. Because the compound forms tight complexes with some metal ions, bound gossypol presumably also includes a fraction of gossypol that is bound as insoluble metal complexes, particularly with iron.

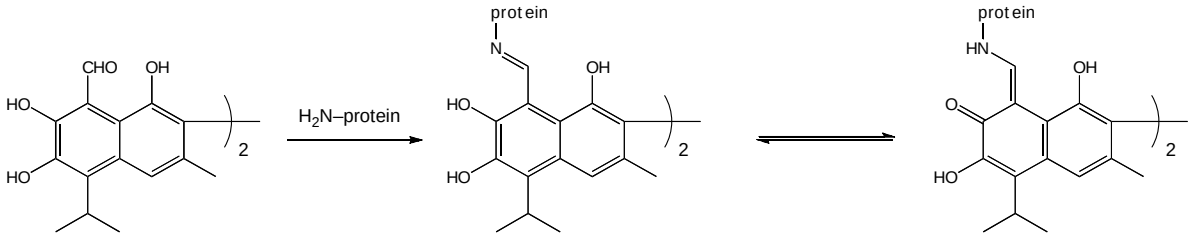


Figure 4. **Conjugation of gossypol with amino groups in proteins**

Part of the bound gossypol can, in principle, be liberated in the digestive tract of animals, but the evidence for this is lacking.

1.2. Gossypol producing plants

Commercial cotton species are *Gossypium arboreum* L. (Tree Cotton, native to the Indian subcontinent), *Gossypium barbadense* L. (American Pima or Sea Island Cotton, native to tropical South America), *Gossypium herbaceum* L. (Levant Cotton, native to southern Africa

and the Arabian Peninsula), and *Gossypium hirsutum* L. (Upland Cotton, native to Central America). *Gossypium hirsutum* is the most commonly grown species worldwide. However, there are many varieties of cotton plants obtained by selective breeding and hybridization of various species in order to improve cotton yield, resistance to diseases, drought-tolerance, etc. These breeding programs are ongoing and include wild cotton varieties.

Moreover, transgenic cotton varieties have been commercialised and dominate the cotton production worldwide. These are herbicide tolerant and/or insect resistant varieties. The insect resistant types are the so-called Bt Cotton, which carries one or several *Bacillus thuringiensis* genes coding for insecticidal proteins that confer resistance to cotton-eating caterpillars and other insect pests. Before approval in the EU, the applicant has to show that no unintended compositional changes have occurred in the genetically modified (GM) plant during the transformation process (European Food Safety Agency EFSA, 2006). Studies on the approved genetically modified cotton and its appropriate non-GM control having a similar genetic background have shown that these are equivalent in terms of gossypol content.

Gossypol is found in both vegetative and reproductive tissues of the cotton plant. The pigment is located almost exclusively in pigment gland structures appearing as dark dots in the plant tissue. Cottonseed (kernels) can contain several percent (up to 10 %, 0.1-100 g/kg) of gossypol. Extracted or pressed cotton kernels are protein-rich by-products of the cotton oil industry. Cottonseed meal contains about 40 % of proteins. The presence of gossypol in cotton protein products is counteracted in part by appropriate processing technologies, and in part by use of glandless cotton varieties, which contain only traces of gossypol. However, glandless cotton varieties are more susceptible to pests.

For many years, plant geneticists have attempted to breed gossypol-free cotton, and in the 1950s researchers developed "glandless cotton", lacking the glands that generate gossypol, using conventional breeding techniques. However this proved to be commercially unviable, since the absence of gossypol in the foliage meant the plants were stripped of their defence to insect pests.

In 2006, researchers in the USA reported that they had successfully genetically modified the cotton plant using ribonucleic acid (RNA) interference to disrupt gossypol biosynthesis specifically in cottonseed tissue by engineering the endogenous terpene pathway (Sunilkumar *et al.*, 2006). It was hypothesized that disruption of the gossypol production exclusively in the seed would not have any inadvertent consequences. A few transgenic lines were followed in the greenhouse up to the T2 generation to ensure that the genetic modification was stable and heritable. Compared with an average gossypol value of 10 g/kg in wild-type seeds, one of these lines produced seeds with 0.2 g/kg gossypol. Further studies are required to establish whether the low gossypol trait is stable also during field conditions.

(+)-Gossypol and (–)-gossypol generally co-occur in the cotton plant, usually with a slight predominance of the (+)-form (Jaroszewski *et al.*, 1992b; Stipanovic *et al.*, 2005, Stipanovic *et al.*, 2006). However, some species and varieties produce almost pure (+)-gossypol, while others accumulate an excess of (–)-gossypol. Commercial Upland Cottons usually have a 3:2 ratio between (+)- and (–)-gossypol (Stipanovic *et al.*, 2006), whereas *G. hirsutum* var. *marie galante* on average contains a much higher proportion of the (+)-gossypol, around 82 %, without having an altered level of total gossypol (Stipanovic *et al.*, 2005, 2006)

In addition to *Gossypium* species, gossypol is found in some other plants belonging to related genera [e.g., *Azanza* (Mociño et Sesse ex A. P. de Candolle) Alef., *Cienfuegosia* Cav., *Gossypioides* Skovsted ex J. B. Hutchinson, *Hampea* Schltdl., *Kokia* Lewton, *Lebronnecia*

Fosberg, and *Thespesia* Sol. ex Corrêa (= *Montezuma* Moc. & Sessé ex DC.) (Jaroszewski *et al.*, 1992b). However, these plants do not have any industrial or agricultural importance.

1.3. Toxicity in laboratory animals and hazard assessment for humans

1.3.1. General toxicity

Gossypol exhibits a variety of biological actions, which range from apparently highly specific pharmacological activities on certain macromolecular targets such as enzymes involved in maturation of mammalian sperm to more unspecific binding to proteins.

Signs of gossypol toxicity are similar in all animals and include laboured breathing and anorexia. Acute toxicity has been shown in the heart, lung, liver, and blood cells, resulting in increased erythrocyte fragility. Post mortem findings include generalised oedema and congestion of lungs and liver, fluid-filled thoracic and peritoneal cavities, and degeneration of heart fibres. Reproductive toxicity is seen particularly in males, where gossypol affects sperm motility, inhibits spermatogenesis and depresses sperm counts, cause Sertoli cell toxicity and may also affect Leydig cells. Gossypol also seems to disrupt oestrus cycles, pregnancy and early embryo development, particularly in the monogastric species studied (Abou-Donia, 1976; Berardi and Goldblatt, 1980; Randel *et al.*, 1992; Dodou, 2005). Acute gossypol intoxication of non-ruminant animals causes circulatory failure. Single-dose oral LD_{50} of racemic gossypol is 2400-3340 mg/kg for rats, 500-950 mg/kg for mice, 350-600 mg/kg for rabbit, 550 mg/kg for pigs and 280-300 mg/kg for guinea pigs. Sub-acute toxicity causes pulmonary oedema and symptoms of malnutrition. Dogs appear to be quite sensitive (repeated oral dose of 10-200 mg/kg for a month was fatal). Compared with rodents, ruminants are less susceptible to cottonseed related toxicity (Abou-Donia, 1976).

The two enantiomers of gossypol have markedly different biological effects. In comparison with (+)-gossypol, the (–)-enantiomer generally exhibits more pronounced effects. For example, (–)-gossypol is more cytotoxic (Band *et al.*, 1989; Benz *et al.*, 1990; Blackstaffe *et al.*, 1997; Shelley *et al.*, 1999), binds more strongly to proteins (Wang *et al.*, 1992; Oliver *et al.*, 2005), is the active anti-spermatogenic agent, and is considered more toxic than the (+)-gossypol (Matlin *et al.*, 1985; Lindberg *et al.*, 1987; Bailey *et al.*, 2000; Lordelo *et al.*, 2005). Most toxicological studies have been performed with racemic gossypol.

Racemic gossypol acetic acid was administered in cynomolgus monkeys at 25 mg/kg b.w. per day for 13 weeks, and most animals died. A variety of symptoms were observed ranging from vomiting, loose faeces, abnormal quietness and change of personality, body weight (b.w.) loss, increase urea and creatinine, to reductions in sodium, potassium and chloride levels. Only one animal survived the 13 weeks of treatment and showed progressive anaemia together with morphological changes in the kidney. Morphological changes in general included necrotic myocardial fibres and fibrosis in the heart, the presence of vacuolar hepatocytes, extensive changes in the kidney including dilation, epithelial vacuolation and degeneration with calcification and dystrophic mineralisation and unilateral testicular atrophy (except in the monkey that survived the experiment for which testicular morphology was normal) (Heywood *et al.*, 1985, Heywood, 1988). At lower doses, reproductive toxicity was observed (see reproductive toxicity section).

The toxicity of (-)-gossypol was investigated in male cynomolgus monkeys dosed orally with 1.5, 4 or 5 mg/kg per day for 4 weeks. At a dose of 4 mg/kg b.w. per day or higher, adverse clinical gastrointestinal signs, including vomiting and decreased body weight gain, were observed. Consistent biochemical changes were observed and included decrease in serum proteins, calcium, inorganic phosphorus, serum cholesterol and hypokalemia in one animal at the lowest dose, while serum urea was increased at all doses. Lower liver weights were also observed, but no morphological changes were found in any organ (Heywood, 1988).

Rhesus monkeys (*Macaca mulatta*) tolerated an oral dose of 4 mg/kg per day of racemic gossypol for 2 years, but gastrointestinal toxicity and weight loss occurred after 4 weeks at 8 mg/kg per day (Sang *et al.*, 1980).

1.3.2. Reproductive toxicity

1.3.2.1. Reproductive toxicity in males

Data from experimental animals

Toxicological effects in rodents after oral administration of gossypol were studied mainly in relation to its reversible inhibition of spermatogenesis. Gossypol caused degeneration of spermatocytes at doses of 10 and 20 mg/kg b.w. per day in hamsters and rats respectively whereas these effects were absent at 40 mg/kg b.w. per day in mice (Hahn *et al.,* 1981). Mice are much less sensitive due to toxicokinetic differences i.e. the mouse eliminates gossypol faster than the rat (Abou-Donia *et al.*, 1989). Oral doses of 25 mg/kg b.w. per day of (±) gossypol acetic acid to male Sprague-Dawley rats for 26 weeks, in addition to testicular pathology including depression of spermatogenesis, Sertoli cell toxicity and degeneration of seminiferous tubules, caused a marked suppression of body weight gain associated with minor biochemical changes (serum alanine aminotransferase) in 6 out of 20 rats. A no adversed adverse effect level (NOAEL) of 5 mg/kg b.w. per day for Sprague-Dawley rats was identified (Heywood *et al.*, 1986). In contrast, other authors found that 2.5 mg/kg per day of racemic gossypol was lethal to Wistar rats (Weinbauer *et al.*, 1983). Daily subcutaneous injections of 1, 5, or 10 mg/kg b.w. per day of racemic gossypol acetic acid for 2 weeks in young rats caused a dose-dependent decrease in the serum concentrations of thyroid hormones, i.e., free thyroxine, triiodothyronine, and reverse triiodothyronine. Histopathological examination revealed focal degeneration of thyroid follicles at 1 mg/kg b.w. per day whereas widespread follicular atrophy occurred at 5 and 10 mg/kg b.w. per day (Rikihisa and Lin, 1989). Recently, proteinaceous fishmeal-based supplements (3, 7 and 10 g per day) were given to groups of rats receiving crude cottonseed oil orally (14 mg free gossypol/kg b.w. per day) for 53 days. At the end of the treatment period, the authors found a dose-dependent decrease in gossypol-induced spermato-toxicity (Akinola *et al.*, 2006).

New Zealand white rabbits were given 4 or 20 mg/kg b.w. per day of racemic gossypol by oral administration for 8 weeks and biochemical, enzymatic, and electrolytic properties of the seminal plasma were studied. The exposure resulted in an increase in seminal plasma total protein, together with a decrease in total lipids and increased activities in seminal lactate dehydrogenase and alanine aminotransferase at both doses. These effects were reversible after withdrawal of gossypol in contrast with the increase in albumin, decrease in seminal plasma

lactate dehydrogenase and aspartate aminotransferase activity and potassium levels which were irreversible (Shaaban *et al.*, 2008).

In several species of monkeys, (±)-gossypol acetic acid has also been shown to inhibit spermatogenesis at oral doses of 0.5 mg/kg b.w. per day 5 days a week in male bonnet monkeys (*Macaca radiata*) after 3 months of treatment (Kalla *et al.*, 1984), and 5 mg/kg b.w. per day in cynomolgus monkeys (*Macaca fascicularis*) after 6 months of treatment (Shandilya *et al.*, 1982). At high doses (25 mg/kg b.w. per day), racemic gossypol acetic was lethal and also induced unilateral testicular atrophy in cynomolgus monkeys after 11–48 days of dosing (Heywood *et al.*, 1985, Heywood 1988). This lethal dose is only 2.5× the dose of 10 mg/kg per day causing inhibition of spermatogenesis in the same species of monkey (Shandilya *et al.*, 1982). Overall, the lowest observed adverse effect level (LOAEL) in monkeys is 0.5 mg/kg b.w. given 5 days per week, corresponding to 0.35 mg/kg b.w. per day.

Human data

In 1957, Liu reported that a village in the Jiangsu province in China did not have any childbirth between the 1930s and 1940s. However, the villagers were fecund before and after that period. This infertility incident was due to a large-scale contamination of cotton oil for human consumption with gossypol (Qian and Wang, 1984; Amini and Kamkar, 2005). Furthermore, in the 1960s, farmers from the Hubei and Hebei provinces in China that ingested homemade unheated cottonseed oil developed fatigue and a burning sensation on the face and other exposed part of the body and these symptoms were qualified as "Hanchuan fever or burning fever" (Wu, 1989). Hence, gossypol attracted a lot of attention as a possible male antifertility agent or a therapeutic agent for some gynaecological diseases, but the research in this area was discontinued due to irreversibility of its anti-spermatogenic effect already at low doses (Dodou, 2005; Waites *et al.*, 1998). Large scale studies involving more than 8000 Chinese males on the use of gossypol as an anti-contraceptive have been carried out using 20 mg (±)-gossypol/day and the study revealed that the drug was efficient and well tolerated, and did not cause changes in blood pressure or biochemical parameters. However, a side effect (hypokalemia) affected around 10 % of Chinese users (National coordinating group on male infertility, 1978; Liu., 1985; Coutinho, 2002). Further trials tested gossypol as a contraceptive agent for men at lower doses enrolling 151 men from various ethnic origins (Brazil, Nigeria, Kenya, and China) which received 15 mg racemic gossypol/day (0.24 mg/kg b.w. per day) for 12 or 16 weeks and 51 control subjects. Subjects were then randomized to either 7.5 or 10 mg/day corresponding to 0.12 and 0.17 mg/kg b.w. per day for further 40 weeks. Spermatogenesis suppression was attained in 81 of the 151 treated subjects and only one subject discontinued treatment (because of tiredness). Potassium levels fluctuated within the normal range, follicle stimulating hormone (FSH) levels increased consistently (indicative of reduced secretion of inhibin from Sertoli cells in the testes), testicular volume decreased, but after discontinuation values returned to control levels. Fifty one percent of the subjects who received 0.12 and 0.17 mg/kg b.w. per day recovered sperm counts to 20 million/mL within 12 months of discontinuing gossypol treatment. However, 18 percent of the remaining 48 patients were still azoospermic one year after termination of gossypol treatment. All men diagnosed with varicocele failed to reverse spermatogenesis suppression. Gossypol blood levels indicated that sperm suppression occurs independently of concentration, whereas spermatogenesis recovery appears to be concentration-dependent (Coutinho *et al.*, 2000).

1.3.2.2. Reproductive toxicity in females

Data from experimental animals

Several studies have also reported toxicity of gossypol acetic acid in laboratory animals. Longer oestrous cycles have been observed at doses of racemic gossypol above 5 mg/kg b.w. per day mainly because of the effects on diestrus. However, rats also suffered body weight loss or a lower body weight gain compared to controls (Gu and Anderson, 1985; Randel *et al.*, 1992). Lower levels of estradiol-17-β were also noticed from day 1 of the diestrus through the oestrus after intramuscular injection of racemic gossypol acetic acid (25 mg/kg b.w.. per day) for 3-5 days (Lin *et al.*, 1985). Another experiment in female hamsters demonstrated elevated serum concentrations of pituitary hormones such as follicle stimulating hormone (FSH) but not of luteinizing hormone (LH). Apparently, gossypol disrupts the oestrous cycle through its effect on hormonal secretion in the pituitary gland and the ovary (Randel *et al.*, 1992).

Racemic gossypol acetic acid treatment before breeding or during early gestation induced lower pregnancy rates at doses of 20, 40, 60 mg/kg b.w. per day in rats (60, 50 and <25 % compared to controls) and at 40 and 80 mg/kg b.w. in mice (60 and 10 % compared to controls). These effects were also associated with lower progesterone and estradiol-17β serum concentrations (Lin *et al.*, 1985; Yang and Wu, 1987). The authors concluded that gossypol is luteolytic and disrupts post-implantation development. Lin *et al.* (1985) also reported that implantation took place at the beginning of proestrus in rats after gossypol treatment (25 mg/kg b.w. per day). Finally, Lagerloff and Tone (1985) showed that the uteri of pregnant rats treated with gossypol were less vascularised than controls.

Human data

In China, women ingesting home made cottonseed oil suffered from burning fever, developed amenorrhoea and physical examination revealed atrophy of the uterus. Human trials have been performed using doses of 20 mg/day of racemic gossypol for 2-3 months followed by a maintenance dose of 40 mg/week for 4-5 months in women with endometriosis, uterine myomas and functional uterine bleeding, gossypol and these resulted in amenomania and atrophy of the endometrium. Examination of uterine biopsies showed a local cytotoxic effect on the uterus together with a systemic effect on the ovarian function (Wu, 1989; Zhu, 1984; Randel *et al.*, 1992).

Embryotoxicity and developmental toxicity

Gossypol treatment has been shown to inhibit the blastocyst formation rate of mouse embryos, which decreased by 50 % at an *in vitro* concentration of 2.5 mg/mL (Jo *et al.*, 2003).

In rats given racemic gossypol as a single gavage dose (10 or 20 mg/kg b.w.) during pregnancy (on one day during days 6-16), neither dose had any adverse effect in the dams. In the pups, the incidence of malformations was very low (Beaudoin, 1985).

In pregnant Balb C mice treated orally with 60 or 120 mg/kg b.w. of racemic gossypol acetic acid on days 6 to 13 of pregnancy and sacrificed on day 18, adverse effects on the dam and offspring were noted, including decreased weight gain during pregnancy, growth retardation of the offspring and also increased resorptions and late fetal deaths. Chick embryos (fertilized hen eggs) injected with 0.25 mg racemic gossypol/egg at 24, 48, 72, or 96 hours of

incubation, had a high mortality. Exencephalic fetuses were observed in one of four litters exposed to the higher dose of gossypol, micromelia was observed in one of 26 chick embryos treated at 24 hours, and gastroschisis was observed in one of 21 chick embryos treated at 72 hours in chick embryos. This study provides evidence that gossypol has embryotoxic and possibly teratogenic activity in mouse and chick embryos (Li *et al.*, 1989).

1.3.3. Genotoxicity and Carcinogenicity

Genotoxicity

Gossypol has been shown to be non-mutagenic in the Salmonella assay, and in the sperm head abnormality assay in mice (Colman *et al.*, 1979; Majumdar *et al.*, 1982; Li *et al.*, 1989; Jo *et al.*, 2003). Gossypol also did not induce chromosomal aberrations in the absence or in the presence of metabolic activation in Chinese hamster cells. Studies on human lymphocytes showed that gossypol had no influence on chromosomal aberrations, sister chromatid exchange and ploidy levels (Cai *et al.*, 1981; Tsui *et al.*, 1983; Liang and Ye, 1985; De Peyster and Wang, 1993).

The incidence of breakage, gaps and polyploidy was also investigated in cultured lymphocytes from males volunteers exposed to gossypol during the early contraceptive trials and no effects of gossypol were noted (National coordination group, 1978).

Carcinogenicity

The tumorigenicity of racemic gossypol was investigated in rats only. In 20 male Wistar rats treated at doses of 5, 15 and 30 mg/kg b.w. per day orally, for one year, no increase in tumours or obvious pathological changes were found in blood or any of the organs with the exception of occasional focal hyperplastic inflammation in the lung, which was also observed in control animals (Gao *et al.*, 1985). The potential for gossypol to induce nodule foci and hepatomas in the liver was studied in 180 Wistar rats given oral doses up to 40 times the contraceptive range (200 mg/kg b.w. per day) for 6 months. No tumours were found and gossypol was concluded to have neither tumour initiating nor promoting effects (Ding *et al.*, 1985). Recently, gossypol has been used in cancer clinical trials, particularly on patients in meta-static refractory cancer, and has shown low, but measurable, responses without serious side effects (emesis, abdominal ileus) such as myelosupression and has now been put forward as a new class off anti-neoplastic agent (Dodou, 2005)

In conclusion, gossypol is not genotoxic and did not induce tumours *in vivo* in rats. However, a full 2-year carcinogenicity study in the rat or in other species is not available. Hence, the available *in vivo* data in laboratory animals and the anti-neoplastic effects in humans as they stand indicate that gossypol is unlikely to be carcinogenic in humans, but the evidence is insufficient to provide a definitive conclusion.

1.3.4. Mechanisms of toxicity

In several studies the mechanisms of toxicity of gossypol have been explored (Ali and El-Sewedy, 1984; Tso and Lee, 1982; Gawai *et al.*, 1995; Kovacic, 2003; Fiorini *et al.*, 2004;

Zhou *et al.*, 2008). Gossypol and its metabolites exert pro- and anti-oxidant potential. Considerable evidence points to oxidative stress, formation of reactive oxygen species, and DNA scission, characteristics of redox-cycling by electron transfer in biosystems (Kovacic, 2003). In rats treated intraperitoneally for 5 days with 5 mg/kg b.w. of racemic gossypol per day biochemical changes in liver enzymes were observed with a decrease in cytochrome P-450, cytochrome b5, NADPH-cytochrome c reductase, aminopyrine N-demethylase and aniline hydroxylase. However, the treatment did not affect levels of cytosolic glutathione S-transferase (GST) and the serum enzymes sorbitol dehydrogenase and alanine aminotransferase, which are indicators of liver damage (Ali and El-Sewedy, 1984). With respect to GST isozymes, gossypol is a reversible inhibitor (Lee *et al.*, 1982). Furthermore, gossypol binds to microsomal membranes, inhibits DNA synthesis, and causes depletion of iron and glutathione in mammalian cells (Gawai *et al.*, 1995).

Gossypol also specifically inhibits the 11β-hydroxy-steroid dehydrogenase (11β-HSD), which oxidises cortisol to inactive cortisone in the kidney and other tissues of importance in electrolyte potassium and sodium regulation. Episodes of hypokalemia observed in clinical trials for antispermatogenic effect are the clinical consequence of inhibition of 11β-HSD (Song *et al.*, 1992; Waites *et al.*, 1998; Reidenberg, 2000).

Another mechanism relevant to the anti-fertility effects of gossypol is the ability to block gap junction intercellular communication (GJIC) in Sertoli cells, required for spermatogenesis, which has been demonstrated in cultured human and rat cells (Herve *et al.*, 1996). *In vitro* experiments in Sertoli cells showed a rapid cytoplasmic delocalization of connexin 43 (a gap junctional protein important for Sertoli cells to regulate spermatogenesis) In addition N-cadherin and connexin 43 protein expressions were decreased (Fiorini *et al.*, 2004; Zhou *et al.*, 2008).

More recently, racemic gossypol has been shown to induce apoptosis and inhibit *in vitro* proliferation of cancer cell types (Dunning, prostate, epithelial (breast), stromal, cervical, colon). These inhibitory effects have been associated with the induction of TGFβ1 in human prostate cancer PC3 cells which in turn influences the expression of the cell cycle-regulatory protein, cyclin D1 (Jiang *et al.*, 2004; Liu., 2005). In addition, (-)-gossypol has been shown to be more potent than the (+)-gossypol to inhibit cancer cell growth. Recently, the pro-apoptotic and anti-tumour effects of gossypol have been attributed to its BH3-mimetic properties through the binding to the BH3 pocket of anti-apoptotic proteins, and thereby displacing the pro-apoptotic factors. In leukaemia cells, gossypol induced mitochondrial outer membrane permeabilisation and production of reactive oxygen species, activation of BAX protein, and release of cytochrome c and apoptosis-inducing factor (Balakrishnan *et al.*, 2008). Finally, gossypol scavenges free radicals, reduces ferric ions, prevents UV-induced deoxyribonucleic acid (DNA) damage and inhibits the growth of *Trypanosoma brucei* cells (Wang *et al.*, 2008).

1.3.5. Evaluations

No acceptable daily intake (ADI), tolerable daily intake (TDI) or equivalent health-based guidance value has been set by any international body. The most important pharmaco-toxicological effect of gossypol has been recognised to be inhibition of spermatogenesis. The lowest dose identified to maintain this effect (induced by a dose of 0.23 mg/kg b.w. per day) in humans is 0.1 mg/kg b.w. per day. The lowest dose identified to cause the same effect in

experimental animals (monkeys) is 0.35 mg/kg b.w. per day. A NOAEL for this effect in humans or experimental animals has not been identified.

2. Methods of analysis

The major issue regarding gossypol analysis is whether "free" or "total" (free and bound) gossypol is to be determined. These forms require different sample preparation techniques. Thus, free gossypol can be extracted with various organic solvents including aqueous acetone, often in the presence of an anti-oxidant such as ascorbic acid, as in the official method of the American Oil Chemists' Society method (AOCS, 1987a,b). Other solvents, such as hexane containing acetone, have also been recommended (Kuk *et al.,* 2005). As the measured content of free gossypol depends on the method of extraction and specificity of subsequent method of analysis used, the definition of free and bound gossypol is largely determined by the method of extraction used. Thus, different solvents can extract, in addition to free gossypol, hydrophilic and lipophilic forms of bound gossypol to various degrees. The official AOCS method of extraction of free gossypol employs acetone-water 7:3 (AOCS, 1987a, b).

Total gossypol is determined after hydrolysis of the sample with acid. It should be emphasized that the conditions of hydrolysis may affect the results of the analysis, because particular forms of bound gossypol may be hydrolysed to different extents, depending on the reaction condition used. Moreover, other forms of protein binding of gossypol, mainly irreversible covalent binding, may occur (Moh *et al.,* 1992).

Cold organic solvent extraction of free gossypol and hot, acidic extraction of bound gossypol are also used in the official EU method of gossypol determination[11]. For extraction of free gossypol, the sample should be extracted at room temperature with an isopropanol-hexane mixture (3:2) containing 0.2 vol. % of 3-aminopropan-1-ol, 0.8 vol. % of acetic acid, and 5 vol. % of water. For extraction of total gossypol, the sample should be heated at 100°C with a solution of glacial acetic acid (10 vol. %) in dimethylformamide containing 2 vol. % of 3-aminopropan-1-ol.

Subsequently, extracted gossypol is determined spectrophotometrically (at 440 nm) as adduct (Schiff base) with aniline, i.e. dianilino-gossypol (Directive 72/199/EEC; AOCS, 1987b). The specificity of this method is questionable, as compounds other than gossypol can contribute to the formation of UV adsorption at 440 nm. However, cotton plants do contain gossypol-like compounds, e.g., gossypol methyl ethers of presumably similar toxicity as gossypol, which will be co-determined with gossypol in the spectrophotometric methods. High-performance liquid chromatography (HPLC) determination of gossypol is preferred as it offers a higher degree of specificity (Abu-Donia *et al.,* 1981; Chamkasem, 1988; Hron *et al.,* 1990; Jaroszewski *et al.*, 1992b; Botsoglou, 1992). Determination of gossypol by HPLC is usually performed using a C18 column with acetonitrile-water or methanol-water, acidified with H_3PO_4, as the mobile phase. Both UV detection (preferably at about 385 nm, which corresponds to the long wavelength absorption maximum of gossypol) and electrochemical detection have been used, with detection limits of about 40 ng/mL and 2 ng/mL in the injected sample, respectively (Jaroszewski, 1998). Validated range of determination of gossypol by LC-MS was 10-2000 ng/mL (Coward *et al.,* 2006); using a standard cottonseed or cottonseed meal sample used in the official EU method (1 g sample extracted with 50 mL of a solvent),

[11] Third Commission Directive 72/199/EEC of 27 April 1972 establishing Community Methods of Analysis for the official Control of Feedingstuffs (OJ L123, 29.5.1972, p. 6)

this allows detection of 0.5 mg gossypol/kg feed. However, this limit can be lowered at least tenfold by increasing the size of the sample extracted.

A HPLC method for determination of gossypol enantiomers in animal tissue based on formation of diastereomers with (*R*)-2-aminopropan-1-ol has also been described (Lee and Dabrowski, 2002).

Recently, methods of determination of gossypol in biological samples based on liquid chromatography-mass spectrometry (LC/MS) (electro-spray ionization in positive-ion mode) have been developed (Coward *et al.,* 2006; Jia *et al.,* 2008). The limit of quantification (LOQ) of gossypol in plasma was 78 ng/mL (Jia *et al.,* 2008). Orth *et al*. (2007) quantified gossypol in budworms feeding on cotton plants by liquid chromatography-mass spectrometry-mass spectrometry (LC/MS/MS) analysis of dianilino-gossypol using deuterated dianilino-gossypol as internal standard.

Gossypol can also be determined by phloroglucinol colorimetry (Tang *et al.,* 2004) and a flow-injection method with chemiluminescent detection based on sensitization by gossypol of the reaction of luminol with ferricyanide (Xue and Liu, 2006). A method based on monoclonal antibodies was developed and showed to correlate well with the results of the official American Oil Chemists' Society method (Wang *et al.*, 2004; Wang *et al.*, 2005).

3. Current legislation

Annex 1 to Council Directive 2002/32/EC[12] contains a list of compounds that are undesirable in animal feed and their maximum levels allowed in different feed commodities. The current EU maximum levels for gossypol in feed materials are given in Table 1.

Table 1. **EU legislation on gossypol containing plant materials used as feed**

Undesirable substances	Product intended for animal feed	Maximum content in mg/kg relative to a feeding stuff with a moisture content of 12 %
Free gossypol	**Feed materials with the exception of:**	**20**
	- cottonseed	**5000**
	- cottonseed cakes and cottonseed meal	**1200**
	Complete feedingstuffs with the exception of:	**20**
	- complete feedingstuffs for cattle, sheep and goats	**500**
	- complete feedingstuffs for poultry (except laying hens) and calves	**100**
	- complete feedingstuffs for rabbits and pigs (except piglets)	**60**

There appears to be a discrepancy between the maximum permitted content of gossypol in cottonseed cakes and in complete feedingstuffs. Thus, use of cake or meal with the maximum

[12] OJ L 140, 30.5.2002, p. 10–22

permitted concentration of gossypol would allow its addition to complete feedingstuffs for cattle, sheep and goats - the most likely users of this feed - at the level of 40 %. However, the maximal recommended inclusion rate is 20 % and the maximal allowed level in the complete feedingstuff would never be reached.

4. Occurrence in feed materials

4.1. Levels of gossypol in cottonseeds

There are many commercial varieties of cotton and as a result the gossypol content in the raw cottonseed can be highly variable. As illustrated in Table 2, whole cottonseed can contain >14,000 and 7,000 mg/kg of total and free gossypol, respectively. However, whole cottonseeds are not normally used as feedingstuffs for livestock in the EU.

4.2. The effect of processing on gossypol concentration in cottonseed meal

Levels of total and free gossypol in cottonseed meal (or cake) are lower than in the parent seed, and the extent of this reduction is influenced by the methods used to process the seed. This includes removal of the fibres (dehulling or decorticating) followed by oil extraction, which is achieved either mechanically (producing pressed cake) or chemically, using a solvent.

During the oil removal process, free gossypol is largely removed with the oil, and the content of free-gossypol is reduced considerably. Subsequent processing can further lower the free-gossypol content of the meals. Exposure to heat alone appears to be ineffective in reducing free gossypol levels with reductions of between 9 % (Barraza *et al.*, 1991) and 12 % (Danke *et al.*, 1965) reported. However, studies have shown that a combination of heat and steam can result in marked reductions in free gossypol levels (Olcott and Fontaine, 1941; Sure *et al.*, 1953; Danke *et al.*, 1965). Danke and Tillman (1965) observed a 62 % reduction in free gossypol content in dehulled cottonseeds after only 10 minutes of exposure to steam at atmospheric pressure. In addition to steam and heat, extrusion also reduces free gossypol concentrations. As a result of these processes, commercial production of cottonseed meals with low levels of free gossypol is now achieved routinely by treating defatted cottonseed meals with moist heat, with only 0.1-0.2 % remaining as free gossypol (Jones and King, 1996). This has been supported by surveys conducted by the National Cottonseed Products Association (NCPA) in the early 1990s showed that the levels of free gossypol in meal manufactured with expander-solvent technology continue to remain low (<0.18 %, Forster and Calhoun, 1995). It should be noted that these processes have the effect of reducing protein digestibility and lysine availability (Damaty and Hudson, 1975), although Bruser and Ababas (2001) reported that extrusion of the cottonseed, which resulted in reductions in free gossypol concentration of 71-78 %, did not adversely affect protein digestibility.

In addition to treatment with moist heat, the addition of iron salts such as ferrous sulphate has been shown to be effective in binding the free gossypol and making it biologically inactive in rations for pigs (Tanksley, 1970) and poultry (Watts, 1970).

More than 97 % of the meal from cotton plants in the United States is partially dehulled, solvent extracted meal (National Cottonseed Products Association, personal communication),

and this is the most common cotton form available in the EU. Table 2 shows typical ranges of free and total gossypol found in whole cottonseed, cottonseed oil, and cottonseed meal.

Table 2. **Levels of gossypol (mg/kg dry weight) in whole cottonseed, cottonseed meal and cottonseed oil (modified from Organisation for Economic Co-Operation and Development OECD, 2004)**

	Gossypol (total)	Gossypol (free)
Whole cottonseed	**5,100-14,300**	**4,700-7,000**
Cottonseed oil (refined)	**0 - 900**	**not detectable**
Cottonseed meal	**9,300 – 14,300**	**200-17,700**

4.3. Reported data from EU Member States

Following a call from EFSA for data on levels of gossypol in whole or processed cottonseed, information was provided by The Czech Republic and France.

From the Czech Republic, EFSA received two results from analysis of cottonseed meal. The two samples contained 430 and 506 mg free gossypol/kg feed with a LOQ of 0.2 mg/kg. A private company from France (INZO) provided data to EFSA (see Table 3) for both whole and processed cottonseeds.

Table 3. **Data provided by INZO. The solvent extraction was performed with hexane and the LOQ was 20 mg/kg**

	Free gossypol content, mg/kg		
	Average	minimum	maximum
Whole cottonseed	**5271 ± 1440 (n = 35)**	**1962**	**8416**
Cottonseed cake, solvent extracted	**1097 ± 865 (n = 3)**	**566**	**2095**
Cottonseed cake, oil extracted, non-decorticated	**558 ± 348 (n = 7)**	**100**	**900**
Cottonseed cake, oil extracted semi decorticated	**901 ± 988 (n = 43)**	**129**	**6968**
Cottonseed cake, pressed, semi-decorticated	**974 ± 401 (n = 21)**	**365**	**1650**

Clearly these data are very limited, and are unlikely to represent the EU market situation. The INZO data show that the mean concentration of free-gossypol content in cottonseeds exceeds the maximum level. It should be further investigated if these concentrations are normally found in feed materials.

No data have been identified on the occurrence of cottonseeds as impurities in feed materials. A cross-contamination is unlikely since cotton is grown as a mono culture, processed

separately and treated as a world commodity like all other crops with strict controls over cross contamination.

5. Estimating the intake by farm livestock

Although cottonseed meal is extensively used as a livestock feed in areas where the cotton is grown and processed, it is not widely imported or used in the EU. Moreover, imports have declined significantly in recent years, due in part to concerns over potential contamination with aflatoxin, particularly following the introduction of legislation on undesirable substances in 1999, which prohibited dilution of contaminated feeds. In 2007/08, 75,000 tonnes of cottonseed was imported into the EU for processing, predominantly into Italy and Greece[13]. In addition, some whole cottonseeds were imported into the EU in 2007. In total approximately 167,000 tonnes of cottonseed meal were used as livestock feed in the EU in 2007/08; this represents less than 1 % of the oilseed meals used in the manufacture of compound feeds[14].

Recommended feeding rates for ruminants suggest a maximum of 20 % of the concentrate mix for growing beef cattle, with lower inclusion rates for younger or milk-producing livestock (Lonsdale, 1989; Ewing, 1997). In the United States, whole cottonseed has been a popular feed for dairy and beef cattle for many years, and diets in which whole cottonseed accounted for up to 10 % of the concentrate component of the ration[15] have been successfully fed (Anderson *et al.,* 1984). Because of the high oil content of whole cottonseed, these authors recommended that the maximum amount to be fed should not exceed 0.5 % of body weight per day for mature cows and 0.33 % of body weight for weaned calves. Whole cottonseed has been imported into the EU from time to time as feed for cattle; although details of quantities imported are not available, it is assumed that amounts are very small.

Cottonseed meal or cake is not widely used as feed for pigs, poultry or fish. Free gossypol is more toxic to monogastric species, and feed manufacturers are therefore cautious about including it in non-ruminant diets. Even with low gossypol meals, the high fibre and moderate energy contents are likely to result in lower feed intakes, feed utilisation and growth rates. Particular care is required with laying hens since comparatively low levels of the meal may cause an olive green discoloration of the yolk in storage.

Cottonseed meal is not used in the manufacture of fish feed, mainly because of the presence of gossypol and the low lysine content[16], which result in reduced weight gain and feed conversion efficiency.

Theoretical exposure to gossypol by farm livestock has been estimated for a number of different classes of livestock based on the maximum permitted level (MPL) of gossypol in complete feedingstuffs (see Appendix 1, table 1).

As discussed in Section 3, the legislation for feedingstuffs gives maximum permitted levels for gossypol in both whole and processed cottonseed, and in complete feedingstuffs. In practice, however, the maximum permitted levels (MPL) set for gossypol in complete feedingstuffs is unlikely to be exceeded with the current MPL for cottonseed meal. In order to

[13] Toepfer International: Statistical Information about the Grain and Feedstuff Market (September 2008 Edition)
[14] FEDIOL Annual Statistics, 2006.
[15] Equivalent to approximately 1.5 kg cottonseed/day.
[16] The available lysine content of cottonseed meal is approximately 40 % and 25 % of that typically found in soya bean meal and fish meal, respectively.

achieve the maximum permitted concentration in a complete feeding stuff for cattle, sheep and goats (500 mg/kg), even with cottonseed meal at its maximum gossypol concentration (1200 mg/kg), the meal would need to be included at >40 % of the diet. As reported above, the maximum recommended inclusion rate for ruminants - in rations for growing cattle - is 20 %, and therefore the maximum permitted level in complete feeding stuffs for livestock is unlikely ever to occur.

Table 4 illustrates the maximum likely exposure of livestock to gossypol when fed rations which include the cottonseed meal at typical maximum inclusion rates, and where the meal contains the maximum concentration of gossypol.

Table 4. **Potential exposure to free gossypol by farm livestock based on the maximum permitted concentration in cottonseed meal (1200 mg/kg at 12 % moisture content), and recommended maximum inclusion rates of cottonseed meal in the complete feeding stuffs (based on Ewing, 1998)**

	Total complete feed (kg/day)	Cottonseed meal inclusion rate (%)	Gossypol intake		
Non-ruminants			mg/day	mg/kg of diet	mg/kg b.w./day
Finishing pigs	**3.7**	**2.5**	**111**	**30**	**1.1**
Sows	**6.5**	**5**	**390**	**60**	**1.6**
Poultry (broilers)	**0.15**	**2.5**	**4.5**	**30**	**2.1**
Poultry (laying hens	**0.115**	**0**	**0**	**0**	**0.0**
Fish	**0.09**	**2.5**	**2.7**	**30**	**0.6**

				Gossypol intake		
Ruminants	Total DM intake, kg/day	Concentrate intake (kg/day)	Cottonseed meal inclusion rate (%)	mg/day	mg/kg of diet	mg/kg b.w./day
Dairy cow	**22**	**14**	**15**	**2864**	**130**	**4.4**
Suckler cow	**16**	**5**	**15**	**1023**	**64**	**1.9**
Growing cattle	**8**	**4**	**20**	**1091**	**136**	**3.6**
Lactating ewe	**1.8**	**1.5**	**10**	**205**	**114**	**2.9**
Growing lamb	**0.6**	**0.45**	**10**	**61**	**102**	**3.1**
Dairy goats	**2.2**	**1.5**	**15**	**307**	**140**	**4.7**

6. Adverse effects on livestock, pets and fish

Fear of toxic effects of gossypol limits the use of cottonseed meal as a feed ingredient. Much effort has been undertaken in order to develop methods to analyse and detoxify gossypol, and to assess its toxic effects and metabolism. Although the acute toxicity of gossypol is low, there are substantial chronic effects, especially in non-ruminant animals. Immature ruminants (with a not functionally developed rumen) are also susceptible to gossypol toxicity (Randel *et*

al., 1992). Ruminants can tolerate higher levels of gossypol as free gossypol seems to be bound to proteins or complexed in the rumen (Coppock *et al.*, 1987; Randel *et al.*, 1992; Schneider *et al.*, 2002). There may also be other incompletely defined mechanisms for rumen detoxification of gossypol involved.

Common clinical symptoms of gossypol intoxication in various species include dyspnoea, inappetence or anorexia, gastro-enteritis, weakness, listlessness and possibly death after several days (Rogers *et al.*, 1975; Randel *et al.*, 1992). High intake may be rapidly lethal. The major lesion on post mortem examination is a generalised oedema with fluid-filled thoracic and peritoneal cavities, congestion of lungs and liver, and myocardial degeneration. Alteration in normal erythrocyte structure and function is also a major effect, most prominent in cows. Therefore red cell fragility has been used as an early sign of gossypol toxicity (Calhoun *et al.*, 1990). At lower feed concentrations, reduced fertility may be predominant in both males and females.

Adverse effects of feeding cottonseed depend on the concentrations of free gossypol, and primarily on the concentration of the most biologically active (-)-enantiomer. In ruminants also the type of cottonseed, particle size and density of the cottonseed, the processing method and the concentration of iron in the diet appears to be of importance (Santos *et al.*, 2005). The plasma concentration is a marker for gossypol bioavailability and may be used to establish limits on amounts of cottonseed products that can be used safely (Santos *et al.*, 2005).

6.1. Ruminants

Male pre-ruminant calves were fed diets containing 0, 100, 200, 400 or 800 mg/kg of free gossypol in cottonseed meals from different sources from day 1 to 120 of age (Risco *et al.*, 1992). Clinical, haematological and blood biochemical parameters were measured regularly. Evidence of adverse effect was found in calves given the two highest doses after 90 days, as some calves showed decreased appetite. They exhibited dyspnoea, coughing and preferred a recumbent posture. Severe inter mandibular swelling and jaundice was also noted. One of 12 calves fed 400 mg/kg and 4 of 12 calves fed 800 mg/kg died as a result of circulatory failure associated with the gossypol consumption. A NOAEL of 200 mg free gossypol/kg diet, corresponding to 4-5 mg/kg b.w. per day, was identified.

In agreement with the study referred to above, lethal effects were also observed upon long-term feeding of male calves with a diet containing 400 mg free gossypol/kg (Velasquez-Pereira *et al.*, 1999).

Male calves were fed free gossypol through cottonseed meal at 9.95 mg/kg b.w. per day for 210 days to study clinico-biochemical parameters and humoral immune response (Pattanaik *et al.*, 2003). No deleterious clinico-biochemical manifestations were found but delayed and depressed humoral immune response against *Brucella abortus* inoculation was noted.

Post pubertal beef heifers were fed diets with cottonseed meal and/or whole cottonseed, containing 0, 0.5, 2.5, 5, 10, or 20 g free gossypol/animal per day for 62 days in order to determine the effects of increased dietary gossypol on metabolic homeostasis and reproductive endocrine function (Gray *et al.*, 1993). The feeding resulted in approximate exposures of 0, 1.3, 6.4, 13, 26 and 51 mg/kg b.w. per day. The two highest dosage levels caused increased osmotic fragility of erythrocyte membranes. At the highest dose, there was also a slight alteration in plasma concentrations of sorbitol dehydrogenase and K^+, as well as

a higher mean concentration of luteinizing hormone (LH). The treatment did not affect average daily weight gain, body condition scores, or plasma concentrations of progesterone during the oestrous cycle. A NOAEL of about 13 mg/kg b.w. was identified.

In another experiment of Gray and coworkers (1993), the long-term effects of feeding a diet resulting in an exposure of 20 mg free gossypol/kg b.w. per day for 33 weeks to lactating beef cows was studied by analysing various metabolic and reproductive endocrine characteristics (Gray *et al.*, 1993). Erythrocyte membrane fragility was increased in cows fed gossypol in comparison with control cows, but reproductive characteristics and body weight condition scores did not differ.

Increased erythrocyte fragility was observed also in beef heifers fed a diet with cottonseed meal for 112 days. The feeding resulted in an exposure corresponding to 12.9 mg free gossypol/kg b.w. per day (Velasquez-Pereira *et al.*, 1998). Vitamin E supplementation reduced the erythrocyte fragility. The gossypol level did not have any detrimental effect on the performance of the heifers.

Three experiments were conducted in diary heifers to determine the effects of feeding diets containing cottonseed with resulting exposures of 0, 20 or 40 mg free gossypol/kg b.w. per day on reproductive parameters, i.e. follicle development, luteal function, embryo quality and embryo development (Coscioni *et al.*, 2003a,b; Villaseñor *et al.*, 2003). The studies indicated that an intake of free gossypol up to 40 mg/kg b.w. did not affect follicle and corpus luteum development in the heifers, but gossypol decreased fertility in a dose-response manner.

Postpubertal heifers were fed cottonseed-containing diets with different contents of free gossypol for 70 days before superovulation and embryo collection to study effects on embryo development (Villaseñor *et al.* 2008). Resulting exposures were 0, 17.8 or 36.8 mg free gossypol/kg b.w. per day. The highest dosage level increased the number of degenerated embryos and reduced the blastocyst development. The embryo diameter was significantly and dose dependently reduced. These effects of gossypol were also observed in dairy cows receiving embryos from heifers fed gossypol (Galvao *et al.*, 2006), which suggests that negative effects in early embryo development persist during maintenance of pregnancy.

A review of several clinical studies in dairy cows fed for a longer time whole cottonseed indicated that no clinical adverse effects were induced up to feed levels of cottonseed corresponding to 15 % of the dry matter (Zhang *et al.*, 2007). The concentrations of free gossypol in these studies were not always presented. Using a cottonseed content of free gossypol commonly reported for the most used cotton variety (Upland) cottonseed, 0.71-0.73 % (Santos *et al.*, 2002), and assuming a mean dry matter intake for lactating cows to be 3.5 % of their body weight, the inclusion would result in a daily free gossypol intake of up to around 40 mg/kg b.w. without apparent adverse clinical effects. However, as indicated below, other studies on beef and dairy heifers and cows have found effects below this dose level.

In a large feeding study, Santos *et al.* (2003) fed pregnant dairy cows isonitrogenous and isocaloric diets with cottonseed containing either 717 or 951 mg free gossypol per kg dry matter diet (corresponding to exposures of 27 and 35 mg/kg b.w. per day) for 170 days. The influence of gossypol on health and reproduction was examined. Cows fed the highest gossypol level showed the lowest conception and pregnancy rate, and an increased abortion rate. No clinical adverse effects were observed. The proportion of (-)-gossypol was higher in the feed with the highest content of free gossypol. The plasma concentrations of gossypol were much higher in cows fed this diet. The investigators suggested this to be due to lack of

lint and decreased particle size in this type of cottonseed, resulting in reduced rumen retention and increased availability of free gossypol in the intestine.

In another study, lactating dairy cows were given diets with different levels and combinations of total and free gossypol via whole cottonseed or cottonseed meal for 84 days (Mena *et al*., 2004). High intake of free gossypol at a level of 16-32 mg/kg b.w. per day, increased dry matter intake and milk production, but also erythrocyte fragility. No effects on erythrocytes were observed at exposure levels of 0 and 3 mg free gossypol/kg b.w. per day. Although clinical health, serum proteins and enzymes were studied, no other adverse effects were observed. The concentration of gossypol in plasma was directly proportional to the intake of free gossypol, and reached a plateau after 28 days of feeding. The plasma level returned to negligible levels 28 days after withdrawal of gossypol from the diet.

Arshami and Ruttle (1988) determined the influence of dietary levels of gossypol as cottonseed products on spermatogenic tissues in yearling bulls. The bulls were fed isonitrogenous diets with free gossypol concentrations at 0, 0.2 or 1.2 g/kg diet for 2 months corresponding to 0, 6 or 36 mg free gossypol/kg b.w. per day. Histological studies of the testis revealed that bulls fed gossypol showed dose-dependent detrimental effects in spermatogenic tissues and associated cells: larger lumens, decreased wall thickness and reduced number of cell layers in the seminiferous tubules. Half the number of bulls in each gossypol fed group were fed a gossypol-free diet for another 2 months to determine if effects induced were reversible. They showed improvements in the histological characteristics, indicating that the effects were partly reversible.

Eleven-month old bulls were fed for 56 days a cottonseed ration resulting in free gossypol exposure of 8 mg/kg b.w. per day in order to study reproductive toxicity (Hassan *et al.*, 2004). At day 28 and 56, scrotal circumference was measured and semen collected to assess sperm motility and morphology. Half of the animals in each group were castrated at day 56 to examine the testes histologically. The other animals were then fed a gossypol-free diet for the next 210 days prior to castration. Significant increases in sperm abnormalities were noted after 28 and 56 days of gossypol feeding but no significant effects were found on sperm motility, scrotal circumference or histopathological characteristics. The sperm abnormalities were still increased four weeks after the end of gossypol feeding but were not significantly different from control samples at subsequent samplings. Chenoweth *et al.* (2000) made similar observations.

Steers receiving diets with 15 % whole cottonseed for 28 days in order to study the influence of iron supplementation, type of cotton variety and pre-treatment of the feed (roasting or extrusion) on the plasma gossypol concentration, reached exposure levels of free gossypol up to 30.7 mg/kg b.w. per day (Santos *et al.*, 2005). The plasma concentration of gossypol depended on cottonseed material, whereas iron supplementation and pre-treatments of the cottonseed reduced plasma gossypol concentration. There was no effect on performance and no adverse clinical effects observed.

In conclusion, a NOAEL of 200 mg free gossypol/kg diet, corresponding to 4-5 mg/kg b.w. per day was identified for clinical effects in pre-ruminant calves. In dairy cows, several studies report no adverse clinical effects of free gossypol up to 40 mg/kg b.w. per day. However, subclinical effects have been found below this level: increased osmotic fragility of erythrocytes was found at 13 mg/kg b.w. and inhibited embryo development at 18 mg/kg b.w. per day. In bulls, adverse effects on spermatogenic tissue have been reported when fed free gossypol at 6 mg/kg b.w. and above.

Sheep

Six-week-old male lambs were for 62 days fed diets with 0, 15 or 30 % cottonseed meal containing 360 mg/kg of free gossypol, resulting in exposures corresponding to approximately 2.5 and 5 mg/kg b.w. per day. No adverse effects on clinical health and blood parameters were found (Kandylis *et al.*, 1992; Nikokyris *et al.*, 1991). Male lambs (about 48 days old) were fed for 28 days isocaloric and protein balanced diets with 0, 5, 10 or 20 % whole cottonseed (2.88 g/kg of free gossypol), and for another 26 days diets containing 0, 10, 20 or 30 % of the same whole cottonseeds, respectively (corresponding to exposures of 0, 6.0, 12.5 and 20 mg free gossypol/kg b.w. per day) (Kandylis *et al.*, 1998; Nikokyris *et al.*, 1999). The daily weight gain and the amount of intestinal fat were significantly increased in lambs fed the two highest dosage levels. Serum lactate dehydrogenase and plasma urea concentrations were significantly increased at all gossypol exposure doses. It should be noted that in these studies it is difficult to determine whether the lambs were pre-ruminants or ruminants at the beginning of the studies.

Eight-week-old lambs were dosed orally with gelatine capsules containing 0, 45, 136 or 409 mg free gossypol/animal for 30 days in order to study clinical and pathological effects of the feeding (Morgan *et al.*, 1988). The feeding resulted in exposures corresponding to approximately 2-3, 5-9 and 16-26 mg free gossypol/kg b.w. per day. All lambs at the highest dosage level died between day 19-30 of the experiment with sudden death or chronic dyspnoea. Lambs in the other groups survived and most of them appeared healthy and had satisfactory weight gain during the experiment. However, at necropsy many of the treated lambs showed macroscopic lesions. They had excessive pericardial and thoracic fluid, heart degenerations, generalized icterus and oedematous lungs. Histopathological examination revealed various degrees of cardiomyopathy, characterized by vacuolation and degeneration of myocardial fibres, separation of fibres by oedema, influx of lymphoreticular cells, and necrosis of myocardial fibres in all gossypol treated lambs. Pulmonary and hepatic lesions reflected progressive cardiomyopathy.

Nagalakshmi *et al.* (2000) fed male lambs (3-4 months of age) diets with 0 or 40 % processed cottonseed meal for 180 days. The cottonseed meal was raw, cooked, treated with $Ca(OH)_2$ or treated with Fe. The intakes of free gossypol were 303, 215, 250 or 222 mg per animal per day, corresponding to 13-35, 9-25, 11-30 and 12-26 mg/kg b.w. per day, respectively. No clinical and gross pathological lesions were found. Histopathological lesions were limited to the testis and epididymis and were most pronounced in lambs fed raw cottonseed meal.

To conclude on sheep, pathological effects in lambs (primarily histopathological effects in the heart) were found when fed free gossypol at 2-3 mg/kg b.w. and above.

Goats

Male goat kids (initial body weight 21 kg) were fed whole cottonseed at 0, 8, 16 or 24 % of the diet for 90 days to study feed intake, digestibility and performance (Luginbuhl *et al.*, 2000). The rations were nitrogen and protein balanced. The doses of free gossypol were 14.8, 29.9 and 40.6 mg/kg b.w. per day, respectively. Both the average daily weight gain and weight gain feed ratio decreased with increasing cottonseed intake. Plasma gossypol concentrations were <4 µg/mL. Because of the low plasma concentrations of gossypol, the authors indicated that the observed decrease in body weight gain was of nutritional origin.

Nubian buck kids (n=12) were fed diets containing 0, 15 and 30 % whole cottonseed for 24 weeks (Solaiman, 2007). The intake of gossypol was 0, 21.2 and 47.7 mg/kg b.w. in the three

groups, respectively. Gossypol in plasma was determined at the end of the feeding period. Total gossypol was 2.66 and 3.37 mg/L, (+)-gossypol 1.35 and 1.76 mg/L and (-)-gossypol 1.31 and 1.61 mg/L in the groups fed 15 and 30 % whole cottonseed in the feed. Inclusion of whole cottonseed in the diet had no effect on feed intake and body weight or body weight gain. Red cell fragility was increased at the higher dose, scrotal circumference, sperm motility and semen concentrations were decreased, and plasma protein was increased by the treatment. Other clinical chemistry tests were not different between the groups.

6.2. Pigs

In pigs, the oral LD_{50} is about 550 mg/kg b.w. for free gossypol (Lyman *et al.*, 1963). In pigs receiving lower doses of gossypol from gossypol containing feed, symptoms usually develop after one or more months.

Hale and Lyman (1957) reported that growing pigs receiving diets with up to 100 mg/kg of free gossypol and 15 % protein for 70 days showed no toxic signs and gained weight as the controls. However, increasing the free gossypol content to 150 mg/kg of the diet resulted in toxicity in some animals. Death occurred above this level. In a second experiment on pigs fed a diet with 30 % protein for 84 days, no toxic symptoms were found on diets containing up to 300 mg/kg of free gossypol. Thus, the NOAEL for clinical effects was 100 mg/kg diet.

Clawson and Smith (1966) fed growing pigs feeds with cottonseed meal having a free gossypol content of 80, 244 or 400 mg/kg diet. The animals had an average weight of 25 kg at the start of the feeding period and were fed gossypol containing diets until they reached about 100 kg b.w. or died due to toxic effects. No effects were observed at the lowest dose (80 mg/kg diet). The higher concentrations of gossypol reduced daily feed intake and weight gain, and several of the pigs died during the experimental period (from day 37). The most prominent clinical symptoms were laboured breathing and purple coloration of the nose and ears. The level of iron necessary in the feed to prevent these effects was also determined. Iron at a 1:1 molar ratio with gossypol in the diet prevented the adverse effects and reduced the accumulation of gossypol in the liver. Other authors have found similar results regarding iron due to inhibited absorption of the gossypol-iron complex (reviewed by Smith and Clawson, 1970; Eisele, 1986).

Growing pigs were fed cottonseed meal as 0, 15, 22.5 or 30 % of the diet starting from a weight of 20-24 kg until they reached a weight of about 75 kg (Fombad and Bryant, 2004). The free gossypol concentrations in the diets were 0, 146, 206 and 348 mg/kg, respectively, resulting in exposures of about 5, 7 or 12 mg/kg b.w. per day, respectively. The weight gain and feed intake were reduced at the two highest gossypol levels. The liver and heart weights increased significantly at all gossypol levels, and the increase was dose-dependent. There was no mortality during the feeding period. The lysine concentration in the diets correlated inversely with the cottonseed cake ratio, and was below recommended level in the experimental diets. Lysine deficiency might have influenced the outcome. The LOAEL was 146 mg/kg diet (5 mg/kg b.w. per day).

In a Chinese study, pigs were fed, from weaning, 8-20 % cottonseed meal containing 690 mg/kg of free gossypol, corresponding to 55-138 mg free gossypol/kg diet to study the effects on reproduction (Ling-yun *et al.*, 1984, in Chinese, information taken from English abstract). Mortality was increased and reproductive performance decreased, particularly in boars, but

also sows showed delayed oestrus. Testosterone concentrations in blood were decreased in boars fed diets containing 110 mg free gossypol/kg and above. Feeding cottonseed meal (concentration of gossypol in the feed not given) to reproducing pigs had no influence on the birth weight of piglets and their weight at 30 days of age.

In conclusion, a level of 100 mg free gossypol/kg diet seems to be a NOAEL for adverse performance effects in growing pigs.

6.3. Poultry

In poultry most of the negative effects caused by feeding *broilers* a diet containing gossypol have been linked to the ingestion of (–)-gossypol. In breeding hens (layers), however, the consumption of (+)-gossypol has been shown to be responsible for decreased feed intake, egg production and egg weight, as well as egg yolk discoloration. Thus, in poultry both isomers of gossypol are toxic (Lordelo *et al.*, 2005). The tolerance concentration of free gossypol in chick ration varies between 1 g/kg diet (Lipstein and Bornstein, 1964, cited in Hermes *et al.*, 1983) to as little as 0.16 g/kg diet (Heywang and Bird, 1955, cited in Hermes *et al.*, 1983) owing to differences in age and strain, protein quality and quantity and content of minerals and in particular iron (Hermes *et al*., 1983).

When purified gossypol was added to poultry feed at 0, 100, 200 and 400 mg/kg feed and fed to day-old broiler chicks for 20 days, feed intake and body weight gain were not influenced (Henry *et al*., 2001). However, at the highest dose of gossypol, the feed conversion ratio was poor in comparison with the other treatments. Mild perivascular lymphoid aggregate formation and bilary hyperplasia in the liver was noted as well. In another study of the same investigators, having approximately the same design (22 instead of 20 days feeding) gossypol at 0, 800 and 1600 mg/kg diet, the highest dose of gossypol resulted in 28 % mortality. In this case, both dietary levels of gossypol resulted in significantly reduced feed intake and decreased body weight gain. Also plasma iron and haematocrit values were significantly reduced by both gossypol doses. The gallbladder was enlarged at the highest dose, whereas severe cases of perivascular lymphoid aggregate formation, biliary hyperplasia, and hepatic cholestasis were observed in broiler chickens fed both gossypol containing diets. No gossypol-related changes were observed in kidney tissues of the birds. Thus, liver toxicity was found to be the critical effect in this study. The no adverse observable effect level was 200 mg/kg diet, corresponding to 20-30 mg/kg b.w. per day.

In a feeding study in New Hampshire chicks recording growth rate, mortality and feed efficiency at a concentration of 0.6 g/ kg of free gossypol or less did not adversely affect these parameters (Couch *et al.* 1955). In 20 day-old New Hampshire chickens fed raw cottonseed in their diets had a borderline effect on growth at an amount equivalent to 0.2 g/ kg diet (Heywang, *et al.* 1966).

In 7 day old Bosbek broiler chickens fed 0, 2.5, 5, 7.5, 10 % raw cottonseed meal in their diets for nine weeks, concentrations of 5 % and above caused a reduced weight gain and feed intake (Atuahene *et al.*,1986). Relative liver and viscera weights as well as haemoglobin and leukocyte concentrations were increased in all groups receiving cottonseed meal.

Dokki 4 and Plymouth Rock chickens fed cottonseed meal up to age 8 weeks at 0, 5, 10, 20 and 25 % in the ration were able to tolerate 0.34 g/kg of gossypol in the diet with 10 % cottonseed meal (Hermes *et al.*, 1983).

Lordelo *et al.* (2007) performed two small experiments in laying hens and broiler breeder hens to determine the relative toxicity of the individual gossypol enantiomers. In the first experiment, 25 individually caged Hy-Line W-36 forty-three-week-old laying hens were fed a standard corn-soy diet supplemented with 0, 200 and 400 mg/kg of the individual gossypol enantiomers for 20 days. In the second experiment, fifteen 44-wk-old broiler breeder hens were fed standard corn-soy-wheat diet supplemented with the individual gossypol enantiomers at 0 or 400 mg/kg diet for 18 days. Feed intake, egg production, egg weight, yolk discoloration and tissue levels of gossypol were determined. Both 200 and 400 mg/kg diet of (+)-gossypol reduced egg production and egg weight. Only laying- and broiler breeder hens fed (+)-gossypol produced eggs with a statistically significant severe yolk discoloration. There was no significant effect on eggs with (-)-gossypol. Total feed intake was significantly reduced in laying hens fed 400 mg/kg diet of (+)-gossypol. Broiler breeder hens fed the diet supplemented with (-)-gossypol consumed less feed than controls. In both experiments, tissue accumulation of (+)-gossypol was higher than the accumulation of (-)-gossypol, with the exception of the bile. No difference in the level of (+)-gossypol and (–)-gossypol was found in the excreta (Lordelo *et al.*, 2007).

In a feeding study, one-day-old broilers were fed 0, 7, 14, 21 and 28 % cottonseed meal in their diets, corresponding to 0, 0.13, 0.26, 0.39 and 0.53 g/kg diet of free gossypol, for 21 days (Gamboa *et al.*, 2001). The diets had equal amounts of lysine and methionine. The expander-solvent-extracted cottonseed meal contained 12.3 g/kg of total gossypol and 1.9 g/ kg of free gossypol. Body weight and cumulative feed to body weight ratios of the broilers were monitored and by 35 days of age the feed-to-body weight ratio of the broilers receiving 28 % seed meal were greater and by 42 days, the body weights were lover than those of the control.

A 16 week feeding trial in Japanese quail (Erturk *et al.*, 2004) soybean meal was substituted by extracted cottonseed meal (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5 %) and effects on laying performance and haemoglobin concentrations were examined. Egg production, egg weight, daily feed intake, feed efficiency ratio did not differ among treatments, whereas haemoglobin concentrations were lower at the two highest inclusion rates.

6.4. Rabbits

The oral LD50 of the (±) gossypol acetic acid complex in rabbits is 350-600 mg/kg b.w. (Abou-Donia, 1976).

The effects of gossypol on semen quality, circulating testosterone and fertility were studied in Dutch-belted male rabbits (Sakesena *et al.*, 1981). Bucks fed daily with 80, 40, 20 mg/kg day gossypol died within 8-17, 23-35 or 35-84 days, respectively. The animals lost appetite and body weight, developed hind limb paralysis, breathing difficulties and collapsed while sitting in their cages. At autopsy, the liver and lungs were congested. Rabbits fed 10 mg/kg/day gossypol exhibited a survival time ranging from 77 to 250 days. Despite the severe toxic effects, weekly semen samples from all treated animals did not show any apparent change in sperm, number, motility and morphology. Likewise, gossypol-treated males mated to estrous

does exhibited fertility comparable to vehicle-treated controls. Gossypol fed at a dose of 10 mg/kg/day for up to 35 weeks failed to induce sterility. By 12-20 weeks surviving male rabbits, fed 20 or 10 mg/kg/day gossypol, had substantially dose dependent reduction testosterone concentration (Sakesena *et al.*, 1981).

In male New Zealand white rabbits treated by gavage every other day with 4 and 20 mg /kg b.w. of gossypol for eight weeks, biochemical parameters such as electrolytes, protein, albumin, cholesterol and enzymes alanin aminotransferase (ALAT), lactate dehydrogenase (LDH) in seminal plasma were influenced by both doses of gossypol. Some of the changes were reversible (Shaaban *et al.*, 2008).

6.5. Dogs and cats

Severe toxicity was observed in dogs fed 19 doses of 50, 100 and 200 mg/ kg b.w. of purified gossypol (Eagle, 1950).

Six dogs died after accidental ingestion of cottonseed bedding (Uzal *et al*., 2005). No clinical signs were observed before death. At post mortem, congested lungs and liver, and ascites were found, and upon histopathological examination multifocal myocardial degeneration and necrosis as well as severe pulmonary oedema and congestion of lungs, heart, liver and kidneys were found. The cottonseed bedding contained 1600 mg/kg of free gossypol. Gossypol was found in the stomach content, but the doses received were unknown.

In a case report, two hound-type dogs were admitted with terminal stages of heart failure. The dogs had received feed containing cottonseed meal daily in a dose of 5.4 to 5.7 mg/kg b.w. of free gossypol for an unknown duration (Patton *et al.*, 1985). One of the dogs had disseminated intravascular coagulopathy.

The renal tubular toxicity of racemic gossypol was examined in 6 mongrel dogs (van Ypersele de Strihou *et al.*, 1988). The dogs were fed a synthetic electrolyte free diet supplemented with 2.5 mmol/ kg b.w. of sodium chloride. A potassium chelating agent was given in a single dose in the control period of 8-16 days after which the experiment started. Three female dogs were given 10 mg/day (about 0.5 mg/kg b.w.) for two weeks followed first by 20 mg/day for two more weeks, secondly by an interruption for ten days and then a dosage of 40 mg/day for an additional two weeks. Three male dogs received 40 mg/day for two weeks followed by 80 mg/day for two more weeks before sacrifice. The dogs tolerated the gossypol well. Pathological examination of the kidneys showed mild proximal tubular vacuolization similar to that seen in animals on a potassium free diet. Liver and heart remained normal. Renal excretion of K, Mg, Ca, phosphate and acid were unchanged and serum K remained within the ranges seen for animals on a K-free diet. Spermatogenesis was arrested in one of the three male dogs.

Cottonseed meal containing 26.6 % (266,000 mg/kg) total gossypol and 0.175 % (1,750 mg/kg) free gossypol was toxic when fed to adult dogs for an unspecified length of time; however, the equivalent oral dosage of free gossypol fed was <6 mg/kg/day (Merck Veterinary Manual, 2008, http://www.merckvetmanual.com/mvm/index.jsp?cfile=htm/bc/211200.htm. (accessed 16.11.08).

No toxicological studies were identified for cats.

6.6. Fish

A purified basal diet supplemented with 0, 300, 600, 900, 1200, and 1500 mg gossypol/kg feed was fed to juvenile catfish twice daily for 12 weeks. The feeding caused final weight gain inversely related to the concentration of dietary gossypol with no effect on fish survival. Whole body moisture increased whereas lipids, body proteins and red blood cell count decreased with increasing dietary gossypol intake. The ratio of (+)- to (–)-gossypol isomers in the liver of the treated catfish was not altered, indicating a similar rate of metabolism of the two isomers. This study concluded that dietary gossypol concentrations of 300 mg/kg diet and above are toxic (Yildirim *et al.*, 2003).

In another study, juvenile catfish were fed for 10 weeks basal diets containing 0, 27.5 and 55 % solvent-extracted cottonseed meal, containing 0, 0.34 and 0.67 g/kg diet of free gossypol, as a replacement for 0, 50 and 100 % of solvent-extracted soybean meal and containing three levels of iron, 40, 336 and 671 mg/kg of ferrous sulphate hydrate (Barros *et al.*, 2002). Replacement by 55 % decreased feed intake, weight gain and feed efficiency ratio. Various haematological parameters were not affected by cottonseed meal. Iron influenced these parameters independently of the cottonseed meal.

Reproductive efficiency was examined in female rainbow trout fed diets containing cottonseed meal for 10 months at concentrations of 0, 0.14, 0,26, 0,42 and 0.62 g/kg feed of gossypol (Blom *et al.*, 2001). Growth and mortality were not affected. Haemoglobin concentrations and haematocrit values were dose-dependently reduced. The number of eggs was unaffected whilst the egg weight was reduced. Eye stage survival of embryos was low in all treated groups and a linear increase in females that produced no viable embryos (from 23.1 to 71.4 %) was observed. Gossypol was transferred to the eggs and high concentrations remained in the juveniles at swimming stage, 0.6 to 20 µg/g of gossypol for the groups fed the lowest and highest concentration of gossypol, respectively.

In another study in rainbow trout from the same research group (Rinchard *et al.*, 2003), the impact of cottonseed-meal containing diets on growth of progenies was examined. Reproductive performance was gender-specific and sperm fertilisation ability was significantly reduced when cottonseed meal exceeded 50 % protein replacement. Progenies from females fed a diet containing 50 % cottonseed meal grew slower than the other groups, whereas progenies from males fed with 25-75 % cottonseed meal grew significantly faster than progenies from males fed with 0 % or 100 % of cottonseed meal. This was not seen with cryo-preserved sperm from cottonseed-meal treated males. Significant amounts (0.001-0.01 g/kg wet b.w.) of gossypol were transferred to the sperm and embryos.

In a long-term feeding experiment with rainbow trout (35 months), fishmeal was substituted with cottonseed meal resulting in free (±)-gossypol concentrations of 0.14, 0.26, 0.43 and 0.62 g/kg in the diet (enantiomer ratio about 1:1), and no adverse effects on growth were found. Neither were concentrations of steroid hormones, reproductive performance, sperm concentration, motility, eyed-stage embryo survival in males affected. In female trout fertility and testosterone concentrations were negatively affected (Lee *et al.,* 2006). Gossypol concentration in wet muscle of trout receiving the highest dietary cottonseed meal ration was less than 1 µg/kg. It has also been reported that 200 µM gossypol inhibit motility and fertilizing ability of sperm of the yellow perch (Ciereszko and Dabrowski, 2000).

7. Toxicokinetics

The toxicokinetics of gossypol has been investigated in several species of experimental animals (rat, mice, dog, pigs, chicken) and in humans. This literature has been reviewed elsewhere and, in summary, there are considerable interspecies differences as well as differences between strains of test species in the absorption, distribution, biotransformation, and elimination of gossypol (Abou-Donia *et al.*, 1976; Wu *et al.*, 1986; Abou-Donia *et al.*, 1989; Jia *et al.*, 2008).

7.1. Absorption and distribution

The oral biovailability of racemic gossypol is 86 % in Fisher rats, 60 % in Sprague Dawley rats, 14 % in B6C3F mice, 12 % in CD2F mice and 31 % in dogs, and the volume of distribution is 200 ml/kg in Fisher rats, 50 ml/kg in Sprague Dawley rats, 1740 ml/kg in B6C3F mice, and 1190 ml/kg in dogs (Wu *et al.*, 1986; Abou-Donia *et al.*, 1989; Othman and Abou-Donia, 1988).

In a balance study, dogs were fed cottonseed meal or free gossypol in capsules (Bressani *et al.* 1964). For the dogs fed cottonseed meal, total gossypol was excreted almost quantitatively in faeces, whereas free gossypol in faeces was about 3.5 times the amount ingested indicating liberation of bound gossypol from cottonseed in the intestinal tract.

Chen *et al.* (1987) found a similar tissue distribution of (+)-gossypol and (-)-gossypol in rats, with highest concentrations in the liver, and smaller amounts in the spleen, lungs, blood, heart, and kidneys. Most of the compound was excreted in faeces. The half-lives of (+)- and (–)-gossypol were 18.4 and 13.5 hrs, respectively, after oral administration, and 7.8 and 4.0 hrs, respectively, after intravenous (i.v.) administration.

The preferential accumulation of gossypol in the liver has also been studied in several livestock animals, such as in sheep (Morgan 1990), swine (Sharma *et al.*, 1966), broiler chickens (Gamboa *et al.*, 2001; Gamboa *et al.,* 2001a) and rainbow trout (Roehm *et al.,* 1967). In broiler chicken, the tissue distribution of gossypol was investigated in broilers fed for 21 days diets with cottonseed meal (total diets containing 0.03-0.18 % of free gossypol (92-504 mg/kg diet) and 0.97-1.46 % total gossypol (2,626-4,085 mg/kg diet), and with similar concentrations of digestible methionine and lysine as in control feed without gossypol. The concentration of gossypol increased linearly in plasma, liver, kidney, and muscle with dietary levels of free gossypol. Liver had the highest concentration of total gossypol (71.4-313.6 mg/kg dry matter) followed by kidney (9.2 to 36.3 mg/kg dry matter), plasma (3.0-14.6 µg/ml), and muscle (2.1-9.8 mg/kg dry matter). The proportion of (-)-gossypol was higher than that of (+)-gossypol by 16-27 % in all tissues (Gamboa *et al.*, 2001; Gamboa *et al.,* 2001a). In catfish, gossypol concentration in the liver was shown to be linearly correlated to the dietary level (300-1500 mg/kg) (Yildirim *et al.,* 2003).Gossypol has also been shown to be transferred from breast milk in rats to the neonatal rat (Lin *et al.*, 1992).

7.2. Biotransformation and elimination

After absorption in the gastro-intestinal tract, gossypol is metabolised in the liver mostly by glucuronidation, sulphation and oxidation. No quantitative excretion data in different species are currently available (Abou-Donia, 1976; Jia *et al.*, 2008).

Jia *et al*. (2008) studied the metabolism and toxicokinetic profiles of gossypol by liquid-chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). (±)-Gossypol and (–)-gossypol showed comparable toxicokinetic profiles and bioavailability (13-18 %) following oral administration to mice. In dogs, rats, and swine, administered gossypol is mainly excreted in faeces as determined by radioactivity measurements following administration of [^{14}C]gossypol (racemate); a minor portion of the radioactivity (10-20 % in rats) was exhaled (Abou-Donia, 1976). The biological half-lives in hens, pigs and rates were 30, 78 and 48 hrs, respectively, being shortened to 23 h when gossypol was coadministered with iron (Abou-Donia, 1976). A proportion of gossypol excreted in urine is small. In urine of pigs fed [^{14}C]gossypol, gossypol was found as unconjugated metabolites, glucuronides, and sulfates. However, no quantitative data on these metabolites were available. After 4 hours incubation, 10-15 % of gossypol remained in mouse and rat plasma, and only 1-4.7 % in human and dog plasma. The *in vitro* metabolism in liver microsomes suggested that gossypol was rapidly degraded: after incubation for 1h at 37°C the remaining gossypol accounted for 19.2 % and 23.9 % in mouse and human liver microsomes, respectively. The relatively long half-life of gossypol observed after oral (*p.o.*) administration to humans and animals (see Table 5) may be explained by high plasma and tissue protein binding that prevents gossypol to be cleared from the blood stream as an unbound free molecule (Abou-Donia, 1976; Jia *et al*., 2008).

Considerable interspecies differences exist in the elimination of gossypol as it is illustrated in Table 5. After oral administration of 20 mg of the gossypol racemate, (+)- or (–)-gossypol in humans, elimination of the racemate and (+)-gossypol was very slow, 286 and 133 hours, respectively, whereas the biologically active (–)-gossypol enantiomer was eliminated very rapidly, having a half-life of 4.6 hours (Wu *et al.*, 1989). In spite of a much shorter half life of the (–)-enantiomer (29 fold less), the differences in the area-under the plasma concentration curve differed much less between (+)- and (–)-gossypol (only 5-fold), indicating a lower plasma concentration of (+)-gossypol (Jia *et al.*, 2008). The terminal half-life of racemic gossypol in F-344 rats administered 10 mg/kg b.w. orally as a single dose, or as daily doses for 14 days was 4.3 and 139 hours, respectively (Abou-Donia *et al.*, 1989). In Sprague Dawley rats, the elimination half-life, after multiple doses of gossypol racemate, has been reported to 102 hours (Othman and Abou-Donia, 1988). Administration of a single oral dose of ^{14}C-labeled gossypol racemate to monkeys (2 mg/kg), dogs (2 mg/kg), rats (15 mg/kg), and mice (40 mg/kg), resulted in plasma half-lives of (±)-gossypol that were 11, 45, 16.5, and 31 hours, respectively (Tang *et al.*, 1980). After *i.v.* injection of free (+)- and (–)-gossypol to rats, the respective half-lives of the free (+)- and (–)-gossypol were 7.80 hours and 3.96 hours (Chen *et al.,* 1987). Another study on mice reported half lives after a single and after multiple oral doses of 10 mg/kg b.w. to be fairly similar, 36 and 20.4 hours, respectively (Abou-Donia *et al.*, 1989). The faeces was confirmed as the primary route for elimination of gossypol in rats by Chen *et al.* (1987). In dogs, oral bioavailability of the racemate was around 30 %, and the half life and volume of distribution of the (+)-gossypol enantiomer were 5-6- fold longer than for the (–)-gossypol enantiomer. The clearance of both enantiomers was similar to the racemate (Wu *et al.*, 1986).

Table 5. **Interspecies differences in the pharmacokinetics of gossypol**

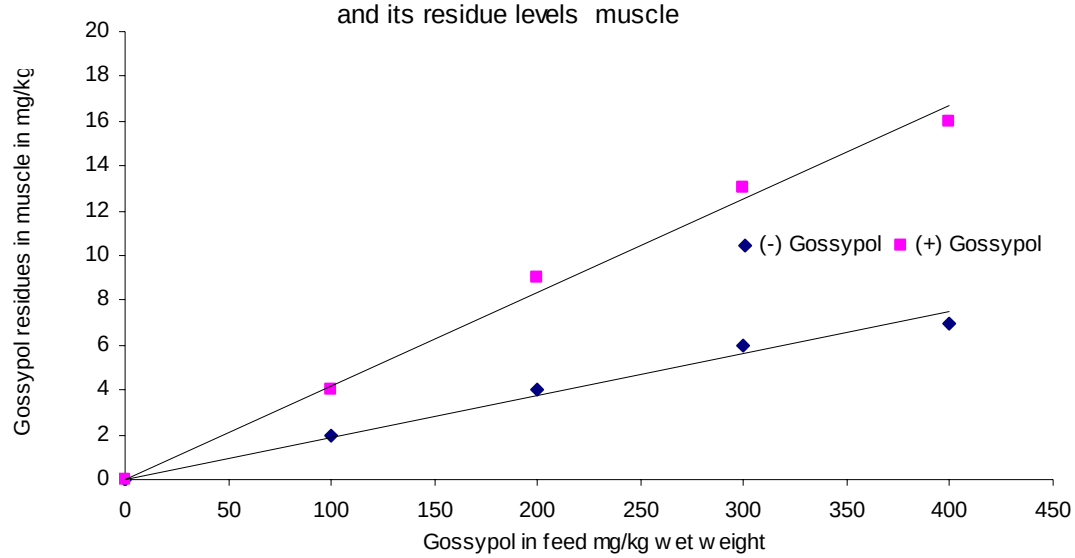
Species	(±)-gossypol			(–)-gossypol			(+)-gossypol		
	F (%)	$T_{1/2}$ (h)	CL iv	F (%)	$T_{1/2}$ (h)	CL	F (%)	$T_{1/2}$ (h)	CL
Human		**286**			**4.55**			**133**	
Rat Sprague Dawley	**60**	**64.8**	**160**						
Rat Fisher	**86**	**4.3**	**1840**						
Mouse B6C3F	**14**	**36**	**1230**						
Mouse CD2F	**12**								
Dog	**31**	**59**	**32**		**16.6**	**50**		**79.5**	**50**
Cows		**40-67**							
Pig		**78**							
Chicken		**30**							

Vd is expressed in ml/kg (intravenous route); CL iv is expressed in ml/h/kg (intravenous route)

8. Carry-over and residues

Several studies have investigated the tissue levels of gossypol in different species after feeding diets containing cottonseed or products thereof. In chickens, gossypol levels in kidney, liver, muscle, and eggs have been reported (Gamboa *et al.*, 2001; Gamboa *et al.*, 2001a; Lordelo *et al.*, 2005; 2007). In broiler chicken fed for 21 and 42 days a standard corn-soy wheat diet supplemented with 0, 100, 200, 300 and 400 mg/kg free gossypol as the racemic mixture or the individual (+)- or (–)-enantiomers, residues (dry weight) were measured in muscle, liver and kidney. A linear relationship between gossypol levels in feed and the remaining gossypol residues (liver, kidney, muscle) at 21 days in the broiler chicken tissues was found (R^2>0.99) as illustrated in Figure 5 for the muscle.

Figure 5- Gossypol intake in broiler chicken and its residue levels muscle



Using the data of Lordelo and coworkers (2005, 2007), tissue concentrations of gossypol after feeding broiler chicken diet containing free gossypol at the maximum permitted levels (MPL) in complete feed (100 mg/kg), and the MPL in cottonseed meal (1200 mg/kg) at the recommended maximum inclusion rates (2.5 %) giving 30 mg/kg feed (see Table 4), can be extrapolated for three theoretical scenarios:

1. Broiler chickens are fed the MPL in complete feed (100 mg/kg), which would correspond to 50 mg/kg of (+)-gossypol and (–)-gossypol as shown in Appendix 1 (wet weight), assuming a ratio of 1:1 between the enantiomers in the feed. Total levels of (+)- and (–)-gossypol in broiler liver, kidney and muscle can be extrapolated with respective values of 32.7, 6.3, 1.1 mg/kg.

2. Broiler chickens are fed the MPL of cottonseed meal (1200 mg/kg) at the recommended maximum inclusion rates of cottonseed meal in the complete feeding stuffs (2.5 %), which would correspond to 30 mg/kg for (+)- and (–)-gossypol, assuming a ratio of 1:1 between the enantiomers in the feed. Total levels of (+)- and (–)-gossypol in broiler liver, kidney and muscle can be extrapolated with respective values of 10.8, 2.1, 0.39 mg/kg.

3. Processing, i.e. treating defatted cottonseed meals with moist heat (which is routinely done during pelleting according to good agricultural practice), will reduce the levels of free gossypol considerably (to values of 0.1-0.2 %). However, such treatment reduces protein digestibility, and protein sparing processing such as extrusion of the cottonseed results in a reduction of gossypol concentration by 71-78 %, leaving 22-29% of the initial concentration in a batch of cottonseed meal. According to the MPL in complete feed (100 mg/kg) and the recent EU statutory limits for free gossypol in cottonseed meal and maximum recommended inclusion in broiler chicken feed (content in total feed 30 mg/kg), and taking into account 29 % remaining free gossypol after processing as the worst case scenario, the residue levels presented in scenario 1 and 2 would be further reduced to approximately one third. These are shown in Table 6 (wet weight) assuming a ratio of 1:1 between the enantiomers in the feed.

Table 6. **Gossypol residues in chicken (wet weight) fed the current maximum levels in cotton and maximum inclusion rates (100 mg/kg) with and without protein sparing processing**

Organ	Total residue levels in mg/kg wet weight after feeding with 100 mg/kg of gossypol in processed feed without protein sparing processing	Total residue levels in mg/kg wet weight after feeding with 30 mg/kg of gossypol in processed feed without protein sparing processing	Total residue levels in mg/kg wet weight after feeding with 30 mg/kg of gossypol in processed feed with protein sparing processing
	Sum of (+) and (-) gossypol	Sum of (+) and (-) gossypol	Sum of (+) and (-) gossypol
Liver	**32.7**	**10.8**	**3.6**
Kidney	**6.3**	**2.1**	**0.7**
Muscle	**1.14**	**0.39**	**0.13**

In the liver of pigs fed an experimental diet containing 80 mg/kg free gossypol for 90 days, the average concentrations were 52.3 and 54.0 mg/kg for the free and bound gossypol, respectively. The concentration of gossypol in feed was above the maximum levels allowed in finishing pigs (36 mg/kg) or sows (60 mg/kg). These levels were higher in animals fed a diet

containing 244 and 400 mg/kg feed of gossypol with 75.3, 67.7 mg/kg wet weight (free form) and 88 and 84 (bound form) mg/ kg wet weight, respectively (Clawson and Smith, 1966).

In cows fed either direct solvent extracted cotton-seed meal or screw-pressed cottonseed meal diets for 14 weeks corresponding to a free/total gossypol intake of 6.6/251 and 42.7/273.3 mg/kg b.w., respectively, resulting concentrations of free/total gossypol in the liver of 59/139 and 94/2030 mg/kg, respectively, were found (Lindsey *et al.*, 1980). There is also evidence for a carry over of gossypol in meat and meat products in other ruminants such as lamb (Kim *et al.*, 1996).

Overall, these studies indicate that gossypol is transferred from feed to edible tissues in food-producing animals.

9. Human dietary exposure

There are no data on gossypol in food of animal origin on the European market. There is also a general lack of quantitative data on transfer rates of gossypol in feed to edible tissues of animals fed gossypol containing feed.

Based on experimental studies on broiler chickens fed a diet containing gossypol, in which gossypol concentrations were determined in various tissues (Lordelo *et al* 2005, 2007; see sect. 8), theoretical calculations for different scenarios of gossypol in feed were performed. The concentrations of gossypol in broiler muscle and liver were then combined with data on meat and offal consumption from the Concise European Food Consumption Data Base (EFSA, 2005; 2008).

Scenario 1

Gossypol concentrations in tissues from broiler chickens (section 8) fed a diet containing the maximum permitted level of free gossypol in complete feed for poultry (100 mg/kg).

Food consumption of the average consumers and the high consumers (95^{th} percentiles) of meat and offal from sixteen member states were available. Overall, average consumers could occasionally be exposed to 0.0109-0.109 mg/kg b.w. per day of gossypol, whereas exposure of high consumers would range from 0.033-0.2 mg/kg b.w. per day with the exception of one data set (Slovakia) with the exposure of 0.33 mg/kg b.w. per day.

Scenario 2

Gossypol concentrations in tissues from broiler chickens (section 8) fed a diet containing the maximum permitted level of free gossypol in cottonseed meal and the maximum recommended inclusion rate (2.5 %) in feed for poultry (30 mg/kg).

Overall, average consumers could occasionally be exposed to 0.0036-0.036 mg/kg b.w. per day of gossypol, whereas for high consumers occasional exposure would range from 0.01-

0.06 mg/kg b.w. per day with the exception of one data set (Slovakia)[17] with 0.11 mg/kg b.w. per day.

Scenario 3

In this scenario, protein sparing processing of cottonseed meal leading to a minimal reduction in free gossypol of 70 % is applied. Using the maximum recommended inclusion rate corresponding to 30 mg/kg feed and outlined in scenario 2, this would lead to intakes of 0.001 – 0.01 mg/kg b.w. per day of gossypol for average consumers, whereas high consumers' occasional exposure would range from 0.003-0.018 mg/kg b.w. per day with the exception of one data set (Slovakia) with 0.033 mg/kg b.w. per day.

In addition, it should be noted that the bioavailability of gossypol in food products of animal origin is not known. Furthermore, food preparation by cooking would most likely cause binding of gossypol to proteins and make the gossypol less bio-available.

Using the data on occasional hypothetical gossypol exposure obtained in scenarios 2 and 3, and taking into account the binding of gossypol during food preparation, it is clear that the exposure would be below the lowest dose of 0.12 mg/kg b.w. per day of free gossypol in humans that resulted in clinical effects upon prolonged exposure.

On the other hand it is clear that the current regulation on gossypol in animal feed does not reflect current practice by feed manufacturers in Europe. Furthermore, the present regulation on gossypol in feed is not adequate with regard to public health.

In some developing countries, live stock is to a large extent fed with cottonseed products with high levels of gossypol and its transfer to edible tissues might represent a hazard for human health.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Chemistry, occurrence in plants and parts used as feed materials

- Gossypol is produced by cotton plants, conferring resistance of the plants to insect damage. Two enantiomers [(+) and (–)] of gossypol exist. (+)-Gossypol has the (*S*) configuration, and (–)-gossypol the (*R*)-configuration. Cottonseeds, a by-product of cotton fibre production, are rich in oil and proteins and are therefore used for oil production and

[17] The consumption of meat in Slovakia is reported to be more than 200 g/day in average and more than 600g per day at the 95th percentile. Such levels are due to specific survey methodology and are unlikely to reflect regular behaviours.

as a feed supplement. Although cotton plants also contain other terpenoids related to gossypol, their contents are low and the biological effect of cottonseed products is practically confined to gossypol.

- Seeds of commercial cotton plants in the form of unprocessed cottonseeds and processed cottonseed meal or cakes are the only important source of potential contamination of feed with gossypol.
- Cottonseed and cottonseed products contain gossypol in two forms: free gossypol, which is readily extractable with solvents, and bound gossypol. The latter form, representing mainly covalent adducts of gossypol to proteins, can be (partially) liberated by heating with acids.
- According to the current official EU method of analysis of gossypol, the compound is quantified in feed by a spectrophotometric method as an adduct with aniline. This method is old and not selective for gossypol.
- Levels of total and free gossypol in cottonseed meal or cake are lower than in the parent seeds, and depend on the processing method used, which includes removal of the fibres and oil extraction. Steam and heat, and oil extrusion reduce free gossypol concentrations, and commercial production of cottonseed meals with low levels of free gossypol is now achieved routinely with only 0.1-0.2 % remaining as free gossypol.

General toxicological effects

- Gossypol shows moderate acute toxicity by the oral route in most species with LD_{50}s of 2400-3340 mg/kg for rats, 500-950 mg/kg for mice, 350-600 mg/kg for rabbit, 550 mg/kg for pigs and 280-300 mg/kg for guinea pigs. Signs of acute gossypol toxicity are similar in all animals and include dyspnoea. Prolonged feeding has been shown to induce anorexia and decreased growth rate. Gossypol is less toxic in ruminants. Generally (–)-gossypol is more biologically active than (+)-gossypol, which is more slowly eliminated.
- The main target organ of gossypol toxicity following repeated exposure is the testis. Dose- and time-dependent reduction in sperm motility, inhibited spermatogenesis and depressed sperm counts have been observed. Suppressed spermatogenesis in humans is irreversible, particularly in males with varicocele. Gossypol can also disrupt the oestrus cycle, pregnancy and early embryo development. Gossypol can also affect the liver, heart and thyroid gland.
- Gossypol is not genotoxic and it did not induce tumours in non standard carcinogenicity studies (six months and one year) in rats.
- No health-based guidance value (ADI, TDI) has been established for gossypol. The lowest doses inhibiting spermatogenesis in humans and monkeys were 0.12 and 0.35 mg/kg b.w., respectively.
- The Panel confirms that the adverse effects of gossypol in animals are associated with its free fraction.

Adverse effects of gossypol in target animals

- Ruminants

Cattle

A NOAEL of 200 mg free gossypol/kg diet corresponding to 4-5 mg/kg b.w. per day was identified for clinical effects in calves. Dairy cows had no clinical adverse effects at doses up to 40 mg/kg b.w. Subclinical effects on erythrocyte fragility and inhibited embryo development occurred at doses of 13 and 18 mg/ kg b.w., whereas in bulls sperm production was adversely affected at a dose of 6 mg kg b.w. of free gossypol and above.

Sheep

A LOAEL in sheep (lambs) of 2-3 mg/kg b.w. per day was identified based on histopathological lesions in the heart.

Goats

Growth was adversely affected in dose-dependent manner in goats fed gossypol for 90 days at dose levels of 15 mg/ kg b.w. per day and above.

- Pigs

A NOAEL of 3 mg/kg b.w. per day was identified based on increased liver and heart weights.

- Poultry

Based on growth in broilers, the NOAEL was 200 mg/kg feed of free gossypol (corresponding to 20-30 mg/kg b.w. per day). At this dose, effects on egg production and egg weight were observed in egg laying hens (non-target species).

- Rabbits

Biochemical changes in seminal fluid were observed at 4 mg/kg b.w. per day.

- Dogs

Severe toxicity and lethality have been reported in dogs following accidental exposure to approximately 5 mg/ kg b.w. per day of free gossypol. No data were available for cats.

- Fish

A LOAEL of 140 mg/kg feed was identified based on reproductive effects.

Gossypol in feed materials

- Current legislation includes maximum content for free gossypol in both cottonseed meal and complete feedingstuffs. Under normal feeding practices, the concentration in complete feedingstuffs will be less than half the maximum permitted level, even assuming the highest permitted concentrations in cottonseed meal and maximum recommended inclusion rates of the meal in livestock diets.
- The concentrations of free gossypol that theoretically could be reached according to the current legislation on maximum permitted concentrations in complete feeding stuffs would lead to an intake of gossypol that could result in adverse effects in livestock. The potential exposure to free gossypol, based on the maximum permitted concentration in cottonseed meal and recommended maximum inclusion rates in complete feed, would not be expected to result in adverse effects in ruminants, poultry and fish. However, not all monogastric livestock animals, *inter alia* pigs, have been fully investigated for potential reproductive effects.
- There is a lack of data on gossypol content (free and bound) in feed materials used for livestock in the EU. Information provided by the livestock feed industry indicates that amounts of cottonseed meal imported into the EU have declined significantly in recent years, and relatively little is now used as a feeding stuff for livestock in the EU. Industry sources confirm that it is not used as a feed for laying hens or fish.

Fate in animals and carry-over

- There are considerable interspecies and intraspecies differences in the absorption, distribution, biotransformation and elimination of gossypol. Free racemic gossypol is readily absorbed, whilst bound gossypol is liberated and absorbed to an unknown extent.. Gossypol is biotransformed in the liver by oxidation/reduction, hydrolytic and glucuronidation reactions. It is mainly excreted in faeces. The racemate and (+)-gossypol have a very long half-life, whereas (-)-gossypol is more rapidly cleared.
- Gossypol is transferred to edible tissues including muscle and offal of ruminants, poultry, and fish. It is also transferred into eggs and probably into cow's milk, as it is transferred to milk in rats. However, there is very little quantitative information on transfer rates. No information was identified on the bioavailability of gossypol (including bound gossypol) in food products from animals fed gossypol containing feed.

Human exposure

- Human exposure to gossypol through the consumption of food products from animals fed cottonseed derived products is probably low and would not result in adverse effects.

RECOMMENDATIONS

- Representative data on occurrence of gossypol in animal feed, using validated analytical techniques, are needed. Because of the variability in content, cottonseed-based feed should be tested regularly.

- The current official EU method of analysis of gossypol should be replaced by a specific analytical method.

- Information on transfer rates of gossypol from feed to animal products for human consumption, including the occurrence of free and bound gossypol in such products, is needed.

- Bioavailability of bound gossypol in edible tissues should be investigated.

DOCUMENTATION PROVIDED TO EFSA

INZO France provided by data on gossypol levels on both whole and processed cottonseeds.

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APPENDIX

Table 1. **Potential exposure to free gossypol based on maximum levels in complete feedingstuffs**

1. Non-ruminants	Total complete feed, kg/day	MPL in complete feeding stuff	Gossypol intake		
	kg/d	mg/kg	mg/d	mg/kg of diet	mg/kg b.w./day
Finishing pigs	**3.70**	**60**	**222**	**60**	**2.2**
Sows	**6.50**	**60**	**390**	**60**	**1.6**
Poultry (broilers)	**0.15**	**100**	**15**	**100**	**7.1**
Poultry (laying hens)	**0.115**	**20**	**2.3**	**20**	**1.2**
Fish	**0.09**	**20**	**1.8**	**20**	**0.4**

2. Ruminants			Gossypol intake		
	Total DM intake, kg/day	MPL in complete feeding stuff (88 % DM basis)	mg/d	mg/kg of diet	mg/kg b.w./day
Dairy cow	**22**	**500**	**12500**	**500**	**19.2**
Suckler cow	**16**	**500**	**9091**	**500**	**16.5**
Growing cattle	**8**	**500**	**4545**	**500**	**15.2**
Lactating ewe	**1.8**	**500**	**1023**	**500**	**14.6**
Growing lamb	**0.6**	**500**	**341**	**500**	**17.0**
Dairy goats	**2.2**	**500**	**1250**	**500**	**19.2**

ABBREVIATIONS

ADI	acceptable daily intake
ALAT	alanin aminotransferase
AOCS	American Oil Chemists' Society
b.w.	body weight
CAS	Chemical Abstracts Service
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
EU	European Union
FSH	follicle stimulating hormone
GJIC	gap junction intercellular communication
GM	genetically modified
GST	glutathione S-transferase
HPLC	high-performance liquid chromatography
HSD	hydroxy-steroid dehydrogenase
i.v.	intravenous
LC/MS/MS	liquid chromatography/mass spectrometry/mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
LD_{50}	Lethal dose – the dose required to kill half the members of a tested animal population
LDH	lactate dehydrogenase
LH	luteinizing hormone
LOAEL	lowest-observed-adverse-effect level
LOQ	limit of quantification
MPL	maximum permitted levels
NCPA	National Cottonseed Products Association
NOAEL	no-observed-adverse-effect level
OECD	Organisation for Economic Co-Operation and Development
p.o.	oral administration
RNA	ribonucleic acid
SCAN	Scientific Committee on Animal Nutrition
TDI	tolerable daily intake

TGF	transforming growth factor
USA	United States of America
UV	ultraviolet