Bioactive compounds in plants – benefits and risks for man and animals

Proceedings from a symposium held at
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Preface I

Geomedicine is the science of environmental impact on health of humans and animals. The Committee for Information and Research in Geomedicine disseminates knowledge about geomedicine and promotes research in this field. This is accomplished through the organising of symposia and through publications.

The present book contains a collection of papers presented at the symposium “Bioactive compounds in plants - risks and benefits for man and animals” held in Oslo 13-14 November 2008. An organising committee was led by Aksel Bernhoft and also consisting of Helle Margrete Meltzer, Jan Alexander and Arne Flåøyen. Aksel Bernhoft has also edited the present book of proceedings. The Committee for Information and Research in Geomedicine would like to thank the organising committee and its chair, as well as the other members of the Geomedicine committee and the Norwegian Academy of Science and Letters for their support and assistance throughout the arrangement.

This symposium was the eighteenth in the series organized by the Committee. Proceedings from previous symposia are listed at the back of this book, and are to be found at http://www.dnva.no/geomed/eng/index.html

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Preface II

The topic of the symposium was secondary metabolites produced by plants and having biochemical effects in man and animals. The huge amounts of such compounds have a broad range of effects, from being acute deadly to being healthy or curative. The symposium presented updated research results on bioactive compounds in plants as the basis of modern pharmacology and medical treatment, and as natural beneficial compounds in vegetable feed, food and supplements. On the other hand, bioactive plant compounds were discussed as acute poisons and as contaminants in feed and food implying risks of adverse effects in animals and man. The ingested dose of bioactive plant compounds is often decisive for whether the effect is beneficial or adverse, and the comprehension of a balanced intake was underlined. The symposium also covered the authorities’ management of bioactive plant compounds as herbal
medicines and as compounds in feed and food at risk or maintained benefit. Finally, agricultural factors influencing the plants’ production of bioactive compounds were discussed. The symposium was open to the public, and most of the 95 seats in the auditorium in the house of The Norwegian Academy of Science and Letters were used. All contributors of lectures and manuscripts are highly appreciated. Thanks are also expressed to Nina Bakkelund at the National Veterinary Institute for contributing to the book layout.

On behalf of the symposium committee,
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Oslo, June 2010
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Some selected plants containing important bioactive compounds
All photos: Finn B. Michelsen
Aconitum septentrionale

Pteridium aquilinium

Taxus baccata

Quercus spp. nuts
A brief review on bioactive compounds in plants

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Abstract

Bioactive compounds in plants can be defined as secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals. Secondary metabolites are produced within the plants besides the primary biosynthetic and metabolic routes for compounds associated with plant growth and development, and are regarded as products of biochemical “side tracks” in the plant cells and not needed for the daily functioning of the plant. Several of them are found to hold various types of important functions in the living plants such as protection, attraction or signalling. Most species of plants seem to be capable of producing such compounds. The main chemical groups of bioactive compounds in plants with their main pharmacological or toxicological effects in man and animal, as well as the main producing plants/plant family are presented here.

Definition of bioactive compounds in plants

Bioactive compounds in plants are compounds produced by plants having pharmacological or toxicological effects in man and animals. Although nutrients elicit pharmacological or toxicological effects when ingested at high dosages (e.g. vitamins and minerals), nutrients in plants are generally not included in the
term bioactive plant compound. The typical bioactive compounds in plants are produced as secondary metabolites. Thus, a definition of bioactive compounds in plants is: secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals.

**Synthesis and purpose in plants**

Secondary metabolites are produced within the plants besides the primary biosynthetic and metabolic routes of compounds aimed at plant growth and development, such as carbohydrates, amino acids, proteins and lipids. They can be regarded as products of biochemical “side tracks” in the plant cells and not needed for daily functioning of the plant. A survey of the pathways for the synthesis of secondary metabolites in the plants is presented in Figure 1. Phylogenetically, the secondary bioactive compounds in plants appear to be randomly synthesised – but they are not useless junk. Several of them are found to hold important functions in the living plants. For example, flavonoids can protect against free radicals generated during photosynthesis. Terpenoids may attract pollinators or seed dispersers, or inhibit competing plants. Alkaloids usually ward off herbivore animals or insect attacks (phytoalexins). Other secondary metabolites function as cellular signalling molecules or have other functions in the plants. That plants producing bioactive compounds seem to be the rule rather than the exception. Thus, most plants even common food and feed plants are capable of producing such compounds. However, the typical poisonous or medicinal plants contain higher concentrations of more potent bioactive compounds than food and feed plants.

**Main groups of bioactive compounds in plants**

Bioactive compounds in plants are classified according to different criteria. A presentation based on clinical function – their pharmacological or toxicological effects – is relevant for the clinician, pharmacist or toxicologist. An approach based on biological effects is complicated by the fact that the clinical outcome is not exclusively connected to chemically closely related compounds; even chemically very different molecules might produce similar clinical effects. A botanical categorisation based on families and genera of the plants producing the bioactive compounds might also be relevant, as closely related plant species most often produce the same or chemically similar bioactive compounds. However, there are also ranges of examples that species even genetically less
related produce similar secondary compounds. The main focus of this book are the bioactive chemical compounds, therefore it is useful to categorise them according to biochemical pathways and chemical classes.

The following is a brief presentation of the main chemical groups of bioactive compounds in plants:

**Glycosides**
The glycosides consist of various categories of secondary metabolites bound to a mono- or oligosaccharide or to uronic acid. The saccharide or uronic acid part is called glycone, and the other part the aglycone. The main groups of glycosides are cardiac glycosides, cyanogenic glycosides, glucosinolates, saponins and anthraquinone glycosides. Furthermore, flavonoids frequently occur as glycosides. Following ingestion the glycosides usually hydrolyse in the colon, and the more hydrophobic aglycone might be absorbed.

The aglycones of **cardiac glycosides** have a steroidal structure. Their effect is inhibition of Na+/K+-ATPase-pumps in the cell membranes. These pumps are concentrated in and critical for the functioning of the cardiac cells and the effects from these compounds are very pronounced in the heart, resulting in increased contractility and reduced rate. The cardiac glycosides are present in plants of *Scrophulariaceae* (figwort family) particularly *Digitalis purpura* (foxglove) and in *Convallariaceae* (convall family) with *Convallaria majalis* (lily of the valley) as a typical example.

The **cyanogenic glycosides** have aglycones derived from amino acids. Several of these compounds can interfere with the iodine utilisation and result in hypothyroidism. The other important effect is via their release of hydrogen cyanide, which is very toxic being lethal at high dosages. Cyanogenic glycosides are present in species of *Rosaceae* (rose family) in particular in *Prunus* spp.

The **glucosinolates** contain sulphur-containing, pungent amino acid-derived aglycones. The compounds show a complex set of effects on cytochrome P450 isoforms in various cells and tend to decrease hepatic bioactivation of environmental procarcinogens. The glucosinolates can be skin irritating and also induce hypothyroidism and goitre. The **Brassicaceae** (brassica family) is the family mainly associated with glucosinolate production.

Most **saponins** – “soap forming compound”- occur as glycosides. The aglycones consist of either pentacyclic triterpenoids or tetracyclic steroids. They are structurally distinct, but have main functional properties in common. The saponin glycosides are large molecules with a hydrophilic glycone and a
hydrophobic aglycone, which give emulsifying properties and can be used as detergents. Saponins show immune modulating and antineoplastic effects. A common in vitro effect is haemolysis of red blood cells. However, this effect does not seem to be an in vivo problem. Some saponins induce photosensitisation and jaundice. Saponins are present in a range of plant families. Among them is *Liliaceae* (lily family) with the important sheep toxic plant *Narthesium ossifragum* (bog asphodel).

**Anthraquinone glycosides** show a relatively limited distribution within the plant kingdom. In *Polygonaceae* (dock family) they are present for instance in *Rumex crispus* (curly dock) and *Rheum* spp (rhubarbs). Their primary effect is induction of water and electrolyte secretion as well as peristalsis in colon.

**Flavonoids and proanthocyanidins**

Flavonoids consist of a central three-ring structure. Proanthocyanidins are oligomers of flavonoids. Both groups of compounds can occur as glycosides. All compounds contain phenol-groups involved in an effect as general antioxidant. Other actions are diverse – several structures reduce inflammation or carcinogenicity. The group isoflavones are primarily known as phytoestrogens. Flavonoids and proanthocyanidins are all pigments occurring in a long range of plant families. Isoflavones are produced by species of *Fabaceae* (bean family).

**Tannins**

There are two distinct types of tannins. Condensed tannins which are large polymers of flavonoids and hydrolysable tannins which are polymers composed of a monosaccharide core (most often glucose) with several catechin derivatives attached. The two types of tannins have most properties in common, but hydrolysable tannins are less stable and have greater potential to cause toxicity. The water solubility is restricted and decrease in general with the size of the tannin molecule. Tannins indiscriminately bind to proteins and larger tannins are used as astringents in cases of diarrhoea, skin bleedings and transudates. Tannins are very widely distributed in the plant kingdom. Examples of plant families associated with presence of tannins are *Fagaceae* (beech family) and *Polygonaceae* (knotweed family).

**Mono- and sequi-terpenoids, and phenylpropanoids**

The terpenoids are synthesized via the five-carbon building block isoprene. Monoterpenoids consist of two isoprene units and sesquiterpenoids of three units. They are referred to as low-molecular-weight terpenoids and represent the most diversely category of plant constituents with more than 25,000 individual
compounds identified. The less diverse phenylpropanoid are based on a nine-carbon skeleton and are synthesised via another pathway. Compounds of all three groups are lipophilic and tend also to volatilise readily. They have strong odours and flavours. Their actions vary greatly, a range of which have been utilised in herbal remedies. Of particular importance are antineoplastic, antibacterial, antiviral effects as well as gastrointestinal stimulation. However they are not associated with toxicity unless they are concentrated as volatile oils. The plant family best known for these compounds is Lamiaceae (thyme family) but are also present in a range of other families.

**Diterpenoids**
Diterpenoids are composed of 4 isoprene units (20 carbons). They are very lipophilic and tend to have strong flavours, but are not volatile and thus, odourless. Much less toxicological information are available on the diterpenoids than on the lower molecular terpenoids. Several of the compounds possess antineoplastic activity. Diterpenoids are found in several plants, among them Coffea arabica (coffee). Diterpenoids are also typically present in resins (see below).

**Resins**
The resins are complex lipid-soluble mixtures – usually both non-volatile and volatile compounds. The non-volatile fraction may consist of diterpenoid and triterpenoid compounds, and mono- and sequiterpenoids predominate in the volatile fraction. Most typical are resins secreted by wood structures, but resins are also present in herbaceous plants. They are all sticky and the fluidity depends on their contents of volatile compounds. When exposed to air they harden. Most resins are antimicrobial and wound healing, but their actions depend on the composition of the chemical mixture. Resins are generally safe, but contact allergy may occur.

**Lignans**
Lignans are composed of two phenylpropanoid units to form an 18-carbon skeleton, with various functional groups connected. They are generally lipophilic and have structural functions within the plant cell membranes. Lignans are present at highest concentrations in oil seeds, but are also found in other parts of a long range of plants of different families. Several lignans show clinical activity as phytoestrogenic, cathartic or antineoplastic effects.
**Alkaloids**
The alkaloids are heterocyclic, nitrogen containing compounds, usually with potent activity and bitter taste. They are of limited distribution in the plant kingdom. The various groups have diverse clinical properties.

**Tropane alkaloids** are present in Solanaceae (nightshade family) for instance Atropa belladonna (deadly nightshade) Datura spp (thorn apples), and Hyoscyamus niger (henbane). The compounds have anticholinergic activity (muscarine receptor antagonists) and are used medically to reduce smooth muscle spasms, hypersecretion and pain.

**Pyrrolizidine alkaloids** are produced in Asteraceae (daisy family), particularly in Senecio spp. (Ragworts) and in Boraginaceae (borage family). Their adverse effect in man and animals are hepatotoxicity after bioactivation.

Papaveraceae (poppy family) and Berberidaceae (barberry family) produce **isoquinoline alkaloids** which have a range of biochemical effects relevant for medical use, as inhibition of various conditions as pain, cancer cells and bacteria, and stimulation of bone marrow leucocytes as well as myocardial contractility.

The main producers of **methylxanthine alkaloids** are Coffea arabica (coffee) and Theobroma cacao (cacao). Methylxanthines to a various extent bind to adenosine receptor and elicit neurological effects in man and animals which may be regarded stimulating at low to moderate intake. In rodents high intakes of methylxanthines show reduced sperm production and testicular atrophy.

Compounds called **pseudoalkaloids** have chemical properties close to alkaloids and are produced by species in Apiaceae (carrot family) for instance Cicuta virosa (cowbane) and Conium maculatum (hemlock), and in Taxaceae (yew family) for instance Taxus baccata (yew). The pseudoalkaloids in Cicuta virosa and Conium maculatum have effects on the central nervous system and taxine in yews like T. baccata inhibits the ion transport of the hearth.

**Furocoumarines and naphthodianthrones**
Furocoumarines in Apiaceae (carrot family) particularly in Heracleum spp (cow parsnips) have photosensitizing properties. The naphthodianthrones for instance in Hypericum spp (St. John's-worts) of Clusiaceae (garcinia family) and in Polygonaceae (dock family) e.g. Fagopyrum esculentum (buckwheat) have similar effects. The compounds in Hypericum spp. have an antidepressant effect.
Proteins and peptides
Proteins from plants are an important source in food and feed. Amino acids thereof are absorbed from the intestine of man and animals and are built up into adapted proteins. Nevertheless, there are also plant proteins and peptides with bioactivity. They are often not hydrolysed in the digestive tract, but may to a certain extent be absorbed and exert their specific action in the body. Euphorbiaceae (spurge family) include plants producing such proteins, for instance ricin in seeds of Ricinus communis (castor bean). The very potent little protein (lectin) ricin inhibits protein synthesis and induce systemic effects in animals and humans, with gastrointestinal symptoms dominating. Far less potent lectins are also present in seeds of several species of Fabaceae (bean family). Colic and other gastrointestinal symptoms may occur if seeds are eaten without sufficient heat treatment, which inactivates many lectins.

Concluding remarks
Plants, including most food and feed plants, produce a broad range of bioactive chemical compounds via their so called secondary metabolism. These compounds may elicit a long range of different effects in man and animals eating the plants dependent on plant species and amount eaten. Plants with potent bioactive compounds are often characterised as both poisonous and medicinal, and a beneficial or an adverse result may depend on the amount eaten and the context of intake. For typical food and feed plants with bioactive compounds with less pronounced effects, the intakes are usually regarded as beneficial.

Literature

Highlights through the history of plant medicine

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Abstract

As long as human beings have been living on Earth they have used remedies from nature to improve their health or to cure illnesses. Documentation of this can be found as far back in time as approximately 6000 years. It is interesting to note that certain plants that are described in plates being that old are still in use both in what today is called traditional medicine, but also the active ingredients from these plants are used as single compounds in modern medicine. Documents of major importance for the development of plant based medicine are mentioned in this review, as well as some of the most important persons in the history of pharmacy and medicine. Certain plants have been chosen as examples of plants with a long history of usage, and modern use of products from these plants is also described.

Documentation on the use of medicinal plants in prehistoric times

It is said that the development of Western civilisation emerged in the areas around Eufrat and Tigris. From this region one has found old clay plates from around 4000 B.C. containing drawings and scripts showing that the culture already in those days knew about the use of medical plants also known today as opium, thyme and liquorice. From Babylon there are also old engravings in rocks of a lot of plants that most probably were used as medicinal plants. Documents showing the use of senna, coriander, saffron, cinnamon and garlic,
as well as certain preparations used as liniments externally and elixirs for internal use were also available.

The Egyptian culture has given us a great number of documentations on their large knowledge on the use of plants for medicinal purposes. These go back to at least 3000 B.C. and the best known is probably Papyrus Ebers, given the name after the German Egyptologist Georg Ebers who came across a great number of old documents found by an Arab living in Luxor. The documents contained at least 800 recipes and around 700 medicinal plants of both local and foreign origin which were also known among the Babylonians. Amongst those mentioned are aloe, absinth, peppermint, colocynth, Indian hemp (cannabis) as well as garlic, opium poppy, juniper, cumin, ricinus seeds and Arabic gum.

It is not only in our part of the world that old documents have been found containing information on the use of medicinal plants. The Chinese emperor, Shen Nung, living around 3000 B.C., left a document given the name Pen tsáo that contained descriptions of plants such as opium poppy, liquorice, ergot, rhubarb, gentian and valerian, showing that some of the same plants were used in cultures far apart during the same period, and several of these are still in use today. Also in the Indian system of Ayurveda the use of medicinal plants is frequently mentioned, and most of those being used from the very beginning are still used in today’s traditional medicine as practiced in India (1, 2).

The Greek Roman period

In Europe, the so-called antique period has left a large number of scripts dealing with medicinal plants used for treatment of illnesses. They describe both the plants to be used and recipes and production method for the remedies used (1, 2).

Hippocrates from Kos, Greece, living in the period 460-377 B.C., is often called The Father of the medicinal art. After him we have 60 documents describing some of the plants he used.

Theophrastus (370-287 B.C.) from Eressos in Lesbos was an important successor of Hippocrates and took over the position he had as a philosopher and medical person. He wrote the important documents “De causis plantarum”, 8 volumes of which 6 is available today, and “Historia plantarum”, both describing plants, their growth and uses, including their use as medicinal plants.
Coming into the period of the Roman Empire, Plinius the elder, (23 A.D. – August 25, 79) wrote “Historia naturalis” describing the use of 250 medicinal plants. This document was used as a reference source during several centuries by various types of scholars, especially those dealing with medicine, plants and plant products. The reason for knowing his exact death date is that he died on the same day as Vesuvius had an outburst.

Aulus Cornelius Celsus (ca 25 B.C.—ca 50 A.D.), wrote a reference work, De medicina, describing the use of 250 medicinal plants. This book was for a long time used as a source for knowledge on diet, pharmacy, surgery and other related topics.

Pedanius Dioscorides living during the period 40-80 A.D., is one of the best known medical persons from the Roman period. He wrote “De Materia Medica” describing 600 medicinal plants. This document is reckoned to be the precursor of the later documents on medicinal plants and medicines called Pharmacopeias. He was a celebrated Greek physician, botanist, pharmacologist and surgeon and travelled with the armies of the Roman Emperor Nero. Dioscorides used extracts from mandragora to induce anesthesia when people underwent surgery. This did not leave the persons unconscious, but gave them a feeling of not being present. Plants of the same family will be described later, being the source that took witches to Blocksberg.

Claudius Aelius Galenus (129 – 200 A.D.), also know as Galen of Pergamum, was a well recognised Roman physician and philosopher and is looked upon as one of the best medical scientists of the Roman period. His theories dominated Western medical science for well over a millennium. He was amongst others one of the first to make preparations of the medicines of those days in dosage forms, he described how to make coatings on pills and he has given the name to one of the most important pharmaceutical sciences of today, Galenical pharmacy, dealing with the production and the processes behind the production of medicinal preparations. Galen left documentations on 130 medicinal plants and recipes.

The Arabic and Monastery period

Avicenna (980-1037A.D.) was a philosopher and physician and is best known for his document Canon Avicenna, also called Canon of medicine, describing systematically the knowledge of medicine and pharmacology of that period. In this period they created a system for pharmacies, that amongst other remedies
sold several being known today as camphor, mastix, rhubarb, saffron and aloe. The document also describes the production of juices, tinctures, extracts of herbs, pills with coatings and distillation of alcohol as some of the first in pharmacies. Avicenna also introduced systematic experimentation and quantification in physiology, introduced experimental as well as evidence-based medicine, he recognised the importance of a proper diet and also the influence of climate and the environment on health.

Further into the middle Ages, the Monastery period was important for the health care system in several of the countries in the Mediterranean region. The Salerno-school understood the importance of good sanitation and made rules that can be found in documents like “Regimen sanitatis salernitanum”. Most of the plants used for the benefit of health were of Mediterranean origin and was thus not easily available north of the Alps. Charles the great (742-814 A.D.), an emperor of France and the Roman empire, asked the monk Ansegis to work out methods for growing important medicinal plants north of the Alps. This was the origin of the Monastery gardens that spread all over Europe where monks and nuns settled. Ansegis wrote the text ”Capitulare de villis” that contained rules on how to live and how to grow medicinal plants in the monasteries. He was so successful with all his work that he after his death was canonised and is now known as Saint Ansegisus (1, 2).

The great reformer Paracelsus
Phillipus Aurelius Theophrastus Paracelsus Bombastus von Hohenheim (11 November or 17 December 1493 – 24 September 1541), better known as Paracelsus, was an important medicinal reformer in his time. He said that he did not want to recommend a remedy for treatment of illnesses that he had no proof for was working. He was one of the first to perform clinical trials in order to verify the effect of the products he used as a doctor before applying them on the patients. He was a pioneer in the area of clinical studies. He is still remembered for the development of surgical methods, medicinal chemistry and his theory for development of medicines and their properties. In addition to being a famous doctor, he was also an alchemist and an astrologer. Paracelsus had some interesting theories about illnesses and how to cure them, and his definition of what could be a poison is still valid. Below, translated into English, one can find some of Paracelsus´ important thesis:

“The body is a conglomerate of chemical compounds that have to be in equilibrium with each other. If they are not so, the body is ill, and other chemical components must be added to get the body in balance again.”

“The healing chemical products should be found in plants by e.g. extractions, alchemi, distillation and pyrolysis.”
“Everything is poison; it is just the concentration that will decide if something is nontoxic.”

The “scientific” period

After Paracelsus, the “scientific” period started. Modern science is often referred to the period that started around year 1600 and goes up till present, i.e. the period that has laid the ground for science as we know it today. Some names will forever be remembered because their achievements are so important that they are still great examples for modern scientists.

The Swedish botanist from Uppsala University, Carl von Linné (23 May 1707 – 10 January 1778), realised that a unique and specific identification and systematisation of living species was important. He is best known for the introduction of plant systematics that included rules for how to determine different features of the plant and then use these features to put the plants into a system that made it easier to identify them. His system is valid today, and a great number of plants that were given the names after “the system of Linné” still carry his name. Today gene technology has been developed to be used for classification. In most cases the system of Linné has been shown to be correct.

Another Swedish scientist, the pharmacist Carl Wilhelm Scheele (9 December 1742 – 21 May 1786) is best known for the discovery of oxygen and nitrogen, as well as a lot of other elements, and thus laid an important basis for the modern chemistry.

Friedrich Wöhler (31 July 1800 – 23 September 1882) was a German chemist, and he is best known for being the first to synthesize urea, and he was also the first to isolate several chemical elements.

Paul Ehrlich (14 March 1854 – 20 August 1915) was a German scientist working on hematology, immunology, and chemotherapy, and he obtained the Nobel price in medicine for his research in autoimmunity, that he called "horror autotoxicus". In the pharmaceutical world he is best known for being involved in the synthesis of salvarsan in 1910. This was the first drug against syphilis as well as the first synthetic drug.

The next person that must be mentioned is Sir Alexander Fleming (6 August 1881 – 11 March 1955), a Scottish biologist and pharmacologist. He discovered the enzyme lysozyme in 1923, but he is best known for the re-discovery of the
antibiotic substance penicillin from the fungus *Penicillium notatum* in 1928, for which he earned the Nobel Prize. This lead to a change in the treatment of illnesses caused by bacteria and has revolutionized the treatment of a several illnesses that prior to this discovery caused a great number of deaths world wide (1, 2).

**From plants to pure bioactive compound**

Medicinal products had up to the beginning of the 20th century mainly been produced by using extracts or powder of medicinal plants as the main active ingredient. The chemical and biological achievements mentioned above, were amongst those that were important for the development of more modern medicines based on pure compounds isolated from plants or micro-organisms. In order to decide what plants to use for isolation of active ingredient the scientific discipline Ethnopharmacology evolved.

Ethnopharmacology involves studies of cultures still using traditional medicines and can be defined as the observation, identification, description and experimental investigation of the ingredients and the effects of such indigenous drugs. This is a truly interdisciplinary field of research which is very important in the study of traditional medicine. Ethnopharmacology is reckoned to be the most efficient method for detection of products that can form the basis for new compounds leading to new and better medicinal compounds than those available today. This has been realized by the modern pharmaceutical industries and most of the new medicines registered over the last 10-year period have their origin from the traditional use of medicinal plants.

After a plant has been identified as a source for new products, the bioactive compounds are located in the relevant fractions using so called “Bioassay guided isolation”. In this process the plants are successively extracted with solvents of increasing polarity and tested by a range of bioassays relevant to the activity of the compounds one is trying to isolate by using the relevant enzyme systems, micro-organisms, organs, etc. When the bioactivity has been located to a specific fraction, further separation is performed by a combination of chromatographic methods based on separation by size, charge and hydrophobicity. When a pure compound has been obtained, structural elucidation is performed using a variety of spectroscopic methods like NMR, IR and MS and in certain cases x-ray crystallography. After toxicity tests, the last, but the most important part, is the clinical trial to test effect, efficacy and doses
to investigate if the new medicinal product is better than those already available on the market.

Examples of important plant compounds developed for the benefit of human health

Opium alkaloids

Opium poppy, *Papaver somniferum*, has given rise to four important medicines. The plant is a very old painkiller and a sedative medicinal plant. The use of this plant can be found in several of the old documents referred above. The four important alkaloids found in opium that are used as medicines today are morphine, codeine, noscapine and papaverine.

Morphine is used as a painkiller, and is a very good one. The problem with this compound is that it leads to addiction and has been the origin of a great deal of drug misuse over the centuries. This goes at least as far back as to the so called “Opium wars” in China during the 18th century, causing great problems for the local population in China. Morphine has also, unfortunately, a structure that makes it easily transformed into heroin, diacetylmorphine, being one of the greatest drug problems worldwide. The political situation in Afghanistan today and also in the Golden Triangle as the situation was around 1960, could have been a very different one if the plant opium poppy did not exist.

Codeine, another of the compounds found in opium, is primarily used against cough and is mainly present in cough remedies. In addition, codeine has also painkilling properties and is used as a compound in combination with other painkilling agents to give a better effect than with only one of them. Unfortunately, codeine can also give addiction.

Noscapine is also an agent used against cough. This compound has fortunately no adverse effects and is present in formulations both as tablets and mixtures as remedies to stop cough.

Papaverine was originally a remedy primarily used against the treatment of visceral spasm, vasospasm (especially those involving the heart and the brain), and occasionally in the treatment of erectile dysfunction. The latter is a relatively new usage of the papaverine.
Both morphine and codeine are lead compounds for important derivatives used as painkillers, especially for products used in relation to cancer and other illnesses causing great pain.

Abuse is a great problem for morphine, codeine and their derivatives, and for this reason the search for new non-opiate painkillers is important.

It can be mentioned that the compound epibatidine, present in the skin of a frog *Epipedobates tricolour* living in Equador, has been shown to be 200 times stronger painkiller than morphine. This was discovered because natives used the extract of the skin as an arrow poison. The most interesting aspect was that the compound has a totally different mechanism of action than morphine. This is of great importance as it will not give the same type of addiction as opiates, i.e. it is non-addictive. Due to this, science is now trying to find a derivative that can be used as a new type of painkiller. Epibatidine itself is too poisonous to be used, but certain derivatives are promising. Structurally, it is different from opiates, this being the reason for the different mode of action (3, 4).

**Acetyl salicylic acid**

Acetyl salicylic acid is a compound derived from salicin that is present in *Salix*-species. In the documents left by Dioscorides it can be found that the bark of *Salix* was described used against warts and thorns, it was especially the ash from the bark that had this use. Other peculiar uses were noted down by the Dane Henrik Smith (ca.1560) and some of these were the use against greediness and laxity. Anders Ulfkjær, 1858, says that the bark of *Salix* sp was used against mental illnesses and toothache and also against “unwanted love”, whatever that may be. He also said that a decoction of *Salix* bark was used against fewer and this use getting nearer to the use of the product we know today.

Salicin was first isolated in 1838 and soon converted into salicylic acid. This compound was used as a good remedy against rheumatism, but a lot of the people using this got severe stomach problems, and gastric ulcer developed quite frequently. As salicylic acid, a compound with effects needed against rheumatism, being a problem for many people, the search for how to convert the compound into one with less stomach problem started, and acetyl salicylic acid was invented (1898). This was made into products more known as Aspirin, Albyl and Dispril and is used as a painkiller, a fever reducing agent and is today also frequently used as an anti-coagulant (1, 3, 4).
Tubocurarine and strychnine
When Europeans came into contact with the Indians in South America, especially in the upper Amazonas, they noticed that extracts from *Chondrodendron tomentosum* were used as arrow poison when hunting. When tested in biological systems it was shown that the material had muscle relaxant effects in small doses and that it was a short time effect. The active compound was tubocurarine.

Strychnine was isolated from the plant *Strychnos nux-vomica*, a plant originating from South East Asia, but was early grown around the Mediterranean. This compound was shown to have the same type of effect as tubocurarine and structurally they had certain similarities. Both of them were used as lead compounds for the medical compound Atrakurium, registered as a medicine used as a muscle relaxant in connection with operations. *Strychnos nux-vomica* has a long tradition in Europe as an agent for killing animals, e.g. foxes, and in Norway the local name of the seeds from this plant has got a name that translated into English is equivalent to “fox cake”. A weak extract was in former days used in elixirs used as tonic. A small amount was not poisonous and due to the bitter taste it enhanced the gastric juice production leading to a better appetite (3, 4).

Atropine, hyoscynamine and scopolamine
Atropine, hyoscynamine and scopolamine are all present in the plants *Atropa belladonna* (deadly nightshade), *Hyoscyamus niger* (henbane) and *Datura stramonium* (thorn apple or jimson weed). The plants are all recorded in old medicinal books, both as hallucinogenic plants and plants that had other medical properties.

The Oracle in Delphi inhaled the smoke of burning seeds of henbane, provoking hallucinations that led her to “talk in tongues” and she then gave so called good advices for what to do in certain cases of importance for those taking part in the ceremonies. Henbane is the name given for *Hyoscyamus niger*, as hen thieves in Great Britain used to mix the seeds with the grain given to the hens so that they became dizzy and unconscious during the night and did not wake up when the thieves came to collect them. Thus the farmer was not woken up by the hens and that was then the bane of the hens. In the Norwegian tradition, the leaves of the same plant was mixed with fat and made into an ointment. This was put by women onto places on the body with thin skin and the active ingredients were absorbed and caused hallucinations. They sometimes also put the ointment on the handle of broomsticks, and when sitting on this, the alkaloids were absorbed via the vagina (5). The women thought that they flew to “Blocksberg”, the home of witches, and during the Middle Ages, at least in Norway; these women were
termed as witches and burned on the fire. The agents causing these effects were the alkaloids present in the plants.

During the reign of Louis XIV, the ladies should have large, dark eyes to be attractive. To obtain this kind of eyes, they put a drop of the juice of the berries of deadly nightshade into their eyes. The pupils then became large; this is the effect that is caused by atropine when the ophthalmologist today is using eye drops in the eyes of the patient in order to study the inner parts of the eyes. This compound is also used in connection with operations to reduce the production of secreted juices.

The compound scopolamine is today used in travel sickness plaster. This is placed behind the ear and will stabilise the balance system in the brain, thus a lesser degree of travel sickness will be the case.

*Datura stramonium* leaves were in former times used for relaxing the smooth muscle of the bronchial tubes, and cigarettes made from *D. stramonium* leaves were smoked in order to treat an asthmatic's bronchial spasm. The plant was grown during the Second World War, also in Norway, for the purpose of producing “Asthma-cigarettes” (2-4).

**Cardiac glycosides**
Foxglove, *Digitalis purpurea*, was one of 20 plants that in Scotland were used as a remedy against dropsy. The Scottish doctor William Withering became in 1775 interested in a herbal mixture as he saw that it had effect. He identified all the plants and performed a clinical trial on each of them, and this way he found that it was the foxglove that was responsible for the activity of the mixture. Foxglove was also used as an emetic and as a wound healer. The compounds responsible for the cardiac activity were later shown to be the products named cardiac glycosides. One of them, digitoxin, is still used against certain heart conditions (3, 4).

**Quinine and artemisinin**
Malaria is one of the most fatal diseases world wide. An old well know remedy for treatment of malaria is the quinine isolated from the bark of the tree *Cinchona pubescens* and other *Cinchona* species. The use of the china-bark as a fever reducing agent, one of the symptoms following malaria, came from the local population in South America. This knowledge was brought to Europe with Columbus. Quinine was used for a long time, and was also a model compound for the synthesis of chloroquine being in use for the treatment of malaria. This compound developed resistance in various *Plasmodium* species and this effect
has given quinine a renaissance as a remedy for treatment of and prophylaxes against malaria. One of the other alkaloids present in the cinchona bark, quinidine, was shown to have an anti-arrhythmic effect and is used today for special types of heart diseases.

*Artemisia annua* is an old Chinese anti-malaria remedy called quinhao. This was shown to be an effective remedy both as prophylaxis and in the treatment of malaria. Modern science showed that the active ingredient is artemisinin. This product had to be modified in the laboratory to gain the best stability and bioavailability to be used as an effective drug and is today one of the most used anti-malaria products (3, 4).

**Podophyllotoxin**
Podophyllotoxin is a resin compound isolated from the roots of *Podophyllum peltatum* (May apple). This remedy had a traditional use amongst others against constipation, warts, internal worms and as bile stimulator. It has often been noted that natural remedies used against warts might have an antiviral effect. So was also the case for podophyllotoxin which today is used against *Condylomata acuminata*, caused by the papilloma virus. Podophyllotoxin was also the lead compound giving rise to the development of the medical drugs teniposide and etoposide, today used against several types of cancer (3, 4).

**Vinblastine and vincristine**
The Madagascar periwinkle, *Catharanthus roseus*, had a traditional use against diabetes. It has been shown that the alkaloids present in the plant has hypoglycaemic effect and can thus explain the traditional use of the plant in Madagascar. In a screening study of a great number of medicinal plants it was unexpectedly found that the alkaloids from the Madagascar periwinkle had an anti-neoplastic effect. This led to the discovery of vinblastin and vincristin, which both have been important for curing different types of cancer. Vinblastin has successfully been used against Hodgkin’s disease and vincristin against leukaemia, lymphomas and small cells lung cancer. Vincristin is such a good remedy for the treatment of leukaemia in children that approximately 80 % survive today, while earlier most of the children died. Unfortunately it is impossible to synthesize these compounds yet and the demand is great. 500 kg plant material is necessary to obtain 1g pure alkaloid and the isolation and purification process is complicated.
The Hardanger plain story
Not all new medicinal compounds from nature have the origin from plant with a long traditional use as medicinal remedies. One of the large international medicinal companies sent sterile test tubes with their employees when they went on holiday abroad. They were asked to take a soil sample with them back home, and then they investigated the micro-organisms from the sample for their ability to produce interesting compounds that could have medicinal properties. From a sample that originated from the Hardanger plain in Norway, the compound ciclosporin A was isolated from the microfungus *Tolypocladium inflatum*. This compound turned out to be a polypeptide consisting of 11 amino acids and it was shown that it had immunosuppressive properties. This product revolutionized the survival period for people needing transplantation of organs and has given a large number of people an elongated life (4).

What will the future bring?
No one really knows the number of plants on earth, but a conservative estimate is approximately 250 000 higher plants. About 10 % of them have been investigated for their content of bioactive compounds. It is also so that most of these plants have only been investigated for a smaller part of their chemical compounds. Lower plants, micro- and macro- algae are organisms that also have been studied only to a small extent and are candidates for investigations for new natural compounds for better health of the populations on earth. The same concerns micro-organisms like bacteria and moulds, as well as marine organisms as fish and sponges. This means that mankind can still look forward to a great variety of new compounds which can be used for better health in the future. There are still several plants in active use by traditional healers in Asia, Africa and South-America which have not been thoroughly investigated, and studying these will most probably give the fastest route to new interesting compounds.

References
Main plant poisonings in livestock in the Nordic countries

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Abstract

The main plant poisonings in livestock in the Nordic countries are reviewed. Data are mainly derived from a literature search of the Nordic national veterinary journals from the last three decades. The most important plant poisoning in Nordic countries, from both an animal welfare and an economic perspective, is photosensitization of lambs (‘alveld’) caused by ingestion of bog asphodel (Narthesium ossifragum). The economic losses and animal suffering caused by this disease have been so serious in certain regions of Norway, that several farmers have been forced to abandon sheep husbandry. With the exception of ‘alveld’, plant poisonings appear to occur only sporadically, and, in comparison with other regions of the world where livestock management is far more extensive, cases are considered to be rare in the Nordic countries. However, efforts to obtain estimates of the prevalence and geographical distribution of plant poisonings are hampered by the fact that very few cases are reported. Furthermore, it is difficult to assess the full effects of plant poisonings, as the toxic compounds may cause only indistinct signs such as mild digestive disturbances or reduced fertility, that pass more or less unrecognized. Thus the economic losses caused by plant toxins are probably largely underestimated. Changes in climatic conditions and agricultural practices may alter the extent to which livestock species in Nordic countries are exposed to poisonous plants. Establishment of a systematic registration of plant poisonings of livestock is highly recommended and could be used as a knowledge base for providing farmers with prophylactic advice.
Introduction

The scientific literature contains a variety of definitions for poisonous plants. One such definition could be: "a poisonous plant is a plant which, when ingested, may result in illness or death", while another may read: "poisonous plants are plants which, when eaten, can give rise to a departure from normal health". These rather vague definitions indicate that plant poisoning incidents present with clinical signs that span a wide range of severity. The course may be acute and dramatic, with convulsions and seizures resulting in death of the animal, or the signs of poisoning may be so indistinct that the event passes more or less unrecognized.

The consequences of plant poisoning depend, of course, on how severely the animal is affected. The sufferings of the animal together with the production losses for the farmer receive most emphasis. However, in addition to the issues of animal welfare and economy it should not be neglected that, like in other diseases, animal poisoning may also represent a serious emotional problem for the farmer.

The toxic principle of a poisonous plant may be either a single substance, a number of substances with different chemical properties, or the toxic compound(s) may not have yet been characterized. Poisonous substances in plants are often termed secondary plant metabolites. The vast variety of chemical compounds synthesised within the plant kingdom may be divided into primary metabolites and secondary metabolites. Primary metabolites are essential for basic biochemical reactions, while secondary metabolites do not appear to have any obvious function in growth and development. However, the division between these two categories is not clearly defined and secondary metabolites, often previously described as waste products, are now considered to be essential for plant life, some of them acting as defensive agents against pathogenic microorganisms, and herbivorous insects and mammals.

Toxin content may vary considerably between the different anatomical parts of a poisonous plant. Furthermore, genetically determined variations exist in toxin content between different populations (cultivars) of some plant species. It should also be noted that soil, climate, and other environmental factors might modify the plant toxin content. Finally, one should be aware that in addition to their intrinsic toxins, plants may also acquire potentially harmful compounds from external sources. For example, some plants might accumulate nitrate, copper, and various pesticides, or they may be contaminated by mycotoxins.

Different animal species often vary in their susceptibility to a specific poisonous plant. This may be due to differences in anatomy of the gastrointestinal tract,
feeding patterns, or, probably more importantly, species differences in detoxification mechanisms. Ruminant species such as cattle and sheep have large forestomachs, where ingested toxic substances are considerably diluted. Additionally, the passage of the stomach contents into the intestines, from where the compounds are absorbed, is much slower than in non-ruminant species such as the horse and pig. Moreover, microbial action in the forestomachs may destroy the ingested toxins, although the breakdown of plant material may also lead to the release of poisonous compounds.

Following absorption from the intestine to the blood, the poisonous principles are transported to the liver, where they may undergo enzymatic transformation. These metabolic processes can for convenience be considered as being divided into two phases. In the first phase, reduction, oxidation, or hydrolysis results in the formation of a primary product. This primary product may be eliminated unaltered, or it may be further metabolised by phase II enzymes to more water-soluble conjugates that are readily excreted. However, it should be noted that these enzymatic transformations do not inevitably result in detoxification; indeed the converse might occur, as the enzyme action may generate a toxic principle from an initially harmless compound. The diverse battery of enzymes involved in these two phases of metabolic processes differs genetically with animal species, regarding quantity, structure, specificity, and activity. The composition and activity of enzymes may also differ between individuals of the same species, and could in addition be affected by environmental factors, such as season, diet, and the amount of toxic compound absorbed (1).

The majority of modern textbooks classify poisonous plants according to their known toxic constituents, and the following chemical groups are often employed: alkaloids, glucosides, proteins, peptides and amino acids, oxalates, and phenolic compounds. This approach for classification of poisonous plants is used in the present study.

Unfortunately plant poisoning in livestock is not systematically registered in any of the Nordic countries. Consequently no precise data are available, and in order to obtain an estimate of the prevalence and geographical distribution of plant poisonings, other sources of information are necessary. In this respect case reports and inquiries published in veterinary journals are of considerable importance. This presentation is restricted to poisonings in farm animals caused by vascular plants (Tracheophyta), and having been reported in the Nordic national veterinary journals during the last three decades.
Plants containing alkaloids

Alkaloid-containing plants that have been reported to be responsible for livestock poisonings are listed in Table 1. Those most commonly causing poisoning in the Nordic countries are described briefly below.

Table 1. Alkaloid-containing plants that have been reported to cause livestock poisoning.

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>English</th>
<th>Danish</th>
<th>Swedish</th>
<th>Norwegian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conium maculatum</td>
<td>Hemlock</td>
<td>Skarntyde</td>
<td>Odört</td>
<td>Giftkjeks</td>
</tr>
<tr>
<td>Oenanthe aquatica</td>
<td>Fine-leaved water-dropwort</td>
<td>Billebo-klaseskærm</td>
<td>Vattenstäkra</td>
<td>Hestekjærvel</td>
</tr>
<tr>
<td>Laburnum anagyroides</td>
<td>Laburnum</td>
<td>Almindelig guldregn</td>
<td>Sydgullregn</td>
<td>Gullregn</td>
</tr>
<tr>
<td>Solanum dulcamara</td>
<td>Woody nightshade</td>
<td>Bittersød natskygge</td>
<td>Besksöta</td>
<td>Slyngsøtvier</td>
</tr>
<tr>
<td>Solanum nigrum</td>
<td>Black nightshade</td>
<td>Sort natskygge</td>
<td>Nattskatta</td>
<td>Svartsøtvier</td>
</tr>
<tr>
<td>Colchicum autumnale</td>
<td>Meadow saffron</td>
<td>Høsttidløs</td>
<td>Tidlösa</td>
<td>Tidløs</td>
</tr>
<tr>
<td>Aethusa cynapium</td>
<td>Fool’s parsley</td>
<td>Hundepersille</td>
<td>Vildpersilja</td>
<td>Hundepersille</td>
</tr>
<tr>
<td>Hyoscyamus niger</td>
<td>Henbane</td>
<td>Bulmeurt</td>
<td>Bolmört</td>
<td>Bulmeurt</td>
</tr>
<tr>
<td>Senecio spp.</td>
<td>Ragwort</td>
<td>Eng-brandbæger</td>
<td>Stånds</td>
<td>Landøyda</td>
</tr>
<tr>
<td>S. jacobaea</td>
<td>Marsh ragwort</td>
<td>Vand-brandbæger</td>
<td>Vattenstånds</td>
<td>Dikesvineblom</td>
</tr>
<tr>
<td>S. aquaticus</td>
<td>Eastern groundsel</td>
<td>Vår-brandbæger</td>
<td>Vårkorsört</td>
<td>Vårsvineblom</td>
</tr>
<tr>
<td>S. vulgaris</td>
<td>Groundsel</td>
<td>Almindelig brandbæger</td>
<td>Korsört</td>
<td>Åkersvineblom</td>
</tr>
<tr>
<td>Cicuta virosa</td>
<td>Cowbane s. Water hemlock</td>
<td>Gifttyde</td>
<td>Sprängört</td>
<td>Selsnepe</td>
</tr>
<tr>
<td>Taxus baccata</td>
<td>Yew</td>
<td>Almindelig taks</td>
<td>Idegran</td>
<td>Barlind</td>
</tr>
<tr>
<td>Aconitum spp.</td>
<td>Northern wolfsbane</td>
<td>Nordisk stormhat</td>
<td>Nordisk stormhatt</td>
<td>Tyrihjelm</td>
</tr>
<tr>
<td>A. septentriionale</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

The main toxic components of Senecio spp. (Family: Asteraceae), commonly known as ragwort, are pyrrolizidine alkaloids. These plants have a worldwide distribution and cause major problems in grazing animals, particularly in regions with extensive livestock management. The four species listed in Table 1 are native to the Nordic countries and have all caused livestock poisoning (seneciosis), mainly in cattle and horses. Pyrrolizidine alkaloids remain unaltered in cured hay and silage and are not themselves toxic, but are bioactivated to pyrrolic metabolites by the hepatic phase I enzymes, i.e. the...
MFO (mixed function oxidase)-or CYP (cytochrome P-450)-enzymes. The resultant reactive pyrrolic metabolites are electrophilic and bind covalently to cellular macromolecules in hepatocytes, causing liver injury. The poisoning usually develops slowly and may not become apparent until long after ingestion of the plant. Initial clinical signs are associated with the digestive system, and may include abdominal pain and diarrhoea or constipation. In later stages there may be progressive weight loss and jaundice, haemorrhages, and oedema. Post mortem findings include acute centrilobular necroses in the liver, hepatic fibrosis, and cirrhosis. In addition to digestive system disturbances, there may also be neurological signs including restlessness, uncoordinated movements, and partial paralysis. This condition, called hepatic encephalopathy, is apparently due to an increase in blood ammonia and occurs particularly in horses. Sheep, unlike cattle and horses, are relatively resistant to ragwort poisoning, probably due to the protection conferred by their ruminal metabolism. In the Nordic countries seneciosis occurs sporadically, predominantly in cattle (2-4).

*Cicuta virosa* (Family: *Apiaceae*), commonly known as cowbane or Northern water hemlock, grows in shallow water or marshland. The plant is regarded as highly toxic and ingestion of only part of a root may be sufficient to kill a cow or a horse. The thick root, which is divided internally with cross-walls into hollow compartments, is particularly rich in an unsaturated aliphathic alcohol, cicutoxin. Cicutoxin acts as a GABA-receptor antagonist, and its action in the central nervous system (CNS) is considered responsible for the highly dramatic and often fatal intoxication following ingestion. The clinical signs may appear within an hour, and include salivation, dilated pupils, abdominal pain, vomiting, muscular spasms, and convulsions. Death from respiratory failure may occur within a few hours. Due to the acute nature of the poisoning, animals are often found dead not far from the habitat of the plant, where the plant’s roots may have become exposed following a previous drop in the water level. There are no characteristic pathological findings at post mortem. Incidents, mainly involving cattle, occur sporadically in the Nordic countries (5).

*Taxus baccata* (Family: *Taxaceae*) or European yew is an evergreen tree or bush, which may grow wild or be cultivated as hedges. Nearly all parts of the plant contain a complex mixture of alkaloids, of which the Taxines A and B have been characterized structurally. Taxine A is the major constituent and considered the most important from a toxicological perspective. It is absorbed rapidly and interferes with calcium channels in cardiac myocytes, resulting in cardiac arrest and death from circulatory collapse. The taxines, as well as other alkaloids in yew, may also affect the CNS causing signs like muscular trembling, excitability, and uncoordinated movements. Collapse and death may occur within a few hours after ingestion. Horses are more sensitive than cows, and lethal doses of plant material for cattle and horses have been estimated to be
between 1 and 10 g and between 0.5 and 2 g per kg body weight, respectively. Yew has been long and widely recognised as poisonous and most cases have resulted from accidents, such as animals gaining access to yew hedges through broken fences during winter when green feed plants are scarce. The acute nature of the intoxication means that animals are often found dead with no evidence of poisoning, apart from plant material in the mouth, stomach, or rumen. Inflammation of the mucous lining of the digestive tract may also occur. Cases of yew poisoning occur sporadically in horses, cattle, and sheep (5, 6).

*Aconitum* spp. (Family: *Ranunculaceae*), commonly known as monkshood or wolfbane, comprise cultivated and naturalized varieties as well as wild species, subspecies, and hybrids. In the Nordic countries various cultivars of *A. napellus* are garden plants, while *A. septentrionale* (northern wolfsbane) grows in the wild. The plants contain the highly toxic diterpene alkaloid aconitine as well as several other alkaloids with similar structures. The alkaloid content varies widely according to plant part, growing conditions, and season. The effect of the alkaloids on the CNS is initially stimulatory, followed by depression of activity, and death from respiratory and circulatory failure may occur within an hour after ingestion. The toxic potential of *A. septentrionale* is currently controversial, but there is evidence of intoxication occurring sporadically in cattle (2, 7).

**Plants containing glucosides**

Plants containing glucosides are listed in table 2. Livestock poisonings by plants containing cardiac glucosides have not been reported in recent decades except for an incident in sheep where foxglove could have been responsible (8). Similarly, intoxications with cyanogenic glucosides after ingestion of *Sambucus* spp. or *Prunus* spp. have been reported very rarely. However, fatal cyanide poisoning in sheep following ingestion of laurel cherry (*Prunus lauroceracus*) has recently been reported (9). This plant was recently introduced into Norway and is cultivated for hedges. The reported incident is an example of poisoning due to accidental exposure to “new” plant species introduced into regions previously unfamiliar with them.

Hepatogenous photosensitization “alveld” (elf fire), in sheep associated with grazing on *Narthecium ossifragum* (Family: *Liliaceae*), commonly known as bog asphodel, occurs mostly in Norway, and is the most important livestock poisoning in the Nordic countries, both from an economic and an animal welfare point of view. In addition to “alveld”, which mainly affects lambs of 2-6 months
of age, ingestion of bog asphodel may also cause toxic nephrosis in several ruminant species, particularly cattle.

**Table 2.** Glucoside-containing plants that have been reported to cause livestock poisoning.

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Type of glucosides</th>
<th>English</th>
<th>Danish</th>
<th>Swedish</th>
<th>Norwegian</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Digitalis purpurea</strong></td>
<td>Cardiac glucosides</td>
<td>Foxglove</td>
<td>Almindelig fingerbol</td>
<td>Fingerborgsblomma</td>
<td>Revebjelle</td>
</tr>
<tr>
<td><strong>Convallaria majalis</strong></td>
<td>Cardiac glucosides</td>
<td>Lily of the valley</td>
<td>Liljekonval</td>
<td>Liljekonvalj</td>
<td>Liljekonvall</td>
</tr>
<tr>
<td><strong>Nerium oleander</strong></td>
<td>Cardiac glucosides</td>
<td>Oleander</td>
<td>Nerie</td>
<td>Oleander</td>
<td>Oleander</td>
</tr>
<tr>
<td><strong>Narthecium ossifragum</strong></td>
<td>Saponic glucosides</td>
<td>Bog asphodel</td>
<td>Benbræk</td>
<td>Myrlilja</td>
<td>Rome</td>
</tr>
<tr>
<td><strong>Prunus spp.</strong></td>
<td>Cyanogenic glucosides</td>
<td>Prunus</td>
<td>Prunus</td>
<td>Prunus</td>
<td>Prunus</td>
</tr>
</tbody>
</table>

The steroidal saponins in bog asphodel are believed to be hepatotoxic, causing liver damage. This interferes with the biliary excretion of phylloerythrin, a photoactive compound that is a normal metabolic product of chlorophyll, and therefore accumulates in the blood. Upon exposure to sunlight, this photosensitizing compound causes skin damage, particularly in poorly pigmented regions of the skin not protected by wool. The photosensitizing process involves generation of reactive oxygen species that cause membrane damage. It should be noted that difficulties in reproducing the liver damage experimentally using saponin extracts may be indicative of a more complex aetiology. Despite the pathogenesis of the hepatotoxicity in “alveld” being unresolved, it is generally agreed that the sapogenins play an important aetiological role.

The signs of photosensitization include oedema and erythema of the skin, particularly in the head region, followed by itching, pain, exudation, and secondary infections. Blindness may also occur, as well as jaundice due to liver failure. Usually only white lambs are affected but also black lambs, even protected against developing photosensitization by their pigmentation, may show signs of liver failure. In contrast to the skin necrosis and secondary infections of affected lambs, gross pathological lesions of the liver are seldom observed. However, microscopic examination reveals histopathological changes, including necrosis of hepatocytes, portal fibroplasia, and bile duct proliferation.
Sapogenin crystals are characteristically present in the bile duct epithelium and hepatocytes.

For “alveld”, the most serious and common plant poisoning in Norway mortality rates of up to 50% have been recorded. The prevalence varies greatly from one year to the next, and also between regions, and may reach several thousand cases annually (10, 11).

During the summer of 1992, which was unusually dry and hot, a serious disease outbreak occurred in western Norway involving about 250 head of cattle, of which more than 100 died. The affected animals showed signs of uraemia, such as depression, anorexia, dehydration, and diarrhoea. Clinical blood chemistry also indicated kidney failure, and autopsy revealed degeneration and necrosis of the renal tubular epithelium. Pasture inspection indicated that bog asphodel was probably involved, and this was subsequently confirmed in experimental studies where the condition was reproduced using an aqueous extract of the plant. A furanone compound is currently believed to be the causative agent. Similar cases had been observed prior to 1992 when the climatic conditions resulted in elevated numbers of cases, and a few cases of toxic nephrosis in cattle, probably due to ingestion of bog asphodel, have occurred annually thereafter (12, 13).

Plants containing proteins, peptides and amino acids

*Equisetum* spp. (*E. arvense, E. pratense, E. Palustre*, Family: *Equisetaceae*), commonly known as horsetails (DK: Padderok, NO: sneller, SW: fräken), contain a variety of toxic constituents, the most important being thiaminase, an enzyme which destroys thiamine (vitamin B$_1$), and thus causes vitamin B$_1$ deficiency. The plants are unpalatable when fresh, and therefore poisoning is unlikely to occur on pasture. However, the toxic principle is not destroyed by drying and storage, and 5% horsetails in hay may result in clinical signs in horses. The symptoms usually develop over several weeks and include weakness and uncoordinated movements, particularly of the hind limbs. Posterior paralysis and inability to stand upright may occur. In fatal cases convulsions and coma may precede death. Post mortem findings are nonspecific, but degenerative changes may be found in the brain. Although previously more common, poisoning is of minor importance today (2).

*Pteridium aquilinium* (Fam.: *Dennstaedtiaceae*) or bracken (DK: almindelig ørnebregne, NO: einstape, SW: örnbäken) contains several toxic compounds,
three of which, a cyanogenic glucoside, a thiaminase, and a carcinogen, have been studied and characterised.

The amount of the cyanogenic glucoside prunasin in bracken is normally too low to cause poisoning and is thought to protect the plant against grazing, as non-cyanogenic populations of the plant are often grazed heavily, while cyanogenic cultivars are not. Thus bracken is an interesting example of how a biochemical polymorphism might affect feeding behaviour.

The enzyme thiaminase, probably together with a heat-stable anti-thiamine factor, may cause thiamine (vitamin B1) deficiency in non-ruminant species. The symptoms resemble those described for intoxication with *Equisetum* spp. Thiaminase is unlikely to induce vitamin B1 deficiency in ruminants as thiamine is synthesized by rumen bacteria, and breakdown of thiamine in the rumen will be compensated for by increased bacterial synthesis.

The carcinogenic compound ptaquiloside (PTQ) is a water-soluble, norsesquiterpene glycoside, and is involved in several disease conditions. It may induce tumours in the gastrointestinal tract and urinary bladder. It also depresses bone marrow activity significantly, and reduces the production of red and white blood cells and blood platelets, resulting in anaemia, immunosuppression, and impaired blood coagulation, respectively. Immunosuppression, together with oncogenic papillomavirus, probably contributes to the induction of neoplasms. Furthermore, PTQ is involved in acute haemorrhagic syndrome and enzootic haematuria in cattle and retinal degeneration in sheep. Poisonings associated with the effects of PTQ on the bone marrow may occur sporadically (14).

**Plants containing oxalates**

Several of the *Rumex* spp., commonly known as docks and sorrels (DK: skræppe, NO: syrer; SW: skräppsläktet), as well as *Rheum rhabarbarum* or rhubarb (DK: rabarber, NO: rabarbra, SW: rabarbra), contain oxalic acid. Soluble oxalates are usually detoxified in the digestive tract, particularly in ruminants. However, if ingested in large quantities they may be absorbed and bind blood calcium, thus causing hypocalcaemia. Furthermore, calcium oxalate crystals may accumulate in the kidneys, blocking tubules and leading to renal failure. Oxalate crystals may also form in the brain resulting in disorders of the CNS. During the last three decades there have been very few reports on this type of poisoning in livestock in the Nordic countries (15).
Plants containing phenolic compounds

*Hypericum* spp. (*H. perforatum, H. maculatum*), commonly known as St John’s wort (DK: perikon, NO: perikum, SW: Johannesört), contain a polyphenolic compound, hypericin, which is a red, fluorescent pigment. If hypericin is ingested it may cause photosensitization in unpigmented areas of skin that are exposed to sunlight. Although the lesions resemble those associated with “alveld”, the mechanism of development differs. In “alveld” liver injury is a prerequisite for the disease to develop, and is therefore known as hepatogenous or secondary photosensitization. Hypericin however causes primary photosensitization, as it occurs without any preceding liver damage. Although poisoning by St John’s wort has been rarely reported from Nordic countries (16), these plants may possibly contribute to the incidents of primary photosensitization of unknown aetiology that are sporadically observed in cattle.

All parts of *Quercus* spp. or oak (DK: eg, NO: eik, SW: ek) contain gallotannins, with the highest levels in young leaves and green acorns. The tannins are metabolised by the intestinal flora of herbivorous animals to low molecular weight phenolic compounds, such as gallic acid and pyrogallol. These compounds may be absorbed and are believed to be responsible for gastrointestinal haemorrhages and kidney and liver damage. It has also been suggested that mycotoxins present in acorns may contribute to their toxicity.

Although ingestion of small quantities of acorns or oak leaves is usually harmless, intake of larger amounts may cause serious disease. Cattle and horses are the species most often affected, and in Nordic countries such incidents occur primarily in the autumn when animals feed on fallen acorns. In cattle, there may be a time lag between ingestion and the occurrence of clinical signs. Initially the symptoms of oak poisoning in cattle are related to the gastrointestinal tract, and include cessation of rumination and lack of appetite. Following initial constipation, small quantities of dark coloured faeces are passed. In chronic cases there is considerable wasting. In serious acute cases, kidney damage may be so severe that it causes death, and abdominal pain and convulsions may be followed by sudden death. In horses, clinical signs may include depression, loss of appetite, abdominal pain, and initial constipation followed by diarrhoea. Post mortem findings are the same for all species, and include intestinal inflammation and haemorrhages. Degenerative changes in the kidneys are characteristic, and liver enlargement may also be observed. Oak poisoning occurs sporadically in cattle, horses, and sheep, but pigs are regarded as being relatively resistant (17,18).
Plants containing grayanotoxins

Several diterpenoids, called grayanotoxins, are found in various species of flowering plant including Rhododendron spp. (DK: rhododendron, NO: rhododendron, SW: rhododendron), Pieris japonica, commonly known as Japanese andromeda (DK: almindelig rosmarinlyng, NO: pyramidelyng, SW: japansk buskrosling), and Andromeda polifolia, commonly known as bog rosemary (DK: almindelig rosmarinlyng, NO: hvitlyng, SW: rosling). After ingestion adverse effects develop rapidly, and this may prevent the animals from consuming a lethal dose. Clinical signs are similar for all animal species and include digestive tract symptoms such as salivation, vomiting, abdominal pain, and diarrhoea. CNS associated symptoms may also occur, including trembling, uncoordinated movements, and exhaustion. In fatal cases death is due to respiratory failure. The only clues found at post mortem examination may be plant fragments in the rumen. Poisoning occurs sporadically, mainly in sheep and goats (5, 19).

General discussion

In the intensive farming practices of Nordic countries, fodder that is provided for livestock kept inside is generally well regulated. Furthermore, pastures for animals grazing outside are usually cultivated with feed crops of high nutritive value grown from quality-assured seeds. These practices restrict the exposure of livestock to toxic plants, and therefore cases of plant poisoning occur considerably less frequently than in other regions of the world practising extensive livestock management, and where the free-ranging animals must eat whichever plants are available, usually non-cultivated, and often under highly unfavourable climatic conditions including periods of drought.

There is, however, one important exception. Sheep management is extensive in Nordic countries, particularly Norway, and photosensitization of lambs on pasture (‘alveld’ or ‘elf fire’), caused by ingestion of bog asphodel (Narthecium ossifragum), is, without doubt, the most important plant poisoning that occurs there. In certain regions of Norway, the extent of economic losses and animal suffering caused by ‘alveld’ has resulted in some farmers abandoning sheep husbandry.

The present review is mainly based upon case reports and inquiries published in the national veterinary journals of Denmark, Finland, Norway, and Sweden. It might be disputed whether a representative overview of the significance,
prevalence, and geographical distribution of cases of plant poisoning is provided by this approach, since there are clearly associated shortcomings. Scientific journals generally require that data meet certain criteria regarding diagnostic confirmation. The fulfilment of these criteria for a diagnosis of plant poisoning is dependent on knowledge and experience from several disciplines, including chemistry, comparative clinical toxicology, and pathology, as well as botany. In addition, for a proper and often time-consuming investigation to be carried out, special laboratory facilities with expensive equipment are necessary. The majority of veterinary practitioners have neither the facilities nor the time to investigate cases of suspected plant poisoning sufficiently thoroughly to be able to confirm a diagnosis definitively, and therefore relatively few incidents are published in the scientific literature. Thus, in order to obtain a more reliable overview of the situation, additional sources of information must be accessed. In this respect, results from inquiries published in professional journals, notes in the local public press, and personal communications from veterinary practitioners, farmers, and colleagues in the scientific community are especially valuable. These sources have largely confirmed that the plant species selected for this presentation are those that most often cause livestock poisoning in the Nordic countries.

The toxic effects of secondary plant metabolites that cause death or severe illness in livestock obviously attract the most interest. In such cases, the cause of the deterioration in health can usually be identified. However, it is probable that considerably greater economic losses are attributable to less obvious effects of plant toxins. These effects include transient digestive disturbances, which can affect weight gain and the production and/or quality of meat, milk and wool, and interference with reproduction, which may reduce fertility. For example, it has long been recognised that phytoestrogens may cause both temporary and permanent infertility in sheep (20), and subclinical plant poisoning caused by plants containing non-steroidal phytoestrogens, mainly belonging to the biochemical classes isoflavones and coumestans, is of considerable economic importance on the Australian continent. Additionally, feeding experiments conducted in Sweden and Finland have confirmed that red clover (*Trifolium pratense*) may contain sufficient quantities of phytoestrogens to affect reproductive performance in cattle (21). However, whether this is of clinical significance under normal (and intensive) management conditions has yet to be investigated.

Currently there is no systematic registration of cases of plant poisoning of livestock in any of the Nordic countries. Ongoing changes in agricultural practices, including less intensive production, reduction in the use of herbicides, and more frequent pasturing of livestock outside, in association with climatic changes that might facilitate the introduction and spread of “new” plant species
into regions previously unfamiliar with them, have created a demand for prophylactic advice for farmers. A databank based on systematic registration of plant poisonings would obviously represent a most valuable advisory tool for this purpose.

Finally, it should not be forgotten that information derived from cases of plant poisoning of animals may provide unexpected benefits to man. For example, the discovery in North America in the 1920s that feeding cattle with ‘mouldy’ sweet clover (Melilotus officinalis) caused death from uncontrollable internal bleeding, provided the origin for today’s oral anticoagulation therapy (22). The causative agent for ‘sweet clover disease’ was identified as dicoumarol, and following its isolation, several related compounds were synthesized. The most useful of those to date has been warfarin; national sales statistics suggest that about 1% of the population of Nordic countries currently use warfarin therapy (23).

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Human plant poisoning in the Nordic countries – experiences from the Poisons Information Centres

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Abstract

Ingestions of plants by children are quite common in the Nordic countries and represent approximately 6-7 % of the total inquiries about acute exposures to the Poisons Centres. Exposures to plants are most often accidental in nature, and the amounts ingested are usually low. When treatment is recommended decontamination and observation is often sufficient. Risk of moderate to severe poisonings was estimated in only 3 % of the cases of plant ingestions, where medical attention and possible hospital treatment were recommended. Symptoms were most frequently caused by the group of plants with irritating effect on mucous membranes. The treatment initiated at home is often sufficient in these cases. Other common plants as reflected by the number of inquiries to the Poisons Centres, which may cause poisoning due to their toxicity and widespreadness are Convalia majalis (lily of the valley), Taxus baccata (yew), Digitalis purpurea (foxglove) and Rhododendron. The sporadically reported severe plant poisonings in humans are most often self-harm in adults.
Introduction

Plants are widely available in the human environment. In the Nordic countries the high season for outdoor plants is during the summer months, from May to September. According to the inquiries to the Poisons Centres, plant material is most frequently ingested in the late summer and autumn (1, 2). In the winter season indoor plants are a common source of exposures to small children. A wide variety of plants produces compounds that may cause clinical symptoms in humans, some of which have caused severe poisonings. A few plants give rise to serious poisoning after ingestion even of a limited amount of plant material (3). There are few reports on serious or fatal human plant poisoning in the Nordic countries (4).

The Poisons Centres in the Nordic countries offers a 24 hour telephone emergency service to both the general public and health care professionals, where they provide information, risk assessment and treatment advice in cases of acute poisoning and exposure to drugs, chemicals, and plant materials. The Poisons Centres also provides treatment guidelines for the hospitals concerning poisonings. The total number of inquiries to the Norwegian Poisons Information Centre (Norwegian PIC) was 41,074 in 2008 (1). Eighty-four percent of these inquiries where caused by acute exposures (1). The following statistics about plant poisonings from Norway were derived from the database of the telephone service (GISBAS).

The experiences from Norway are representative for the status of human plant poisonings in the Nordic countries. The Nordic Poisons Information Centres collaborate and meet annually to share experience and discuss topics of common interest. We observe that human plant poisonings show much the same patterns in the Nordic countries regarding incidence, age of the exposed, and cause and severity of the exposures. There are climatic differences, however, and the northern areas have limited vegetation, including toxic plants, compared to the southern areas. Furthermore there are imported foreign garden and indoor plants with no natural habitat in our climate. The number and types of plants that can cause poison are thereby increased.

Acute plant exposures

Exposures to plants are most often accidental in nature. The Norwegian PIC received 4,354 inquiries about plants in 2008, 11 % of the total number of inquiries. Of these, 3,613 contacts were cases of exposures to plants. The
Swedish Poisons Information Centre received 4,514 inquiries (6% of the total) about children exposed to plants in 2008 (2). Outdoor plants which produce fruits and berries are of special interest to small children. Usually the amount of plants ingested in such cases is small. Plants of known identity frequently involved in exposures leading to contacts with the Norwegian PIC, are listed in Table 1 (garden/wild plants) and Table 2 (indoor plants).

Table 1. Garden/wild plants frequently involved in the exposures leading to contact with the Norwegian PIC (data from the inquiry statistics 2007).

<table>
<thead>
<tr>
<th>Scientific plant name</th>
<th>Common plant name (English/Norwegian)</th>
<th>Number of inquiries to the Norwegian PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Convallaria majalis</td>
<td>Lily-of-the-valley / Liljekonvall</td>
<td>89</td>
</tr>
<tr>
<td>Cotoneaster spp.</td>
<td>Cotoneaster / Mispelarter</td>
<td>83</td>
</tr>
<tr>
<td>Taxus baccata</td>
<td>Yew / Barlind</td>
<td>56</td>
</tr>
<tr>
<td>Digitalis purpurea</td>
<td>Foxglove / Revebjelle</td>
<td>46</td>
</tr>
<tr>
<td>Rhododendron spp.</td>
<td>E.g. Azalea / Rhododendron</td>
<td>46</td>
</tr>
<tr>
<td>Laburnum spp.</td>
<td>E.g. Golden chain / Gullregn</td>
<td>35</td>
</tr>
<tr>
<td>Symphoricarpos albus</td>
<td>Snow berry / Snøbar</td>
<td>23</td>
</tr>
<tr>
<td>Daphne mezereum</td>
<td>Mezereon / Tysbast</td>
<td>17</td>
</tr>
<tr>
<td>Heracleum laciniatum</td>
<td>Tromsøpalme</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 2. Indoor plants frequently involved in the exposures leading to contact with the Norwegian PIC (data from the inquiry statistics 2007).

<table>
<thead>
<tr>
<th>Scientific plant name</th>
<th>Common plant name (English/Norwegian)</th>
<th>Number of inquiries to the Norwegian PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spathiphyllum wallisii</td>
<td>Peace lily / Fredslilje</td>
<td>120</td>
</tr>
<tr>
<td>Zamioculcas zamifolia</td>
<td>Smaragd</td>
<td>94</td>
</tr>
<tr>
<td>Orchidaceae</td>
<td>E.g. Phalaenópsis / Orkidé</td>
<td>85</td>
</tr>
<tr>
<td>Euphorbia pulcherrima</td>
<td>Poinsettia / Julestjerne</td>
<td>80</td>
</tr>
<tr>
<td>Crassula spp.</td>
<td>E.g. Tykkblad</td>
<td>77</td>
</tr>
<tr>
<td>Epipremum aureum</td>
<td>Devils ivy / Gullranke</td>
<td>35</td>
</tr>
<tr>
<td>Dieffenbachia spp.</td>
<td>Dumb cane / Dieffenbachia</td>
<td>13</td>
</tr>
</tbody>
</table>

A typical case of plant exposure in a child

The following case is an example of an inquiry from the public to the Poisons Centre regarding an outdoor plant exposure in a child. The case illustrates how the Poisons Centres work to give tailormade and proper advice to the client, collect anamnestic information, help with identification of toxic agent, bring about knowledgebased documentation, perform risk assessment and give treatment advices.
A one-year-old-girl ate 3-5 berries from an unknown plant 10 minutes ago. The parents called the Norwegian PIC. There were no symptoms. The parents could describe the plant, and they also immediately sent a picture of the plant by e-mail to PIC. The plant was identified as *Arum orientale* (Figure 1), which is a toxic plant with mostly irritating effects on mucous membranes and eyes. In the case of this particular toxicity and the amount of berries ingested, we expect none or minor symptoms. The treatment advice was administration of active medical charcoal and observation at home.

![Figure 1. Picture of the plant (*Arum orientale*) in this case. Photo: Norwegian PIC.](image)

**Severity of plant exposures**

To what extent does human symptomatic plant poisoning occur? None or minor effects were expected in 80 % of the cases in which the Norwegian PIC were contacted in 2008. No treatment or treatment at home was the advice in these cases. The risk of moderate to severe poisoning was estimated in 3 % of the cases, where medical attention and possible hospital treatment were recommended (1, 5). Manifested severe poisonings following plant exposures are extremely rare in our material. A limitation with the PIC data is that these centres are not a place where poisonings should be reported, but a place to get advice. Therefore, poisonings which do not come to our knowledge may occur. In addition, the Poisons Centres may lack complete follow-up information from the cases.
Irritating effects are most frequent

The group of plants that most often caused symptoms, are plants with irritating effect (1, 5). Examples of common irritating plants in our material are Zamioculcas zamifolia (smaragd), Spathiphyllum wallisii (peace lily / fredslilje) and Dieffenbachia (dumb cane). The mechanisms of action of the irritating plants are not fully understood. A wide variety of chemical substances most probably contributes to the toxicity, including calcium oxalate (3). This type of plant exposure is most common in children, and after intake of indoor plants. When the juice of these plants comes in contact with mucous membranes, for instance when chewed, it can give rise to irritating effect in the mouth and the upper gastrointestinal tract. Clinical symptoms are burning sensation, increased salivation, reddening, nausea, vomiting, diarrhoea, and in more serious cases difficulty in swallowing (3). There is pronounced variation to which extent the different plants in this group cause irritation. Exposures to irritating plants do seldom need medical attention. Treatments that can be performed at home, as rinsing of skin and eyes, giving something to drink and general observation, are often sufficient. Administration of activated charcoal may be recommended in some cases. Manifested serious poisonings after exposures to irritating plants are rare. Both the low toxicity of most of the plants in this group and the small amount of plant normally ingested, contribute to the low incidence of poisonings induced by irritating plants.

Common plants with the potential to cause serious poisonings

From the list of outdoor plants that frequently cause inquiries to the Poison Centre are several toxic species (Table 1). These plants have potential to cause serious poisoning if the amount ingested reaches the toxic threshold. Convalla majalis (lily-of-the-valley) contains cardioactive glucosides like convallatoxin, which can give cardiotoxic effects like ECG- disturbances and arrhythmias. The most common situations concern small children ingesting a small number of the red berries, present in the late summer and autumn. Because of the limited amount ingested, such intakes are usually benign. However, Convalla majalis may sporadically give rise to more serious poisonings (6).

Taxus baccata and other Taxus spp. (yew) are other temptations for curious children. The sweet tasting, red coloured non-toxic aril surrounding the toxic seeds is a common source of accidental exposures in the autumn (3). However, a toxic dose is nearly never reached in children. In contrast to children, who seldom taste leaves or bark, there are a few reports of serious or fatal cases in
adults, most often in connection with self-harming (7). The *Taxus* spp. contain different toxins (e.g. taxine B) and the symptoms and clinical signs will arise from the gastrointestinal tract, central nervous system, heart, circulation and respiratory tract.

*Digitalis purpurea* (foxglove) is a toxic plant that grows in the southern parts of the Nordic countries. The toxicity of Foxglove is well known, a fact that probably contributes to the relatively high number of inquiries to the Nordic PICs (Table1). The amounts ingested are usually small. The plant contains cardiotoxic glycosides (digitaloides), and may cause serious cardiac arrhythmias (3).

Rhododendrons (e.g. azalea) are common outdoor and indoor plants and have attractive and often generous flowers. The toxicity varies between the different species. Grayanotoxin is one of the known toxins in *Rhododendron* spp. (3). The clinical effects are irritation, often pronounced, in the gastrointestinal tract. In more serious cases it may give rise to symptoms and clinical signs from the central nervous system and possible organ toxicity (3).

Some other plants that elicited toxic symptoms after intake in Sweden in 2008 were *Datura* spp. and *Aconitum* spp. (5).

In spite of the potential toxicity and availability of all the plants mentioned above, manifested poisonings in humans are very rare. Children are often accidentally exposed to these toxic plants, but the amounts taken are usually small, and do seldom reach toxic levels. When treatment is recommended, decontamination with activated charcoal and observation at home is usually sufficient.

### Phototoxic plants

*Heracleum laciniatum* (tromsøpalme) and *Heracleum mantegazzianum* (giant hogweed/kjempebjørnekjeks) are common plants that give rise to a phototoxic skin reaction in humans. Skin exposures to phototoxic plants are often accidental in both children and adults. During 2008 the Norwegian PIC got 40 inquiries after human exposures to phototoxic plants with estimated risk for moderate symptoms in 15 % of the cases. The toxic effects develop after contact with the skin followed by exposure of ultra violet radiation from the sun light. Skin reactions develop in 1-3 days after the plant exposure and look like serious sun
burn with erythema, vesicles and burning pain. The acute skin reaction is often followed by persistent hyper pigmentation lasting for months (8).

**Intentional plant exposures**

Nutmeg (*Myristica fragrans*), is an example of a plant that can be used experimentally. It has an anticholinergic and psychotrophic effect. The experimental use has been sporadic, but from time to time we see more clustered cases (15 enquiries to the Norwegian PIC in 2007). We also experience a few cases of herbal medicine overdose and misuse, and also cases of mistaken plants as ingredients in food, all occurring in adults. Moderate to severe intentional poisonings with herbal medicine are rare (2 cases at the Norwegian PIC in 2007).

**Written information regarding plant poisoning from the Poisons Centres**

The Norwegian PIC has a homepage for the public (www.helsedirektoratet.no/giftinfo). The homepage contains a list of 120 poisonous plants or plant groups with a brief piece of information on each. To the public, the Norwegian PIC also distributes four different folders with pictures and brief information about poisonous plants as well as a textbook on poisoning in children.

In Sweden, the website for the public is www.giftinformastionscentralen.se. It contains an alphabetic list of plants describing both poisonous and non-poisonous plants.

The Danish PIC has a similar list and information about plant exposures on their website, www.giflinjen.dk.

Hospitals and health care professionals can find four Norwegian guidelines for treatment of acute plant poisonings in the website for health care professionals (www.helsebiblioteket.no/forgiftninger). A website for poisoning information intended for health care professionals in Sweden is www.giftinfo.se.
Conclusion

Accidental plant exposures in children are frequent in the Nordic countries. The ingested dose is often small and insignificant, and the risk of clinical symptoms is low. No treatment or treatment at home is usually sufficient. Moderate to severe plant poisonings in humans are rare.

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Role of dietary phytochemicals in oxidative stress

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Abstract

A plant based dietary pattern with high intake of fruits, berries, vegetables and whole grains, and low intake of red meat and energy-dense foods has been associated with reduced risk of cancer, cardiovascular diseases and other chronic diseases. The mechanisms and the active compounds involved in the protective effects of plant foods have not been clearly identified. As a common factor in pathogenesis of chronic degenerative disease is the involvement of oxidative stress, it is hypothesised that this healthy dietary pattern may dampen oxidative stress and thereby reduces the risk of diseases. Our major aim is to identify bioactive plant compounds (i.e. antioxidants and other phytochemicals), plant extracts as well as specific plant foods with ability to dampen oxidative stress. Cellular protection against oxidative damage is mainly provided by either direct antioxidants, which are redox active molecules that are consumed in the process of their antioxidant actions or indirect antioxidants that may or may not be redox active. Our projects include an extensive chemical screening of several thousand foods, testing of bioactivity in cell culture systems, preclinical studies in transgenic reporter mice models, randomized clinical trials as well as epidemiological studies. The present chapter discuss the role of oxidative stress and the presence of redox active plant compounds in the diet.
Plants harvest energy from sunlight and produce energy-rich molecules, such as glucose. The plant cells use the energy-rich sugar for their own metabolism and function, including converting the energy into other sorts of molecules. Animals obtain energy from plants to maintain themselves, and for their development, by oxidizing reduced feedstuffs. This is a chemical process where oxygen reacts with the reduced energy-rich molecules. Following many intermediate steps the reactions will end up in oxidized forms of matter such as carbon dioxide and water (1-2).

The energy involved in these chemical reactions comes from the movement of electrons from oxidizable organic molecules to oxygen. Thus, there is a transfer of electrons from one compound to another. The loss of electrons from a substance is called oxidation; the addition of electrons to another substance is called reduction. Not all reduction-oxidation reactions (redox reactions) involve complete transfer of electrons from one substance to another; some change the degree of electron sharing between atoms or molecules. A redox reaction that relocates electrons closer to oxygen, such as the burning of carbohydrates, releases chemical energy that can be put to work. Oxygen therefore contains much energy that can be released when carbohydrates, fats and proteins are catabolized in cellular reactions (1-2).

However, oxygen is a double-edged sword. Oxygen – or byproducts of these reactions - also plays a significant role in the breakdown of our body's functionality – which is reflected in our aging as times passes by. This negative effect of oxygen is called oxidative stress. There are also a few other molecules or compounds, such as nitrogen or iron, that can cause oxidative stress by causing unwanted oxidation. For simplicity, I will just use the term Reactive Oxygen Species or ROS for these compounds in the present chapter.

Oxidative stress is a relatively new term. While a handful scientific reports started to show interest in oxidative stress in the nineteen seventies and eighties, the field exploded during the nineties, and today it is one of the fastest growing topics in modern biomedical science.

Oxidative stress is a general term used to describe the level of oxidative damage in a cell, tissue, or organ, caused by ROS. Oxidative stress is a sort of chemical stress induced by the presence in our body of abnormal quantities of ROS. This condition may be the consequence of an increased production of ROS and/or of a decreased efficiency of the antioxidant defence systems. Thus, when the critical balance between generation of ROS and the antioxidant defence is unfavourable, oxidative damage can accumulate (3-6). Oxidative stress can
scientifically be defined as “a condition that is characterized by accumulation of non-enzymatic oxidative damage to molecules that threaten the normal function of the cell or the organism” (4).

Oxidative damage can affect a specific molecule or the entire organism. ROS represent a class of molecules that are derived from the metabolism of oxygen and exist inherently in all aerobic organisms. There are many different sources by which the ROS are generated. Most ROS come from endogenous sources as by-products of normal and essential metabolic reactions, such as energy generation from mitochondria or the detoxification reactions. Exogenous sources include exposure to cigarette smoke, environmental pollutants such as emission from automobiles and industries, consumption of alcohol in excess, asbestos, exposure to radiation, and bacterial, fungal or viral infections. When ROS are formed, these highly reactive compounds will react non-enzymatically and potentially alter the structure and function of several important components such as cell membranes, signalling molecules, lipoproteins, proteins, carbohydrates, RNA, and DNA (3-6).

Not surprisingly, the organism has developed an efficient defence system to buffer these products (4, 7-13). This is a complex defence system, made up of various sorts of antioxidants, in order to prevent or to lessen the potential damaging action of ROS. The antioxidant system include a diverse series of substances such as enzymes, vitamins, vitamin-like substance, trace elements, metal-binding proteins and various kinds of repair systems, all with the capacity to hinder or repair the damaging effects of ROS.

**Oxidative stress is a common cause of most age-related diseases**

ROS are the major causes of DNA damage, and a slow, steady accumulation of DNA damage has been considered as the major theory of aging (5). Evidence for a role of oxidative stress in aging comes from genetic manipulation of antioxidant enzymes and oxidative DNA damage repair enzymes in simple organisms such as bacteria, yeast, fruitfly, worms and even rodents. For example, improved antioxidant defence or reduced ROS production increases lifespan, while the opposite manipulations decrease lifespan. Many mutations related to oxidative stress, or DNA damage and repair, have also been identified in human syndromes with premature aging (3-6). Recently, it was demonstrated that over expression of antioxidant enzymes in mice increases lifespan by 20% and reduces the incidence of age related diseases (14).
To date, it has been discovered that oxidative stress may be involved in more than 100 common diseases (5-13) such as:

- acquired immunodeficiency syndrome (AIDS),
- alcohol-related diseases
- all inflammatory diseases (arthritis, vasculitis, glomerulonephritis, lupus erythematosus, adult respiratory distress syndrome),
- autoimmune diseases
- cancer,
- diabetes,
- emphysema,
- eye diseases like macular degeneration and cataract
- gastric ulcers,
- hemochromatosis,
- hypertension
- heart disease
- stroke
- lung disease
- neurologic diseases (multiple sclerosis, Alzheimer’s disease, Parkinson disease, amyotrophic lateral sclerosis, muscular dystrophy),
- rejection of organ transplants
- preeclampsia,
- smoking-related diseases

In these diseases, oxidative stress can have different roles. For some, oxidative stress in a specific organ can be the major cause of the disease. Other diseases may have other basic causes, but these other causes or injuries to the organs may eventually lead to oxidative stress, which in turn will further fuel the development of the disease.

**Inflammation – a major cause of oxidative stress**

Inflammation is a complex set of interactions among soluble factors and cells that arise in response to injuries, infections, toxic damages or other sorts of injuries (10-12). Inflammation is the response that leads ultimately to the restoration of tissue structure and function after the damage or injury. If prolonged, however, inflammation can also contribute to breakdown of tissues and structures and development of diseases. This is the case in many infectious diseases, cancer, cardiovascular diseases, neurodegenerative diseases, diabetes,
chronic respiratory diseases, chronic liver disease, chronic renal failure and rheumatic diseases. In all of these chronic degenerative diseases is inflammation causally linked to the progression of the disease (4, 15-17).

Inflammation is closely related to oxidative stress. White blood cells and many other cells produce ROS in large amounts during inflammation. Thus, a common aspect of inflammation is oxidative stress, characterized by accumulation of non-enzymatic oxidative damage. In fact, inflammation is one of the major sources of oxidative stress in our body.

What is an antioxidant?

As the name implies, antioxidants are substances that are capable of counteracting the damaging oxidation reaction, without affecting the normal life-essential oxidation reactions that are taking place in our body. The term ‘antioxidant’ cannot be defined purely chemically; it is always related to the biological context and to oxidative stress. Scientifically, an antioxidant is defined as “a redox active compound that limits oxidative stress by reacting non-enzymatically with a reactive oxidant”, while an antioxidant enzyme is “a protein that limits oxidative stress by catalysing a redox reaction with a reactive oxidant” (4). In human and animal biology, an antioxidant is therefore any compound or mechanism that dampens or counteracts oxidative stress – either by reducing the cause or the consequences of oxidative stress (4, 12-13).

All cells in the human body produce antioxidants

A complex endogenous antioxidant defence system has been developed to counteract oxidative damage and oxidative stress. Such an antioxidant defence is essential for all living organisms exposed to oxygen. The endogenous antioxidant defence system has both enzymatic and non-enzymatic components that prevent ROS formation, remove ROS before damage can occur, repair of oxidative damage and elimination of damaged molecules. Mutations in DNA or genes coding for components of the antioxidant defence system often lead to increased risk of oxidative stress-related diseases as well as premature death. The coding in the genes is not identical from one person to another. There are tiny differences in the DNA sequence which have been inherited from the parents. If these differences in sequence are located within the genes that
contribute to the antioxidant defence system, an altered capability to defend against oxidative stress may appear (12-13).

The antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase serve as a primary line of defence in destroying free radicals. Together, they repair oxidized DNA, degrade oxidized protein, and destroy oxidized lipids. Many other enzymes act as a secondary antioxidant defence mechanism to protect from further damage (12-13).

**Diet can induce the cells to produce more antioxidants**

An important antioxidant defence mechanism involves detoxification enzymes. These enzymes are referred to as phase 2 enzymes or cytoprotective proteins because they catalyze conversion of xenobiotics, drugs and toxic compounds to compounds that are more readily excreted from the body. It is believed that if non-damaging plant compounds induce the phase 2 enzymes, cells are more readily able to neutralize toxic agents when they appear (18-20).

The major plant compounds able to support the antioxidant defence via this mechanism include the glucosinolates and several other sulphur containing plant compounds. Glucosinolates are widespread plant constituents, and it is believed that glucosinolate breakdown products (such as the isothiocyanate sulphoraphane) induce phase 2 enzymes and are therefore responsible for the protective effects shown by Broccoli or similar vegetables. Onion also contains a number of sulphur containing compounds that may induce phase 2 enzymes. Dietary plants rich in compounds that induce phase 2 detoxification enzymes include the vegetables:

- broccoli,
- Brussels sprouts,
- cabbage,
- kale,
- cauliflower,
- carrots,
- onions,
- tomatoes,
- spinach
- garlic

Not only dietary plants, but also physical activity seems to be able to increase the antioxidant defence. It is well known that excessive physical activity may
induce oxidative stress, and it is believed that normal “healthy” physical activity represents a mild oxidative stress that is not damaging in itself, but is able to induce the antioxidant defence. Thus, a little stress is good for your health – while too much may be damaging.

A plant based diet contains thousands of antioxidants

It is widely accepted that a plant based diet with high intake of fruits and vegetables may reduce the risk of oxidative stress-related diseases such as cancer and cardiovascular diseases. Understanding the complex role of diet in chronic diseases is challenging since a typical diet provides more than 25,000 bioactive food constituents, many of which may modify a multitude of processes that are related to these diseases.

Most bioactive food constituents are derived from plants and are collectively called phytochemicals. The majority of these phytochemicals are antioxidants. Since antioxidants can eliminate and counteract the formation of ROS, and these reactive species can contribute to most chronic diseases, we hypothesize that a complex network of many different antioxidants may contribute to the observed protective effects of a plant based diet. In contrast to the hypothesis that high doses of one or a few supplemental antioxidants might be beneficial, we suggest that low concentrations of a large combination of different redox-active phytochemicals explain the observed effects of a plant-based diet (4).

There are several hundred antioxidants belonging to the carotenoids, and a very large class of compounds is called phenols or polyphenols. They are synthesized in large structural varieties belonging to several molecular families such as benzoic acid derivatives, flavonoids, proanthocyanidins, stilbenes, coumarins, lignans and lignins. Over 8000 plant phenols have been isolated. Plant phenols are antioxidants by virtue of the hydrogen-donating properties of the phenolic hydroxyl groups (21-24).

A complete characterization of the total concentration of antioxidants in various foods and food groups is therefore essential. While only some smaller antioxidant food tables (10-100 items) have previously been available, we have now developed a complete antioxidant food table, covering more that 3,500 different foods from many areas of the world (4, 25-27, Carlsen et al, unpublished results). Thus, for the first time it is now possible to identify antioxidant-rich foods, and an antioxidant-rich diet – and study whether such a diet is associated with reduced risk of oxidative stress-related diseases.
Antioxidants defence networks in plant and animal cells

The collection of solar energy and its conversion into chemical energy in plants would not have been possible without a mechanism that effectively eliminates hazardous excess energy and prevents oxidative damage of the plant cell. The role of direct antioxidants in plants is to reduce oxidative stress and thereby keep the plant cells healthy (8).

Many of the antioxidants are produced by plants in response to oxidative stress. If the plant experiences stress (from for example sunlight, drought, microorganisms) they speed up their antioxidant production. Stressed plants therefore contain the most antioxidants (8).

In nature, most antioxidants are produced in plants. Plants are therefore, in general, characterized by high concentrations of numerous antioxidant compounds. Animal cells have a much more limited production of antioxidants. Oxidative damage can therefore more easily accumulate in animal cells when the critical balance between generation of ROS and antioxidant defence is unfavourable. It is suggested that the antioxidants that are essential for the plant cells also may be beneficial for animal cells if the plants are consumed by the animals. It is also suggested that the large and unique patterns of antioxidants that are normally present in plant foods may be necessary for obtaining a health beneficial effect in humans. That is, high concentrations of one single antioxidant (for examples a supplemental antioxidant) can not replace the large spectrum of antioxidants found in plants. Thus, combinations of antioxidants in dietary plants may be more likely to protect against human diseases than antioxidant supplements (4).

Antioxidants have their different functions and cooperates in an integrated manner in plants cells. Many different antioxidants are needed to keep the plant cells healthy. Thus, a network of antioxidants with different chemical properties is needed for proper protection against oxidative stress in plant cells. We suggest that a similar situation exists in human cells and other animal cells. We suggest that a concerted action of a number of dietary antioxidants might be needed for animal cells, exactly as in plants (4).

All parts of the human body and the human cells needs to be protected from oxidative stress. And it is well known that antioxidants cannot replace each other in a biologic system. For example, if a human develops scurvy (also called vitamin C-deficiency), he should be treated with vitamin C. Vitamin E is also a good antioxidant, but it has no effects in treatment of scurvy. The same holds for vitamin E-deficiency which should be treated with vitamin E, not with vitamin C. Thus, vitamin C cannot be replaced by vitamin E, and vice versa (28).
Some of the classical, well-known antioxidants

Most scientific investigations into the role of antioxidants have been focusing on vitamin C, vitamin E, beta-carotene and selenium (28).

- Vitamin E is a term that refers to all entities (eight found so far) that exhibit biological activity as the alfa-tocopherol. Alpha-tocopherol has the highest biological activity in most biological systems. However, recent studies suggest that other forms of vitamin E may be more efficient in specific biological mechanisms. Because it is fat-soluble, alpha-tocopherol is in a unique position to safeguard cell membranes which largely composed of fatty acids from damage by ROS. Alpha-tocopherol also protects the fats or lipid particles in the blood such as low-density lipoproteins (LDLs, or the "bad" cholesterol) from oxidation. Vitamin E is found in vegetable oils, walnuts, peanuts, almonds, seeds, olives, avocado, wheat germ, liver, and leafy green vegetables.

- Vitamin C (ascorbic acid) is a water-soluble vitamin. As such, it scavenges ROS that are in an aqueous environment, such as inside the cells or in the blood. Vitamin C works synergistically with vitamin E to quench ROS and free radicals. Vitamin C also regenerates the reduced form of vitamin E. Good sources of vitamin C are citrus fruits (like oranges and grapefruit), broccoli, leafy green vegetables, tomatoes, peppers, potatoes, cantaloupe (rockmelon), and strawberries.

- Beta-carotene is also a lipid-soluble antioxidant. It is the most widely studied of the 1000 carotenoids identified to date. It is thought to be the best quencher of singlet oxygen (an energized but uncharged form of oxygen that is toxic to cells). Beta-carotene is also excellent at scavenging free radicals in low oxygen concentration. Common sources of beta-carotene include cantaloupe, mangoes, papaya, pumpkin, peppers, spinach, kale, squash, sweet potatoes, and apricots.

- Selenium is a trace element. It is a mineral that we need to consume in only very small quantities, but without which we could not survive. Selenium is not an antioxidant in itself, but is a cofactor for antioxidant enzymes. It forms the active site of several antioxidant enzymes including glutathione peroxidase. Rich sources of selenium include whole grains, seafood, beef, pork, chicken, Brazil nuts and brown rice.

The phenols

Phenols are of great importance in plant physiology with their role in pigmentation, flavour, growth, reproduction and resistance to pathogens and
predators. Several thousand phenols with at least one aromatic ring (phenolic ring) bearing hydroxyl groups have been characterized. The phenols can be divided into subgroups based on the structural components. Distinctions are thus made between the phenolic acids, flavonoids, stilbenes, and lignans (21-24).

Most phenols have antioxidant properties and are the most abundant antioxidants in our diet. The estimated intake is suggested to be as high as 1 g/day where phenolic acid account for about one third of the total intake and flavonoids account for most of the remaining two thirds. The main dietary sources of phenols are fruits and beverages (fruit juice, wine, tea, coffee and chocolate) and, to a lesser extent vegetables, dry legumes and cereals. Certain phenols are found in a wide variety of plants, while others are specific to particular plants. In most cases, foods contain complex mixtures of phenols. Environmental factors such as soil type, sun exposure, temperature and rain fall have major effects on the phenol content of plants. The degree of ripeness also affects the concentration and proportion of phenol, where phenolic acid concentration decrease during ripening, whereas anthocyanin concentrations increase (21-24).

Flavonoids are the most common phenol compound in our diet and human intake is estimated to be between a few hundred mg/d to 650 mg/d. Flavonoids can be divided into 6 major subclasses: flavones, flavonols, flavanones, flavanols (cathechins and proanthocyanidins), anthocyanins, and isoflavones. Flavonoids provide flavour and colour to fruits and vegetables (21-24).

Flavonols are the most widely represented flavonoids in the food. Fruits often contain 5-10 different flavonol glycosides (glycosylated form of flavonols). The flavonols accumulate in the skin and leaves of the fruit because their biosynthesis is stimulated by light. Thus the flavonol concentration of fruits and vegetables depend on the exposure to light.

Phenolic acids can be divided into two subclasses: hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acids are found only in a few plants eaten by humans such as certain berries and onions. Hydroxycinnamic acids are more common and are found in flour, coffee, aubergine, blueberry, kiwi and other fruits and vegetables.
<table>
<thead>
<tr>
<th>Phenolic class</th>
<th>Chemical structure</th>
<th>Dietary sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavonols</td>
<td>![Flavonol Structure]</td>
<td>Quercetin, Kaempherol Cherry tomato, onion, broccoli, tea, red wine, berries</td>
</tr>
<tr>
<td>Flavones</td>
<td>![Flavone Structure]</td>
<td>Apigenin, Luteolin Cereals, parsley, celery</td>
</tr>
<tr>
<td>Flavanones</td>
<td>![Flavanone Structure]</td>
<td>Hesperetin, Naringenin Citrus fruits</td>
</tr>
<tr>
<td>Flavanols</td>
<td>![Flavanol Structure]</td>
<td>Catechins Chocolate, beans, apricot, tea, red wine, cherry, apple</td>
</tr>
<tr>
<td>Anthocyanidins</td>
<td>![Anthocyanidin Structure]</td>
<td>Cyanidin Aubergine, berries, red wine, red cabbage</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>![Isoflavone Structure]</td>
<td>Daidzein, Genistein Soy products, peas</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phenolic acid</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxybenzoic acid</td>
<td>![Hydroxybenzoic Acid Structure]</td>
<td>Gallic acid Berries Onion</td>
</tr>
<tr>
<td>Hydroxycinnamic acid</td>
<td>![Hydroxycinnamic Acid Structure]</td>
<td>Caffeic acid Ferulic acid Blueberry, kiwi, cherry, plum, apple, grains</td>
</tr>
<tr>
<td>Lignans</td>
<td>![Lignan Structure]</td>
<td>Matairesinol Linseed, lentils, cereals, garlic</td>
</tr>
<tr>
<td>Stilbenes</td>
<td>![Stilbene Structure]</td>
<td>Resveratrol Wine, grapes, blueberries</td>
</tr>
</tbody>
</table>
The carotenoids

Another class of antioxidant compounds is the carotenoids. The carotenoids represent a large group of pigments that are widespread in nature and responsible for the yellow, orange, red or purple colours of many vegetables, fruits and flowers. As many as 1000 naturally occurring variants have been identified. Their major role in plants is related to light collecting as auxiliary components and quenching of excited molecules that might be formed during photosynthesis. Carotenoids are lipid soluble pigments that also are widespread in some microorganisms (21-24).

Many of these carotenoids can be absorbed and stored in animals, and often to such a degree that they give color to animal tissues. For example, lutein and zeaxanthin are concentrated in human macula (in the eye), lycopene in human prostate, beta-carotene in bovine corpus luteum (surrounds the ovum) and chicken egg yolk, astaxanthin and canthaxanthin in salmon flesh and flamingo feather.

At least 60 carotenoids occur in fruit and vegetables commonly consumed by humans. Some of the carotenoids can be converted to vitamin A by animals. The most important carotenoids in the diet are:

- \( \alpha \)-carotene
- \( \beta \)-carotene
- b-cryptoxanthin
- lycopene
- lutein
- zeaxanthin

Carotenoids occur in many fruits and vegetables including tomatoes, carrots, watermelon, kale, spinach, see table below.
Table 2. Carotenoids

<table>
<thead>
<tr>
<th>Carotenoids</th>
<th>Dietary sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>lutein</td>
<td>spinach, kale,</td>
</tr>
<tr>
<td></td>
<td>broccoli, brussel</td>
</tr>
<tr>
<td></td>
<td>sprouts</td>
</tr>
<tr>
<td>zeaxanthin</td>
<td>egg yolks, maize,</td>
</tr>
<tr>
<td></td>
<td>spinach</td>
</tr>
<tr>
<td>b-cryptoxanthin</td>
<td>citrus fruits,</td>
</tr>
<tr>
<td></td>
<td>avocado, papaya,</td>
</tr>
<tr>
<td></td>
<td>pepper</td>
</tr>
<tr>
<td>alfa-carotene</td>
<td>carrots, pumpkin,</td>
</tr>
<tr>
<td></td>
<td>maize</td>
</tr>
<tr>
<td>beta-carotene</td>
<td>carrots, spinach,</td>
</tr>
<tr>
<td></td>
<td>parsley</td>
</tr>
<tr>
<td>lycopene</td>
<td>tomato and its</td>
</tr>
<tr>
<td></td>
<td>products, water</td>
</tr>
<tr>
<td></td>
<td>melon, guava</td>
</tr>
</tbody>
</table>
Some additional antioxidants

Several other families of compounds may also function as antioxidants. Depending on the cellular context they may act either directly as antioxidants, or they may act indirectly by enhancing the production of the antioxidant defence. These include:

- Allyl sulfides found in onions, garlic, leeks, chives
- Curcumin found in turmeric
- Indoles found in broccoli, cauliflower, cabbage, russels sprouts, bok choy
- Isothiocyanates (e.g., sulforaphane) found in broccoli, cauliflower, cabbage, brussels sprouts, bok choy
- Lignans: Seeds (flax seeds, sunflower seeds)
- Monoterpenes: Citrus fruit peels, cherries, nuts
- Phytic acid: Whole grains, legumes
- Saponins: Beans, legumes

Antioxidant supplements can be harmful

The health authorities do not recommend using antioxidant supplements but instead recommend that people daily eat a variety of foods from all of the basic food groups. Moreover, in April 2000, the Food and Nutrition Board of the Institute of Medicine, an advisory group that is part of the National Academy of Sciences, USA, reported that Vitamin C, vitamin E, selenium, and carotenoids like beta-carotene should come from food, not supplements. After examining available data on the beneficial and harmful health effects of antioxidants, the panel concluded that there is no evidence to support the use of large doses of these nutrients to combat chronic diseases. In fact, the group warned that high doses of antioxidants may lead to health problems and the risk of toxic reactions (28).

Results from intervention trials with single compounds such as vitamins E, vitamin C or beta-carotene have not supported protective effects against cancer and cardiovascular diseases. Indeed, supplementation with large doses of beta-carotene has resulted in adverse disease outcomes in clinical trials. Firstly, in the ATBC study 29,133 Finnish men aged 50-69 years who smoked five or more cigarettes daily were randomly assigned to receive alfa-tocopherol (50 mg), beta-carotene (20 mg), or a placebo daily for 5-8 years. Disappointingly,
however, the results showed that beta-carotene supplementation was associated with about 20 % increase in lung cancer risk (29-30).

Secondly, similar finding were observed in the CARET study testing 30 mg beta-carotene taken daily against placebo in 18,314 men and women at high risk of developing lung cancer. The CARET intervention was stopped 21 months early because of clear evidence of no benefit and substantial evidence of possible harm; there were 28% more cases of lung cancers and 17% more deaths in the active intervention group that was administered 30 mg beta-carotene per day (31-32). The CARET study also observed that the active-treatment group had a 26 % higher relative risk of death from cardiovascular disease. Thus, after an average of four years of supplementation, beta-carotene had no beneficial effect and in fact appeared to have an adverse effect on the incidence of lung cancer and on the risk of death from lung cancer and cardiovascular diseases in smokers (31-32).

When the frequency of major coronary events in 1,862 men enrolled in the ATBC study (smokers aged between 50 and 69 years who had a previous myocardial infarction) was studied, there were significantly more deaths from fatal coronary heart disease in the beta-carotene groups than in the placebo group – all in agreement with the CARET study.

It is worth noticing that both the ATBC and the CARET studies included only smokers and workers exposed to asbestos. The results of these studies strongly suggest, however, that high doses of supplements containing beta-carotene (20-30 mg) should not be recommend for any group or individuals until the safety of such doses can be established.

Taken together, the data today suggest that a combination of direct antioxidants is healthy, while high doses of single supplemental antioxidants may cause harm. Antioxidant rich foods which contains low doses of a large number of antioxidants is therefore believed to dampen oxidative stress more efficiently than large doses of single antioxidants.

**Determination of the amount of total antioxidants in foods**

To determine the total content of antioxidants in various foods, we used an assay that measures the total concentration of redox active substances with reduction potential above a certain limit. Before we made the determinations, the assay was validated with regard to its ability to measure different antioxidant and non-antioxidant compounds. We have determined the total content of antioxidants of
about 3,500 different foods, beverages, spices, herbs and supplements from around the world (4, 25-27, Carlsen et al, unpublished results).

As shown in the tables we detected large variations both between and within each food category, and all of the food categories contain products almost devoid of antioxidants. The categories “Spices and herbs”, “Herbal medicine” and “Supplements” include the most antioxidant-rich products analyzed. The categories “Berries and berry products”, “Fruit and fruit juices”, “Nuts and seeds”, “Breakfast Cereals”, “Chocolate and sweets” and “Vegetables and vegetable products” include most of the common foods and beverages which have medium to high antioxidant values (Carlsen et al, unpublished results).

The average antioxidant value of all the products in the database is 11.6 mmol/100g, while the median value is 0.502 mmol/100g. The high average value is due to a minority of products with very high antioxidant values, found among the herbal medicines, spices, herbs and supplements. If we exclude these categories from our calculations the mean and median values are 2.2 and 0.4 mmol/100g, respectively. In addition we find that plant based foods are much higher in antioxidant content than animal based products; with average antioxidant values of 3.4 and 0.1 mmol/100g, respectively (Carlsen et al, unpublished results).

Our results show that antioxidant rich foods originate from the plant kingdom while meat, fish and other foods from the animal kingdom are low in antioxidants. Comparing the mean value of the ‘Meat, meat products and substitutes’ category with plant based categories, fruits, nuts, chocolate and berries have 4.7, 15.3, 16.3 and 33.0 times higher average antioxidant content than the average meat product, respectively, showing the important dietary antioxidant contribution from these plant based foods. Diets comprised mainly of animal based foods are thus low in antioxidant content while vegetarian diets and diets based mainly on a variety of plant based foods are antioxidant rich, due to the thousands of bioactive antioxidant phytochemicals found in plants which are conserved in many foods and beverages.
Table 3. Summary of antioxidant analysis (Carlsen et al, submitted)

<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>mean</th>
<th>median</th>
<th>min</th>
<th>max</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plant based foods</strong></td>
<td>1943</td>
<td>11.57</td>
<td>0.88</td>
<td>0.00</td>
<td>2897.11</td>
</tr>
<tr>
<td><strong>Animal based foods</strong></td>
<td>211</td>
<td>0.18</td>
<td>0.10</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>Mixed foods</strong></td>
<td>854</td>
<td>0.91</td>
<td>0.31</td>
<td>0.00</td>
<td>18.52</td>
</tr>
</tbody>
</table>

**Categories**

<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>mean</th>
<th>median</th>
<th>min</th>
<th>max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berries and berry products</td>
<td>119</td>
<td>9.86</td>
<td>3.34</td>
<td>0.06</td>
<td>261.53</td>
</tr>
<tr>
<td>Beverages</td>
<td>283</td>
<td>8.30</td>
<td>0.60</td>
<td>0.00</td>
<td>1347.83</td>
</tr>
<tr>
<td>Breakfast cereals</td>
<td>90</td>
<td>1.09</td>
<td>0.89</td>
<td>0.16</td>
<td>4.84</td>
</tr>
<tr>
<td>Chocolates and sweets</td>
<td>80</td>
<td>4.93</td>
<td>2.33</td>
<td>0.05</td>
<td>14.98</td>
</tr>
<tr>
<td>Dairy products</td>
<td>86</td>
<td>0.14</td>
<td>0.06</td>
<td>0.00</td>
<td>0.78</td>
</tr>
<tr>
<td>Desserts and cakes</td>
<td>134</td>
<td>0.45</td>
<td>0.20</td>
<td>0.00</td>
<td>4.10</td>
</tr>
<tr>
<td>Egg</td>
<td>12</td>
<td>0.04</td>
<td>0.04</td>
<td>0.00</td>
<td>0.16</td>
</tr>
<tr>
<td>Fats and oils</td>
<td>38</td>
<td>0.51</td>
<td>0.39</td>
<td>0.19</td>
<td>1.66</td>
</tr>
<tr>
<td>Fish and seafood</td>
<td>32</td>
<td>0.11</td>
<td>0.08</td>
<td>0.03</td>
<td>0.65</td>
</tr>
<tr>
<td>Fruit and fruit juices</td>
<td>278</td>
<td>1.25</td>
<td>0.69</td>
<td>0.03</td>
<td>55.52</td>
</tr>
<tr>
<td>Grains and grain products</td>
<td>227</td>
<td>0.34</td>
<td>0.18</td>
<td>0.00</td>
<td>3.31</td>
</tr>
<tr>
<td>Herbal / traditional plant medicine</td>
<td>59</td>
<td>91.72</td>
<td>14.18</td>
<td>0.28</td>
<td>2897.11</td>
</tr>
<tr>
<td>Infant foods and beverages</td>
<td>52</td>
<td>0.77</td>
<td>0.12</td>
<td>0.02</td>
<td>18.52</td>
</tr>
<tr>
<td>Legumes</td>
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<td>0.48</td>
<td>0.27</td>
<td>0.00</td>
<td>1.97</td>
</tr>
<tr>
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<td>0.31</td>
<td>0.32</td>
<td>0.00</td>
<td>0.85</td>
</tr>
<tr>
<td>Miscellaneous ingredients, condiments</td>
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<td>0.77</td>
<td>0.15</td>
<td>0.00</td>
<td>15.54</td>
</tr>
<tr>
<td>Mixed food entrees</td>
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<td>0.19</td>
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<td>0.03</td>
<td>0.73</td>
</tr>
<tr>
<td>Nuts and seeds</td>
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<td>0.03</td>
<td>33.29</td>
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<td>0.15</td>
<td>0.05</td>
<td>1.00</td>
</tr>
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<td>0.00</td>
<td>1.17</td>
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<tr>
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<td>0.00</td>
<td>4.67</td>
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<tr>
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<td>0.08</td>
<td>465.32</td>
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<td>0.00</td>
<td>1052.44</td>
</tr>
<tr>
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<td>0.80</td>
<td>0.31</td>
<td>0.00</td>
<td>48.07</td>
</tr>
</tbody>
</table>

**Conclusion**

It is suggested that the total antioxidant content of dietary plants may be a useful tool for testing the antioxidant hypothesis. Several berries, fruits, nuts, seeds, vegetables, drinks and spices have been found to be high in total antioxidants. Additionally, some compounds found in brassica and allium vegetables may improve the endogenous antioxidant defence through induction of antioxidant
and phase 2 enzymes. Further research is needed to clarify if such dietary plants can reduce pathogenesis related to oxidative stress-related diseases.

References


Compounds that may be responsible for reduced risk for cardiovascular diseases related to consumption of vegetables, fruit and berries

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Abstract

An increased intake of fruit, berries and vegetables is usually recommended for subjects with high risk for coronary heart disease. These commodities generally have high content of vitamin C, nitrate, flavonoids and carotenoids that may preserve the endothelium capacity to produce nitric oxide (NO); a vasodilator with anti-atherogenic properties. Components in fruit, berries and vegetables that are involved in the NO metabolism need further investigation to elucidate their possible role in risk reduction for cardiovascular disease. We report here results of studies on effects of fruit, berries and vegetables in subjects with high risk for coronary heart disease with focus on our own trials. In the first study, change in dietary intake of vitamin C was inversely associated with intima media thickness (IMT) progression \( r = -0.181, P = 0.006 \) as was increased intake of fruit and berries \( r = -0.125, P = 0.056 \). In multivariate analyses adjusted for confounders, the increased intake of vitamin C explained 4 %, and fruit and berries explained 3 % of the variation in the progression of carotid IMT after three years. In the second study, a 500 g increase in daily intake of vegetables and fruit resulting in increased concentrations of plasma \( \alpha \)-carotene and \( \beta \)-carotene, led to a 2.5 kg weight loss and reduced systolic and diastolic blood pressure compared to controls during three months. We conclude that in subjects with increased risk for coronary heart disease, consumption of vegetables, fruit and berries has beneficial effects on end points related to cardiovascular risk factors.
**Introduction**

Even a small reduction in risk of cardiovascular disease has major public health implications. A number of meta-analyses have shown reduced risk with increased intakes of fruit and vegetables (1-3). Consuming more than five servings of fruit and vegetables per day has been associated with substantial decreased risk for coronary heart disease and stroke (2, 3). Blood pressure is a strong, independent and causal risk factor for stroke, and it has been estimated that a reduction of systolic blood pressure (BP) of only 3 mmHg can reduce mortality of stroke by about 8 % (4). Thus, an increased intake of vegetables and fruit is usually recommended to subjects with high risk for coronary heart disease. However, the compounds responsible for the beneficial effects are not elucidated. In addition to vitamin C, folate, potassium and soluble fibre, fruit and vegetables also have high contents of nitrate, flavonoids and carotenoids that may have favourable effects on vascular health. These compounds appear to preserve the endothelium’s capacity to produce nitric oxide (NO) (5-10).

The NO is an endogenous gas synthesized by NO synthase and has important effect as endothelium derived vasodilator. It regulates vascular tone and BP. In addition, NO has shown anti-atherogenic effects on smooth muscle cell proliferations, platelet aggregation and inflammation (5). However, NO is a reactive compound that rapidly may be transformed to peroxynitrite by superoxide anions or other endogenous oxidants that favour NO synthase uncoupling (9). Thus, increased intake of components that allow for higher bioavailability of NO by decreasing endogenous oxidant formation or by increasing precursors for NO formation may increase vascular protection.

The thickness of the carotid intima media is closely correlated to atherosclerosis (11) and coronary artery disease (12) and is used as a surrogate end point for cardiovascular disease. Ultrasound scanning is a non-invasive method to detect structural changes in the carotid artery.

In the following we will focus on the results of two studies with the aim to investigate effects of intake of vegetables, fruit and berries on cardiovascular risk factors and carotid intima media thickness (IMT). Furthermore, we will discuss the possible impacts of antioxidants assessed or measured in these studies on NO.
The effect of fruit, berries and vegetables on thickness of carotid intima media

The Diet and Omega-3 fatty acid Intervention Trial (DOIT) showed that increased intake of vitamin C was inversely associated with IMT progression ($r = -0.181, P = 0.006$) as was increased intake of fruit and berries ($r = -0.125, P = 0.056$) among participants in the intervention group (13). The DOIT was a randomised, controlled, clinical trial that determined the effect of atherosclerosis in a cohort of elderly men followed for three years (14). The men participated in the Oslo Diet and Antismoke Trial in 1972. Of the original cohort of 1272 men, 910 men were invited to participate in the DOIT study and 563 men with a mean age of 70 years were included in the present study. They were randomised to one of four groups: dietary intervention, omega-3 supplementation, both or neither.

No effect of the omega-3 intervention on carotid progression was seen and the subjects were pooled to dietary intervention or no dietary intervention. In the dietary intervention, the participants were instructed to follow a Mediterranean-like diet based on usual Norwegian foods. They were asked to increase their intake of fresh fruit and salads, to vary their diet with as many different fruits, berries and vegetables as possible and to drink a glass of orange juice every morning. Cold pressed, rapeseed oil (Askim Frukt og Bærpresseri AS, Askim, Norway) was provided and the participants were recommended to use an amount of 10-20 ml per day of this oil. Wine limited to 1 glass per day was the preferred choice of alcohol in the dietary intervention.

After three years, 233 men in the intervention group and 231 in the control group completed the study. The dietary intervention group showed less progression of the carotid IMT compared to the control group. In multivariate analyses adjusted for confounders, the increased intake of vitamin C explained 4% and fruit and berries explained 3% of the variation in the progression of carotid IMT. However, in the intervention group the change from baseline was 13 mg vitamin C, giving a total intake of about 145 mg per day. This intake is well above recommended intake in the Nordic countries (15), but less than the dose of 200-1000 mg per day that is necessary to produce plasma saturation of 80 to 100 % (16). The relatively low increased intake of vitamin C may be too small to affect atherogenesis by itself. We expect that the increased intake of vitamin C rich fruit, berries and vegetables also increased intake of other components such as carotenoids and flavonoids. In combination these components of vegetables and fruit may influence atherosclerosis by impacts on endothelial function via bioavailability of NO. In line with this, a three-year controlled intervention study in 19 patients with coronary artery disease showed
reduction in carotid IMT, LDL oxidation and BP by daily intake of 50 ml pomegranate juice that was high in vitamin C, tannins and anthocyanins (17). A limitation in the DOIT was that no plasma concentrations of vitamin C, carotenoids or flavonoids were measured.

The effect on fruit and vegetables on blood pressure

In a three-month dietary intervention including obese subjects with sleep related breathing disorders (SRBD), an increased intake of fruit and vegetables of about 500 g per day resulted in increased concentrations of plasma carotenoids, weight loss and reduced systolic and diastolic BP compared to controls (18). The mean group difference in weight loss between the intervention group (n = 68) and the controls (n = 57) was 2.5 kg. The between group difference in systolic and diastolic BP was –7 mmHg (P = 0.0022) and –4 mmHg (P = 0.0120), respectively.

In this study weight loss was achieved by using increased intake of vegetable and fruit as a tool to decrease energy density in the diet. Weight loss will reduce BP and this may be the most likely explanation for the BP reduction seen. However, plasma concentrations of carotenoids used as biomarkers for the reported intake of fruit and vegetables were increased for α-carotene and β-carotene. Increased concentrations of carotenoids have shown beneficial effects on cardiovascular disease (19) and BP (20, 21).

In a six-month randomized, controlled, intervention study in 690 healthy subjects, systolic and diastolic BP fell more, and plasma concentrations of α-carotene, β-carotene, β-cryptoxanthin together with vitamin C increased more in the intervention group compared to the control group (20). Recently, it was reported that among more than 4000 young adults followed for 15 years, those with higher concentrations of carotenoids (α-carotene, β-carotene, lutein/xeaxanthin and cryptoxanthin) had 16 % lower risk for hypertension (21).

SRBD have been associated with increased risk for hypertension and cardiovascular disease (22). Increased sympathetic activity, endothelial dysfunction and inflammatory processes have been associated with SRBD in clinical studies. Thus, patients with SRBD may show sympathetic over-activity and increased oxidative stress, insulin resistance and a pro-thrombotic state (23-25). Indeed, oxidative stress has recently been identified to have a possible role in the pathogenesis of hypertension. An imbalance between superoxide and NO production may account for reduced vasodilatation and in turn the development
of hypertension (26). Hence, an increased intake of antioxidants may reduce the amount of free radicals and increase the NO formation. Increased plasma concentrations of α-carotene and β-carotene were seen in the study (18) but total antioxidant capacity measured by the ferric reducing antioxidant power (FRAP) assay was not increased. This may be because the FRAP method is better to measure antioxidant capacity in foods than in plasma (27), better to measure bioavailability or the total amount ingested.

The intervention group reported higher intake of vitamin C and β-carotene. Unfortunately, other antioxidants than vitamin C and carotenoids were not recorded in our study. However, estimation of the antioxidant content of the Dietary Approaches to Stop Hypertension diet; a diet rich in vegetables and fruit showed higher contents of flavones, flavanones, flavan-3-ols, β-carotene, β-cryptoxanthin, lycopene, phytosterols and lutein/xeaxanthin compared to the control diet (28).

Recently, a favourable effect of berry consumption on systolic BP in hypertensive was reported (29). Berries are high in antioxidants and the authors hypothesized that the effects could be partly explained by change in NO metabolism. Recent research has demonstrated that NO can be generated from nitrite via several routes involving enzymes, proteins and vitamins (9). Indeed, a BP lowering effect of beetroot juice was recently demonstrated in healthy males. The authors explained the effect by the increase in generation of NO from increased levels of plasma nitrate and nitrite, causing vasodilatation in resistant vessels and a consequent lowering of BP (10). In general, dark green and leafy vegetables have higher content of nitrate than fruit (30). However, it has been shown that ingestion of modest amounts of a concentrate of fruit- and vegetable juice low in nitrate has increased the serum concentration of nitrate (31).

**Conclusion**

We have shown that in subjects with increased risk for coronary heart disease, increased intake of fruit and vegetables have beneficial effects on end points related to cardiovascular risk factors. Increased intake of vitamin C was associated with less progression of IMT. During weight loss achieved by an increased intake of vegetables and fruit, concentrations of plasma α-carotene and β-carotene was higher and BP lower compared to controls. Which components in vegetables and fruit that is responsible for the beneficial effects on the endothelial and vascular system involved in atherogenesis and BP regulation is yet not known, but mechanism involving NO needs further investigation. In the
mean time, the message to eat at least five servings of different fruit, berries and vegetables per day will increase the diet with compounds that have beneficial health effects.

References

28. Most M. Estimated phytochemical content of the Dietary Approaches to Stop Hypertension (DASH) diet is higher than in the control study diet. *J Am Diet Assoc* 2004; **104**: 1725-1727.
Phytoestrogens and human health

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Abstract

A brief overview of the associations between phytoestrogens, particularly isoflavones and lignans, and human health is presented. The results of studies on isoflavones and lignans and the role of formation of active metabolites in the gut are discussed. Isoflavones particularly present in soy and lignans particularly in the bran fraction of cereals and in flax and sesame seed seem to be associated with risk for several diseases. Lignans may reduce the risk for breast and colon cancer as well as for coronary heart disease. Finally a new group of compounds in whole grain wheat and rye, the alkylresorcinols (ARs), are presented. Determination of these compounds or their metabolites (DHBA and DHPPA) in blood or urine may be used for the evaluation of cereal fiber intake in human subjects. Our first results indicate that AR metabolites in urine are low in breast cancer, indicating that the consumption of cereal fiber and lignans from wheat and rye is low in these subjects. This was confirmed by recording fiber intake during all four seasons in all subjects. It is concluded that phytoestrogens, particularly associated with cereal fiber may have an important role for our health, but much work is still needed. This role is partly mediated by the activities of the colonic bacteria producing equol and enterolactone from their respective precursors in the plants and being important for the fermentation of fiber and starch. The assay of ARs and their metabolites in blood or urine is a good tool in the work on the relation between cereal fiber and human disease.
Introduction

Phytoestrogens are weakly estrogenic compounds in plants abundant in our diet. They bind to estrogen receptors and function both as estrogens and antiestrogens and have numerous other biological effects today still largely unknown. The two main groups are isoflavones and lignans, but also coumestans and many flavonoids are included because of their estrogenic activity. They are all polyphenols and have antioxidative properties. This brief overview on the possible associations between phytoestrogens and human health is restricted to certain topics that may be of general interest and is not a comprehensive discussion.

In 1979 we detected in collaboration with Ken Setchell two unknown compounds in the urine of green monkeys and later in human subjects (1) and their identification revealed that they were lignans (2). In the same number of Nature another group had reached the same conclusion with regard to the identity of the two unknown compounds (3). Because they are formed from plant precursors in the large bowel by bacteria they are nowadays called enterolignans (former mammalian lignans) and named enterodiol and enterolactone (4). The first small clinical study (5, 6) revealed that healthy breast cancer survivors had significantly lower urinary enterolactone and equol (not significant) than the controls, and vegetarians had the highest values. This was the start of studies on phytoestrogens and breast and other cancer still going on.

Equol from isoflavones – important for human health?

Isoflavones have been well known from veterinary medicine, particularly the problem with the clover disease in grazing sheep in Australia (7). Some clover species contain considerable amounts of formononetin that is converted in the gut to daidzein and further to equol. A special type of clover has very high content of formononetin (Trifolium subterraneum) (8). The high equol levels cause infertility in sheep and this is due to its high estrogenicity. However in human subjects such problems have not been documented (9) and it is known that are species difference regarding sensitivity to the estrogenic effects of isoflavones.

Equol was first detected in urine of pregnant mares (10) and then in many other animals species, but first 50 years later equol was identified in human urine (5, 11). The amount excreted in urine in non-vegetarians consuming a Western diet is small and is derived mainly from milk products that contain equol formed in the rumen of cows grazing clover or receiving soy in their feed.
Later on it became important to know more about the formation of equol in the gut. Several gut bacteria are able to produce equol from daidzein (12). It was found that those women who could convert daidzein to equol had a benefit from consuming soy products. Those who were producers of equol had less menopausal symptoms, osteoporosis, and less cardiovascular problems and may be cancer (13). Only 25-30 % of subjects consuming a Western diet are able to produce significant amounts of equol, but more than 50 % of Japanese subjects. If small amounts of soy is given to human subjects the equol producers can be distinguished from the non-producers by measuring equol in urine (14) or the equol/daidzein ratio in plasma (15).

Equol is the most potent antioxidant of all isoflavones (13, 16) and may be responsible for the observed decrease of cholesterol and LDL cholesterol in hypercholeolemic subjects, for increased LDL oxidation resistance, for decrease of circulating oxidized LDL particles, and plasma F2-isoprostanes and finally for improving endothelial function. However, the soy isoflavone, genistein, may also be involved because it is almost as estrogenic as equol.

The highest concentrations of equol in urine were from chimpanzees in captivity (almost 100 μmol/l). In Japanese humans we found a mean value of 2.8 μmol/24h urine and similar amounts in macrobiotics, a certain type of vegetarians, living in USA. It is of interest to note that non-human primates are very resistant to breast cancer even after administration of carcinogenic compounds (17) and we have suggested that the extremely high production of equol in these animals could be involved (18,19).

Antibiotics reduce equol production in monkeys by 80-90 %. Some of the antibiotics caused an increase in plasma levels of daidzein, genistein and glycine in which can be explained by the destruction of the gut bacteria metabolizing these isoflavones (20). The human microflora can exclusively produce S-equol enantiomers. S-equol has high affinity for the estrogen receptor ERβ, and R-equol has much less affinity (13, 21). Thus it was suggested that S-equol is biologically more active. However recently, it is found that racemic equol has strong antigenotoxic effects in contrast to the purified S-equol enantiomer suggesting that the R- rather than the S-enantiomer as being responsible for the strong antioxidant effects of equol (22).

We obtain isoflavonoids from food practically only from soy and small amounts from milk products. Some bread may also contain soy protein containing some isoflavones. Nowadays many companies produce tablets and other supplements containing relatively high amounts of isoflavones. There has been much discussion on the positive and negative effects of soy intake. The literature is
extensive (see (9, 23) and it is not the topic of this publication. However, one problem I like to deal with is the possible effect of soy isoflavones on the fetus in pregnant women (24). We found that genistein and daidzein and in particular equol concentration in maternal plasma in Japanese women at delivery showed a positive correlation with those in cord plasma. However, the incidence of hypospadias, a malformation in newborn boys caused by estrogens, is remarkably low in Japan compared to England and France suggesting that the continuous consumption of soy products in pregnancy is not causing this malformation. Environmental estrogens are the likely cause in industrialized countries.

Breast cancer has in the past been a relatively rare cancer in Japan and we suggested in the beginning of the 1980ies that phytoestrogens could be protective. In experimental studies, Lamartinier et al. showed that soy intake during adolescence reduced the incidence of breast cancer in rats (25, 26). Only a few days of soy feeding were enough. The isoflavones affect the breast duct end buds making them less sensitive to toxic compounds causing breast cancer. Several studies have now confirmed this observation also in women. Some soy in the food 1-3 times per week in adolescence may give protection against breast cancer (27). It is not necessary to consume soy products every day.

Another interesting topic is the role of isoflavones in prostate cancer. In rats equol administration reduces the weight mainly of the ventral prostate and protects against prostatitis (28). Equol binds to 5α-dihydrotestosterone (DHT), the most biologically active androgen. When coupled to equol, DHT cannot anymore bind to the androgen receptor and the androgen activity in the prostate decreases (29).

Several epidemiological studies suggest that equol non-producers have greater risk for prostate cancer than equol producers (30-32). This suggests that equol or some other metabolites of daidzein is responsible for the low prostate cancer incidence in Asian countries.

Lignans and cancer

Lignans are polyphenols and are regarded as phytoestrogens because some of them bind to the estrogen receptor and have estrogenic activity. They are minor components in numerous edible plants and form the building blocks for the fiber lignin. In the gut both lignin and lignans can be degraded by intestinal bacteria to enterolactone (33), the main lignan metabolite in the urine of mammals. The history of the detection, identification and first studies on lignans during almost 10 years has been described in (34). Because first recently methods for
quantification of other lignans than secoisolariciresinol and matairesinol in edible plants were developed very little is known about the plant lignans (35). Now methods for 4 or more lignans in food are available (36-39). Our proposed metabolic scheme for some of the lignans in the gut is shown in Fig. 1. This is also the biosynthetic pathway for these compounds. The conversion of secoisolariciresinol to enterodiol and enterolactone occurs in the gut as well as the conversion of matairesinol to enterolactone occur in the gut (40).

![Chemical structures](image)

**Fig. 1**
Proposed metabolism of lignans in the gut, modified from reference (40).

Recently, 24 lignans were identified and determined in 18 bran extracts from 16 cereal species, in four nut species and in two oilseeds (sesam seed and linseed). Of these 18 lignans were previously unidentified in cereals (41). The role for all these cereal lignans for our health is unknown but for some there is some information (42). Wheat and rye bran have the highest content of lignans, but the two oilseeds have even higher concentrations (Table 1). Because it was thought that they are only formed by bacteria in the intestinal tract of mammals the enterolignans have previously been called “mammalian lignans”. Now it has been shown that they also occur in water and in plants (43-45) and that plants can take up lignan-containing water.
Table 1. Concentrations of lignans in whole-grain cereals and oil seeds (μg/100 g wet weight). Data from (39, 60).

<table>
<thead>
<tr>
<th>Lignan</th>
<th>Flax</th>
<th>Sesame</th>
<th>Rye</th>
<th>Buckwheat</th>
<th>Oat</th>
<th>Wheat</th>
<th>Barley</th>
<th>Millet</th>
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<td>323670</td>
<td>14</td>
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<td>131</td>
<td>19</td>
<td>35</td>
<td>30</td>
<td>67</td>
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<tr>
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<td>5202</td>
<td>734</td>
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<td>1</td>
<td>71</td>
<td>3</td>
<td>3</td>
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<td>183</td>
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<td>85</td>
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<td>37</td>
<td>72</td>
<td>85</td>
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<td>0</td>
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<td>33</td>
<td>40</td>
<td>30</td>
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<td>8</td>
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<td>1891</td>
<td>867</td>
<td>859</td>
<td>507</td>
<td>370</td>
<td>245</td>
</tr>
</tbody>
</table>

Abbreviations: SEC = secoisolariciresinol; MAT = matairesinol; LAR = lariciresinol; PIN = pinoresinol; MED = medioresinol; SYR = Syringaresinol; SES = sesamin (lignan precursor).

The determinants of plasma enterolactone in man are different in various countries. Determinants in populations of Finland, USA and the Netherlands are shown in (Table 2). It seems that the origin of the lignans in the food is associated, in addition with the plasma concentration, with disease risk. In many studies the association between low breast cancer risk and high enterolactone levels in plasma could not be found, however in other there has been an association. The same seems to be true for the association between enterolactone and other cancers like prostate and colon cancer. If the high enterolactone level is particularly associated with high cereal fiber intake a protective effect can be observed. This means that additional factors in the fiber fraction or the fiber itself play a role. The activity of the microflora is likely to be the major determinant of plasma enterolactone.

Consequently, we suggested that the source of enterolactone that was responsible for high blood level of enterolactone is important (46). This is because it seems that fiber is needed in addition to enterolactone. In Finland, one study in Eastern area where whole grain rye intake is at the highest, an inverse correlation between breast cancer risk, and the lignan level was found (46). In Western and Central Finland, however, with known lower intake of whole grain rye no association between enterolactone and breast cancer risk was found (47).
Table 2. Determinants of plasma enterolactone in populations of various countries.

<table>
<thead>
<tr>
<th>Country and ref.</th>
<th>Food</th>
<th>Positive (pos) or negative (neg) association</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Finland</strong> (76)</td>
<td>Whole-grain products</td>
<td>pos</td>
</tr>
<tr>
<td></td>
<td>Water-insoluble fiber</td>
<td>pos</td>
</tr>
<tr>
<td></td>
<td>Rye products</td>
<td>pos</td>
</tr>
<tr>
<td></td>
<td>Berries</td>
<td>pos</td>
</tr>
<tr>
<td></td>
<td>Total vegetables</td>
<td>pos</td>
</tr>
<tr>
<td></td>
<td>Tea</td>
<td>pos</td>
</tr>
<tr>
<td></td>
<td>Body mass index</td>
<td>neg</td>
</tr>
<tr>
<td></td>
<td>Smoking</td>
<td>neg</td>
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<tr>
<td></td>
<td>Constipation</td>
<td>pos</td>
</tr>
<tr>
<td></td>
<td>Antibiotics</td>
<td>neg</td>
</tr>
<tr>
<td><strong>USA</strong> (77)</td>
<td>Insoluble fiber</td>
<td>pos</td>
</tr>
<tr>
<td></td>
<td>Plant food</td>
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</tr>
<tr>
<td></td>
<td>Total fruit and vegetables + fruit juice</td>
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</tr>
<tr>
<td></td>
<td>Tea and Coffee</td>
<td>pos</td>
</tr>
<tr>
<td></td>
<td>Alcoholic beverages</td>
<td>pos</td>
</tr>
<tr>
<td><strong>The Netherlands</strong> (78)</td>
<td>Grain</td>
<td>pos (9% from bread)</td>
</tr>
<tr>
<td></td>
<td>Fruit</td>
<td>pos</td>
</tr>
<tr>
<td></td>
<td>Wine, tea and coffee, juices (main part)</td>
<td>pos</td>
</tr>
</tbody>
</table>

Some particularly interesting enterolactone and breast cancer studies will be discussed:
In an intervention study with pure enterolactone in rats, plasma levels of enterolactone was 400 nmol/l and this was sufficient to inhibit the growth of 7,12-dimethylbenz(a)anthracene-induced breast cancer (48). In women with breast cancer, intake of 25 g of flaxseed/day during 32 days led to a decrease in markers of proliferation and an increase in apoptosis by 31 % (49). In women with one or two A2 alleles of CYP 17, high level of enterolactone was inversely related to breast cancer risk (50, 51). High lignan intake in girls during adolescence reduced breast cancer risk with a significant trend (52). In a large study in Sweden (53), the authors concluded: 1) The study supports the conclusion that enterolactone is a biomarker of a healthy lifestyle. 2) The protective association between enterolactone and breast cancer was significantly different between ERβ(-) and ERβ(+) tumors and most evident in tumors that express ERα but no ERβ. 3) Plasma enterolactone showed statistically significant correlations with total fiber, fruit and berries, and high-fiber bread intake.
Results from *in vitro* studies indicate that lignans, particularly matairesinol and enterolactone may protect against prostate cancer (54). However, the majority of case-control and prospective studies have given negative results (55-58). One possibility is that the enterolactone concentrations have been too low because few subjects in the Western world have optimal plasma levels of enterolactone (30-80 nmol/l). Some studies have found an inverse association between consumption of lignan-rich food and prostate cancer (see literature in (59)). But usually the materials have been small and the amount of various lignans in the food has been unknown because measurements have not been done. No overall association between dietary intake of total or individual lignans and prostate cancer could be found (59), but high intake of food items rich in phytoestrogens was associated with a decreased risk of prostate cancer. These food items were flax seed, sunflower seed, berries, peanuts, beans and soy. Soy beans contains in addition to isoflavones relatively much lignans (60). Intermediate plasma levels of enterolactone (15-24 nmol/l) have been associated with a decreased risk of prostate cancer (59). In this study the material was larger and the number of lignans determined in the food higher. It is concluded that evidence for a role of lignans in prostate cancer is scanty but lignans may afford protection for some men (see (61)).

Only few studies on lignans and colorectal cancer have been carried out. Studies in rodents using rye bran and flax seed have shown very variable results. The use of Apc<sup>min</sup> mice may not be a good model because most of the tumors are found in the small intestine and enterolactone is formed in the upper part of the large bowel. High plasma enterolactone and particularly enterodiol were found to be inversely associated with colorectal adenoma risk (62). Later the same group came to the opposite conclusion in a prospective study after adjustment for known colorectal cancer risk factors (63). Current smokers had increased risk but never smokers had decreased risk. Smoking is associated with lower enterolactone levels in blood but the mechanism is unknown. Because fiber in itself is believed to protect against colorectal cancer, a possible role of lignans is difficult to separate from the fiber effect. Another study found no interactions between lignans and polymorphic genes that could encode enzymes possibly involved in metabolism of lignans, and colorectal cancer risk. However, calculating the intake of secoisolariciresinol and matairesinol, they found that high intake was associated with considerable reduction in colorectal cancer risk (OR = 0.73). The same was true also for isoflavonoids intake and when all phytoestrogens were summed the OR decreased to 0.71 (64).
Lignans and coronary heart disease

Recently I have discussed the possible role of lignans in cardiovascular disease (61). It has been shown that cereal fiber is more protective than vegetable and fruit fiber. In smoking middle-aged men, a 10 g greater intake of fiber reduced the risk of coronary death by 18 % (65). Further support for the view that whole grain cereals are protective with regard to atherosclerosis was obtained in a recent study showing that cereal fibre and whole-grain intake are associated with reduced progression of coronary artery atherosclerosis in postmenopausal women with coronary heart disease (CHD) (66).

Because enterolactone production in the gut is associated, as found in Finland, particularly with cereal fibre intake, we carried out a prospective study in 167 middle-aged men living in the Kuopio area in Northeast Finland following them for an average of 7.7 years to an acute coronary event. In this region cereal fiber intake is relatively high because of high consumption of whole grain rye bread. The men in the highest quarter of the enterolactone distribution (>30.1 nmol/L) had a 58.8 % (95 % CI 24.1-77.6, P=0.005) lower risk of acute coronary events than men in the lowest quarter. After adjustment for the nine most strongly predictive risk factors, men in the highest enterolactone quarter had a 65.3 % (11.9-86.3, P=0.03) lower risk than men in the lowest quarter (67).

In another study in the same region, the serum enterolactone concentration and cardiovascular risk factors were determined in 1889 men aged 42 to 60 years. In an average follow-up of 12.2 years, 70 CHD-related, 103 cardiovascular (CVD) related, and 242 all-cause deaths occurred in participants free of prior CVD. Multivariate analyses showed significant associations between elevated serum enterolactone concentration and reduced risk of CHD- and CVD-related mortality, but weaker associations in relation to all-cause mortality. In the Cox proportional hazards regression model adjusting for the most potent confounding factors, the risk of CHD-related (P=0.03 for trend) and CVD-related (P=0.04 for trend) death decreased linearly across quartiles of serum enterolactone concentration (68). In addition, the correlation coefficient for association between serum enterolactone and F-2-isoprostane (a measure of lipid oxidation) concentrations was -0.30 (P<0.003). Serum F-2-isoprostane levels decreased linearly across quintiles of serum enterolactone concentration (P=0.008 for a linear trend) (69). In a multivariate model, enterolactone persisted as a significant predictor after adjustment for vitamins and other variables, with the strongest associations with F-2-isoprostanes (69).

Many other studies have been carried out and the protective role of whole-grain cereals against cardiovascular disease in man is well established. There are also studies with negative results (70). We think that this is due to the fact that
enterolactone in these investigations are mainly derived from other components in the diet than cereal fiber, probably wine, tea and coffee. However, the mechanism by which cereal fiber protects is unknown. Lignans may play a role but other phytochemicals must be involved. It is likely that the whole fiber complex with all its antioxidants is responsible for the protection.

## Alkylresorcinols – biomarkers of whole-grain intake and breast cancer risk

Alkylresorcinols (ARs), a group of phenolic lipids, are 1,3-dihydroxy-5-alkylbenzene derivatives with an odd-numbered alkyl chain from 15 to 25 carbons long. They occur particularly in the bran fraction of whole-grain rye and wheat and in smaller amounts in triticale and barley grains but not in highly refined flour (71). The structure of the ARs and their two metabolites (DHBA = 3,5-dihydroxybenzoic acid and DHPPA = 3,5-dihydroxyphenyl-1-propanoic acid) are seen in Fig. 2. They cannot be regarded as phytoestrogens, but no studies on the estrogenicity of ARs or their metabolites have been carried out. All can be determined in plasma and urine (70-72). Cereal fiber consumption correlates highly significantly with individual ARs in plasma and with the metabolites in plasma and urine. The best correlation is obtained between cereal fiber intake and urinary DHPPA (r=0.48; P=0.002). Alkylresorcinols are antioxidants and may also be involved in the protection afforded by whole-grain products against disease.

Our hypothesis since many years has been that cereal fiber protects against breast cancer (72,73). This has also been proposed by others (74). Now we had the possibility to study cereal fiber intake by measuring alkylresorcinol metabolites in urine. We have studied 3 groups of women, vegetarians (n = 20), omnivores (n = 20) and healthy breast cancer survivors (n = 16) and found that the excretion of DHBA and DHPPA was highly significantly lower in the healthy breast cancer survivors indicating low intake of whole-grain bread and cereals. We measured the compounds 4 times during one year in all subjects. The P-values for differences were all 0.003 or lower with the highest values found for the vegetarians (accepted for publication). The usefulness of this new method will be great because lack of cereal fiber in the diet may be involved in the etiology of many diseases.
Figure 2. Structure of alkylresorcinols and their two known metabolites in plasma and urine: 1,3-dihydroxybenzoic acid = DHBA and 3-3-(3,5-dihydroxyphenyl)-1-propanoic acid = DHPPA. Ross et al.; *J Chromatogr B* 2004.

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Risk of adverse effects of phytoestrogens in animal feed

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Abstract

The main phytoestrogens, also classified as isoflavones, that have been isolated and identified from subterranean clover are formononetin, biochanin A, genistein, and daidzein. Soy and soy products are the most significant sources of daidzein and genistein. Biological activities of phytoestrogens are highly variable, complex and also species specific. Isoflavones resemble oestradiol-17β and may act as oestrogen agonist or antagonist through oestrogen receptor α and β. Ingestion of phytoestrogens may have impact on secretion of several hormones, such as prolactin, LH, testosterone, insulin growth factor-I, prostaglandin F₂α, and thyroid hormones in different species. The complexities of biological effects after ingestion of phytoestrogens and the differences in their metabolism indicate that interpretation of either risk or benefits need to be made with caution.

Introduction

Phytoestrogens, a group of chemicals produced naturally by a number of edible plants, exert various effects on the development and physiology of mammals (1). The presence of oestrogenic substances in plant extracts was first recognised
in the late 1920s. Infertility in female sheep grazing on oestrogenic subterranean clover (Trifolium subterraneum L.) was described as part of the condition known as "clover disease". Exposure of ewes to subterranean clover produced pathophysiological and morphological changes in the reproductive tract, as well as in the pituitary, adrenal and thyroid glands (2). The main phytoestrogens which have been isolated and identified from subterranean clover are formononetin, biochanin A, genistein, and daidzein. Soy and soy products are routinely fed to domestic farm animals as an important source of protein. These products are among the most significant sources of daidzein and genistein (3). All the above-mentioned phytoestrogens are classified as isoflavones, which resemble oestradiol-17β and activate oestrogen receptor α (ERα) and β (ERβ) (4). Whitten and Patisaul (1) concluded that genistein might more effectively induce ERα-mediated actions, despite having a higher affinity for ERβ. This compound, although slightly more potent via ERβ-linked reporters, was less efficacious, displaying only partial agonism via ERβ but full agonism via ERα.

In addition to a significant oestrogenic activity, genistein inhibits activity of tyrosine-specific protein kinases and DNA topoisomerases, and possesses antioxidant activity both in vitro and in vivo. Furthermore, genistein inhibits cyclooxygenase-2 (COX-2), an inducible enzyme that converts arachidonic acid to prostaglandins (5, 6). In general the phytoestrogens, except genistein, are aromatase inhibitors at low concentrations (less than 1 μM) but estrogenic at higher concentrations (higher than 1 μM), resulting in U-shaped dose-response curves (6). Conceivably, this phenomenon might contribute to the cancer-protecting properties of phytoestrogens (6).

Metabolism of phytoestrogens

The isoflavones occur in intact plants predominantly as glycosides and are hydrolysed by plant enzymes or by microorganisms in the gastrointestinal tract (GI-tract) to free aglycones (7). The metabolic conversions of the estrogenic isoflavones formononetin and biochanin A are similar in most animal species, including human beings (7-10). Since the demethylation and other metabolic transformations are performed by microorganisms in the gastrointestinal tract, the ultimate product formed depends on which type of microorganisms have colonised the GI-tract. Setchell et al. (11) have shown that about 50-70 % of the adult human population excrete equol in urine after consumption of soy isoflavones. This may be explained by differences in microflora in the GI-tract. Furthermore, equol is unique in having a chiral centre due to lack of a double bond in the heterocyclic ring, unlike genistein and daidzein from soy and formononetin and biochanin A from red clover. Therefore, two distinct optically active equol isomers occur. However, according to chiral HPLC analysis, the
equol present in the human (12) and ovine serum (13) was solely of the (S)-form.

Gu et al. (14) have shown that ingested daidzein and genistein from soy protein isolates contributed to majority of the summed isoflavones (more than 80 %) found in serum and the urine of pigs and women. A contribution of daidzein and genistein to the summed isoflavones in serum and urine of rats and monkeys was relatively low i.e. 30 %. In addition, daidzein was mainly metabolized to equol in rats and monkeys whereas humans and pigs have a very low metabolic conversion rate of this isoflavone. On the other hand, Lundh (10) has shown that considerable proportions of equol are produced from formononetin and daidzein in pigs fed 20 % red clover silage during 7 days. Such contradictory results may very likely depend on differences in microbe diversity in different groups of animals.

The isoflavones and their metabolites are conjugated to glucuronic acid already in the intestinal mucosal cells (15, 16) and only 1-5 % of the total phytoestrogen concentration in blood plasma is in unconjugated (free) form in sheep and cows. In pigs, the free amount of equol constitutes about 30-50 % of total amount (10). Since glucuronides are less active than their aglycones, the degree of conjugation activity is of very great importance in the risk assessment of phytoestrogens to different animal species. Cassidy et al. (17) concluded that bioavailability and pharmacokinetics of isoflavones are influenced mainly by the type of food matrix or the form in which they are ingested. Other factors such as gender and physiological status may also affect the metabolism of isoflavones. Early pregnant heifers, fed a single dose of bruised soy, had higher blood concentrations of daidzein, equol, genistein and P-ethylphenol, in comparison with late pregnant heifers fed the same diet (18).

**Effects in cattle**

We have studied the influence of phytoestrogens in ovariectomized Swedish Friesian heifers fed 20 kg of 100 % red clover silage per animal daily for 14 days (19). The daily intake of phytoestrogens was 34.9 g, of which 20 g was formononetin. The heifers exhibited oedema and mucous discharge in the vulva, presence of milky fluid in the mammae, and increased teat size. By means of ultrasonography fluid accumulation in the uterus was visualized for a long period; even 30 days after the red clover silage had been withdrawn. Red clover silage appeared to reduce the magnitude and duration of the pituitary response to GnRH injections (19).
Woclawek-Potocka et al. (20) reported that phytoestrogen metabolites (equol and para-ethyl-phenol) stimulated prostaglandin (PG) F$_{2\alpha}$ secretion in a dose-dependent manner in bovine epithelial and stromal cells. Interestingly, according to studies by Kotwica et al. (21) a diet containing phytoestrogens can be an important part of a feeding strategy to prevent reproduction disorders in domestic animals elicited by environmental pollutants. These authors showed that polychlorinated biphenyls (PCBs) stimulated PGF$_{2\alpha}$ but not PGE$_2$ secretion from endometrial cells. A high ratio of PGF$_{2\alpha}$/PGE$_2$ as a consequence of PCB exposure can impair both ovarian functioning and uterine contractility. The proper ratio of PGF$_{2\alpha}$/PGE$_2$ secreted by bovine endometrium could be restored by phytoestrogens (21). On the other hand, phytoestrogens and their metabolites may disrupt corpus luteum function by inhibiting PG- and LH-stimulated progesterone secretion in heifers and cows fed a soy bean diet at a level of 2.5 kg/animal/day (22). These authors concluded that a high soybean diet given to cows may produce disorders in the oestrous cycle, ovarian dysfunctions during early pregnancy and early embryo mortality.

**Effects in sheep**

In our own previous studies, we have observed different effects of phytoestrogens on physiological processes in sheep. Feeding of red clover silage to ovariectomized ewes caused an increase of teat length and circumference, and even stimulated a milky fluid secretion (23). The daily ingestion of phytoestrogens was 81-95 mg per kg body weight during an experimental period of 14 days. These changes were similar to those when treated with oestradiol-17β implant. Ingestion of phytoestrogens also stimulated secretion of thyroid hormones and tended to increase follicle size and ER$\alpha$ immunoreactivity of thyroid glands of ovariectomized ewes (24). We have also demonstrated that centrally administrated genistein stimulates the secretion of prolactin in ewes during seasonal anoestrus (25). The response of prolactin was related to the increasing doses of genistein. Polkowska et al. (26) studied the effect of genistein infused into the third ventricle of the brain on the ER$\alpha$ immunoreactivity and secretory activity of gonadotrophic cells in the adenohypophysis of the ewe. Their data indicate that genistein stimulates the expression of ER$\alpha$ in the LH$\beta$-positive cells, decreases the pool of secretory granules stored in LH-producing cells, and augments the synthesis of $\beta$-subunit for LH.

Pace et al. (27) reported that subterranean clover with low content of formononetin (Table 1) did not affect ewe reproduction but improved the
animal’s weight gain. The new cultivar of subterranean clover contains only 0.36 % of the formononetin and 2.14 % of the biochanin A present in the original red clover cultivars (Table 1). It is interesting to note that genistein is not removed from these new cultivars and represents almost the total amount of phytoestrogens (27). These authors concluded that foders containing such cultivars are beneficial for growth of animals maintaining good characteristics of carcass and meat.

**Table 1.** Isoflavone concentrations (mg/g dry weight) in leaves of the early buds (EB) of 13 red clover cultivars (adapted from (28)) and a new cultivar of subterranean clover (Trikkala) (adapted from (27)).

<table>
<thead>
<tr>
<th>Clover</th>
<th>Daidzein</th>
<th>Genistein</th>
<th>Formononetin</th>
<th>Biochanin A</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB</td>
<td>0.60</td>
<td>0.63</td>
<td>8.22</td>
<td>7.94</td>
<td>20.39</td>
</tr>
<tr>
<td>Trikkala</td>
<td>0.00</td>
<td>0.98</td>
<td>0.03</td>
<td>0.17</td>
<td>1.18</td>
</tr>
</tbody>
</table>

**Effects in goats**

A study was undertaken to investigate whether a low addition of phytoestrogens to a normal diet affects thyroid hormone secretion, the establishment of testosterone production and thyroid gland activity during puberty in male goat kids (29). Four male kids were given a standard diet and 3 were given an addition of 3-4 mg phytoestrogens/kg body weight in tablets containing genistein, daidzein, biochanin and formononetin. The treatment commenced at 3 month of age and continued until they were slaughtered at 6 months of age. Testosterone and total and free triiodothyronine (T<sub>3</sub>) were determined weekly in blood samples collected from the jugular vein. The results from this study are presented in Table 2. The total and free T<sub>3</sub> concentrations were significantly higher in the phytoestrogen treated animals than in the control group at week 5 and weeks 8-9, respectively. The plasma testosterone concentrations were significantly higher in the phytoestrogen treated animals than in the control group at week 7.
Table 2. Effects of phytoestrogens on the plasma concentrations of testosterone and total and free T<sub>3</sub> in male goat kids. Bold numbers indicate significant difference (P ≤ 0.05) between control and treated animals (adapted from (29)).

<table>
<thead>
<tr>
<th></th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
<th>Week 7</th>
<th>Week 8</th>
<th>Week 9</th>
<th>Week 10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Testosterone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>24.5±</td>
<td>23.3±</td>
<td>20.0±</td>
<td><strong>19.1±</strong></td>
<td>28.2±</td>
<td>28.2±</td>
<td>26.5±</td>
</tr>
<tr>
<td>Phytoestrogens</td>
<td>21.2±</td>
<td>25.3±</td>
<td>25.1±</td>
<td><strong>37.5±</strong></td>
<td>31.5±</td>
<td>22.9±</td>
<td>24.7±</td>
</tr>
<tr>
<td></td>
<td>3.7</td>
<td>1.9</td>
<td>8.3</td>
<td><strong>9.9</strong></td>
<td>9.6</td>
<td>5.4</td>
<td>4.7</td>
</tr>
<tr>
<td><strong>Total T&lt;sub&gt;3&lt;/sub&gt;</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.7±</td>
<td>1.2±</td>
<td>1.6±</td>
<td>1.5±</td>
<td>1.7±</td>
<td>1.7±</td>
<td>1.6±</td>
</tr>
<tr>
<td>Phytoestrogens</td>
<td>1.9±</td>
<td><strong>2.3±</strong></td>
<td>1.7±</td>
<td>1.6±</td>
<td>2.1±</td>
<td>1.7±</td>
<td>1.6±</td>
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<tr>
<td></td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td><strong>Free T&lt;sub&gt;3&lt;/sub&gt;</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.1±</td>
<td>2.9±</td>
<td>2.3±</td>
<td>2.7±</td>
<td><strong>2.5±</strong></td>
<td>6.0±</td>
<td>9.7±</td>
</tr>
<tr>
<td>Phytoestrogens</td>
<td>3.9±</td>
<td>2.6±</td>
<td>3.1±</td>
<td>3.7±</td>
<td><strong>5.1±</strong></td>
<td>8.8±</td>
<td>8.2±</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

Effects in pigs

Scholzahrens <i>et al.</i> (30) studied the mechanism mediating the effect of dietary casein or soy protein on serum cholesterol concentrations and followed the endocrine response to the intake of these dietary proteins in minipigs. They found that total and free thyroxine concentrations were 34 and 26 % higher on average when fed soy protein when compared with casein.

A dietary supplement to sows of daidzein at a dosage of 8 mg/kg feed from day 85 of gestation resulted in down-regulation of ERβ gene expression in the hypothalamus of the newborn pigs (31). This indicates a possible effect of daidzein on the neuroendocrine system. Ren <i>et al.</i> (31) also reported that the piglet survival and the birth weight of male piglets was higher in sows fed daily with 40 mg daidzein that than that of the control group. The intramuscular administration of daidzein (0.5 mg/kg body weight) to male 5-6 weeks old piglets, every 3<sup>rd</sup> day during 3 weeks, stimulated body weight gain and serum insulin growth factor-I (32). However, these effects were seen only between days 14 and 28 from the start of daidzein injections.

Ford <i>et al.</i> (33) reported that uterus and cervix of ovariectomized gilts responded to genistein treatment via intramuscular injections (100-200 mg/pig per day; 2-4
mg/kg body weight per day) in a manner qualitatively similar to oestrogen treatment. After 10 days of treatment, uterine and cervical wet weights were increased by the higher dosage level but not by the lower level compared with those of control gilts. Injections of 400 mg genistein per day also resulted in an increase of the height of epithelial cells lining the uterine glands and the lumen of uterus and cervix when compared to the untreated gilts.

Recently, Norrby et al. (34) reported that when genistein (2 mg/kg body weight per day) was given orally to gilts, their ability to communicate with a boar during oestrus expressed as vocalization rate was decreased, basal secretion of LH was inhibited and plasma level of PGF$_{2\alpha}$ increased at the insemination time.

**Tissue distribution of ingested phytoestrogens**

The other important aspect is the distribution of phytoestrogens and their metabolites in different organs and body fluids. Urpi-Sarda et al. (35) reported that long-term exposure to red clover silage in ewes (daily intake of 157.6 mg/kg body weight of phytoestrogens) results in the highest accumulation of equol and daidzein in kidney, followed by liver, reproductive tract, thyroid and muscle. According to these authors a following level of around 10 $\mu$g of daidzein and 80 $\mu$g of equol in 100 g of meat could not be considered as an appreciable source for isoflavones.

Antignac et al. (36) in France and Hoikkala et al. (37) in Finland analysed commercial milk products. Both studies revealed that milk from organically managed dairy farms had a higher content of isoflavonoids than conventionally produced milk (i.e. 191 and 411 $\mu$g/l of equol vs. 36 and 62 $\mu$g/l of equol, respectively). Steinshamn et al. (38) reported that cows fed silage containing red clover produce milk with increased content of flavonoids and decreased content of nonflavonoids such as mammalian lignans, when compared with silage containing white clover. Their study also revealed that the highest concentration of equol in milk (364 $\mu$g/l) was related to the cows fed the silage with highest content of formononetin and daidzein (3.1 and 1.75 mg/kg dry matter, respectively).
Conclusion

Biological activities of phytoestrogens are highly variable, complex and also species specific. Ingestion of phytoestrogens may have impact on secretion of several hormones, such as prolactin, LH, testosterone, insulin growth factor-I, prostaglandin F$_{2a}$ and thyroid hormones. In total, an estrogen-like effect in often dominating but interestingly, a stimulatory effect of phytoestrogens on thyroid hormones is also seen in different species like sheep, goat and minipigs. The complexities of biological effects after ingestion of phytoestrogens and the differences in their metabolism indicate that interpretation of either risk or benefits need to be made with caution.

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References


Glucosinolates in *Brassica* – health risks but also benefits

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Abstract

Health risk and health benefits associated with consumption of glucosinolates and glucosinolate derived components are defined by structure including stereochemistry of the dietary compounds and their concentrations in the diets. Both intact glucosinolates and their metabolites have the ability to give various types of biological effects. This is well documented by the comprehensive number of in vivo and in vitro experiments performed with individual compounds and with more or less complex mixtures and matrix systems. The variations in sensitivity of the humans, the test animals, target organisms, organs or assay systems are, however, key factors which need to be considered in relation to conclusions concerning health risks and health benefits of the bioactive compounds. It is also found, that structural variations among the glucosinolates and some of their metabolites in the diets give the basis for differences in their transformation in digesta and the bioactivities as a consequence of absorption from the digestive tract and/or the lungs. In the animals these xenobiotica have potential effects on internal organs and on the xenobotica metabolism, where the biological value (BV) and true protein digestibility (TD) from balance trials are found to be valuable in vivo measures.
for such effects. The structures of the actual compounds causing the effects on the target molecules and xenobiotica metabolism as well as the mechanism behind these effects are unsatisfactory known and call for attention. In the present work evaluations of the health risks and health benefits are based on data obtained with studies of bioactive effects from use of brassicaceous material in food and feed as well as from studies of bioactive effects from sixteen structurally different Brassicaceae glucosinolates tested individually in balance trials. The pure glucosinolates were tested in different concentrations in standard diets with or without active myrosinase, but otherwise devoid of other cruciferous compounds. Based on the results obtained, it seems that glucosinolate levels below $1 \mu\text{mol} \cdot \text{g}^{-1}$ of feed dry matter, corresponding to 10 $\mu$mol per rat per day or 120 $\mu$mol·kg$^{-1}$ of body weight per day do not trigger antinutritional or health risks. This level varies, however, for structurally different glucosinolates.

### Introduction

Glucosinolates are a group of allelochemicals with well defined structures consisting of alkyl aldoxime-O-sulphate esters with a $\beta$-D-thioglucopyranosyl group at the aldoxime carbon (C-0) in cis-[Z]-configuration to the sulphate group (1-7). These compounds co-occur with myrosinase isoenzymes (thioglucoside glucohydrolases; EC 3.2.1.147) (8-11) in all plants of the order Capparales comprising the family Brassicaceae with the genus Brassica and only in some few other plants (1, 4, 5, 12-16). The number of structurally different glucosinolates comprise more than 140 compounds with differences in physico-chemical properties, which also are reflected in their diverse biological activities (1, 17-21).

The occurrence of glucosinolates in plants used in feed, food and for non-food applications e.g. Brassica crops as oilseed rape (B. napus; B. rapa) and B. oleracea vegetables as broccoli, Brussels sprouts, cauliflower, cabbage and kale, are of special interest owing to the characteristic and unique taste and smell but also due to various other bioactivities resulting from glucosinolates and transformation products of glucosinolates (22-31). The accumulation of glucosinolates in the plant and in different plant compartments is chemotaxonomic defined, and is most often limited to cover few compounds. Moreover, in total, approximately 20 % of the known 140 glucosinolates are accumulated in plants of the Brassica genus (1, 6, 29, 32, 33).

The physico-chemical-biological properties of the glucosinolates are defined by the hydrophilic character of the thioglucopyranoside group and the strongly acid
or anionic sulphate group, but these properties are more or less moderated by the properties of the side chain structure (R-group). The structural variations caused by substituents on the thioglucoside group and especially the size and type of functional groups in the side chain contribute to the various biochemical functions of the glucosinolates and especially the derived reaction products (1). Beside the type of glucosinolates and myrosinase isoenzymes (1, 7-11), the reaction conditions are essential for the type of products formed during glucosinolate transformations (6, 9, 32, 34, 35).

Based on recently performed experiments, a proposal for the likely reaction mechanism in the myrosinase active site has been suggested to explain the formation of different glucosinolate products as a direct result of the reaction conditions (8, 9, 34). Non-enzymatic glucosinolate transformations may also occur at temperatures of 20-40 °C in reducing reaction media containing e.g. Fe$^{2+}$, ascorbic acid and thiol groups (36). Ascorbic acid, thiol group and other antioxidants are, in addition to nucleophiles in the reaction media, responsible for further transformations of the very reactive glucosinolate intermediate products as thiohydroxamate-O-sulphonates, isothiocyanates (ITCs) and indole-3-ylmethylcarbonium ion/indole-3-ylcarbinols which may lead to complex mixtures of ITCs, thiocarbamates/5-substituted oxazolidine-2-thiones (OZTs), dithiocarbamates (DTCs), thioureas (TUs), nitriles, epiprogroitrin, thiocyanates, thionamides, amines, thiocyanate ion, ascorbigens and other indolyls as well as some other bioactive products (1, 9, 17, 19, 21, 34-43).

Additional information within these areas are warranted to elucidate associations between structure and bioactivities of glucosinolates and their metabolites, which also include an augmented need for analytical methods and bioassays to characterise the allelochemicals and xenobiotica responsible for health risks and/or benefits (37-43). This include determination of their bioavailability and potential inter-conversion of compounds as ITCs, nucleophiles, thiocarbamates, OZTs, DTCs, TUs and indolyls (17, 19, 21-23, 37-47). The present work especially focus on the specificity of glucosinolates and glucosinolate derived products in relation to their effects on the xenobiotica metabolism using biological value (BV) as in vivo measure. This comprises comparison of effects from the stereoisomeric pairs; 2-hydroxy-2-phenylethylglucosinolates and 2-hydroxybut-3-enylglucosinolates, epiprogroitrin and progoitrin. The latter is quantitatively dominating in some of the Brassica vegetables together with sinigrin, gluconapin, (R)-methylsulfinylalkylglucosinolates and especially indol-3-ylmethylglucosinolates (1, 21, 24, 26-33). Evaluation of the influence on health risks and benefits are based on in vivo tests including determination of the BV and true protein digestibility (TD) from balance trials with young growing rats (22, 23, 45). Balance trials have been shown to be valuable test systems in studies of xenobiotica with pronounced bioactive effects on xenobiotica
metabolism (23, 45). These metabolic effects seem to be especially pronounced in the liver, lungs and intestines, the latter two representing the entrance points where the uptake of bioactive xenobiotica as glucosinolates and glucosinolate derived compounds occur. Our current new knowledge on this subject enables us to reanalyse and update our previous finding.

Thus, the aim of the present work is to determine the dose dependent effects of structurally different glucosinolates in complex mixtures and matrix systems and given individually in different concentrations to the feed for young rats to evaluate the doses resulting in adverse health risk as well as safe levels that may in turn be beneficial for the health.

**Materials and methods**

The applied materials, methods and techniques have been described in details elsewhere and comprise: Procedures for isolation of intact glucosinolates and myrosinase isoenzymes and the bioassays of these compounds (7, 9, 20, 23, 24, 30, 31, 45, 48-50). Quantitative determinations of individual intact glucosinolates, desulfoglucosinolates and transformations products of glucosinolates have been based on LC procedures including reversed phase high performance liquid chromatography (RP-HPLC) (50-52), micellar electrokinetic capillary chromatography (MECC) (50, 53-56), supercritical fluid chromatography (SFC) as enhanced fluid liquid chromatography with evaporative light scattering detection (EFLC-ELSD) (40-43).

**In vivo rat balance study**

In vivo evaluation of bioactivity from glucosinolates and glucosinolate derived products have been performed with young rats in balance studies according to described standard procedures (23, 45). In short, five male Wistar rats (70 g) per group, were fed a standard diet consisting of potato starch (441.8 g), casein added methionine (54.5 g), minerals (20 g) and vitamins (8.0 g) with dry matter (DM) content of 94.9 % and containing 1.43 % N (Diet 1; control). Each rat was fed 150 mg N and 10 g DM per day. Diets 2 to 5 were added glucosinolates in different doses and with or without added myrosinase. Diet 2 was diet 1 added 0.5 μmol glucosinolate/g DM; Diet 3 was diet 1 added 2.5 μmol glucosinolate/g DM; Diet 4 was diet 1 added 12.5 μmol glucosinolate/g DM and Diet 5 was diet 1 added 2.5 μmol glucosinolate/g DM + 0.15 myrosinase units. The glucosinolates tested up to now by use of this procedure have comprised the compounds shown in Figure 1. All diets were analytically checked for the intact glucosinolates to ensure that they contained the correct concentrations. The
preliminary period lasted 4 days followed by 5 days balanced intervention period, where the rats were housed in individually metabolic cages. During the balance period, urine and faeces were collected separately and any feed residual were taken into considerations. The calculation of BV, TD and net protein utilization (NPU) were based on nitrogen contents (Kjeldahl analysis) and calculated by the following equations:

True protein digestibility: 

\[
TD \% = \frac{N_{\text{consumed}} - (N_{\text{faeces}} - N_{\text{metabolic}})}{N_{\text{consumed}}} \times 100
\]

Biological value: 

\[
BV \% = \frac{N_{\text{consumed}} - (N_{\text{faeces}} - N_{\text{metabolic}}) - (N_{\text{urine}} - N_{\text{endogene}})}{N_{\text{consumed}} - (N_{\text{faeces}} - N_{\text{metabolic}})} \times 100
\]

Net protein utilization: 

\[
NPU \% = \frac{TD \times BV}{100}
\]

**Statistical analysis**

Statistical analysis was performed as described in Bjerg *et al.* (45). One factor analysis of variance was used for each glucosinolate to detect significant differences in mean values of the measured parameters. Duncan’s multiple range test and Tukey’s Studentized range (HSD) test using SAS data program were applied (SAS Institute, Inc., Cary, NC).

**Results and discussions**

Figure 1 shows the structural different glucosinolates selected, to investigate and compare their biological effects when added to the diet in different doses and used in rat balance studies. The structure of the glucosinolates define their physico-chemical properties and the potential breakdown products formed both in non-enzymatic and in the myrosinase catalysed reaction. The structure of the compounds in the digesta or absorbed from digesta are considered to be responsible for the differences in biological effects of different glucosinolates and mixtures of the transformation products. Figure 1 shows the structural different glucosinolates selected, to investigate and compare their biological effects when added to the diet in different doses and used in rat balance studies.
Figure 1. Structures of selected glucosinolates used in rat balance trials. Numbering according to Bellostas et al. 2007 (1).
Structures and physico-chemical properties of glucosinolates

Glucosinolates are strongly acidic allelochemicals as revealed from their structures shown in Figure 1 and 2. They occur as salts in all physiological solutions and in conjunction with the thioglucoside group, glucosinolates become hydrophilic compounds (Figure 2). Glucosinolates are relatively stable at room and body temperature even in aqueous solution without catalyst that affects the functional groups (6, 34-36). Desulfoglucosinolates will be the hydrolytic products when glucosinolates without substituents on the thioglucose part react with active sulphatase (Figure 2). In the presence of active myrosinase, however, a very fast hydrolysis of the glucosinolates give the highly reactive thiohydroxamate-O-sulphonate intermediate if the thioglucose is unsubstituted (R’2=R’3=H; Figure 2).

Figure 2. Initial steps in degradation of glucosinolates. Enzyme catalysed degradation by sulphatase results in desulfoglucosinolates whereas myrosinase creates thiohydroxamate-O-sulphonates. Non-enzymatic catalysed degradation of glucosinolates can depending on the conditions create carboxylic acids, amino acids, nitriles, or thiocarbamides.

Non-enzymatic transformation of glucosinolates can also occur at different condition resulting in various products (6, 35). Carboxylic acids are, thus, the main product in acid catalysed hydrolysis at elevated temperatures. In alkaline catalysed transformations of glucosinolates at elevated temperatures, amino acids among other products are produced as recently described in an excellent
review on non-enzymatic and enzyme catalysed glucosinolate transformations (35).

In metal ion catalysed reactions, glucosinolates can be transformed into nitriles and some few other products at reducing and acidic condition at room and body temperature (6). With 2-hydroxy-substituted glucosinolates in a solution containing Fe$^{2+}$, the 2-hydroxy-substituted thionamides will be one of the products formed at room and body temperatures (36). The possibilities for non-enzymatic glucosinolate transformations are of special interest in connection with identifications of the actual compounds responsible for health risks and/or health benefits of glucosinolates.

**Structures and physico-chemical properties of thiohydroxamate-O-sulphonate derived products**

Specific and detailed knowledge on the transformations of thiohydroxamate-O-sulphonates are especially relevant for the elucidation of the actual compounds produced and their effects on mechanisms related to health effects (Figure 3). The transformation of thiohydroxamate-O-sulphonates in the reactive sites of myrosinase isoenzymes is highly dependent on specific reaction conditions and the presence of co-factors, which together with the glucosinolate structure define the product type formed (8-11). The formation of thiohydroxamate-O-sulphonates are immediately followed by its transformation into structurally different compounds (Figure 3) as reactive ITCs, epithionitriles, nitriles (if redox conditions are present) (9) and amines if geminal dithiol groups are available for the reaction with ITCs (17, 57-59). The reaction between gem dithiols and ITCs result in dithiocarbamates and then the cyclocondensation releases the amine (9, 17, 57-59). Thiocyanates, epithionitriles and nitriles are formed when a limited Lossen rearrangement occurs and appropriate co-factors are present in the enzymes active site. The optical active 5-substituted oxazolindine-2-thione (OZT) is produced in very fast reactions if a 2-hydroxy group is present in the glucosinolate (1) where the (2R) chiral center in e.g. progoitrin yield the (5S) center in goitrin ((5S)-5-vinyloxazolidine-2-thione (VOT)).

Isothiocyanates of indol-3-ylmethylglucosinolates are extremely unstable and release in fast reaction the thiocyanate ion and a carbonium ion, which react with available nucleophiles. With water this reaction generates the carbinols. The carbinols are, however, very reactive in weak acidic solutions, comprising the stomach conditions, and depending on reactivity and available nucleophiles, complex groups of indolyls will be formed (1, 40-43).

Other types of ITCs are, depending on their side chain (R-group) more or less lipophilic and partly volatile. They have, thus, limited solubility in aqueous
systems, but formation of thiocarbamates change their aqueous solubility and reactivity and they will also be easy to dissolve in lipophilic media including biological membranes, as well as in oils, emulsions and food matrix systems. Nucleophilic thiols present together with ITCs and/or its thiocarbamates, will give dithiocarbamates (DTCs) and reactions between ITCs and free amines will result in thioureas (TUs) (1, 21, 37). All of the reactions occur easily at room temperature and can be followed by new analytical techniques which allow detection of the transformation of substrates and simultaneous detection of product types and amounts (44, 58).

**Figure 3.** Reactions of thiohydroxamate-O-sulphonates that may occur in biological materials.

**Dose-dependent effects on biological value**
The obtained effects in dose-response studies of individually glucosinolates (Figure 1) on BV are summarised and compared in Table 1 for sixteen structurally different but related glucosinolates. The data are shown as % reduction in BV relative to BV of the control group. It is thus a direct effect
from addition of different doses of glucosinolates to the animal feed and the glucosinolates are ranked according to their effects on percent reduction in BV from the high dose group. The results show that intact sinigrin and progoitrin given in the high dose (12.5 μmol/g DM) have the largest negative effect on BV and that the results on BV are dose related. This dose dependent effect on reduced BV was commonly observed with the exception of glucocheirolin, glucotropaeolin, and glucosibarin where the low dose did not result in smaller effects on BV than obtained with the middle dose (Table 1). The effects on BV from added myrosinase to the diet were more pronounced for progoitrin than for epiprogoitrin, glucosibarin, and glucobarbarin. However, pronounced effects on BV from added myrosinase were also seen for the non-OZT producing sinigrin, glucocheirolin, and glucotropaeolin, indicating effects of glucosinolate derived products on the xenobiotica metabolism.

The BV values (vide supra) are found to be an in vivo sensitive measure for effects of bioavailable xenobiotica – glucosinolates and glucosinolate derived products – on the xenobiotica metabolism (23, 45-47). With data as shown in Table 1, it is surprising to see the pronounced differences in bioactivity between sinigrin and glucotropaeolin and their higher homologues gluconapin and gluconasturtiin. It is also remarkable that sinigrin and glucotropaeolin are much more active than the other compounds, although it is closely followed by progoitrin, epiprogoitrin, glucoiberin, glucocheirolin and glucotropaeolin in their activity on BV or xenobiotica metabolism. Comparing the effects of ± myrosinase for the same glucosinolate dose (2.5 μmol/g DM) it is seen that higher percent reduction in BV is found for most glucosinolates when myrosinase is included, but this is not the case for all as e.g. sinalbin. The increased effects caused by added myrosinase activity are also quite different for the structural different glucosinolates, especially as seen for the oxazolidine-2-thione producing glucosinolates, progoitrin and epiprogoitrin (vide infra). The general large effect on BV from glucosinolates without the presence of myrosinase indicates that the effects are a result of bioactive intact glucosinolates (60-62; vide infra) and/or effects from products on non-enzymatic glucosinolate transformation (6, 35, 36).
Table 1. Percent reduction of biological value (BV) caused by selected glucosinolates fed to rats in a standard diet with or without addition of 0.15 U myrosinase/g diet (Myr). Each test group consisted of five male Wistar rats each weighing 70 g.

<table>
<thead>
<tr>
<th>No.</th>
<th>Glucosinolate</th>
<th>Dosage (μmol/g diet)</th>
<th>12.5</th>
<th>2.5</th>
<th>2.5 + Myr</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>Sinigrin&lt;sup&gt;A&lt;/sup&gt;</td>
<td>16.0</td>
<td>3.3</td>
<td>9.4</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>1.1</td>
<td>Progoitrin&lt;sup&gt;A&lt;/sup&gt;</td>
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<td>2.4</td>
<td>7.4</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>Glucocheirolin&lt;sup&gt;B&lt;/sup&gt;</td>
<td>12.6</td>
<td>3.1</td>
<td>10.1</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>Glucoiberin&lt;sup&gt;B&lt;/sup&gt;</td>
<td>11.5</td>
<td>5.1</td>
<td>7.2</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>Epiprogoitrin&lt;sup&gt;B&lt;/sup&gt;</td>
<td>10.5</td>
<td>6.1</td>
<td>7.2</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>10.1</td>
<td>Glucotropaeolin&lt;sup&gt;B&lt;/sup&gt;</td>
<td>9.8</td>
<td>4.0</td>
<td>8.2</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
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<td>9.6</td>
<td>6.9</td>
<td>6.7</td>
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<td>Glucosibarin</td>
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<td>1.4</td>
<td>2.3</td>
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<td>4.8</td>
<td>2.1</td>
<td>3.5</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>11.5</td>
<td>Glucobarbarin</td>
<td>4.6</td>
<td>1.3</td>
<td>4.0</td>
<td>1.0</td>
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<td>1.2</td>
<td>1.9</td>
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<td>3.5</td>
<td>4.3</td>
<td>2.8</td>
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<td>1.0</td>
<td>0.3</td>
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</tr>
<tr>
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<td>0.6</td>
<td>-0.1</td>
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</tr>
</tbody>
</table>

<sup>A</sup>: Bille et al. 1983 (23); <sup>B</sup>: Bjerg et al. 1989 (45).

Biological effects on internal organs

The effects of glucosinolates/glucosinolate derived products on the size of internal organs are shown in Table 2 which summarizes the physiological effect on BV, TD, liver, kidney, testicles and thyroid reported from rat balance studies of the tested glucosinolates (Figure 1) (23, 45). In general, all the tested glucosinolates affected the BV. However, the table also shows that the effects on internal organs vary depending on the glucosinolate studied. The activity of progoitrin with respect to xenobiotica metabolism is, in part, explained as a result of effects on the thyroid gland and its hormones (Table 2). Also glucosibarin and sinalbin are shown to increase the weight of the thyroid glands. Glucobarbarin affected all the organs investigated as well as BV and TD whereas glucocheirolin only affected BV and TD. It is noteworthy that stereoisomeric glucosinolates e.g. progoitrin and epiprogoitrin results in highly diverse biological effects on organ weight and the effect of products from myrosinase catalysed glucosinolate transformation on BV. The structural influence on the biological effects is therefore highly relevant to study (vide infra).
Table 2. Physiological effect of selected glucosinolates and products formed from transformation of glucosinolates. Numbering according to Figure 1.

<table>
<thead>
<tr>
<th>No.</th>
<th>Glucosinolate</th>
<th>BV</th>
<th>TD</th>
<th>Liver</th>
<th>Kidneys</th>
<th>Testicles</th>
<th>Thyroid</th>
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<td>↑</td>
<td>(licts)</td>
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<td>*</td>
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<td>*</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<td>Epiprogoitrin&lt;sup&gt;B&lt;/sup&gt;</td>
<td>*</td>
<td>(licts)</td>
<td></td>
<td></td>
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<tr>
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<td>*</td>
<td>(*)</td>
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<tr>
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BV: Biological value; TD: true protein digestibility; *: negative effect; (*): weak negative effect; ↑: increased weight; (↑): slightly increased weight; ↓: reduced weight; (↓): slightly reduced weight. NA: not analysed. <sup>A</sup>: Bille et al. 1983 (23); <sup>B</sup>: Bjerg et al. 1989 (45).

In vivo balance trials to reveal important bioactivity or xenobiotica effects from glucosinolates

With the increasing knowledge obtained during the last few decades concerning bioactivities of glucosinolate containing plants and isolated compounds from the plants, increased focus have been given to the potential health risks and health benefits related to glucosinolates/glucosinolate derived compounds. These types of research have especially been based on the progress in analytical and preparative techniques and the sensitivity and specificity of both in vivo and in vitro bioassays. Some decades ago focus was mainly on the health risks of glucosinolates present in diets including the glucosinolate containing plant materials (23, 25, 60). In the following period, the relevance of studying health risks from individual isolated intact glucosinolates ± myrosinase added to a standard diet devoid of other cruciferous compounds were recognized (23, 45, 49). Highly valuable and novel findings showed that intact glucosinolates in diets without added myrosinase was indeed degraded to a great extent and/or absorbed from the intestinal tract (60-62). Equally important, it was also shown that intact glucosinolates were absorbed by facilitated transport across the intestinal epithelium but with differences in velocity depending on the glucosinolate structure (62). However, identification and quantitative determination of the types of degradation products of the glucosinolates formed
in the digesta either by non-enzymatic or enzyme catalysed reactions, or both were not properly documented.

The present focus has therefore been directed at identification of glucosinolate degradation products (21, 30, 31, 46, 47) and their potential absorption from digesta. Recently performed experiments have confirmed that intact glucosinolates can be absorbed to the body from the intestinal digesta (61, 62). It is also well documented that glucosinolates and/or the derived products have various effects on the xenobiotica metabolism depending on dietary concentrations and structures of the actual xenobiotica (17, 19, 21-23, 25, 28, 45-47, 49, 58, 60-63). The present work aims thus at giving renewed focus on the glucosinolates mentioned in Figure 1, their transformation products and on the results obtainable from balance trials with rats. The structural variations in the selected glucosinolates give the opportunity to compare more or less specific effects on the xenobiotica metabolism originating from the compounds with different structures and functional groups.

**Specificities of OZTs**

For most of the glucosinolates tested it is seen (Table 1) that the bioactive effects are increased when concentrations of 2.5 μmol intact glucosinolates/g diet with added myrosinase were compared with similar glucosinolate concentrations without myrosinase. Even as structural different glucosinolates give relatively great variations in their effects on BV, and thereby on the xenobiotica metabolism, it seems to be acceptable, to recommend total glucosinolates levels below 1 μmol/g diet DM as safe in relation to avoid antinutritional effects for monogastric animals (64-69). However, this recommendation need to be modified in accordance with variations in function of animal species and developmental stage of the animals (23, 45, 64-69), where pregnant and young animals are the most sensitive.

Evaluation of biological effects specifically turn the attention to the 2-hydroxy-substituted glucosinolates, which in myrosinase catalysed reactions are transformed into OZTs as discussed previously (44). Glucosibarin, glucobarbarin, progoitrin and epiprogoitrin are examples of glucosinolates forming OZTs in such reactions (23, 44, 45). With conservation of the chirality at the carbon with the 2-hydroxy group, the (2R)-configuration in the glucosinolates glucosibarin and progoitrin give the (5S)-configuration in the 5-substituted OZTs and the (2S)-configuration in glucobarbarin and epiprogoitrin give the corresponding (5R)-OZTs. Evaluating the effects of the same dose (2.5 μmol/g DM) of glucosibarin (2R) ± myrosinase on the thyroid, a 5 % reduction in the relative weight of the glands was shown with myrosinase present (Table 1). Considering the corresponding effects in the balance trials with diets
containing $2.5 \mu mol$ glucobarbarin (2S)/g DM ± myrosinase, a 7 % increase in the relative thyroid weight was found with myrosinase present (Table 1).

The corresponding effects on thyroid weight in the trials with epiprogoitrin (2S) (45) was a 10 % reduction in thyroid weight with addition of myrosinase and for progoitrin (2R) (23) myrosinase addition gave a 67 % increase in the relative thyroid weights. Given the possible reaction mechanism, these effects are expected to result from production of 5-substituted OZTs (44). It is, thus, an absolute specificity of (5S)-5-vinyloxazolidine-2-thione ((5S)-5-vinyl OZT) as neither the enantiomer nor the two structural isomeric OZTs give corresponding effects. With (2R)-progoitrin the weight of thyroids were also increased as a linear function of the glucosinolate concentration in the diet without myrosinases, where the diet with $12.5 \mu mol$ glucosinolate/g DM resulted in 110 % increase in the relative weight of the thyroids compared to that of the control without glucosinolates. The effects on the thyroid glands from (5S)-5-vinyl-OZT result in corresponding effects on the thyroid hormones T3 and T4, which in turn have pronounced effects on the metabolism and thereby also on BV. In addition to effect from (5S)-5-vinyl-OZT, the thiocyanate ion has as well a known effect on the uptake of iodine (69) to the thyroid. This effect is also seen from feeding trials with sinalbin (Figure 3, Table 1 and 2) (23).

**Toxicological tests; health risks – health benefits**

Evaluation of biological activities from xenobiotica resulting in health risks are for various reasons often performed by *in vitro* and *in vivo* tests at xenobiotica concentrations far from lethal doses. However, toxicological test with determination of LD$_{50}$ values (lethal dose for 50 % of the tested organisms) after subcutaneous treatment have been reported for some selected glucosinolates and their derived products e.g. some few ITCs, OZTs and nitriles (70-75). These types of tests are of value in relation to determination of ultimate concentrations of bioactive compounds resulting in severe health risks. Great variations were, however, found in rat trials for the structurally different compounds.

For the ITC’s LD$_{50}$ values were found for allyl-ITC from the glucosinolate sinigrin (0.93 mmol/kg BW), 3-methylsulfinylpropyl-ITC from the glucosinolate glucoiberin (0.55 mmol/kg BW) and phenethyl-ITC from the glucosinolate gluconasturtiin (0.31-5.15 mmol/kg BW).

For structural different nitriles LD$_{50}$ values were found for glucobrassicin, gluconapine and progoitrin derived nitrils. These values were as follows: Indol-3-ylmethylnitril (1.55 mmol/kg BW), 1-cyano-2-hydroxy-3,4-epithiobutane (1.86 mmol/kg BW), 1-cyano-3,4-epithiobutane (0.97 mmol/kg BW), 1-cyano-
2-hydroxybut-3-ene (1.75 – 2.06 mmol/kg BW) and for (R)-goitrin (the OZT of epiprogoitrin (2S)) (0.77 – 10.97 mmol/kg BW).

The values thus obtained by subcutaneous injection of the individual and selected glucosinolate products with the indicated LD\textsubscript{50} values are at the same level as the highest tested level of glucosinolates, added to the feed (0.063, 0.31 and 1.56 mmol/kg BW/day). In these feeding trials the highest level resulted in appreciable biological effects but without any lethal effects or serious health risks. This indicates that the glucosinolate/glucosinolate products give much less health risks when administered with the feed than what is the case of subcutaneous injection of the same products.

The values obtained for glucosinolate concentrations in feed to rats where it seems to be without any health risks are at levels below 125 $\mu$mol/kg BW/day. This is in agreement with results obtained with use of glucosinolate containing feed to farm animals (64-69). If we consider the human intake of glucosinolates, it is difficult to obtain reliable data (76), but a rough evaluation can give values of 0.25 $\mu$mol/kg BW/day for The Netherlands, 0.5 – 0.6 $\mu$mol/kg BW/day for Canada and USA, and 1.6 $\mu$mol/kg BW/day for UK.

At elevated intake of crucifers with high levels of glucosinolates as e.g. Brussels sprouts (28) and broccoli (24, 26, 27, 30, 31) the levels can be about 10 $\mu$mol/kg BW/day. At such a human intake level of \textit{Brassica} vegetables, it can be important with identification of the structural types of glucosinolate/glucosinolate derived products which give the health benefits (24, 30, 31, 46, 47, 61, 63, 77).

\textbf{Conclusion}

The presented data show that ingested levels of glucosinolates below 1 $\mu$mol/g DM feed or 125 $\mu$mol/kg body weight (BW) per day was not associated with antinutritional or health risk. At higher concentrations, adverse effects were observed for some glucosinolates. The structural type of the glucosinolate and/or the possibility to transform the highly reactive isothiocyanates (ITCs) into thiocarbamates/5-substituted oxazolidine-2-thiones (OZTs), dithiocarbamates (DTCs) and thioureas (TUs) can be correlated to the observed difference in biological activity. The biological values (BV) are found to give important \textit{in vivo} information of the acceptable xenobiotica concentrations in relation to health risk or antinutritional effects. OZTs have been shown to give a specific effect on the thyroid gland with respect to both structure and stereospecificity with (5S)-5-vinyloxazolidine-2-thione (5-VOT) directly linked to the weight of
the gland and to the production of the hormones T3 and T4. The thiocyanate ion seems to exert activity toward the thyroid gland and this ion is quantitatively produced from the ITC of indol-3-ylmethyl compounds and to some extent also from sinalbin. Among the earlier tested glucosinolates sinigrin has much higher activity on the xenobiotica metabolism and BV than seen for the other tested glucosinolates.

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Cyanogenic glycosides in food, feeding stuffs and green medicine

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Abstract

Cyanogenic compounds are compounds that upon degradation release hydrogen cyanide HCN a highly toxic clear to pale blue liquid or gas. Naturally occurring cyanogenic compounds includes the groups of (a) cyanogenic glycosides, (b) cyanogenic lipids, (c) 2,3-epoxynitriles and (d) cyanohydrins. Of these a-c are chemically more or less stable, giving rise to the formation of cyanohydrins (group d) as a result of their degradation. One may classify the food related use of cyanogenic plants as follows: the plant commodity is (a) a staple food component, (b) a minor food component (of sporadic use), (c) a component in the production of beverages, pastry or sweets, or (d) used in medical treatments, usually within the sector of green/alternative medicine. Cyanogenic plants and plant parts also are grazed by animals, and relatively often deliberately included into feeding stuffs used for our husbandry. The released HCN from the degradation of cyanogenic glycosides shows acute and chronic toxicity. In general, safety regulations have been implemented in the EU and in most other industrialized countries; for foods as well as feeding stuffs. Food based on highly cyanogenic plant parts such as bitter cassava roots can be made chemically safe (based on the levels accepted in existing standards) by processing. However, food safety authorities and the general population must be aware that changing food habits, e.g. as a result of new health trends, may cause risks for hitherto unseen exposure to cyanogenic glycosides; as in the case
of an increased intake of linseed. The use of cyanogenic glycosides e.g.
amygdalin or products based on cyanogenic compounds (as Laetrile) for the
treatment or prevention of different forms of cancer has no support in the
scientific medicinal literature.

Cyanogenic glycosides: why and what?

Cyanogenic glycosides (if the sugar part is glucose also called cyanogenic
glucosides) and cyanogenic compounds in general, are discussed in this review
due to the fact, that they are poisonous, but they do occur in a number of
important food crops, crops used for the production of feeding stuffs and plant
materials used in health products of different kinds. Furthermore, claims have
been and are being made that they may contribute positively to the treatment of
cancer in a broad sense.

Cyanogenic compounds are chemical compounds that upon degradation can
release hydrogen cyanide (HCN; historical common name Prussic acid). This
process is called cyanogenesis (from cyano – Greek [kyanos = blue] and genesis
– Greek [creation]. Hydrogen cyanide is a highly toxic clear to pale blue liquid
or gas. It melts at -14 °C and boils at 25.6 °C. It is miscible in water or ethanol
and is slightly soluble in ether. Its water solution, called hydrocyanic acid, is a
weak acid and partly ionizes to give the cyanide anion, CN⁻. The salts of
hydrogen cyanide are known as cyanides. Hydrogen cyanide has a faint, bitter,
almond-like odour that some people are unable to detect due to a genetic trait
(1).

Industrially, HCN is obtained by the reaction of ammonia with carbon monoxide
(Andrussow process) or with natural gas (methane) in the presence of
rhodium/platinum catalyst at approximately 1100 °C. The principal use of HCN
is in the manufacturing of acrylates, synthetic fibres (as a starting material for
nylon 66), plastics and cyanide salts, especially sodium cyanide to extract gold
from ore.

Naturally occurring cyanogenic compounds includes the chemical groups of (a)
cyanogenic glycosides, (b) cyanogenic lipids, (c) 2,3-epoxynitriles and (d)
cyanohydrins (2). Of these a-c are chemically more or less stable, giving rise to
the formation of cyanohydrins (group d) as a result of their degradation. The
cyanohydrins, some of which also are naturally stored in a number of organisms
including plants, fungi and millipedes, are rather stable at low pH but
decompose into HCN and an oxo compound (aldehyde or ketone) at neutral to
alkaline pH. The same decomposition may be facilitated enzymatically by so-
called cyanohydrin lyases. In Figure 1, the process of degradation of linamarin (the dominating cyanogenic glucoside in the cyanogenic crop *Manihot esculenta* = cassava) to form HCN via cyanohydrin is illustrated.

![Image of Linamarin degradation](image)

**Figure 1.** Degradation of the cyanogenic glucoside linamarin to acetone cyanohydrins and further to HCN.

Stable cyanogenic compounds such as glycosides, lipids and 2,3-epoxynitriles are biosynthesised by, and therefore found in, many terrestrial plants (3) and a small number of animals, e.g. some bugs and the larvae of certain moths such as the burnet moth (*Zygaena filipendulae*) (3-5). The latter is not only able to *de novo* biosynthesise the two cyanogenic compounds linamarin and lotaustralin but also to sequester these from its preferred feed plant *Lotus corniculatus* L. (Birdsfoot Trefoil) (5).

Some centipedes and millipedes can protect themselves by spraying HCN formed from the cyanohydrin R-mandelonitrile by enzymatic decomposition (enzyme: cyanohydrine lyase) on enemies (4, 6) while a smaller number of fungi (including some mushrooms) are also able to produce hydrogen cyanide from unstable precursors such as e.g. the cyanohydrins of pyruvic acid and glyoxylic acid (7, 8). Also a number of bacteria have been demonstrated to be able to release HCN formed from different precursors of amino acid nature (9).

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**Hydrogen cyanide and its toxicity**

The acute toxicity of hydrogen cyanide in humans is relatively well known and described in a large number of scientific reviews as well as in books and other material concerning industrial work hygiene or the diagnosis and treatment of
intoxications. Actually this information has been gained from fatal as well as non-fatal poisonings from oral as well as respiratory exposure to HCN, KCN and NaCN. Clinical symptoms reported include: anxiety and excitement, rapid breathing, faintness, weakness, headache (pulsating), constricting sensations in the chest, facial flushing, dyspnoea, nausea, vomiting, diarrhoea, dizziness, drowsiness, confusion, convulsions, incontinence of urine and faeces, irregular respiration and coma. In case of large, lethal doses, convulsions are immediately seen, followed by coma and death. At longer exposure to lower doses resulting in death, one will see the symptoms described above develop in an orderly fashion. A characteristic early feature of acute cyanide poisoning is tachypnoea and hyperpnoea, resulting in an increased tidal volume (10).

Cyanide is well absorbed via the gastrointestinal tract. Once absorbed, it is rapidly and ubiquitously distributed throughout the body, although the highest levels are typically found in the liver, lungs, blood, and brain. There is no accumulation of cyanide in the blood or tissues following chronic or repeated exposure. Approximately 80% of absorbed cyanide is metabolized to thiocyanate in the liver by the mitochondrial sulfur transferase enzyme rhodanese and other sulfur transferases. Minor pathways for cyanide detoxification involve reaction with cystine to produce aminothiazoline- and iminothiazolidine carboxylic acids and combination with hydroxycobalamin (vitamin B12a) to form cyanocobalamin (vitamin B12). These other end-products are excreted in the urine together with the thiocyanate (11).

The toxic effects of cyanide ion in humans and animals are generally similar and are believed to result from inactivation of cytochrome oxidase and inhibition of cellular respiration and consequently histotoxic anoxia. The primary targets of cyanide toxicity are the cardiovascular, respiratory, and central nervous systems (11). Chronic exposure to lower (non-fatal) concentrations of hydrogen cyanide is known to affect the central nervous system of both animals and man.

In production animals, cyanide thus for a long time has been associated with syndromes affecting CNS and giving rise to ataxia in sheep, cattle, and horses grazing on another cyanogenic crop; namely sorghum (*Sorghum bicolor* (L.) Moench), which contains the cyanogenic glucoside dhurrin (12). Histopathological examinations of affected horses and cattle have shown spheroids in the white matter of the spinal cord, mostly in the ventral funiculi, and in the cerebellar peduncles. The lesions reported in lambs are dominated by the formation of spheroids in the brain stem, cerebellum and ventral horn grey matter of the spinal cord, and mild gliosis. A Brazilian research group led by professor Soto-Blanco, recently demonstrated a number of neuropathological lesions in goats experimentally fed different concentrations of KCN for five months. Investigators found the presence of spheroids in the pons, medulla
oblongata, and ventral horn of the spinal cord. In addition gliosis and spongiosis in the medulla oblongata, gliosis in the pons and damaged Purkinje cells in the cerebellum from the goats receiving 3.0 mg KCN/kg b.w. per day (corresponding to 1.25 mg HCN). Goats on the lower dose of 1.2 mg (corresponding to 0.5 mg HCN) had congestion and haemorrhage in the cerebellum, and spheroids in the spinal cord, all as reviewed in 2007 (13).

A restricted number of controlled long term studies in traditional laboratory animals such as rats as well as studies in pigs and dogs have also demonstrated CNS damages as supplemented by finding of slower reaction time and reduced explorative behaviour etc. In a recent conclusive analysis of these studies, the highest No Observed Adverse Effect Level (NOAEL) value and all reliable LOAEL (Lowest Observed Adverse Effect Level) values for neurological effects in different species and for different durations of exposure were calculated and reported by The Agency for Toxic Substances and Disease Registry (ATSDR), Department of Health and Human Services (Atlanta, Georgia, USA) (14).

Among a variety of neuropathies reported from regions of Africa with populations that consume a high level of the tuberous starchy root of cassava (*Manihot esculenta* Crantz) at least Konzo is generally believed to be caused by cyanide from the monotonous consumption of insufficiently processed bitter cassava (15). It should be mentioned, however, that suggestions have been made as to the coumarin compound scopoletin – also present in cassava – being another causative factor (16). The edible parts of cassava contain the two cyanogenic glucosides linamarin and lotaustralin (17), bitterness being – at least to a certain extent - a function of the content of these compounds (18, 19). Konzo is a distinct upper motor neuron disease characterised by the sudden onset of varying degrees of symmetric, isolated, non-progressive spastic paraparesis (15).

**Cyanogenic compounds in plants used for food and green medicine**

In general, one may classify the food related use of cyanogenic plants as follows: the plant commodity is (a) a staple food component, (b) a minor food component (of sporadic use), (c) a component in the production of beverages, pastry or sweets, or (d) used in medical treatments, usually within the sector of green/alternative medicine.
Staple food components

Staple food components which are cyanogenic includes the root of cassava (*Manihot esculenta* Crantz; cassava, mandioc, manioca, manihot, manioc, tapioca, yuca) and the lima bean (*Phaseolus lunatus* L.; lima bean, butter bean, sieva bean, butter pea). Also bamboo shoots and seeds of a number of other leguminous plants, i.e. plants belonging to the plant family *Fabaceae* (= *Leguminosae*), could be mentioned.

Cassava originates in or nearby the Amazonian region of Brazil. Domesticated within South America it is today grown worldwide in the tropics. It was estimated by FAO the year 2002 to be the staple food for 600 million people. The plant is a perennial shrub which forms root tubers rich in starch. The tubers as well as the leaves are eaten by man. Cassava is the only domesticated staple crop for which a significant part of the production is of the bitter (i.e. toxic) type (20). In some geographical regions in Africa and elsewhere, the production, proportion and importance of the bitter varieties are increasing and outnumber the non-toxic varieties (21). This phenomenon is due to a number of reasons among which we find taste preferences, resistance to certain pests and a stronger social control of (especially African) female farmers over the food resources of the family. The latter should be understood in the way that the processing necessary to render the final food product non-toxic most often is done by the women (18, 22-23).

The lima bean is native to tropical Central and South America, and is an internationally important legume crop. Guatemala is the general region of origin. Dispersal of the crop was undoubtedly due to Native American trade and travel routes. Lima beans were well established in the southwestern and southeastern United States long before the early explorers arrived. Although a tropical perennial in the wild, lima beans have been developed as annuals for production in the United States. Wild lima beans often support themselves by climbing on other plants. Climbing lima bean varieties known as Pole Beans because of the pole that supports their growing, are a very popular item in home and garden market settings. However, the varieties developed for commercial production have more determinate growth habits. Smaller, bush types are necessary for mechanical harvest (24). Lima bean contains the cyanogenic glucoside linamarin as the main cyanogen in its seeds (25). The content of cyanogenic compounds (the toxicity) varies to a very high extent among different cultigroups (26, 27).

With bamboo shoots we mean the edible shoots (new bamboo culms that come out of the ground) of the two bamboo species (family *Poaceae*) *Bambusa vulgaris* Schrad. ex J.C. Wendl. and *Phyllostachys edulis* (Carrière) J. Houz. (Synonym *Bambusa edulis* Carrière). As an example one can find the following about *Phyllostachys edulis* as a food item:
Young shoots are cooked. They are very palatable when cooked but acrid when raw. Their large size makes them very popular. Extensively eaten in China, they are usually cooked in one change of water. The shoots are harvested in the spring when they are about 8 cm above the ground, cutting them about 5 cm below soil level (28). The dormant young shoots, harvested in the winter before they emerge above the ground, are especially relished as a delicacy (29).

The bamboo shoots contain the cyanogenic glucoside taxiphyllin. Recently the Food Standards Australia New Zealand (30) made a risk assessment concerning the consumption of cassava roots and bamboo shoots in the two countries. The assessment concluded that: "On the basis of information on cassava and bamboo products currently available in Australia and New Zealand and the levels of consumption, the likelihood of cyanide intoxication from consumption of cassava or bamboo shoots is low, but due care in preparation remains necessary. While the current users have adequate knowledge regarding the risks associated with consumption of cassava and bamboo shoots, more widespread use in the community would increase the public health risks".

Of the different beans other than the lima bean, especially seeds of P. aureus Roxb. (Mung bean; synonym Vigna radiata (L.) R. Wilcz.), Cajanus cajan (L.) Millsp. (Pigeon pea; synonym Cajanus indicus Spreng.), Canavalia gladiata (Jacq.) DC. (Sword jackbean) and Vigna unguiculata (L.) Walp. (Cowpea; synonym Vigna sinensis (L.) Savi ex Hassk.) are of interest. The identity of the cyanogenic principle(s) (probably cyanogenic glycosides) is not known in detail for all species (31).

**Minor food components**

Minor food components (of sporadic use) that are cyanogenic include flax seed (linen seed; Linum usitatissimum L.) and a smaller number of edible mushrooms.

Flax seed store as the main cyanogenic compound the diglucoside linustatin and to a lesser extent neolinustatin (25). The seeds of 10 flax cultivars (Andro, Flanders, AC Linora, Linott, McGregor, Noralta, NorLin, NorMan, Somme, and Vimy) grown at different Canadian sites (Portage la Prairie, MB, in 1987, 1988, and 1989 and at Beaverlodge, AB, and Indian Head, SK, in 1989 were analysed for their content of cyanogenic glucosides (32). The content of linustatin varied from 213 to 352 mg/100 g of seed, accounting for 54-76 % of the total content of cyanogenic glucosides. The content of neolinustatin ranged from 91 to 203 mg/100 g of seed. Whole flax seed has gradually become more and more used in certain forms of bread ("healthy fiber rich wheat as well as rye breads") and parallel with this trend also a rising demand for flaxseed meal, which has
retained the beneficial nutrients, protein, fat, fibre, and lignans at the same level as flaxseed. Hence, research into the preparation of such meals with a reduced content of the toxic cyanogenic glycosides has been reported (33).

The edible cyanogenic mushrooms in general are seen as causing no health concerns since even mild processing, such as drying at temperatures over 50 °C, and culinary preparation, e.g. cooking and frying, completely destroyed the cyanogenic compounds. Consequently, HCN in edible mushrooms does not present any health hazard to the consumer (8).

**Components in the production of beverages, pastry or sweets**

Component in the production of beverages, pastry or sweets/confectionary include almond (*Prunus dulcis* var. *amara*; synonyms *Prunus amygdalus* var. *amara, Amygdalus communis* var. *amara, Amygdalus dulcis* var. *amara, Prunus communis* var. *amara*) which exists in a sweet and a bitter variety (with a high content of amygdalin), seeds of apricot (*Prunus armeniaca*), peach (*Prunus persica*), and stone fruits such as cherry (*Prunus avium/cerasus*) and plum (*Prunus domestica*). All belong to family *Rosaceae*.

Almonds, and seeds of apricot and peach are used in the production of marzipan and persipan, respectively. Persipan (from Persicus (peach) and Marzipan; also known as Parzipan) is a material used in confectionery. Both normally consist of around 40 % ground kernels and 60 % sugar. The crushed kernels have a strong bitter flavour caused by the release of HCN and benzaldehyde from the degradation of amygdalin, a cyanogenic glycoside which has to be at least partly removed before the kernels can be used. The seed of apricot and peach are normally not used otherwise, making persipan lower-priced than marzipan; although peach kernel oil is increasingly used in the cosmetic industry as a base oil for skin oils and creams. Persipan has a somewhat different taste from that of marzipan, and is sometimes preferred by people who do not like the taste of marzipan. Persipan often contains 0.5 % starch and can be differentiated from marzipan with an Iodine test. According to an opinion from the European Food Safety Authority (EFSA) presenting an investigation from the UK, marzipan and similar products made from apricot kernels were found to contain cyanogenic compounds and HCN corresponding to a total release of 15-20 mg HCN/kg in retail products, 30-35 mg HCN/kg in higher grade and manufacturing products and about 50 mg HCN/kg in baker’s raw paste (34).

Stone fruits are among others used for the production of stone fruit spirits by fermentation and distillation (35). Scientifically the fruit type is called a *drupe*, i.e. a fruit in which an outer fleshy part (exocarp, or skin; and mesocarp, or flesh) surrounds a shell (the pit or stone) of hardened endocarp with a seed.
inside. In the traditional production of stone fruit spirits, the stones are included when making the fermentation mash. The seed of all Prunus species contains the cyanogenic glycoside amygdalin (36). During the processing (mash making and fermentation), stones/seeds may be damaged giving rise to release of cyanogenic compounds and the formation of HCN (37). HCN is easily distilled over during the subsequent distillation to enhance the alcoholic percentage. This is why e.g. the EU has legislative regulation for the content of HCN (cyanogenic compounds) not only in products such as marzipan, but also in distilled spirits.

A special problem related to the production of spirits from stone fruits is the formation of ethyl carbamate. Ethyl carbamate (synonyms: urethane or carbamide acid ethylester) occurs at low level, from ng/kg to mg/kg, in many fermented foods and beverages. Ethyl carbamate is genotoxic and a multisite carcinogen in animals as well as probably carcinogenic to humans. It has been discussed as consumer risk in fermented food and alcoholic beverages for more than twenty years. Distilled spirits, particularly stone fruit and stone fruit mash spirits, contain ethyl carbamate in manifold higher concentrations than other fermented foods and beverages. The formation of ethyl carbamate in stone fruit spirits is linked to the presence of cyanogenic glycosides in the stones of the fruits, and their further hydrolysis by enzymes to sugar and hydrogen cyanide, as already discussed. Thus hydrocyanic acid can be formed quickly in the mash, distilled over during the distillation, and transferred in catalytic pathways by reaction with ethanol into ethyl carbamate. Certain environmental conditions such as light exposure or copper ions promote the formation process in the distillate (38).

**Use in medical treatments**

Cyanogenic plant parts used in medical treatments, usually within the sector of green/alternative medicine, first of all include apricot seeds and secondly bitter almond both of which already have been mentioned above as a components in persipan and marzipan, respectively.

If one today search the world wide web for information on “cancer and apricots” or “cancer and almonds” a lot of hits are coming up, e.g. one saying “"six almonds a day keeps cancer away" (39), another stating “almonds don’t prevent cancer, apricot kernels prevent cancer”. Since the two look very alike, almost the same, people confused the two. The one contains lots of amygdalin (B-17) and is thus bitter; the other does not contain B-17 and thus does not taste bitter. You’ve been warned; don’t accidentally eat 3 almonds a day instead of 3 apricot kernels and then end up having cancer” (40). Why these statements? According to the web-published historical review “The Rise and Fall of Laetrile” (41) we will have to go back to the early 1950’s to understand. This was when Ernst T.
Krebs, Sr., M.D., and his son Ernst, Jr., began using what they called “a purified" form of amygdalin to treat cancer patients. Since then a number of alternative therapists, health networks and shops has promoted amygdalin, amygdalin-containing-extracts or apricots/bitter almonds for the treatment of cancer in general. Often the products are marketed or sold under the brand name of "Laetrile” originally registered by Ernst T. Krebs Jr. for laevo-mandelonitrile-beta-glucuronoside, a substance he allegedly synthesized for the treatment of "disorders of intestinal fermentation" and later promoted as an anticancer drug. The designation B-17 was also given by the commercial company owned by Krebs Jr. and Sr., in order to indicate that the cyanogenic compound is a vitamin. This claim can by no means be supported scientifically (41).

Since the beginning of the 1980’s a number of reviews on treatments already undertaken as well as clinical trials has been published. They all concluded that amygdalin has no value as an anticancer drug. Still, however, promotion has taken place through different unauthorised channels – especially from Mexico towards the US market - which is why Milazzo et al. (42) recently published a new systematic review of the existing clinical evidence for the effect (if any) of Laetrile against cancer. The aim of this review was to summarize all types of clinical data related to the effectiveness or safety of Laetrile interventions as a treatment of any type of cancer. All types of clinical studies containing original clinical data of Laetrile interventions were included. The authors searched the Cochrane Central Register of Controlled Trials (CENTRAL), MEDLINE (from 1951), EMBASE (from 1980), Allied and Complementary Medicine (AMED), Scirus, CancerLit, Cumulative Index to Nursing and Allied Health (CINAHL; all from 1982), CAMbase (from 1998), the MetaRegister, and the National Research Register. For reports on the safety of Laetrile, they also searched the Uppsala database. No language restrictions were imposed. Thirty six reports met the inclusion criteria. No truly controlled clinical trials were found. Three articles were nonconsecutive case series, 2 were consecutive case series, 6 were best case series, and 25 were case reports. None of these publications proved the effectiveness of Laetrile. Therefore, the claim that Laetrile has beneficial effects for cancer patients was not supported by sound clinical data according to the authors (42).

Cyanogenic compounds in plants used in feeding stuffs

The early literature about the toxicity of cyanogenic plants to grazing animals is to quite an extent identical with the articles describing the first isolation and structure identification of different cyanogenic glycosides. Thus, the first cyanogenic constituents to be isolated and structure elucidated were plant
glycosides like amygdalin (1830), sambunigrin (1928) and acacipatalin (1935). The structure of the latter was slightly revised in 1977. Pioneers within this development was an Australian group led by Dr. H. Finnemore (43, 44) and the South African scientists Steyn and Rimington (45). Both groups were investigating the principle behind the toxicity to cattle and sheep of different *Acacia* species.

In a modern European agricultural context, seeds and byproducts from crops such as apricot (*Prunus armeniaca*; contains amygdalin) and flax (*Linum usitatissimum*; contains linustatin and neolinustatin)) and chips made from cassava root (*Manihot esculenta*; contains linamarin) are the main sources of concern (46). However, also sorghum (*Sorghum bicolor*; contains dhurrin) must be mentioned (12). Cassava in the form of imported dried chips and root meal has for long been used as animal feed (e.g. for pigs and poultry) in the husbandry sector of Europe, however, the import has fluctuated greatly during the last several decades (47-51). Problems with intoxications or reduced productivity are today seldom seen due to a quite restrictive legislation (see below) as well as the implementation of good knowledge based agricultural practices.

**Risk analysis and current European legislation**

Risk analysis consists of risk assessment, risk management and risk communication. The human use of cyanogenic plant materials obviously calls for risk analysis in order to avoid casualties.

Several decades ago national legal restrictions have been put on the use of certain cyanogenic plant materials as feeding stuffs for husbandry within Europe and elsewhere. In the EU today the *directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed* regulates this area. Regulation is implemented by setting maximum levels for the total amount of HCN that can be released from different feedingstuffs (Table 1).

When it comes to food, the corresponding document, namely the *Commission Regulation (EC) No 1881/2006 of 19 December setting maximum levels for certain contaminants in foodstuffs* regulates mycotoxins, metals such as mercury etc., dioxins and benzo(a)pyrene among others, but not the content of cyanogenic substances. This is due to the fact that HCN (and thereby cyanogenic compounds) in the EU traditionally has been, and still are, regulated as a flavour compound, which means that we find the legally approved
maximum levels for HCN in the Annex II of Directive 88/388/EEC on flavourings. The actual limits are as follows: (a) 1 mg/kg in foodstuffs and beverages (with the exception of 50 mg/kg in nougat, marzipan or its substitutes or similar products), (b) 1 mg per percent volume of alcohol in alcoholic beverages and (c) 5 mg/kg in canned stone fruit (34; 52).

However, during the last few decades the cyanogenic root crop “fresh cassava roots” gradually has entered the European food market. In order to facilitate the international trade with this commodity, the UN organisation Codex Alimentarius Commission (CAC) by 2003 finally launched a so-called Codex Standard for sweet cassava. According to this, sweet cassava roots are those with a total cyanogenic potential less that 50 mg/kg fresh weight (53).

Also the EU has recognised, that not only cassava and cassava products but indeed a number of food commodities (in the EU terminology “novel foods”) that earlier were not produced or imported into the EU, gradually are entering the European market. By January 1995 the EU AIR Concerted Action NETTOX was initiated to compile and evaluate data on natural food plant toxicants, to assess the risk to human health and to identify strategies to minimize such risk (54). This initiative was soon followed by the introduction by the EU of the Regulation No 258/97 of January 1997 of the European Parliament and of the Council concerning Novel Foods and Novel Food Ingredients, which provided a tool for the national authorities to regulate the introduction of novel foods based on a formalized safety assessment. The regulation applies to the placing on the market within the Community of food and food ingredients which has not hitherto been used for human consumption to a significant degree within the Community (55).

In 1998 NETTOX and subsequently in the year 2005 The Nordic Committee of Senior Officials for Food Issues, published risk assessments of raw cassava roots as a “new” food commodity on the European market (56, 57). The NETTOX assessment suggested a lower limit than the CAC limit for sweet cassava varieties, namely a limit of 50 mg CN equivalents/ kg dry weight (of which maximally 10 mg/kg in the non-glucosidic form) to avoid acute toxicity as well as the development of Konzo. However, it was also stressed that setting an ADI or product requirements on the basis of mammal detoxification rates may be inappropriate for long term exposure, as this might result in depletion of cofactors for detoxification and, therefore, in gradually increasing toxicity of cyanide (56). In further comments to this assessment, it was among others said (quotation) “There are problems with cassava sold in the Netherlands, since raw cassava contains enough cyanide to be lethal. Still some would eat it unprocessed. Whenever selling raw cassava a processing instruction should be included” (56). The Nordic assessment from 2005 did not propose any limit(s)
for the content of these naturally inherent toxicants but concluded (quotation) “As illustrated in the cassava case (i.e. “in populations where cassava does not have a tradition of use and where the community may not possess adequate knowledge regarding the risks of insufficient preparation, widespread use in the community would increase the public health risks”), novel plant foods may need special attention when introduced in countries and regions, where there is no tradition for their use” (57, p. 49).

No initiative has yet been taken to change the legislation as a result of e.g. the NETTOX assessment of raw cassava roots as a novel food. However, recently EFSA has carried out two risk assessments concerning cyanogenic constituents (HCN) in feeding stuffs (13) and in flavourings and other food ingredients with flavouring properties (34). One conclusion of the latter is as follows: “The Panel concluded that the current exposure to cyanide from flavouring ingredients (97.5th percentile) is unlikely to give rise to acute toxicity. For chronic exposure the overall data were not considered adequate to establish a numerical no-observed-adverse-effect level (NOAEL) or Tolerable Daily Intake (TDI) in humans. In view of the lack of adequate data on chronic toxicity, the Panel supports the continued application of limits for the presence of HCN in foods and beverages”.

**Table 1.** Cyanogenic feed materials and the maximum levels expressed as mg/kg of HCN released. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed.

<table>
<thead>
<tr>
<th>Undesirable substance</th>
<th>Products intended for animal feed</th>
<th>Maximum content in mg/kg (ppm) relative to a feedingstuff with a moisture content of 12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocyanic acid (HCN)</td>
<td>Feed materials with the exception of:</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>- linseed</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>- linseed cakes</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>- manioc products and almond cakes</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Complete feedingstuffs with the exception of:</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Complete feedingstuffs for chicks</td>
<td>10</td>
</tr>
</tbody>
</table>
Cases to illustrate the levels of cyanogenic glycosides in foods exported to, available in or recommended by health promoters from the EU

(a) Apricot kernels withdrawn over cyanide concern (2006)
In 2006 an announcement from the UK Food Standard Agency (FSA) that eating too many bitter apricot kernels found at the market during April presented a possible health hazard, led to a voluntary withdrawal of the ingredient in the UK. The FSA judges that 2 kernels could mean a health risk, while at the same time a so-called dosage sheet following the product advocated that consumers did not consume more than 10 kernels per day (58).

(b) Linseed supplementation (2008)
During the year 2008 a new book on how to eat in a healthy way as based on a family’s private experiments (and experiences) was published in Denmark under the title of “Kernsund familie, sådan!” (Healthy family, just like that!). Supplementation of the daily food with 4 to 6 table spoons of crushed linseed (flax seed) was among others recommended. This prompted the Danish food authorities to quickly make an assessment of the recommendation. A worst case scenario calculation showed that the recommended intake could be equivalent to around 0.5 mg/kg body weight of HCN (theoretically released in the intestines) which, when compared to the reported lethal dose for man being 0.5 to 3.5 mg/kg b.w., made the authorities react with a risk communication through all media including the internet. The latter notice said: “Linseed has a high content of natural toxicants; i.e. cyanogenic glycosides). These compounds may be split in the intestines to release HCN. In principle one can risk an acute intoxication if greater amounts of linseed are consumed. In addition nervous system damages are a possible effect.” (59).

(c) The content of cyanogenic glucosides in cassava roots in retail shops in Copenhagen (2009)
An increasing number of retail shops in Copenhagen sell fresh cassava roots. A survey was made concerning the shop characteristics, the origin of the roots, the buyers and the shop owner knowledge of the actual toxicity levels. Shops selling fresh cassava were mostly owned by persons originating in the Middle East or Afghanistan, buyers were found predominantly to be of African origin and the salesmen’s knowledge about the potential toxicity was found to be very restricted. A high proportion (60%) of the 25 roots purchased had total cyanogenic potentials higher than the 50 mg HCN equivalents/ kg d.w. proposed
as acceptable by an EU working group (NETTOX). The mean and maximum content were 110 and 351 mg HCN equivalents/ kg d.w., respectively (60).

Conclusion

Cyanogenic glycosides occur widespread in the plant kingdom. Both animal feed and human food resources may contain such compounds. The released hydrogen cyanide from the degradation of cyanogenic glycosides may produce acute and chronical toxicity. This means that regulations must be in place to ensure the chemical safety of food and feed for man and animals. In general such regulations - as based on risk assessments – have already been implemented in the EU as well as in most other industrialized countries. In their text most of the risk assessments behind the regulations do recognize the risk of central nervous system damages as a result of long term exposure to relatively low concentrations of HCN. However, due to individual variation of sensitivity, relationship with other factors such as nutritional deficiencies and increased sensitivity over time, an Acceptable Daily Intake (ADI) has not been made.

Food based on highly cyanogenic plant parts such as bitter tuberous cassava roots can be made chemically safe (as based on the levels accepted in the existing standards) by different means of processing, that at the same time improves shelf life. On the other hand, national and international food safety authorities, as well as the general population, must be aware the fact, that changing food habits, e.g. as a result of new health trends, may cause risks for hitherto unseen exposure to cyanogenic glysosides; as in the case of an increased intake of linseed.

The use of e.g, amygdalin (or products based on cyanogenic compounds; Laetrile) for the treatment or prevention of different forms of cancer has no support in the scientific medicinal literature.

References


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Bioactive plant compounds in social non-alcoholic drinks – a risk for human reproduction?

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Abstract

Consumption of foods and beverages containing the methylxanthines caffeine, theobromine and theophyllin could, particularly in combination with the use of drugs containing caffeine and theophylline result in a comparatively high exposure to methylxanthines. Such exposures are well known to result in pharmacological effects which some persons find to be advantageous and others adverse. Methylxanthine concentrations ten times higher than those resulting in pharmacological effects, produce irreversible damage of the sperm producing cells of the testicles in rats exposed chronically. During the last seventy years a reduction in sperm count, and sometimes also in sperm quality, has been reported in several human populations. Many different hypotheses to explain this reduction in sperm count has been suggested, the most popular one being that environmental factors act in utero on the developing foetus in an estrogen-like or anti-androgen-like manner. Here an alternative hypothesis is presented for testing: High intakes of methylxanthines from cocoa, coffee, tea, cola and other foods and drugs, although being substantially lower than the doses required to induce the toxic effects observed in rodents, may be sufficiently high to influence the testicular Sertoli cells in such a way that they in adult men are able to produce a sub-optimal number of mature sperms.
Introduction

It is quite remarkable that nearly all of the social non-alcoholic drinks used by man over the world (Table 1) contain one and the same or closely related alkaloids, methylxanthines (1). Tea was discovered as a beverage in China, and coffee in Arabia, whereas the habit of consuming cocoa, mate, guaraná and yoco originate from Latin and South America. Cocoa, coffee and tea are used so extensively around the world that these products have become leading products of international trade, and thus important factors in world economy. With a single exception these beverages owe their stimulatory qualities to their content of caffeine (Figure 1). The exception is cocoa, which contain some caffeine but significantly more theobromine.

Table 1. Sources of methylxanthine exposure from beverages

<table>
<thead>
<tr>
<th>Beverage</th>
<th>Source</th>
<th>Area of cultivation</th>
<th>Methylxanthine content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocoa</td>
<td><em>Theobroma cacao</em> L. (seeds)</td>
<td>West Africa</td>
<td>0.2-2.7 % theobromine 0.1-0.8 % caffeine</td>
</tr>
</tbody>
</table>
| Coffee   | *Coffea arabica* L.  
*Coffea liberica* Hiern  
*Coffea canephora* Pierre ex. Froehner (*C. robusta* L) (seeds) | South America | 0.8-2.4 % caffeine |
| Guaraná  | *Paullinia cupana* Kunth. (seeds) | Amazonas | 2-6 % caffeine |
| Maté     | *Ilex paraguayensis* St. Hill. (leaves) | SW South America | 1.1-1.9 % caffeine |
| Tea      | *Camellia sinensis* (L.) Kuntze (leaves) | SE/E Asia | 3-4 % caffeine |
| Yoco     | *Paullinia yoco* R.E. Schultes & Killip (bark) | NE South America (not cultivated) | 2.7 % caffeine |
| Cola     | *Cola nitida* (Vent.) Schott. & Endl. (seeds)  
*Cola acuminata* (P. Beauv.) Schott. & Endl. (seeds) | West Africa, West Indies, South America | 1.5-3.5 % caffeine |
Figure 1. Chemical structure of xanthine and some common methylxanthines. Caffeine = 1,3,7-trimethylxanthine; theophylline = 1,3-dimethylxanthine, and theobromine = 3,7-dimethylxanthine.

Cocoa
The Cacao tree (Theobroma cacao) originates from South America. It is believed that the Maya Indians in Central America were the first to acknowledge the cacao beans. Our word chocolate is derived from the language spoken by the Mexican Indians. However, they mainly used cocoa as a beverage consisting of the ground cured beans whipped up in hot water and flavoured with pepper and other spices – a royal drink for the Incas and Aztecs (e.g. Montezuma). *Theobroma* means ‘food for the gods’. Christopher Columbus brought the cacao beans to Spain, and Cortes was the first European to introduce the hot beverage with added vanilla and sugar in the early 1500’s. In 1580 it was in common use in Spain and one hundred years later it was well known in most European countries.

The Cacao tree was introduced into the African Gold Coast (Ghana) in 1879. Today it is mainly cultivated in Africa. The Ivory Coast, and Ghana together with Indonesia are producing nearly three quarters of the world production of cacao (2).

Coffee
The coffee plants (*Coffea arabica, C. canephora, C. liberica*) are native to Africa. The coffee tree was introduced into the West Indies and Brazil in the
early 1700s, and this region is now the centre of coffee production in the world. Brazil and Columbia produce more than a third of the World production of Coffee, but production is increasing in regions such as Indonesia and Vietnam.

The custom of consuming the hot coffee beverage comes from the Middle East. Nowadays, coffee is the most common social non-alcoholic beverage in most parts of Europe with highest consumption in the Nordic countries, particular in Finland and Sweden. The majority of coffee consumed is prepared from the beans of Coffea arabica or Coffea canephora, the latter often called Robusta coffee. As Arabica coffees are milder (contain less caffeine), and are commonly considered better tasting than the coffee from Robusta beans, almost 75 % of the coffee produced in the world is Arabica coffee. The more bitter (higher caffeine content) coffee from Coffea canephora is produced because the tree is more robust, and can be cultivated under a wider range of conditions. These beans are commonly used in instant coffee. Coffea liberica beans, grown in Liberia, Surinam and Malaysia, are less commonly used for coffee production. Most cultivars of C. arabica contain about 1.0 % caffeine, C. canephora 1.7 %, and C. liberica 1.4 %, but it must be noted that the methylxanthine content is influenced by where it is cultivated, the variety of the plant, the climate, and cultural practices (3, 4).

Tea
Tea brewing originates from Eastern Asia. Today, China and India stands for around 50 % of the tea produced in the world, and tea is now the most common social beverage in the world. The drink is prepared by brewing dried young leaves and shots of the plant Camellia sinensis. There are five types of tea – green tea, paocong, oolong tea, pu-erh (red teas) and black tea. These varieties are characterized by different oxidation states of the flavonols. In green tea they are reduced, whereas they to a large extent are oxidized in the fermented pu-erh and black tea. Paochong and oolong tea are products where the flavonols are only partly oxidized due to a short fermentation process. Flavoured teas are produced by spraying flavours on traditional tea. Herbal teas are not ‘real teas’ as they do not contain the tea plant.

Colas and others
The colas, which are economically important carbonated soft drinks, do contain caffeine but in this case the compound has been added to the drink during the manufacturing. The most famous cola, Coca-Cola, received its name from the two plants that originally contributed with its two key constituents, cocaine and caffeine. The cocaine was derived from the Coca leaf (Erythroxylum coca) and the caffeine from the Kola nut (Cola acuminata or Cola nitida). In the 18th
century a glass of Coca-Cola contained around 9 mg cocaine, but in 1903 it was decided that this compound should not be allowed in the beverage and had to be removed (5). Today, one single company is allowed to produce the important flavours of ‘used’ Coca leaves, after the cocaine has been removed. An additional contribution to the flavour of Coca-Cola comes from the Kola nut, which also supply caffeine (Table 1). The stimulating and fatigue-preventing properties of the cola nut were discovered by the natives of West Africa, who chewed the nuts rather than using them for the preparation of a beverage.

As shown in Table 1 guaraná is the plant material used for beverage production that contains the highest amount of caffeine. Extracts of guaraná is used in modern types of beverage sold as energy or smart drinks, which are consumed chilled. When these drinks contain caffeine it can be of either natural (extract of guaraná) or synthetic origin. The content may be higher than in traditional colas (Table 2).

**Table 2.** Average contents of caffeine in various beverages commonly consumed in the Nordic countries (from 1)

<table>
<thead>
<tr>
<th>Beverage</th>
<th>Caffeine content per serving</th>
<th>Theobromine content per serving</th>
<th>Volume per serving</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot cocoa</td>
<td>5</td>
<td>57</td>
<td>150 ml</td>
</tr>
<tr>
<td>Chocolate milk</td>
<td>3</td>
<td>39</td>
<td>150 ml</td>
</tr>
<tr>
<td>Instant Coffee</td>
<td>53</td>
<td>Not stated</td>
<td>150 ml</td>
</tr>
<tr>
<td>Brewed (dripoliated) coffee</td>
<td>103</td>
<td>Not stated</td>
<td>150 ml</td>
</tr>
<tr>
<td>Guaraná</td>
<td>Variable</td>
<td>Low</td>
<td>variable</td>
</tr>
<tr>
<td>Maté</td>
<td>33</td>
<td>Low</td>
<td>150 ml</td>
</tr>
<tr>
<td>Tea</td>
<td>38</td>
<td>3</td>
<td>150 ml</td>
</tr>
<tr>
<td>Soft drinks/Cola drinks</td>
<td>36</td>
<td>0</td>
<td>360 ml</td>
</tr>
<tr>
<td>Energy drinks</td>
<td>80</td>
<td>0</td>
<td>250 ml</td>
</tr>
</tbody>
</table>

**Methylxanthine exposure in man**

With the exception of soft drinks and energy drinks that are sold in bottles and cans of defined sizes and containing regulated levels of added caffeine, it is very difficult to estimate the methylxanthine content of standard servings of methylxanthine-containing beverages. The amount of caffeine and theobromine present in one cup of cocoa, coffee and tea, or the drinks guaraná, maté and yoco (less consumed in the Nordic countries), depends on a number of factors which differ both depending on the plant source used for the beverage production and on individual preferences and habits. As a consequence the methylxanthine content of individual beverages will vary considerably.
Based on a large number of analytical data available in the published literature, Andersson et al. (1) estimated the average caffeine and theobromine content of a standard serving of different beverages (Table 2). Compared to these methylxanthines, the theophylline content is low and mainly comes from tea and beverages containing guaraná.

The consumer is exposed to methylxanthines not only though beverages, but also though consumption of chocolate products (theobromine and smaller amounts of caffeine) and pharmaceuticals (caffeine and theophylline). Milk chocolate, which contains between 10 and 15% cocoa on average, contains around 1530 mg theobromine/kg product (range 1350-1860), whereas dark chocolate, that contains between 30 and 80% cocoa, contains on average 4600 mg theobromine/kg product (range 3600-6300) (6). Chocolate is also used in cakes, cookies and desserts, but the amounts and type of chocolate used varies. One normal serving of a chocolate cake or chocolate biscuits may contain around 75 mg theobromine and 10 mg caffeine.

The exposure to methylxanthines from pharmaceuticals, for obvious reasons varies considerably between individuals, and depends on the type of medicinal preparation used. Whereas both caffeine and theophylline are used in medicines, there is no current therapeutic use of theobromine for humans. Drugs used for treatment of migraine may at the maximum dose advised result in an exposure of 600 mg caffeine per day, whereas other drugs commonly results in exposures around 200-400 mg caffeine/day. Although the per capita intake of caffeine from medical remedies is a very artificial exposure parameter, it has been calculated in Iceland and found to range from 36 to 41 mg/day during the 1990s (1).

In order to assess whether exposure to methylxanthines may have biological effects, reliable data on the intake of methylxanthine-containing foods, beverages and pharmaceuticals are needed. This would require chemical analytical data and/or information from databases on the actual methylxanthine content in foods and beverages (and remedies), combined with information on intake of these foods, beverages and pharmaceuticals, and/or biomarkers of exposure. However, biomarker studies, such as measurements of the plasma concentration of methylxanthines, are very rare due to the high costs and difficult logistics in collecting the blood. When it is feasible to measure plasma levels as an exposure parameter, a single biological sample per participant is frequently available. As the consumption of coffee, tea, and cocoa usually varies over time, for example between the various days of a week (weekdays/weekend), the limitation of the exposure assessment to one single point in time questions the representativeness of the sample.
Most intake data on methylxanthines is per capita data or average level limited to a single methylxanthine (usually caffeine), and this is not enough. Of the around 20 studies available on the total daily intake of caffeine from foods and beverages (1), four discuss the European situation. The average daily intake of caffeine was estimated to 280-444 mg/day in United Kingdom, 370 mg/day in Iceland, 490 mg/day in Denmark, and 425 mg/day in Sweden. Unfortunately, none of these studies gives data on individual consumers. The importance to consider the intake of individuals instead of average intake may be illustrated by looking at the caffeine intake in Nordic countries where the intake is heavily influenced by coffee consumption. A Swedish study presented intake data of coffee on 202 individuals. Only 5% of the participating females and 0.7% of the males did not drink coffee (7). Among participants in the age group 30-59 years, which had the highest coffee intake, males on average consumed 600-650 ml coffee/day (412-446 mg caffeine), whereas females consumed 415-540 ml/day (285-371 mg caffeine). Although males drank significantly more coffee than females, the caffeine exposure from coffee, calculated on per kg body weight basis, was only slightly higher in males than in females. The participant with the highest intake of coffee consumed 2240 ml a day (1538 mg caffeine). Sixteen percent of the participants consumed more than 900 ml coffee/day (>618 mg caffeine/day). These high consumers at risk for adverse effects will not be identified by the average data.

If the exposure data to caffeine is limited, the exposure data to theobromine is rudimentary and the exposure data to theophylline non-existent. There are data on the daily per capita intake of theobromine in the United States via foodstuffs and beverages in 1980, which indicate an intake of around 39 mg (8). Again, these average data are not useful to estimate potential risks.

An informed guess on the total daily methylxanthine intake in individuals with a high exposure from the diet and pharmaceuticals would be around 25 mg/kg body weight. In pregnant women, however, the methylxanthine exposure is likely to be lower than in other women. This hypothesis is based on data on caffeine exposure before, during and after pregnancy (1). These data show a reduction in caffeine exposure, particularly during the first two trimesters of pregnancy, mainly caused by a reduced coffee consumption. It is not known whether the change in caffeine exposure has a biological reason, or if it is a result of responding to advice from child health care authorities. However, it is clear that a number of women experience nausea during pregnancy, a plausible reason to reduce coffee consumption.
Biological dose of methylxanthines

There are several studies on the pharmacokinetics (absorption, distribution, metabolism, and excretion) of methylxanthine in man (1, 3). Following intake, methylxanthines are rapidly and essentially completely absorbed from the gastrointestinal tract and peak concentrations in the blood are usually reached within 0.5-2 hours. The binding to plasma proteins is low. The half-life of caffeine, theobromine and theophylline in the plasma of healthy adult humans is 3-6 h, 7-12 h and 8-9 h, respectively (9). An ordinary dietary portion of methylxanthine-containing beverages or chocolate may result in plasma levels of at least pharmacological significance (1, 10). The methylxanthines cross the placenta, enter gonadal tissue and are secreted into milk (9). Caffeine, and to a lesser extent theophylline, also diffuse through the blood/brain barrier (11). There is no accumulation of methylxanthines in any tissue or organ (12).

In adults, biotransformation takes place in the liver and is performed by cytochrome P-450 (CYP) isoforms with minimal first pass elimination (12). Although less is known about the enzymatic machinery responsible for the metabolism in test species (rat, mouse, rabbit, dog), the major routes of metabolism of the methylxanthines seem to be the same as in humans, leading to demethylation (3- and 7-N-demetylation) and, for caffeine, the formation of paraxanthine (1,7-dimethylxanthine), and 1-, 3- and 7-methylxanthine, compounds which are further oxidised to their respective methyluric acids. The methylxanthines may also be C8-oxidised to dimethyluric acids or ring-opened methyluracils (1, 3). Urinary excretion patterns of methylxanthines and metabolites are qualitatively similar among species but the quantity of the excreted compounds may vary. It should be noted that a genetic polymorphism of cytochrome P-450’s, particularly the most important enzyme CYP1A2, has been reported in humans and dogs (13-15). Such metabolic polymorphisms might play a role for the sensitivity to methylxanthines.

As reviewed by Andersson and coworkers (1), the half-life of methylxanthines in human plasma is dependent on a number of additional factors, including age, hormonal status and smoking habits. Thus, in the foetus and newborn, which do not possess the liver enzymes that metabolise the drug, caffeine and other methylxanthines have a much longer half-life. However, the foetus seems able to convert some of the theophylline to caffeine (9). Due to the hormonal changes resulting from pregnancy, or by the use of oral contraceptives, the half life of caffeine in the women’s plasma is about doubled. Smoking increases methylxanthine metabolism and reduces the plasma half-life. Taken together, these data on the pharmacokinetics of methylxanthines identify the foetuses of women having a high intake of methylxanthine-containing beverages and foods as being of particular risk for obtaining potential adverse effects.
Potential adverse effects of methylxanthines

The pharmacological effects include stimulation of the central nervous system, complex effects on the cardiovascular system, increased renal excretion of sodium and water, and stimulated gastric excretion of hydrochloric acid and pepsin. As it would carry too far to review these and other actions here, the reader is referred to general (1, 16) or more detailed reviews by others (17-19). Whereas some consumers would define those effects as advantageous, others would categorize them as adverse.

While the main molecular target of caffeine and theophylline is their antagonistic effect on adenosine receptors, in particular $A_1$, $A_{2A}$ and $A_{2B}$ subtypes, theobromine is a weak antagonist with a two- and threefold lower affinity to $A_1$ and $A_{2A}$ receptors than caffeine (3, 19). At considerably higher doses than those associated with adenosine receptor antagonism methylxanthines may have other mechanisms of action, e.g. inhibition of phosphodiesterase (19).

Acute and subacute toxicity

Acute toxic effects of methylxanthines include hypotension, palpitations, tachycardia, arrhythmias, slushing and marked circulatory failure, sweating, trembling, nausea, diarrhoea, severe headache, epigastric pain and occasionally peptic ulcer and haematemesis (17, 20). High intakes of cocoa products (containing theobromine) may be fatal, particularly in sensitive animals such as horses, dogs and birds (2). But deaths have also been reported in pigs and rabbits, and in wild animals such as coyotes, the red fox and the European badger. A summary of the chronic toxicity of methylxanthines can be found elsewhere (1, 16, 21).

In 1978 Weinberger and co-workers (22) performed an interesting experiment in which they investigated whether methylxanthines may become nitrosated \textit{in vivo} and produce toxic nitrosamines able to damage DNA and induce tumours. In this experiment, they fed Osborne-Mendel rats a diet with large quantities of caffeine, theobromine, or theophylline for 14 to 75 weeks resulting in exposures of approximately 250 mg/kg b.w. per day. The rats had lower weight gains than the controls, but when the investigators studied the hypothesized target organs, mainly the gastrointestinal tract and the liver, no sign of carcinogenicity was found. Instead they made the unexpected finding of severe testicular atrophy, particularly in rats exposed to caffeine or theobromine. In rats that had been given caffeine or theobromine for at least a year, more than 94 % of the animals showed testicular atrophy and more than 82 % aspermatogenesis, sometimes
accompanied by thymus atrophy. In the theophylline-treated rats the corresponding figures were lower, 50 and 17 %, respectively.

Understandably, these observations initiated a cascade of studies on the effect of methylxanthines on the male reproductive tract (23-27), for example aiming at identifying the critical exposure and the sensitive species. It was confirmed that caffeine had similar or possibly stronger effects than theobromine (27), and that theophylline was substantially less potent (28). Exposure times of 4 weeks or more were enough to induce the condition, which after doses of 300 mg/kg b.w. or more were irreversible (24). The most striking morphological observation was a retarded release of late spermatids into the tubular lumen, indicating selective interference with germ cell kinetics possibly due to Sertoli cell toxicity (29-31). Mice, hamsters and rabbits were less sensitive to methylxanthines than rats but the target organs seemed to be identical (31-33).

**Human sperm count**

The sperm count in humans has been monitored on a population level for more than seventy years, and is one of the factors investigated in infertility situations. In 1992 a Danish research team lead by Skakkebæk reviewed the published controversial data on sperm counts in the United States, Latin America, Western Europe, India, China, Australia and the former Soviet Union, based on 61 investigations, and concluded that the sperm count in semen has declined between 1939 and 1992, from 113x10^6/mL in 1940 to 66x10^6/mL in 1990 (34). A worrying signal about this downward trend is that having a sperm count less than 20 million sperms per mL would define you as infertile. If the downward trend in sperm counts continues, values less than those defining infertility will be reached in these populations in a not too distant future.

The Danish study attracted a lot of attention and resulted in a multitude of comments. The critics claimed that the different studies in the meta-analysis were very heterogenous in terms of fertility, age and socioeconomic status, that the amount of information available on the study subjects varied considerably, and that the statistical methodology was not adequate (fundamental flaw in controlling for confounders) (35, 36). Other investigators published new data or new meta-analysis of data that supported the Danish findings (37-41). The observation that the sperm counts varies between and within countries or regions appears to be much less controversial than the claimed decline in sperm counts (42, 43).
An additional scientific observation is that not only the quantity of sperm might be decreasing, but also the quality of the sperm. For example, Pajarinen and co-workers (44) reported that the proportion of normal sperm from Finnish males in Helsinki fell from about 50% in 1980 to about 25% by 1992.

Interestingly, the decline in sperm counts is observed concurrently with an increase in the occurrence of testicular germ cell cancer, cryptorchidism and hypospadiasis. The Danish investigators suggested that the cause of all these adverse effects is an increased exposure to an environmental factor that acts in utero on the developing foetus in an estrogen-like or anti-androgen-like manner (45, 46). Thus, hormone-mimicking chemicals in the mother's blood affects male children before they are born in such a way that they come to life with fewer Sertoli cells in the testicles. As these Sertoli cells later in life orchestrate and control the process of spermatogenesis, the number of Sertoli cells present puts a 'ceiling' on the maximum attainable germ cells being produced (47).

The main support for the ‘endocrine disruption’ hypothesis was originally obtained from experimental or wildlife data (47-50), but later human observations were added. The in utero exposure hypothesis could be exemplified by data on environmental pollutants (e.g. dioxins) or on sons of women who were given the synthetic hormone diethylstilbestrol (DES) during the 1950s and 1960s. About a million American women were given this substance as a ‘morning after’ pill to be taken after an intercourse to reduce the likelihood of pregnancy. In cases it did not function, their sons have shown an increase in genital tract abnormalities, and reduced sperm count (46). Some investigators have confirmed that fertility treatment of women during pregnancy is linked to a reduced reproductive health of male offspring, including a lower sperm count (51).

Exposure to ‘endocrine disruptors’ during foetal development is far from the only hypothesis suggested to explain the decline in sperm count over the last seventy years. Also the life-style of the mothers, or the men themselves, has been suggested to be important. A non-exhaustive list of factors hypothesized to contribute to the lowering in sperm count would include: genital/sexual transmitted disease (52), dietary insufficiencies, malnutrition and obesity (53-55), smoking (e.g. 55), alcohol consumption and use of other drugs (e.g. 56, 57), testicular overheating (e.g. due to fever incidents, sauna baths, vigourous exercise, too slim trousers, or driving motor vehicles) (e.g. 58-60), environmental polutants (e.g. dioxins) (61-63), and ionizing and non-ionizing radiation, e.g. from cell phones (64, 65).
**Caffeine and reproductive toxicity**

Considering the toxicological profile of higher doses of methylxanthines summarized above, targeting the Sertoli cells in the testis, an additional hypothesis for the reduction in sperm count could be considered. In summary, this hypothesis would suggest that high intakes of methylxanthines are sufficient to influence the testicular Sertoli cells of adult men in such a way that sub-optimal numbers of mature sperm are produced. Although there are no studies devoted specifically to the influence of methylxanthine intake in human males on sperm counts, several investigations have observed that high exposures to caffeine increase the likelihood of genetic damage in sperm cells (e.g. 66-68).

To assess the value of these hypotheses, it seems apparent that well-designed prospective studies in defined cohorts of men living in various parts of the world are required to evaluate the potential effect of external factors, such as exposure to ‘endocrine disruptors’ or methylxanthines early in life, on male reproductive health.

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Management and regulation of certain bioactive compounds present as inherent toxins in plants intended for feed and food

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Abstract

Directive 2002/32/EC of 7 May 2002 of the European Parliament and of the Council on undesirable substances in animal feed is the framework for the EU action on undesirable substances in feed, including bioactive compounds present as inherent toxins in plants. Council Regulation (EEC) 315/93 of 8 February 1993 laying down Community procedures for contaminants in food (12) is the basic regulation governing the measures on contaminants in food. The framework legislation provides, inter alia, for a mandatory consultation of a scientific body (European Food Safety Authority) for all provisions which may have an effect upon public health or animal health or on the environment. Following requests of the European Commission, the Panel on Contaminants in the Food Chain (CONTAM) from the European Food Safety Authority (EFSA) has completed recently a series of risk assessments undertaken on undesirable substances in animal feed, including bioactive compounds present as inherent toxins in plants, reviewing the possible risks for animal and human health due to the presence of these substances in animal feed. The paper describes the main outcome of the risk assessments and the measures taken following these assessments. The management and regulation of certain bioactive compounds
present in feed and food as inherent plant toxins, are at EU-level more developed in the feed than in the food legislation. However, for a comprehensive legislation covering all relevant inherent plant toxins in feed and food there is still a long way to go, whereby the risk assessor and risk manager are confronted with a lot of obstacles to move forward.

Regulatory framework for EU feed and food safety legislation

The Regulation (EC) 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety (1) provides for the general principles of food law, which must be followed when measures to ensure feed and food safety are taken. This Regulation is more commonly known as the “General Food Law”.

General principles and objectives of food law

General food law applies to all stages of the production, processing and distribution of food and also of feed produced for, or fed to, food producing animals.

Food law shall pursue one or more general objectives of a high level of protection of human health and the protection of consumers’ interests and of, where appropriate, the protection of animal health and welfare, plant health and the environment.

Food law shall aim to achieve the free movement in the European Union of feed and food manufactured or marketed according to the general principles and requirements of food law.

When international standards exist or their completion is imminent, they shall be taken into consideration in the development of food law, except where such standards would be an ineffective or inappropriate means for the fulfilment of the legitimate objectives of food law.
In order to achieve the general objective of a high level of protection of human health, the General Food Law lays down that EU food legislation shall be based on risk analysis except where this is not appropriate to the circumstances or the nature of the measure (e.g. labelling).

Risk assessment shall be based on the available scientific evidence and undertaken in an independent, objective and transparent manner.

Risk management shall take into account the results of risk assessment, other factors legitimate to the matter under consideration and the precautionary principle where appropriate.

Under the precautionary principle, the Member States and the Commission may take appropriate provisional risk-management measures when an assessment points to the likelihood of harmful health effects and there is a lack of scientific certainty.

There is a requirement for transparent public consultation, directly or through representative bodies, during the preparation, evaluation and revision of food law. When a food or feed product is deemed to constitute a risk, the authorities must inform the general public of the nature of the risk to human or animal health.

**General requirements**

Food must not be placed on the market if it is unsafe, i.e. if it is harmful to health and/or unfit for consumption. In determining whether any food is unsafe, account is taken of the normal conditions of use, the information provided to the consumer, the likely immediate or delayed effect on health, the cumulative toxic effects and, where appropriate, the particular health sensitivities of a specific category of consumers. If food which is unsafe forms part of a batch, lot or consignment, the entire quantity is presumed to be unsafe.

Feed must not be placed on the market or given to any food-producing animal if it is unsafe. Feed is deemed to be unsafe if it has an adverse effect on human or animal health. The entire quantity of a batch, lot or consignment is considered unsafe if any part of it fails to satisfy the requirements.

At all stages of the food production chain, business operators must ensure that food and feed satisfy the requirements of food law and that those requirements are being adhered to. The Member States enforce the law, ensuring that
operators comply with it and laying down appropriate measures and penalties for infringements.

The traceability of food, feed, food-producing animals and all substances incorporated into foodstuffs must be established at all stages of production, processing and distribution. To this end, business operators are required to apply appropriate systems and procedures.

If an operator considers that a food or feed product which has been imported, produced, processed, manufactured or distributed, is harmful to human or animal health, steps must be taken immediately to withdraw the product from the market and to inform the competent authorities accordingly. In cases where a product may have reached consumers, the operator must inform them and recall the products already supplied.

EFSA and Standing Committee

EFSA
Regulation (EC) 178/2002 established the European Food Safety Authority (EFSA). The Authority provides scientific advice and scientific and technical support for the Community's legislation and policies in all fields which have a direct or indirect impact on food and feed safety. It provides independent information on all matters within these fields and communicates on risks.

The scientific opinions provided by the Authority serve as the scientific basis for the drafting and adoption of Community measures related to feed and food safety and to other matters relating to animal health and welfare and plant health.

Standing Committee
The Regulation (EC) 178/2002 establishes furthermore the Standing Committee on the Food Chain and Animal Health, hereinafter referred to as the "Committee".

The Committee is made up of representatives of the Member States and is chaired by a representative of the Commission.
The Committee's mandate covers the entire food supply chain, ranging from animal health issues on the farm to the product that arrives on the consumer's table, thus significantly enhancing its ability to target risks to health wherever they arise in the production of our food.

The Committee is a regulatory committee. The Commission may adopt the implementing measures only if they obtain a favourable opinion from the committee, given by a qualified majority of the Member States. Failing that, the proposed measure is referred to the Council, which takes a decision by a qualified majority. However, if the Council fails to reach a decision, the Commission adopts the implementing measure unless the Council opposes it by a qualified majority.

Qualified majority voting is based on the principle of the weighting of votes. Under the current weighting system, the Member States with the largest populations have 27-29 votes, the medium-sized countries have 7-14 votes and the small countries 3 or 4 votes. In the EU with 27 Member States, a proposed measure requires at least 255 out of 345 votes in favour of the proposed measure, expressed by a majority of the Member States (at least 14 delegations) in order to enable the Commission or the Council, if the case occurs, to adopt the measure.

The Committee is organised in sections to deal with all the relevant matters. These sections are as follows:

- General Food Law
- Biological Safety of the Food Chain
- Toxicological Safety of the Food Chain
- Controls and Import Conditions
- Animal Nutrition
- Genetically Modified Food and Feed and Environmental Risk
- Animal Health and Animal Welfare
- Phytopharmaceuticals

The section on “Toxicological safety of Food Chain” is responsible for measures related to bioactive compounds in food and the section “Animal nutrition” for measures related to bioactive compounds in feed.

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1 EU Member States with number of votes between brackets: Germany (29), France (29), United Kingdom (29), Italy (29), Spain (27), Poland (27), Romania (14), Netherlands (13), Greece (12), Portugal (12), Belgium (12), Czech Republic (12), Hungary (12), Sweden (10), Austria (10), Bulgaria (10), Denmark (7), Slovakia (7), Finland (7), Ireland (7), Lithuania (7), Latvia (4), Slovenia (4), Estonia (4), Cyprus (4), Luxembourg (4), Malta (3)
Regulatory framework for contaminants in feed


"Undesirable substance in feed" is defined as any substance or product, with the exception of pathogenic agents, which is present in and/or on the product intended for animal feed and which presents a potential danger to animal or human health or to the environment or could adversely affect livestock production. The Regulation does not apply to contaminants or undesirable substances covered by more specific legislation.

It provides:

- that products intended for animal feed may enter for use into the Community, be marketed and used in the Community only if they are sound, genuine and of merchantable quality and therefore do not represent any danger to human health, animal health or to the environment or do adversely affect livestock production.

- that maximum levels and action levels can be set for contaminants in all products intended for animal feed:
  * maximum levels: feed containing an undesirable substance above the maximum level cannot be marketed and/or used for animal feeding.
  * action level: in case a feed is containing an undesirable substance at a level above the action level, Member States, in cooperation with the economic operators concerned, must carry out investigations to identify the sources of the substances concerned. They must then inform the Commission of the outcome of these investigations and the measures taken to reduce the level of the substances or eliminate them.

- that products intended for animal feeding containing levels of an undesirable substance that exceed the maximum level may not be mixed for dilution purposes with the same, or other, products intended for animal feed.

- that detoxification is allowed by chemical treatment and that Member States shall ensure that measures are taken to guarantee the correct application of these detoxification processes and to guarantee the conformity of the
detoxified products intended for animal feed with the provisions of the Directive.

- for an obligatory consultation of the European Food Safety Authority (EFSA) - Scientific Panel on Contaminants in the Food Chain before provisions having effect upon public or animal health or on the environment shall be adopted.

The Directive furthermore foresees that Member States may not prohibit trade in feeds which comply with this Directive for any reason related to the aspects covered by the provisions of this Directive. A safeguard clause is also included enabling Member States to temporarily suspend or restrict marketing on their territory of any feedstuff suspected of containing contaminants which would endanger human or animal health or the environment.

**Procedure for setting regulatory limits for contaminants in feed**

The scientific risk assessment which is the assessment of the risks related to the presence of a contaminant in feed for animal and human health, is the basis for the measures to be taken.

In this scientific assessment the toxic exposure level for different animal species is determined above which harmful animal health effects can occur. Given the different sensitivity of the animal species, the toxic exposure level can differ significantly from one animal species to another. In order to assess the risks of the presence of an contaminant in feed for human health, the carry over of the undesirable substance from feed into food of animal origin (meat, edible offal, milk, eggs) is assessed, where possible quantitatively. Also the contribution of the presence of the contaminant in food of animal origin to the total human exposure is assessed.

The animal exposure assessment does also enable to identify the feeds/feed groups significantly contributing to the exposure. From these feed and feed groups, the occurrence data, obtained following the application of good practices are used to determine the appropriate maximum level to protect animal and public health.

Maximum levels are set at a strict level which is reasonably achievable by following good agricultural, fishery and manufacturing practices and taking into account the risk related to the consumption of the feed.
Such approach ensures that feed business operators apply measures to prevent and reduce the contamination as far as possible in order to protect animal and public health.

**Contaminants/ undesirable substances currently regulated by Directive 2002/32/EC**

**List of undesirable substances covered by Directive 2002/32/EC**

The following contaminants/undesirable substances were in 2002 covered by Directive 2002/32/EC:

- **Inorganic contaminants** (arsenic, lead, mercury, cadmium, etc.)
- **Mycotoxins** (aflatoxin B1, rye ergot)
- **Organochlorine compounds** (dioxins, dieldrin, endosulfan, etc.)

**Inherent plant toxins:**
- hydrocyanic acid
- free gossypol
- theobromine
- volatile mustard oil
- vinyl thiooxazolidone

**Harmful botanical impurities:**
- weed seeds and unground and uncrushed fruits containing alkaloids, glycosides or other toxic substances separately or in combination including *Lolium temulentum, Lolium remotum and Datura stramonium*
- seeds and husks from the castor oil plant, *Ricinus communis*, as well as the processed derivatives separately or in combination
- *Croton tiglium* as well as the processed derivatives
- *Crotalaria spp.*
- Apricots – *Prunus armeniaca*, as well as the processed derivatives
- Bitter almond - *Prunus dulcis var. amara* (= *Prunus amygdalus var. amara*), as well as the processed derivatives
- Camelina – *Camelina sativa*, as well as the processed derivatives
- *Mowrah, Bassia, Madhuca*, as well as the processed derivatives
- unhusked beech mast, *Fagus silvatica*, as well as the processed derivatives
- purghera, *Jatropha curcas*, as well as the processed derivatives
- Indian mustard, *Brassica juncea ssp integrifolia*, as well as the processed derivatives
- Sareptian mustard, *Brassica juncea ssp juncea*, as well as the processed derivatives
- Chinese mustard, *Brassica juncea ssp juncea var lutea*, as well as the processed derivatives
- Black mustard, *Brassica nigra*, as well as the processed derivatives
- Ethiopian mustard, *Brassica carinata*, as well as the processed derivatives

**Recent review of the provisions as regards undesirable substances covered by Directive 2002/32/EC**

In 2002, the Commission made the commitment to review the provisions provided for in the Annex to Directive 2002/32/EC based upon updated risk assessments.

It was e.g. not always possible to track the background to or scientific justification of the provisions as regards inherent plant toxins and botanical impurities. Why are certain botanical impurities listed and others not? Possibly, however not documented, are certain provisions related to (isolated) poisoning incidents (animal health) which happened in the past.

Following requests of the European Commission, the Panel on Contaminants in the Food Chain (CONTAM) from EFSA has completed a series of 30 risk assessments undertaken in the time period 2004-2009 on undesirable substances in animal feed reviewing the possible risks for animal and human health due to the presence of these substances in animal feed. These opinions were the basis for several changes to the provisions on undesirable substances in feed.

In the Annex to Directive 2002/32/EC several plant species, or seeds and fruits of these plant species as well their processed derivatives are listed and may in many cases only be present in feed in trace amounts not quantitatively determinable. This can currently only be controlled by microscopic detection and on the condition that the feed is not finely milled or ground. Therefore EFSA was asked to perform risk assessments on the inherent plant toxicants presumed to be responsible for the toxicity of the plants for animal and human and consequently, maximum limits for botanical contaminants of particular concern should be set on the basis of their known toxicants or maximum levels for the toxicants itself in addition or in replacement to the provisions on the botanical impurities.

However a major drawback to move forward in that direction is the lack of validated routine method for the analyses of these toxicants and the lack of a comprehensive dataset on the presence of these toxicants in feed.
In the following chapter, attention will be paid individually to the outcome of risk assessments on inherent plant toxins and harmful botanical impurities and to the management measures taken as a consequence.

Inherent plant toxins/harmful botanical impurities in feed

Hydrocyanic acid/cyanogenic glycosides

Outcome of the risk assessment by the CONTAM panel from EFSA (3)

Hydrogen cyanide (HCN) is formed following the enzymatic hydrolysis of cyanogenic glycosides, which are produced as secondary metabolites by various plant species. In the intact plant these cyanogenic compounds are stored separated from hydrolytic enzymes. Crushing of plant materials either by technical processes or by chewing by animals obliterates this separation and initiates the enzymatic hydrolysis of cyanogenic compounds, resulting ultimately in the formation of HCN. Hydrolysis to release HCN can also be accomplished by micro-organisms in the digestive tract.

Cyanogenic glycosides are widely distributed in the plant kingdom. Typical feed materials that contain cyanogenic glycosides are linseed (flax), cassava root and the green parts of sorghum species. Linseed is presented as animal feed mainly as pressor extraction cake, both being by-products in the production of linseed oil. The same applies to the seeds of various Prunus species, of which the press- or extraction cake is used as feed material. Cassava roots are commonly processed into chips, which are exported to Europe as feed material for pigs.

Depending on the pH, HCN may also occur as the cyanide anion. Both HCN and cyanide are toxic to all animal species. Exposure to HCN may lead to acute, fatal intoxications. More frequently, however, chronic intoxications are observed, characterized by growth depression and neurological symptoms resulting from tissue damage in the central nervous system. Ruminants, in which the forestomach flora contributes to the hydrolysis of cyanogenic glycosides, are considered to be more vulnerable to such compounds than monogastric animals and humans. This results in a higher prevalence of clinical cases of intoxication in ruminant species following exposure to cyanogenic glycosides.

No systematic experimental studies exist on the carry over of cyanide or its precursors into edible products such as meat, offals and eggs. Based on kinetic considerations and a common metabolic pathway of degradation of cyanide, the
levels in meat, milk, or eggs intended for human consumption can be expected to be very low in all food producing animals.

The Panel noted the need for more data on the toxicology and the presence of cyanogenic glycosides in feeding stuffs. The Panel also identified the need for up-to-date analytical methods that allow the determination of the total cyanogenic potential.

Management /regulatory measures taken following EFSA’s risk assessment

The maximum levels for hydrocyanic acid remain unchanged (see table 1) for the time being. However in the near future a reliable method for the analysis of cyanogenic glycosides needs to be validated and after collection of occurrence data, the current maximum levels need to be re-assessed.

Table 1. Maximum levels for hydrocyanic acid in feed

<table>
<thead>
<tr>
<th>Products intended for animal feed</th>
<th>Maximum content in mg/kg (ppm) relative to a feed with a moisture content of 12 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed materials</td>
<td></td>
</tr>
<tr>
<td>with the exception of</td>
<td></td>
</tr>
<tr>
<td>- linseed</td>
<td>50</td>
</tr>
<tr>
<td>- linseed cakes</td>
<td>250</td>
</tr>
<tr>
<td>- manioc products and almond cakes</td>
<td>350</td>
</tr>
<tr>
<td>- manioc products and almond cakes</td>
<td>100</td>
</tr>
<tr>
<td>Complete feed</td>
<td></td>
</tr>
<tr>
<td>with the exception of</td>
<td></td>
</tr>
<tr>
<td>- complete feed for chicks</td>
<td>50</td>
</tr>
<tr>
<td>- complete feed for chicks</td>
<td>10</td>
</tr>
</tbody>
</table>

Apricots (*Prunus armeniaca*) and bitter almond (*Prunus dulcis* var. *amara* or *Prunus amygdalus* var. *amara*) were included in the Annex to Directive 2002/32/EC and the seeds and fruits of these plant species as well as their processed derivatives were only allowed to be present in feed in trace amounts not quantitatively determinable. According to the abovementioned EFSA opinion these plant species do contain significant amounts of cyanogenic glycosides. However also other plant species, such as *Prunus persica*, *Prunus laurocerasus* and *Prunus serotina* can contain very significant amounts of cyanogenic glycosides.

Maximum levels have been established for hydrocyanic acid in all feed materials and complete feed. From the EFSA opinion it can be concluded that the requirement for absence of quantifiable amounts of apricots and bitter almond and their derivatives is not necessary for the protection animal and public health insofar the maximum levels for hydrocyanic acid in feed materials and complete feed are not exceeded. It has therefore been decided to delete
apricot and bitter almond and their processed derivatives from the Annex to Directive 2002/32/EC.

The existing official EC-method for the analysis of hydrogen cyanide in feed has been deleted in legislation as it has been demonstrated that the method is generating false positives. The European Standardisation Committee (CEN) Technical Committee (TC) CEN/TC 327 Animal Feedingstuffs – methods of sampling and analysis is currently working on the elaboration and validation of an updated method for the determination of hydrocyanic acid, in execution of the mandate from the Commission to CEN.

**Free gossypol**

Outcome of the risk assessment by the CONTAM panel from EFSA (4)

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 ingredient. Storage, steam and 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 LD50s 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.

No health based guidance value (ADI, TDI) has been established for gossypol. Monogastric animals appear to be more susceptible to gossypol toxicity than ruminants. However, not all monogastric livestock animals, e.g. pigs, have been fully investigated for potential reproductive effects.
Current legislation includes maximum limits for free gossypol in both cottonseed meal and complete feed. Under normal feeding practices, the concentration in complete feed 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 feed 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.

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. 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.

Management /regulatory measures taken following EFSA’s risk assessment

From the EFSA opinion it can be derived that the existing maximum levels of free gossypol in feed for sheep including lambs, and goats including kids, were not sufficiently protective against adverse animal health effects, and therefore, a significant lowering of the maximum level of gossypol in feed for these animal species was appropriate (see Table 2).

Another conclusion from the abovementioned EFSA opinion is that the specificity of current official spectrophotometric method of analysis of gossypol in EU is questionable, as compounds other than gossypol can contribute to the formation of UV adsorption at 440 nm (5). 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 current official method. High-performance liquid chromatography (HPLC) determination of gossypol is preferred as it offers a higher degree of specificity. Therefore it should be considered to replace the current official EU method of analysis of gossypol by a specific analytical method (such as HPLC).
Table 2. New maximum levels for free gossypol in feed

<table>
<thead>
<tr>
<th>Products intended for animal feed</th>
<th>Maximum content in mg/kg (ppm) relative to a feed with a moisture content of 12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed materials with the exception of:</td>
<td></td>
</tr>
<tr>
<td>- cottonseed</td>
<td>20</td>
</tr>
<tr>
<td>- cottonseed cakes and cottonseed meal</td>
<td>5000</td>
</tr>
<tr>
<td>- the exception of:</td>
<td>1200</td>
</tr>
<tr>
<td>Complete feed with the exception of:</td>
<td></td>
</tr>
<tr>
<td>- complete feed for adult cattle;</td>
<td>20</td>
</tr>
<tr>
<td>- complete feed for sheep (except lambs and goats (except kids);</td>
<td>500</td>
</tr>
<tr>
<td>- complete feed for poultry (except laying hens) and calves;</td>
<td>300</td>
</tr>
<tr>
<td>- complete feed for rabbits, lambs, kids and pigs (except piglets).</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>60</td>
</tr>
</tbody>
</table>

Theobromine

Outcome of the risk assessment by the CONTAM panel from EFSA (6)

Theobromine (3,7-dihydro-3,7-dimethyl-\(H\)-purine-2,6-dione) is a 3,7-dimethylxanthine with a slightly bitter taste naturally present in the cacao tree (\(Theobroma cacao\)) and its seeds, and consequently in cocoa products and by-products. It is also a metabolite of caffeine in mammals.

As a feed material, cocoa pod husk may be used in developing countries where cacao is grown. The cocoa bean shells, cocoa bean meal, cocoa germs and discarded confectionary are used for feed purposes in Europe.

Theobromine shows moderate acute toxicity and the dog is more susceptible than rodents. When exposed to theobromine, dairy cows and calves showed reduction in milk yield/increase in fat (15 mg/kg b.w. per day) and adverse effects such as hyper-excitability, sweating and increased respiration and heart rates. In horses, which are more susceptible than ruminants to theobromine, the liver and thyroid were affected, while pigs showed growth retardation, diarrhoea and lethargy. Theobromine exposure to laying hens caused liver and kidney toxicity, depressed weight gain and egg-production.

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

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

Management /regulatory measures taken following EFSA's risk assessment

One of the conclusions of the EFSA opinion was that the existing EU regulations on maximum levels (ML) of theobromine in feed (300 mg/kg for complete feed with the exception of 700 mg/kg for complete feed for adult cattle) were not fully protective for some target animal species. It pointed out possible adverse effects on pigs, dogs and horses and on milk production in dairy cows and it was therefore appropriate to establish lower maximum levels (see table 3).

<table>
<thead>
<tr>
<th>Products intended for animal feed</th>
<th>Maximum content in mg/kg (ppm) relative to a feed with a moisture content of 12 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete feed with the exception of:</td>
<td></td>
</tr>
<tr>
<td>- complete feed for pigs</td>
<td>300</td>
</tr>
<tr>
<td>- complete feed for dogs, rabbits, horses and fur animals</td>
<td>200</td>
</tr>
<tr>
<td>- complete feed for dogs, rabbits, horses and fur animals</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 3. New maximum levels for theobromine in feed
Glucosinolates

Outcome of the risk assessment by the CONTAM panel from EFSA (7)

Glucosinolates (alkyl aldoxime-O-sulphate esters with a β-D-thioglucopyranoside group) occur in important oil- and protein-rich agricultural crops, including among others Brassica napus (rapeseed of Canola), B. campestris (turnip rape) and Sinapis alba (white mustard), all belonging to the plant family of Brassicaceae. They are present in all parts of these plants, with the highest concentrations often found in seeds. Several of these Brassica species are important feed ingredients and some species are also commonly used in human nutrition such as cauliflower, cabbages, broccoli and Brussels sprouts. Glucosinolates and their breakdown products determine the typical flavour and (bitter) taste of these vegetables.

The individual glucosinolates vary in structure and the configuration of their side chain. They are hydrophilic and rather stable and remain in the press cake of oilseeds when these are processed and de-oiled. However, glucosinolate producing plants as well as some microorganisms contain specific β-thioglucosidases (denoted as myrosinases). In the intact plant these enzymes are separated from the glucosinolates and sequestered in aqueous vacuoles. Upon plant damage (including chewing during ingestion) the myrosinases are released, and initiate in the presence of water the conversion of glucosinolates into diverse breakdown products, including isothiocyanates, oxazolidinethiones (5-vinyl-2-oxazolidinethione and 5-vinyl-1,3 oxazolidine-2-thione), thiocyanates, nitriles, epithionitriles and other indol-3-ylmethyl derivatives. The biological effects of plant glucosinolates in mammalian species are predominantly related to these glucosinolate-derived compounds.

They interfere with iodine uptake (thiocyanate ion) and the synthesis of thyroid hormones triiodothyronine (T₃) and plasma thyroxine (T₄) (5-vinylazoxazidine-2 thione), leading eventually to hypothyroidism and enlargement of the thyroid gland (goitre). As a consequence of these changes in thyroid function, clinical signs of toxicity described in farm animals include growth retardation, reduction in performance (milk and egg production), impaired reproductive activity, and impairment of liver and kidney functions, the latter are likely being associated with the formation of nitriles. Data on the toxicity of individual glucosinolates for food-producing animal species are very limited. In most cases only the total glucosinolate content in a given feed material, measured indirectly through the quantification of hydrolysable glucose, is available, despite the fact that individual glucosinolates vary in their toxicity. Only for rapeseed meal or press cakes comprehensive feeding trials in farm animals have been conducted, resulting in the recommendation to restrict the total glucosinolate content to 1 –
1.5 mmol per kg feed for monogastric animals, and to even lower concentrations in feeds for young animals.

If the amount of total glucosinolates is measured, impurities caused by the presence of *Brassica juncea*, *Brassica nigra* and *Brassica carinata* seeds, currently allocated to the group of undesirable botanical impurities, would be detected as well. The measurement of 5-VOT (5-vinyloxazolidone-2-thione) does not provide additional information on the toxicity of feed materials, as this compound is volatile and not formed as cleavage product by all glucosinolates.

In recognition of potential adverse effects exerted at high concentrations of glucosinolates, selection of plant varieties with low glucosinolate content (in addition to a low content of erucic acid in the oil) commenced more than three decades ago, resulting in the use of varieties, particularly rapeseeds, with a low glucosinolate content. The common practices of selecting low-glucosinolate plant varieties as forage plants and processing crops with a potential high glucosinolate concentration prior to use, together with experience-based recommendations for maximal inclusion rates into animal diets, have proven to be effective measures to avoid intoxications and production losses in farm animals, and the undesirable fishy taint in animal-derived products.

However, it is recommended that the available advanced analytical techniques should be applied to quantify the major glucosinolates in these forage plants with the aim to more accurately define animal exposure. This applies particularly to new or re-emerging oilseed crops, such as *Camelina sativa*, that may contain long-side chain glucosinolates, which are not detected in other common *Brassica* species.

Following exposure of farm animals to forages and concentrates containing glucosinolates, a carry over of glucosinolates and their associated breakdown products into edible tissues, milk and eggs has been described, but the rate of carry-over is very low. The measurable residues in dairy milk corresponding to approximately 0.1 % of the given glucosinolate dose, the residues in muscle tissues and organs were even lower. In certain breeds of laying hens, excretion of glucosinolate-derived compounds may convey an undesirable fishy taint to the eggs. However, all measured concentrations in animal-derived products are much lower than those found in vegetables for human consumption, and are unlikely to induce adverse health effects in consumers.

Management /regulatory measures taken following EFSA's risk assessment
EFSA concluded that adverse effects in animals are generally correlated to the amount of total glucosinolates in the diet, despite the fact that individual
glucosinolates vary in their toxicity. Maximum levels have been established for volatile mustard oil (expressed as allyl isothiocyanates) and vinyloxazolidone thione (see table 4 and 5). Based on the conclusions of the EFSA opinion, the existing provisions will have to be reviewed in the future once:
- method of analysis for total glucosinolates in feed has been validated
- data on the presence of total glucosinolates in feed have been collected

The review should consist of:
- considering maximum levels related to total glucosinolates instead of the current provisions for volatile mustard oil (expressed as allyl isothiocyanates)
- considering the deletion of the provisions on 5-VOT (5-vinyloxazolidone-2-thione), as the compound is volatile and not formed as cleavage product by all glucosinolates

**Table 4. Existing maximum levels for volatile mustard oil in feed**

<table>
<thead>
<tr>
<th>Products intended for animal feed</th>
<th>Maximum content in mg/kg (ppm) relative to a feed with a moisture content of 12 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed materials with the exception of:</td>
<td></td>
</tr>
<tr>
<td>- rapeseed cakes</td>
<td>100 (expressed as allyl isothiocyanate)</td>
</tr>
<tr>
<td>- complete feed for cattle, sheep and goats (except young animals)</td>
<td>4000 (expressed as allyl isothiocyanate)</td>
</tr>
<tr>
<td>Complete feed with the exception of:</td>
<td></td>
</tr>
<tr>
<td>- complete feed for pigs (except piglets) and poultry</td>
<td>150 (expressed as allyl isothiocyanate)</td>
</tr>
<tr>
<td></td>
<td>1000 (expressed as allyl isothiocyanate)</td>
</tr>
<tr>
<td></td>
<td>500 (expressed as allyl isothiocyanate)</td>
</tr>
</tbody>
</table>

**Table 5. Existing maximum levels for vinyl thiooxazolidone (5-vinyloxazolidine-2-thione) in feed**

<table>
<thead>
<tr>
<th>Products intended for animal feed</th>
<th>Maximum content in mg/kg (ppm) relative to a feed with a moisture content of 12 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete feed for poultry with the exception of:</td>
<td></td>
</tr>
<tr>
<td>- complete feed for laying hens</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>500</td>
</tr>
</tbody>
</table>

Camelina (*Camelina sativa*) was included in the Annex to Directive 2002/32/EC and the seeds and fruits of these plant species as well as their processed derivatives may only be present in feed in trace amounts not quantitatively determinable. According to the EFSA opinion, Camelina does contain significant amounts of glucosinolates but at much lower quantities than e.g. mustard and crambe.
There is a renewed interest in *Camelina sativa* as oil seed crop because of an increasing demand for alternative low-input oilseed crops with the potential for use of the by-products of the oilseed production in animal feed. It can be concluded from the EFSA opinion that the requirement for absence of quantifiable amounts of *Camelina sativa* and their derivatives is not necessary for the protection animal and public health insofar the amount of total glucosinolates in the diet does not endanger animal and public health. It was therefore decided to delete *Camelina sativa* and their processed derivatives from the Annex to Directive 2002/32/EC.


**Tropane alkaloids**

Outcome of the risk assessment by the CONTAM panel from EFSA (8)
The term tropane alkaloids refers to a group of more than 200 compounds best known for their occurrence in the family *Solanaceae* comprising over 100 genera and 3000 plant species. They have in common a two-ringed structure characterized by a pyrrolidine and a piperidine ring sharing a single nitrogen atom and two carbons atoms. The nitrogen atom at the end of the molecule, which characterizes the compounds as alkaloids, is in this group characteristically methylated.

The most important natural tropane alkaloids are (-)-hyoscyamine and (-)-scopolamine (also known as hyoscine). High concentrations of these alkaloids have been found particularly in *Datura stramonium* and *Datura ferox*, as well as in *Datura innoxia*. The pattern of tropane alkaloids differs significantly and in *Datura stramonium* (also known as thorn apple or Jimson weed) hyoscyamine prevails in most parts of the plant, whereas in *Datura ferox* scopolamine is the major alkaloid produced. *Datura* plants are toxic for animals if ingested in larger amounts. Their seeds, which contain significant amounts of hyoscyamine and scopolamine, can be found as botanical impurities in feed materials, particularly in soybean and linseed products.

For the detection of hyoscyamine and scopolamine in plant materials various analytical methods have been described, but most of these methods have not
been validated for feed materials. The same applies for atropine, the racemic mixture of (-)- and (+)-hyoscyamine.

Very little information on the actual contamination of feed materials is available, and previous reports on adverse health effects in animals refer in most cases to accidental intoxications following the consumption of *Datura* plants rather than to the contamination of linseed and soybean containing feed materials. Pigs have been shown to be among the most sensitive species to *Datura* poisoning.

Certain tropanes such as atropine (the racemic mixture of (-)- and (+)-hyoscyamine) as well as scopolamine (mainly as butylscopolamine bromide) are used in human and veterinary therapy. Reports of poisoning of livestock and experimental feeding studies describe as most common symptoms associated with tropane alkaloids exposure dryness of the mucosa in the upper digestive and respiratory tract, constipation and colic (in horses), pupil dilation (mydriasis), alterations in the heart rate and central nervous effects such as restlessness, irritability, ataxia, seizures and respiratory depression.

Tropane alkaloids are readily absorbed following oral ingestion, but have a short biological half-life and are rapidly bio-transformed or excreted. The CONTAM Panel concluded that it is unlikely that residues of tropane alkaloids in edible tissues, milk and eggs constitute a risk for consumers.

**Management /regulatory measures taken following EFSA's risk assessment**

Following the conclusion in the EFSA opinion that a worst case exposure estimate to tropane alkaloids indicated that adverse pharmacological effects in pigs, being among the most sensitive species to *Datura* poisoning, following exposure to *Datura ferox* seeds, mainly containing scopolamine, can not be entirely excluded at the current statutory limits of 3000 mg/kg feed. However, the limited data also suggested that it is not likely that the presence of *Datura stramonium* impurities in animal feed up to the current statutory level of 1000 mg/kg would present a risk to animal health.

It was therefore decided to extend the current limitation of 1000 mg/kg for seeds and unground and uncrushed fruits from *Datura stramonium* to all *Datura* spp. (including *Datura ferox*) (see Table 6). Furthermore work will be undertaken to have routine method available for the analysis of tropane alkaloids (hyoscyamine, scopolamine and atropine - total and individual tropane alkaloid content) in feed in view of a possible future review of the legislation based on these toxic substances.
Table 6. New provisions as regards weed seeds and fruits containing alkaloids, glucosides or other substances

<table>
<thead>
<tr>
<th>Undesirable substance</th>
<th>Products intended for animal feed</th>
<th>Maximum content in mg/kg (ppm) relative to a feed with a moisture content of 12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weed seeds and unground and uncrushed fruits containing alkaloids, glucosides or other toxic substances separately or in combination including - Datura sp.</td>
<td>All feed</td>
<td>3000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
</tr>
</tbody>
</table>

Pyrrolizidine alkaloids

Outcome of the risk assessment by the CONTAM panel from EFSA (9)
The term pyrrolizidine alkaloids (PAs) describes a group of more than 350 individual compounds that share as a basic structure one of the four necine bases platynecine, retronecine, heliotridine, or otonecine. PAs are produced as secondary metabolites of more than 6000 plant species, belonging to the families of Boraginaceae, Compositae (Asteraceae) and Leguminosae (Fabaceae) and occur world-wide. The pattern of PAs in plants varies largely, depending on the plant variety, climatic conditions, period of sampling and part of the plant analysed. Specific and sensitive methods for individual PAs have been developed recently for herbal medicinal products. These methods seem to be applicable to feed analyses as well, to quantify PA levels.

It is assumed that PAs are among the most widely distributed natural toxins affecting wildlife and livestock. Animal exposure to PAs results from the direct ingestion of PA containing plants with fodders. The highest risk to livestock seems to be associated with ingestion of plant materials from Senecio, Heliotropium, Trichodesma and Symphytum species, and in certain geographic regions also from Echium spp. Grazing animals avoid PA containing plants and consume them only if no other feed is available. Therefore in farm animals acute intoxications caused by PAs are rare. The recognition of toxic plant materials is however lost in preserved feeds such as hay and silage.

Contamination of grains and other components used in feed by seeds of weeds containing PAs is the other route leading to animal exposure. Careful mechanical cleaning and sorting of grains used in feed production can reduce the rate of exposure.
At present, data on the exposure of livestock to PAs are lacking, as only the control by microscopic detection of the presence of weed seeds is mandatory within the EU.

The toxicity of PAs is related to secondary structural characteristics such as the composition of the esters, and the number of substituted hydroxyls that impair the metabolism and excretion of PAs. The major toxic metabolites are dehydro-pyrrolizidines and dehydronecines, which readily interact with cellular macromolecules.

Significant differences in the species-specific sensitivity to PAs have been noted, which correlate with the expression of biotransformation enzymes involved in the metabolic conversion of individual PAs. It is generally recognized that rodents, as well as pigs, poultry, cattle and horses are very sensitive to PA intoxication, whilst sheep, goats and rabbits are not.

Major signs of toxicity observed in all animal species include various degrees of progressive liver damage (centrolobular hepatocellular necrosis), and veno-occlusive diseases associated with a direct effect of the toxic metabolites on endothelial cells. Moreover proliferation of the bile ducts, hepatic megalocytosis, and liver fibrosis, resulting in secondary cardiac right ventricular hypertrophy, pulmonary hypertension, degenerative renal injury and signs of hepato-encephalopathy as well as general weight loss, anorexia and depression are regularly reported.

Acute and sometimes fatal intoxications have been incidentally described in livestock and horses. Due to the lag time between exposure and onset of clinical symptoms, chronic PA intoxications remain often undiagnosed, but may result in reduced weight gain and low productivity.

Experimental data on the carry-over into edible tissue suggest that residues of PA metabolites do not occur in muscle tissue, but transfer of PAs and/or their metabolites into milk and eggs, is likely, albeit at a low rate. In addition, honey is found regularly to be contaminated with PAs. The levels of PA metabolites found in milk, eggs and honey are significantly lower than the measurable levels in herbs and spices that are used in the human diet.

In humans, the veno-occlusive disease has been identified as most sensitive endpoint of PA toxicity. Epidemiological data did not provide any evidence for an increased risk of cancer in human populations exposed PAs.
Management /regulatory measures taken following EFSA's risk assessment

In the Annex to Directive 2002/32/EC, a maximum content of 3000 mg/kg is established in general in feed for the presence of weed seeds and unground and uncrushed fruits containing alkaloids, glucosides or other toxic substances separately or in combination (see Table 6). In addition to this general provision, a lower maximum content of 1000 mg/kg was established in the past for the presence of *Lolium temulentum* and *Lolium remotum*.

However, EFSA remarks in its opinion that the justification for specific inclusion of *Lolium* species is unclear. Both *Lolium* species are seldom found in Europe and are not used as pasture grasses, in contrast to other *Lolium* species, such as *Lolium perenne*, which is regularly used in grass mixtures. Moreover *Lolium* species have not been reported to be major pyrrolizidine alkaloids producers. *Lolium temulentum* (Darnel ryegrass) is known to produce corynetoxin, a toxic glycopeptide, not belonging to the group of PAs. *Lolium temulentum* might also contain small amounts of lolin and lolinin. Recent evidence suggests that these compounds do not originate from plant metabolism, but are associated with the invasion of the grasses by endophytes (*Neotyphodium* spp) that also account for the production of lolitrem B, a neurotoxic mycotoxin.

It has therefore been decided to delete the specific reference in the Annex to Directive 2002/32/EC to these two plant species with the lower maximum level.

It was also decided to keep the current provision for *Crotalaria spp* unchanged, by which *Crotalaria spp.* are not allowed to be present in contents above 100 mg/kg in feed. *Crotoloria spp.* represent a prominent class of tropical plants, known to produce high amounts of PAs and having been associated in the past as a source of intoxications in poultry and pigs.

Furthermore, in view of a possible future management measures as regards the presence of pyrrolizidine alkaloids in feed and possibly food, and following the recommendations from the CONTAM Panel from EFSA following initiatives are ongoing:

- Analytical methods suitable for the analyses of individual pyrrolizidine (PAs) as well as total PAs, and the presence of certain individual alkaloids in plant materials, are becoming available through EU funded research projects and these will be validated for the analysis of feed materials.
- Certified materials to be used as standards in analytical procedures need to be made available.
- Given the large variation in the pattern of toxic PAs in plant materials, analytical surveys should focus on selected alkaloids, which have been
identified as major (hepato-)toxic compounds, while at the same time being representative for major PA containing plant families. In a first analytical survey for the presence of PAs in feed materials, the following compounds should be monitored:

- the *Senecio* alkaloids senecionine, seneciophylline and erucifoline, which are found in high concentrations in the *Senecio* species occurring in Europe, and which have been associated with clinical intoxications in livestock.
- the *Crotolaria* alkaloids monocrotaline and trichodesmine.
- the *Heliotropium* alkaloids heliotrine and indicine occurring predominantly in the seeds of plants of the *Heliotropium* species, which might contaminate cereals and grains intended for human and animal consumption.
- the PAs intermedine and lycopsamine, as these are representative for many species of *Anchusa, Borago, Symphytum* and *Eupatorium* species, and may serve as markers for the undesirable presence of these plant species (and/or their seed) in feed materials.

- As reduced weight gain and impaired productivity are associated with exposure to even low concentrations of PAs, the CONTAM Panel recommended that monitoring of feed materials for the presence of PAs should be implemented, at least temporarily, to assess the potential exposure of livestock. This monitoring will be implemented once a routine analytical methodology has been developed and validated.

- Although human exposure resulting from carry-over of PAs from feed into animal derived products seems to be low, the CONTAM Panel recommended that more data should be made available on the potential carry-over into milk, considering that infants have a relatively high consumption per kg b.w. Research on the carry-over of pyrrolizidine alkaloids from feed into milk has been pointed out as a priority for EU-funded research. Moreover, more data are needed to quantitatively assess the contribution of honey to human exposure, as the latter is regularly found to contain residual amounts PA metabolites.

**Ricin**

Outcome of the risk assessment by the CONTAM panel from EFSA (10)

Ricin is a toxic glycoprotein (with several minor variants) belonging to the type II group of ribosome inactivating proteins (RIP type II) found in the seeds (beans) of the castor oil plant (*Ricinus communis* (Euphorbiaceae)). A limited number of other plants in the same family contain type II RIPS, i.a. subtropical leguminous climber *Abrus precatorius*. and, Croton tiglium. which contain abrin
and crotin I, respectively. Croton tiglium has long been known as a toxic plant. The seeds are particularly toxic, containing a number of skin-irritating phorbol esters and showing a strong laxative effect. The seeds also contain crotin I, which is a RIP II protein. The Croton tiglium seeds also contain a number of other toxins which make it unsuitable as a feed for livestock.

Following extraction of castor oil, ricin is left in the press-cake/castor bean meal. Castor oil production mainly takes place outside the EU. Because of its low value as feed no import of the press-cake to the EU is expected. However, exposure of animals can not be excluded if castor oil seeds and other plants containing RIP II proteins are present as botanical impurities in feed materials.

Following cell uptake by endocytosis, ricin causes acute cell death by inactivation of ribosomal RNA. Acute symptoms in humans after intake of castor beans are hematemesis (vomiting containing blood), diarrhoea, haemorrhagic necroses in several organs, renal failure, circulatory collapse and death after 6 to 14 days with a fatal oral dose of about 1 mg/kg b.w. (5-10 castor beans). Because of its destruction in the intestinal tract, ricin is approximately 1000-fold more toxic following parenteral administration or inhalation, than by the oral route.

The very limited data on acute toxicity in target animals comprise mainly information on castor bean products rather than on purified ricin. Amongst ruminants, cattle appear to tolerate higher intakes than sheep. In horses severe colic and death have been observed after a single dose of approximately 7-8 mg ricin/kg b.w. Toxic effects in pigs and birds have been reported as well as accidental poisonings in dogs with vomiting, depression and diarrhoea as the main clinical signs. No- or lowest observed adverse effect levels (NOAELs or LOAELs) for acute effects of ricin could not be identified for any of the animal species.

There are limited data on the toxicokinetics and carry-over of ricin to products of animal origin (milk, meat or eggs). Livestock has a very low tolerance to ricin exposure via feed before clinical symptoms of toxicity are manifest. It is therefore unlikely that highly exposed animals would enter the food chain, and the CONTAM Panel considers the risk of ricin transfer to livestock products to be negligible.

Except for exposures to ricin via the accidental intake of castor beans (Ricinus communis) human exposure via food, under normal circumstances, is unlikely to occur.
Management /regulatory measures taken following EFSA's risk assessment

More information is needed on the occurrence of toxins found in *Abrus precatorius* and *Croton tiglium* in feed before possible maximum levels for these substances can be set in the Annex to Directive 2002/32/EC. Given the similar toxic effects of *Ricinus communis, Croton tiglium and Abrus precatorius*, it has been decided to establish the same provision as already established for *Ricinus communis* for the three botanical impurities, separately or in combination (see table 7) and to delete the requirement for absence of quantifiable amounts of *Croton tiglium* in the annex to Directive 2002/32/EC.

**Table 7.** New provisions as regards *Ricinus communis, Croton tiglium and Abrus precatorius* in feed

<table>
<thead>
<tr>
<th>Undesirable substance</th>
<th>Products intended for animal feed</th>
<th>Maximum content in mg/kg (ppm) relative to a feed with a moisture content of 12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeds and husks from <em>Ricinus communis</em>, <em>Croton tiglium and Abrus precatorius</em> as well as their processed derivatives (<em>), separately or in combination (</em>) Insofar determinable by analytical microscopy</td>
<td>All feed</td>
<td>10</td>
</tr>
</tbody>
</table>

**Saponins in *Madhuca longifolia***

Outcome of the risk assessment by the CONTAM panel from EFSA (11)

Saponins are a diverse group of low molecular-weight secondary plant metabolites that are widely distributed in the plant kingdom. The chemical structure of saponins consists of an aglycone of either steroidal or a triterpenoid nature and one or more sugar chains (glycosides). Saponins can form stable foam in aqueous solutions, hence the name “saponin” from the Latin word for soap (*sapo*). Traditionally, they have been used as detergents, piscicides and molluscicides in addition to industrial applications as foaming and surface active agents.

Apart from *Madhuca* species (genus *Madhuca* Hamilton ex Gmelin), saponins occur in significant concentrations in a number of feed and food plants such as alfalfa (*Medicago sativa*), soybean (*Glycine max*), quinoa (*Chenopodium quinoa* Willd.) and balanites (*Balanites aegyptiaca*).

*Madhuca longifolia* and other *Madhuca* species are large evergreen or semi-evergreen trees with a dense spreading crown extensively cultivated in warm climates for their oil-containing seeds. Saponins of *Madhuca* contain pentacyclic...
triterpenoids as aglycones. The saponin content in the seeds is about 100 g/kg. The seeds of several species of *Madhuca* are rich in oil, and the seed oil is mainly used for non-food purposes such as production of laundry soap and biodiesel. The press cake contains >200 g/kg of saponins and is unsuitable as feed. Therefore, it has traditionally been used only as a fertilizer. There are methods for reducting the saponin content in *Madhuca* seeds.

There are no validated official methods in EU for the determination of saponins in feed. Since most saponins lack chromophores, liquid chromatography – mass spectrometry (LC-MS) techniques would be the analytical methods of choice.

In food and feed, saponins can have an “anti-nutritional” effect and cause toxic effects, but have also been claimed to cause beneficial health effects. In general, saponins, in the form of glycosides, have low oral bioavailability, but may be hydrolysed in the intestinal tract and cause systemic toxicity dependent on the structure and absorption of the aglycone.

Toxicity studies and observations of toxic effects in feeding studies have been reported using crude total saponins or defatted seed meal from various *Madhuca* species. The oral LD50 in mice of crude *Madhuca* saponins was about 1.0 g/kg body weight. In mice and rats *Madhuca* saponins caused local gastro-intestinal toxicity as well as liver and kidney toxicity. At lower doses, *Madhuca* saponins can cause feed refusal and starvation with reduced body weight gain and increased mortality. Because of the limited data available, no health-based guidance value (ADI, TDI) can be established for *Madhuca* saponins.

Results from studies on *Madhuca* seed cakes which contain saponins, on ruminants indicate that they are more tolerant to *Madhuca* saponins than monogastric animals and can tolerate inclusion levels of up to a maximum of 20% of the total diet. Toxicity studies of *Madhuca* seeds on monogastric target animals are scarce. *Madhuca* seed cake in chick mash at approximately 12% level was lethal. No studies have been conducted on horses, pigs, rabbits or dogs. Except for piscicidal effect of *Madhuca* saponins by water exposure in guppy fish, no toxicity studies after dietary exposure were identified in fish.

Data on occurrence of *Madhuca* as a botanical impurity in feed are not available. There is no information on the fate and carry over of *Madhuca* saponins in animals. The CONTAM Panel concluded that human dietary exposure to *Madhuca* saponins in the EU can be considered as negligible.
Management /regulatory measures taken following EFSA's risk assessment

Following the conclusion from EFSA in the abovementioned opinion that no adverse animal health effects are expected because of the negligible exposure of target animals in the Community and that also human dietary exposure to *Madhuca* saponins is negligible it has been decided to delete the provision as regards *Mowrah, Bassia, Madhuca* from the Annex to Directive 2002/32/EC.

EFSA furthermore recommended that before undertaking a monitoring programme for the presence of saponins in plants used for feed, following information should be available a priori:
- information on saponins which are relevant from a feed safety point of view, and
- availability of appropriate analytical methods.

Key saponins for important feed plants should be chosen, and compound-specific analytical methods should be developed. Since pure compounds/saponin standards are generally lacking, such standards should be made available.

**Unhusked beech mast and purghera (*Jatropha curcas*)**

Outcome of the risk assessment by the CONTAM panel from EFSA

In the risk assessment on saponins in feed, the CONTAM Panel also assessed the possible reasons for listing unhusked beech mast as undesirable substance in the Annex to Directive 2002/32/EC. The leaves of *Fagus silvatica* contain triterpenoid saponins with oleanolic acid as the aglycone. In the wood/bark of the plant the free triterpenoids β-amyrin and betulin and β-amyrin as the acetate are present. However, no saponins have been found in the seeds of *Fagus silvatica*. No information is available on the content of saponins of whole unhusked beech mast or on the husk of beech mast. Furthermore, no information has been found in support of the occurrence of cyanogenic glycosides or other cyanogenic compounds in any part of *Fagus sylvatica*, including unhusked beech mast.

Up to the beginning of the 20\textsuperscript{th} century, beech mast was of considerable importance for pig production in many European countries. The beech mast also makes up a considerable part of the diet of wild boar (*S. scrofa*). Likewise deer - such as the sika deer - forage on beech mast as do red deer, roe deer, and fallow deer. Although the above mentioned animal species seem to tolerate beech mast well, evidence exists that beechnuts and beech mast can be toxic to cattle and horses when consumed in larger quantities, the toxic syndrome is likely to be related to the presence of oxalates.
In the risk assessment on ricin and cyanogenic compounds in feed, the CONTAM Panel also assessed the possible reasons for listing purghera (*Jatropha curcas*) as undesirable substance in the Annex to Directive 2002/32/EC.

*Jatropha curcas* is a tree belonging to the *Euphorbiaceae* family. It originated in Central America, but is now found in many tropical and sub-tropical countries in Africa and Asia. The seeds contain 28-40% oil. For many years the oil was used predominantly in the manufacture of soaps and candles, but more recently *Jatropha* oil has become of significant economic importance as a result of its potential as a source of biodiesel. *Jatropha* seed-cake contains toxins, making it unsuitable for animal feed. However, it does have potential as a fertilizer, and if available in large quantities, it can also be used as a fuel for steam turbines to generate electricity. A method has been developed for detoxifying the press-cake through a combination of heat treatment and solvent extraction, but it was not an economically viable option for commercial production.

The plant *Jatropha curcas* was previously believed to also contain RIP II (curcin) in its seeds as it has been reported to be toxic to humans, rodents and livestock. However, more recent research has shown that curcin is a type I RIP being less toxic and point to the irritant phorbol esters as being the major class of toxins. Reports on the intoxication of humans by accidental ingestion of the oil or seeds have appeared in Hawaii, Florida, Philippines and India. The symptoms of intoxication in humans include burning and pain in the mouth and throat, vomiting, delirium, muscle shock, decrease of visual capacity, and an increase in heart rate. A high mortality rate has been reported for rodents (mice, rats) and domestic animals (sheep, goats, calves, and chicks) when fed *Jatropha curcas* seeds. Phorbol esters are present in several *Jatropha* species and different provenances of *J. curcas* contain significantly different levels of these esters.

As regards purghera, *Jatropha curcas*, no indications for being cyanogenic or for the presence of cyanogenic compounds have been found. However, several other species of *Jatropha* (*J. angustidens, J. capensi, J. hieronymi* and *J. macrocarpa*) have been reported to be cyanogenic.

It can therefore be concluded that purghera, *Jatropha curcas*, does not contain a type II ribosomal inhibitory protein (RIP II) neither cyanogenic compounds. The likely cause of toxicity is the significant high content of phorbol esters.

Management /regulatory measures taken following EFSA’s risk assessment
The reasons for listing in the Annex of Directive 2002/32/EC of unhusked beech mast (*Fagus sylvatica*) and purghera (*Jatropha curcas*) as undesirable botanical
impurity in feed with the requirement for absence of quantifiable amounts in all feed of these botanical substances is not clear. However, it is evident that these botanical impurities do contain toxic substances, which might result in adverse animal health effects. It was therefore decided to keep for the time being the current provision for unhusked beech mast (*Fagus silvatica*) and jatropha (*Jatropha curcas*) unchanged, by which these botanical impurities are not allowed to be present in quantifiable amounts in feed.

**Regulatory framework for contaminants in food**

Council Regulation (EEC) 315/93 of 8 February 1993 laying down Community procedures for contaminants in food (12) is the basic regulation governing the measures on contaminants in food.

Contaminant is defined as any substance unintentionally added to food and present therein in the form of a residue from production, manufacture, processing, preparation, treatment, packing, packaging, transport or storage or as a result of environmental contamination. The Regulation does not apply to contaminants covered by more specific legislation.

It provides:
- that food containing a contaminant in an amount which is unacceptable from the public health viewpoint and in particular at a toxicological level shall not be placed on the market.
- that contaminant levels shall be kept as low as can reasonably be achieved following good practices at all stages of production and distribution.
- that, when necessary for protecting public health, maximum levels shall be established for specific contaminants These limits may include a reference to the sampling and analysis methods to be used.
- for an obligatory consultation of the European Food Safety Authority (EFSA) ‘(Scientific Panel on contaminants in the food chain) before provisions having effect upon public health shall be adopted.
Procedure for setting regulatory limits for contaminants in food

The scientific risk assessment, which is the assessment of the risks related to the presence of a contaminant in foodstuffs for human health, is the basis for the measures to be taken. In case it does not concern a genotoxic carcinogen, a health based guidance value is derived.

The human exposure (average and 95 percentile) is assessed in relation to this health based guidance value. Particular attention is hereby paid to vulnerable groups of population and high level consumers. The exposure assessment does also enable to identify the foods/food groups significantly contributing to the exposure. From these food and food groups, the occurrence data, obtained following the application of good practices are used to determine the appropriate maximum level to protect consumer health.

Maximum levels are set at a strict level which is reasonably achievable by following good agricultural, fishery and manufacturing practices and taking into account the risk related to the consumption of the food.

In the case of contaminants which are considered to be genotoxic and carcinogenic or in cases where current exposure of the population or of vulnerable groups in the population is close to or exceeds the tolerable intake, maximum levels should be set at a level which is as low as reasonably achievable (ALARA).

Inherent plant toxins in food

The management of inherent plant toxins in food falls within the frame of the Regulation (EC) 315/93. However for the time being no inherent plant toxin has yet been regulated in the food contaminant legislation contrary to the feed contaminant legislation.

However this might change in the near future. In view of possible future regulation, the Commission has early 2010 asked EFSA to assess the risks for human health related to the presence of opium alkaloids (morphine) in poppy seeds and pyrrolizidine alkaloids in foods, in particular honey and milk.

Also discussions have taken place as regards the need to regulate the marketing of bitter apricot kernels given the risk for human health due to the presence of high level of cyanogenic compounds in these bitter apricot kernels. However these discussions have not yet resulted in a regulatory initiative.
Conclusion

The management and regulation of certain bioactive compounds present in feed and food as inherent plant toxins, are at EU-level more developed in the feed than in the food legislation. However, for a comprehensive legislation covering all relevant inherent plant toxins in feed and food there is still a long way to go, whereby the risk assessor and risk manager are confronted with a lot of obstacles to move forward.

As can also be inferred from the available risk assessments, there is still a very large lack of toxicity data and occurrence data to enable EFSA to perform comprehensive risk assessments on the presence of inherent plant toxins in feed and food. Also for many of these inherent plant toxins, no validated routine methods of analysis are currently available.

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Documentation of beneficial effects of bioactive plant compounds in food and feed

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Abstract

Bioactive plant components are widely used both in food and feed and cover different categories and products. The following presentation outlines the main principles of how the EU authorities regulate the market of such products. Some examples are given on additives that require pre marketing approval. For both food and feed the labelling, presentation and advertising are subject to detailed regulation to secure safe use.

Introduction

The use of bioactive plant compounds has a long tradition in our history both as medicine and as supplement to support normal physiological functions of the body. The number of compounds used is increasing together with the knowledge of their function. This has raised a discussion on the responsibility of the authority to lay down rules for marketing of products used for humans and animals. A harmonised set of rules in this area within the common EU market has been developed, and implemented in Norway by the EEA agreement with some minor derogation. The following presentation gives an overview of the
main areas within feed and food, which are subject to specific regulations. This development of legislation is an evolving process where documentation on beneficial effect may differ widely depending on both type of product and use. Some key issues may be useful to bear in mind in discussion of this topic:

- The responsibility of the authority vs. food and feed business operators.
- Compulsory testing of beneficial effects before marketing vs. self regulation by the market.
- Pre market approval of products vs. control of claims etc. of marketed products.

The EU population covers close to 500 mill people. It is a challenge to make proper rules for testing the beneficial effects of different plant compounds that are put on the market for human and animal use. This presentation will give a short introduction to some relevant regulations that cover different areas and some examples of product classification. Using the “farm to fork” approach, the presentation starts with feedingstuffs.

**Feedingstuffs**

**Additives in feedingstuffs - Categories and documentation of beneficial effects**

Regulation (EC) No 1831/2003 on additives for use in animal nutrition includes the following categories:

1) Technological additives (preservatives, antioxidants, emulsifiers etc.)
2) Sensory additives (colorants, flavouring compounds)
3) Nutritional additives (vitamins, trace elements, amino acids, urea)
4) Zootecchnical additives (digestibility enhancers, gut flora stabilizers, substances which favourably affect the environment, other zootechnical additives)

Authorization of a feed additive, new use or renewal is normally based on an EFSA opinion which also includes documentation of beneficial effects. Regulation (EC) No 429/2008 of 25 April 2008 lay down detailed rules as regards the presentation and preparation of applications and the assessment. The vast majority of additives are chemical substances with a defined chemical composition. All approved substances had to be notified when Regulation (EC) No 1831/2003 came into force, and application for renewal of authorization has to be done before November 2010. The main part of notified substances belongs to the category “Flavouring compounds”, described as “natural products” -
botanically defined”. Another large group is classified as “Natural or corresponding synthetic chemically defined flavourings”. Details on the documentation of beneficial effects have to be clarified.

Some substances have a dual effect as additive, where the main function is responsible for classification. Approved substances are given a number which identify the characteristics of the substance.

Feed materials, compounds and dietetic feed
These sectors have so far been covered by separate legislations, but will now be covered by a horizontal legislation adopted in July 2009 by the Council and the European Parliament (Regulation (EC) 767/2009). The new legislation which covers the placing on the market and use of feed is directed on labelling and claims. A Community Catalogue of feed materials and Codes of good labelling practice have to be established. There is no pre marketing approval so the beneficial effects are regulated by the demands and needs in the market. Medical claims are not allowed, and a list of intended uses gives detailed rules for claims for dietetic feedingsstuffs. Compulsory declarations of nutrients as proteins, fibres, minerals etc. further outline the documentation on beneficial effects. The new legislation on labelling and claims will probably facilitate the distinction between feed materials, additives and medicated feed.

Food

Food additives
Additives are substances that are added intentionally to perform certain technological functions in the food. The categories of additives include colors, sweeteners, preservatives, antioxidants, emulsifiers, stabilizers, etc. They are not normally consumed as a food in itself and not normally used as a characteristic ingredient of food. The Directive does not apply to processing aids, plant protection products, flavourings and substances added to foodstuffs as nutrients.

Food additives thus differ from feed additives as the latter category one also includes substances that have a nutritive value (vitamins, trace elements).

In addition to Council Directive 89/107/EEC there are specific Directives that give more detailed rules on defined areas:
- Directive 94/36/EC on colours for use in foodstuff
- Directive 94/35/EC on sweeteners for use in foodstuffs
- Directive 95/2/EC on food additives other than colours and sweeteners

Additional legislation exists on purity criteria and special conditions are imposed for import of certain additives.

**Flavourings**

Directive 88/388/EEC covers flavourings for use in foodstuffs and to source materials for their production. They are used to give taste and/or smell to food, and could be divided into three different categories:
- Natural, natural-identical or artificial flavouring substances
- Flavouring preparations of plant or animal origin
- Process flavourings which evolve flavour after heating, and smoke flavourings

Regulation (EC) 2232/96 gives rules on the criteria for establishing a Positive list of substances used as flavourings.

Decision 1999/217/EC establishes a register of substances used at national level. About 2800 substances have been notified. As a part of these have been evaluated previously, about 1000 – 1250 substances remain for evaluation.

Regulation (EC) 1565/2000 institutes the establishment of a programmer for evaluation of notified flavourings. The evaluation will be done by EFSA, and is supposed to be finished within the year 2010.

Regulation (EC) 622/2002 concerns submission of information and documentation for further evaluation, and a deadline for sending this.

Regulation (EC) 2065/2003 on smoke flavourings used or intended for use in or on foodstuffs, is an example of specific regulation in the food sector.

"**The food improvement package**"

This term is used to describe a further harmonization of the use of additives in foodstuffs. The package includes:


Steviol glycosides as a novel food additive
- An example on evaluation of a bioactive plant compound as food additive.
  • Steviol glycosides (stevioside, rebaudioside A, rebaudioside C and dulcoside A) are extracted and refined from *Stevia rebaudiana Bertoni* leaves
  • The Scientific Committee on Food (SCF) concluded in June 1999 that ”the substance is not acceptable as a sweetener on the basis of presently available data”
  • There are two new requests for authorisation (November 2007 and June 2008) of steviol glycosides for their use as a sweetener and/or flavour enhancer.
  • The European Commission has requested in 2007 the European Food Safety Authority (EFSA) to provide a global safety assessment on the use of steviol glycosides as a novel food additive (sweetener).

Labelling, presentation and advertising of foodstuffs – Directive 2000/13/EC
The consumers’ right to make their own preference of food creates a need for proper rules on relevant information for being able to do the right choice of products. Documentation of beneficial effects is mainly related to presentation made by labelling and advertising and not to pre marketing approval. Directive 2000/13/EC put forward the general obligations to inform and protect the consumer, and some points are highlighted:

  • It covers all types of foodstuffs including food supplements
  • The labelling and methods used must not mislead the consumer.
  • The information must not attribute to any foodstuffs the property of preventing, treating or curing a human disease, or refer to such properties.

Regulation (EC) No 1924/2006 on nutrition and health claims made on foods
Specific regulations are adopted on nutrition and health claims concerning foods to be delivered as such to the consumer. The key issues are as follows:
• Applies to all nutrition and health claims which are made in commercial communications (vitamins, minerals, fibres, other substances…).
• "Other substance" means a substance other than a nutrient that has a nutritional or physiological effect → bioactive plant compounds.
• A claim should be scientifically substantiated by taking into account the totality of the available scientific data, and by weighing the evidence.
• A food business operator making a nutrition or health claim shall justify the use of the claim.

Nutrition claims are only permitted if they are listed in the Annex and if they are in conformity with the conditions set out in the Regulation.

Article 13
Health claims, also called “functional claims” under this article are describing or referring to:
• The role of a nutrient or other substance in growth, development and the function of the body.
• Psychological and behavioural functions.
• Slimming or weight control or a reduction in the sense of hunger or an increase in the sense of satiety or the reduction of the available energy from the diet.

The Regulation provides for an authorisation of claims for which an application is sent to the national authority before July 2008, and further submitted to the Commission.

By October 2008 the Commission has forwarded approximately 3000 claims for evaluation by EFSA. Those are classified in 9 categories (vitamins, minerals, fibres, probiotics etc.)
A positive list of permitted claims shall be adopted by 31 January 2010 at the latest.

Mint, Menthe piperita, is as an example on forwarded claims.

Article 14
These claims are directed on
• Reduction of disease risk claims
• Claims referring to children’s development and health
Applications shall be sent via the national authority to EFSA. EFSA shall forward its opinion to the Commission and Member States, and a final decision has to be taken.

• The application must contain all pertinent scientific data identified, that form the basis for substantiation for the claim.
• Data from studies in human are required.
• Data from studies in animals or model systems may be included only as supporting evidence because of the scientific uncertainties in extrapolating non-human data to humans.
• A specific food-health relationship is required.

New regulation on food information
A proposal for a new Regulation on general principles, requirements and responsibilities governing food information, and in particular food labelling was sent to European Parliament in January 2008. This will repeal Directive 2000/13/EC and several other Directives. The new proposal will make rules for compulsory declaration of certain nutrients.

Novel foods and novel food ingredients – Regulation (EC) No 258/97
This Regulation covers food and food ingredients that have not been used to a significant degree within the EU Community before 15 May 1997. Novel foods must undergo a safety assessment before placed on the EU market. Only those products that are considered safe for human consumption are authorised for marketing. A request shall be made to the national authority to place a product on the market. This shall be accompanied by the necessary information to demonstrate the safety.

The Commission has drawn up a list of approved products. Some of them have a plant origin shown by the following examples. Phytosterols produced from vegetable oils are used to lower the uptake of cholesterols from the intestinal tract. Hydrolyzed guar gum has specific properties related to fibres. Isomaltulose that contains glucose and fructose, has low absorption compared to other disaccharides. Some interest has been expressed for approval of Stevia rebaudiana, but this is awaiting more documentation.

In addition to the presentation given above, bioactive plant compounds may also be covered by the following Directive:
This Directive concerns foodstuffs to be used by certain categories of persons whose digestive processes or metabolism are disturbed, or are in a special physiological condition. It also covers infants and young children in good health. The groups of foodstuffs included are infant formulae, baby food, energy-restricted diets, food for special medical purposes, and sport nutrition products.

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Does traditional use of herbal remedies guarantee safety?

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Abstract

Herbal remedies are partly regulated as food and partly as medicines. However, the question of safety is equally important irrespective of the legal framework. In the context of medicinal products, traditional use spans from at least 30 years up to several hundred years. Very long traditional use is known for Chinese and Indian herbal remedies. Experience has shown that poisonous and carcinogenic plants may be used in traditional medicine for long periods without its harmful effects being recognized. Preclinical testing may solve some of the problems, but it is impractical to demand extensive toxicological testing of every herbal remedy. Limiting periods of use, avoid use in pregnancy and in small children will minimize possible, but unknown, harmful effects. The risks associated with traditional herbal remedies in general are small, with some notable exceptions. The “Aristolochia disaster” in Belgium serves as an important reminder that herbs may be used in traditional medicine without their toxic properties being recognized. New regulation of herbal medicines in Europe should contribute to the safe use of these products. However, it is a constant challenge that new plants are introduced on the food and drug market without proper scientific evaluation.
Regulation of traditional herbal medicinal products in Europe

The Committee on Herbal Medicinal Products (HMPC) was established in 2004, as laid down by Directive 2001/83/EC as amended by Directive 2004/24/EC. HMPC is a scientific committee under the European Medicines Agency (EMA) and the European Commission. All European Economic Area (EEA) countries are represented in HMPC. Additional experts are also elected to the committee. The main responsibilities of HMPC are to prepare community herbal monographs and documentation for incorporating traditional herbal medicinal products on the community list. Inclusion of traditional herbal medicines on the community list is decided by the European Commission.

Community monographs are not binding, but according to the directive, due consideration should be given to the monographs when a company files for registration of a traditional herbal medicinal product. The community list is binding for member states of the EU and EEA. A company that applies for registration of a product that is on the list is entitled to registration as long as the application is in compliance with the list and pharmaceutical quality is documented.

Well-established and traditional use

According to the European legislation, herbal medicinal products can be approved as ordinary medicines, well-established herbal medicines or traditional herbal medicines. The approval depends on the completeness of documentation and indications for use. Within the scope of this article, only the two latter classifications will be discussed. The directive and subsequent guidelines describes in detail which criteria must be fulfilled in each case.

Well-established use is based on more than ten years of use in the EU and a general acceptance that the drug has a high degree of safety. Further, preclinical and clinical documentation must be comprehensive. Preclinical documentation must as a minimum include appropriate genotoxicity tests. Clinical safety and efficacy must be documented with clinical experience or clinical studies. Well-established use may include indications that require consultation or supervision by healthcare professionals.

Traditional herbal medicinal products are based on documented traditional use for at least 30 years, of which 15 years must be within EU. The clinical effect must be plausible and the risk of severe adverse reactions must be very low. Indications must be suitable for use without consultation or supervision by
healthcare professionals. This will preclude some indications that have a long tradition, like “support of cardiac function”. Cardiac diseases must be diagnosed by a physician.

**Traditional herbal preparations and risk of adverse reactions**

Adverse reactions from traditional herbal medicines can be divided in two main groups: Acute (e.g. acute toxic or allergic) and chronic (e.g. hepatotoxic, carcinogenic or genotoxic) reactions.

Acute reactions are frequently discovered through clinical experience. Herbal preparations with common or severe acute reactions will usually disappear from the market, but it is not difficult to find exceptions to this rule. Ayurvedic medicines that contain heavy metals may cause acute or chronic poisoning (1), but is still widely used, both in India and western countries. It seems that the dangers of heavy metal containing Ayurvedic medicines are only slowly accepted in traditional clinical practice.

Chronic toxicity is more difficult to discover. Standard pharmacovigilance methods like adverse events reporting may prove inadequate. Plants of the genus Aristolochia contain aristolochic acids that are potent nephrotoxic and carcinogenic compounds. Plants from this genus have been used in traditional Chinese medicine (TCM) for a variety of indications. Frequently, misidentification of plants have occurred (2). A herbal slimming preparation containing Aristolochia resulted more than 100 cases of nephrotoxicity in Belgium (3). Subsequently, nephrotoxicity has been reported from several countries, including China, Japan, UK and France (4).

**Genotoxicity testing of herbs and herbal preparations**

For many herbs and herbal preparations an adequate safety profile is documented through the long history of medicinal use. In these cases, no toxicological testing is mandatory. The HMPC monographs will reflect this by recommending short periods of use (up to one or two weeks) and avoiding treatment in pregnancy, lactation and small children.

However, in some cases, safety concerns may be recognised or suspected. In such cases nonclinical testing may be necessary. Unfortunately, nonclinical tests
is often absent in the scientific literature concerning a specific herb or herbal preparation.

HMPC have developed guidelines for the testing of genotoxicity for herbal substances/preparations (5-7). A stepwise approach is recommended (Figure 1). Step 1 is the Ames test. If this test is negative, no further testing is necessary. If the test is positive, further testing is necessary (6). The HMPC is of the opinion that this simplified approach will detect critical problems with DNA-reactive herbal substances.

**Figure 1:** Decision tree on the assessment of genotoxicity of herbal substances (from reference 6).
Conclusions

Longstanding, traditional use of herbs and herbal preparations confers extensive protection against unexpected adverse reactions, but genotoxicity testing may be necessary in some circumstances where the scientific literature is inconclusive or not able to rule out safety problems.

References

Bioactive compounds through food, nutraceuticals or pills?

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Abstract

This article has a somewhat philosophical nature, with the aim to discuss by which means and in which dosages we should provide the body with healthy, bioactive compounds: through foods, nutraceuticals, or pills. A number of studies over the last two decades have shown that moderate amounts of e.g. food antioxidants protect against disease, but more is not necessarily better. Another lesson learnt is that promising results in the laboratory are not necessarily relevant for humans. The philosophy that a healthy and well-balanced diet seems to be the basic strategy to obtain good health has been strengthened. However, nutraceuticals and functional foods have come to stay. The market will probably increase. Without a balanced diet as a basis, nutraceuticals, functional foods and supplements will probably have marginal impact on public health, although vulnerable groups may benefit from selected substances. Health claims should be thoroughly documented. The producers have a special responsibility and should work closely with EFSA and national authorities.
Nutraceutical, functional food, and dietary supplement

To be able to follow the logic of this article, three words/expressions need an explanation: Nutraceutical, functional food, and dietary supplement.

The word nutraceutical is a modern invention and a blend of the two words nutrient and pharmaceutical. It refers to extracts of foods claimed to have a medicinal effect on human health. The nutraceutical can be contained in a medicinal format such as a capsule, tablet or powder in a prescribed dose, or added to a food (which then becomes a functional food). More rigorously, nutraceutical implies that the extract or food is demonstrated to have a physiological benefit or provide protection against a chronic disease. There are roughly eight categories of nutraceuticals:

- Vitamins and mineral elements
- Amino acids, peptides, proteins and derivatives
- Fibres, special carbohydrates, and pre-biotics (i.e. non-digestible materials that promote growth of beneficial micro-organisms in the gastrointestinal (GI) tract)
- Carotenoids (i.e. natural coloured compounds - typically red, orange or yellow - that are fat-soluble)
- Pro-biotics (i.e. living bacteria)
- Special fatty acids and derivatives
- Polyphenols and flavonoids (i.e. natural pigments that are water-soluble)
- Sterols and stanols (which are structurally very similar to each other and to cholesterol)

The expression “functional food” also needs an explanation: All foods are, fundamentally speaking, functional, as they provide taste, flavour, texture or nutritive value. Within the last ten years, however, the term functional food has been adopted and has a different connotation:

- A functional food may be defined as a food having health promoting benefits and/or disease preventing properties above its usual nutritional value
- Will usually be advertised with a health claim
- No internationally agreed definition
- Examples:
  - stanol-enriched margarines
  - folic acid fortified bread or breakfast cereals

A dietary supplement is by definition a supplement to the diet. It is a concentration of substances from foods with nutritional or physiological effect.
It is to be sold in the form of pills, capsules or powder where energy intake does not exceed 50 kcal/day and dosages are clearly described. Lower and upper levels of vitamins and mineral elements are clearly defined.

**We are what we eat**

There has been a thrilling development in scientific understanding of the diet – health relationship during the last 3 – 4 decades: Some 40 years ago, we knew that food was necessary for basic metabolic processes, and focus was on which foods were needed to avoid deficiency diseases. With all essential nutrients being identified, including the latecomer selenium, many scientists of the day though the road had come to an end as to human nutrition research. Today we know that the diet has an impact on all systems of the body, including the foetus, and can modulate different functions far beyond the levels that are connected to malnutrition, e.g. expression of genes, hormone levels, the developing nervous system and risk of diseases later in life (1, 2). Knowledge is also accumulating about the effects of non-nutrient bioactive compounds and toxic dietary substances on health and disease risk. It is thus possible today to say that science verifies the old saying WE ARE WHAT WE EAT. Chemically and biochemically speaking, nutritional science confirms this statement. Part of the challenge is to disentangle and characterize which substances in plant foods that have biological and physiological effects. Another part of our challenge is to get a better understanding of the impact of the administrative route we use to provide the body with bioactive compounds from foods: does it matter whether the exposure is via food, concentrates of food (nutraceuticals/functional foods) or through pills (supplements)? A third issue is the one of dosage. We know the concentrations of nutrients needed to avoid deficiency diseases, but it is still an open question whether more than this dose is better for health. The two latter challenges are the focus of this article.

At first glance it seems intriguing that traditional foods can become even healthier with the addition of bioactive compounds from foods, be it in the form of vitamins, mineral elements or plant extracts. Looking out into the real world, seeing what is on the market, a different impression emerges: The last 50 years have seen a lot of industrial resources and creativity being spent in ”junk food” production and development. Foods rich in sugar, fat and salt have then been given a varnish of health by being added vitamins and minerals. From a nutritional point of view, there has been concern that bioactive compounds added to foods would contribute to increased consumption of unhealthy foods (3)!
However, the market for foods with an additional benefit, be it unhealthy or healthy foods, is on a rapid increase. A gradual shift of industrial focus seems to be becoming apparent: Investments are now put into developing products that are healthy or extra healthy (including functional foods). Foods are given “added value” as a way to increase profits.

The food industry contributes to research on bioactive compounds and wants to take advantage of the development in knowledge, at the same time as complying with what regulations allow them to do.

Health professionals, with nutritionists in the forefront, are watching this development with some concern. An example will illustrate why:

From approximately 1980 until today, our knowledge about how free radical processes in the body are involved in a number of pathological conditions has increased substantially. Based on cell culture and animal studies there is presently solid evidence behind the claim that reactive oxygen species are involved in pathological processes in diseases with inflammation, oxidation and mutation, and thus may influence the development of diseases such as coronary heart diseases and cancer. In the wake of this research there has been a huge increase in interest in dietary factors that may counteract free radical processes, like the vitamins C and E, carotenoids, or the trace element selenium. Vitamin E, which is a chain-breaking antioxidant and prevents the propagation of lipid peroxidation, has been marketed in large doses as an agent to slow down the aging process and as a protective agent against coronary vascular diseases.

Results from two large epidemiological observational studies published in 1993, one including 87,000 women (4) and the other including 40,000 men (5), showed that consumption of vitamin E supplements was associated with 41 and 37 % reduction in the risk of getting coronary heart diseases. Both studies had in common that it was high-dosage supplements that reduced risk, i.e. more than 100 mg E per day, and not vitamin E from the diet or from low-dose multi-supplements. These observational studies were by many taken as “proof” that the results from cell- and animal studies were correct. However, are these findings sufficient to claim that a bioactive compound from food, in this case vitamin E, is warranted in large doses in the form of a pill?

Observational studies have a number of confounding factors which are more or less impossible to control for. Amongst others is the possibility that people taking high-dose vitamin E also in other ways have a lifestyle that reduces the risk of coronary heart diseases. Although the scientists tried to correct for such factors (smoking, age etc), unknown conditions may have influenced the conclusion of the studies.
The last 15 years results from several rather large randomized intervention studies have been published where placebo or vitamin E has been given in dosages from 50 – 800 mg/day (6-14). A meta-analysis of the four first studies showed that the relative risk (RR) for heart infarction, apoplexia or cardiovascular death after 1 – 5 years was 0.97 (0.92 – 1.02), meaning that vitamin E does not have any effect (8). A later meta-analysis came to the conclusion that trials testing high-dosage vitamin E (> 400 IU/d) showed increased risk for all-cause mortality in comparisons of vitamin E versus control (15). Although the latter meta-analysis has been criticized (16), the conclusion is that there is little or no evidence that supports the advice to take vitamin E supplements for healthy people or patients with coronary heart disease in dosages above those that are provided by the normal diet, i.e. 8–10 mg/day.

Similar results to those of vitamin E have also been found for vitamin A: a moderate amount, provided by the diet, protects against disease, but more is not necessarily better. Another lesson learnt is that promising results in the laboratory are not necessarily relevant for humans. The philosophy that a healthy and well-balanced diet seems to be the basic strategy to obtain good health has been strengthened.

From a public health perspective and looking at the global health situation, overweight, obesity, diabetes II, coronary vascular diseases, and cancer are the major challenges. We should not let this situation fade out of focus when discussing dosages and vehicles for providing bioactive substances in the diet.

**Table 1.** Shift of focus when it comes to food supplements and fortification.

<table>
<thead>
<tr>
<th>From</th>
<th>To</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrition</td>
<td>Economy</td>
</tr>
<tr>
<td>Avoid too little</td>
<td>Avoid too much</td>
</tr>
<tr>
<td>Restrictive practice</td>
<td>Increase</td>
</tr>
<tr>
<td>Addition of vitamins and minerals to suitable foods</td>
<td>Addition to unhealthy foods</td>
</tr>
<tr>
<td>Controlled fortification/additions</td>
<td>“Random” additions?</td>
</tr>
<tr>
<td>Transparent market</td>
<td>Non-transparent market</td>
</tr>
<tr>
<td>Good knowledge about effects</td>
<td>Little knowledge about effects</td>
</tr>
</tbody>
</table>
Conclusion

- "FUNCTIONAL FOODS" have come to stay
- The market will probably increase
- Without a balanced diet as a basis, nutraceuticals, functional foods and supplements will have marginal impact on public health
- Vulnerable groups may benefit from selected substances
- Health claims should be thoroughly documented
- The development lays heavy demands on the general knowledge of the public.
- The producers have a special responsibility

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Contents of bioactive compounds in food plants as affected by traditional breeding and environmental factors

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Abstract

Epidemiological studies show an inverse relationship between vegetable consumption and chronic diseases such as cancer and cardiovascular diseases. Bioactive compounds in food plants are believed to be responsible for this effect. There is a very large variation between different varieties of food plants regarding concentration of bioactive compounds. This variation is mainly due to genetic factors but cultivation practices are also of importance. The evidence that bioactive compounds in vegetables have important health related effects associated with their biological activity provides a strong motivation for the manipulation of the concentration in food plants for human consumption.

Introduction

Consumers are becoming more health conscious and the diet is increasingly considered as an important factor which positively influencing the health. Healthy diets have become a social trend in industrial countries, where an accepted estimate is that at least one third of cancer cases and up to one half of cardiovascular disease cases are related to diet (1). Numerous epidemiological
studies have found an inverse relationship between vegetable consumption and chronic diseases such as different types of cancer and cardiovascular disease. So-called bioactive compounds have been demonstrated to be the components responsible for this observed protective effect studied in several cellular and biochemical *in vitro* tests as well as animal experiments.

Generally, overall vegetable consumption in the industrial nations of Northern Europe and North America is relatively low and well under internationally accepted and recommended amounts (i.e. approx. 375 g vegetables/per day) advocated by, e.g. the World Cancer Research Fund/American Institute for Cancer Research.

Reasons for the suboptimal efficiency of current diet campaigns may be consumer complacency with respect to their diet, low income or uncertainty in terms of the interpretation of the recommendations. With regard to current dietary habits, it would be desirable to either increase the contents of phytochemicals in fresh vegetables or enhance supplementation of vegetable products to increase the intake of these health-promoting substances.

One effective way to enhance the intake of bioactive compounds would be to establish production of new plant varieties with enhanced concentrations of bioactive compounds. Another way would be to utilize the production practices eg. nutrient and water supply, production and harvest time, storage etc. to achieve increased concentration of bioactive compounds in the crop.

**Examples of bioactive compounds in food plants**

**Phenolic compounds** are widely distributed in plants. Plant tissue may contain up to several grams per kilogram, and there may be more than 4,000 of such compounds. Phenolic compounds in foods originate from one of the main classes of secondary metabolites in plants. They are produced as a response for defending injured plants against pathogens. Their concentrations in plants are also highly related to the level of stress factors such as UV radiation, intensity of light, low temperature, drought and deficiency of nutrients (2)

In recent years, there is a growing interest in phenolic compounds and their presumed roles in the prevention of various degenerative diseases, such as cancer and cardiovascular diseases. The antioxidant activities of phenolic compounds and their possible usage in processed foods as a natural antioxidant have attained particular interest.
Carotenoids are a class of natural fat-soluble pigments found principally in plants, algae, and photosynthetic bacteria, where they play a critical role in the photosynthetic process. They also occur in some non-photosynthetic bacteria, yeasts, and molds, where they may carry out a protective function against damage by light and oxygen. In fruits such as pepper and tomatoes, carotenoids are synthesised massively during ripening. Mammals appear to be incapable of synthesising carotenoids and may incorporate carotenoids from their diet. When carotenoids are ingested, they show important biological actions such as being antioxidants and free-radical scavengers and reducing the risk of cancer and having a positive effect on the immune response. In addition, several carotenoids are precursors of vitamin A, with β-carotene as the main precursor (3-5).

Glucosinolates are sulphur-containing glucosides (β-thioglucoside $\beta$-N-hydroxysulfates, with an R-group that is an alkyl, alkenyl, thioalkyl, thioalkenyl, aryl, arylalkyl or indoyl moiety), and occur in cruciferous crops. In recent years, several epidemiological studies have suggested that isothiocyanates resulting from the hydrolysis of alkyl glucosinolates found in cruciferous vegetables may play a chemo protective role in the human diet by reducing the risk of cancer (6). Among the isothiocyanates, indole-3-carbinol (from glucobrassicin), phenylethyl isothiocyanate (from gluconasturtiin) and sulphoraphane (from glucoraphanin) are shown to regulate detoxification enzymes and protect against cancer.

Michaud et al. (7) have reported results from a large cohort study in which there was a significant correlation between cruciferous vegetable consumption and reduction in the incidence of bladder cancer. Other studies provide evidence that cruciferous vegetable consumption reduces the risk of cancers of the colon/rectum (8), prostate (9), breast (10), and lung (11), as well as non-Hodgkins lymphoma (12). Consequently, protective effects derived from consumption of *Brassica oleracea* L. vegetables such as broccoli (*Brassica oleracea*, Italica group) have attracted increasing attention.

**Genetic variation**

Studies show that the contents of bioactive compounds vary widely among varieties of food plants (13-15). This variation is caused by several factors such as environmental factors as climate, soil and fertilization, however, the most important factor determining the content of bioactive compounds is the genetic variation (16-17). Thus, breeders have the opportunity and challenge to produce
new varieties of food plants with an improved content of health-related bioactive compounds.

In raspberry there was a significant variation in phenolic compounds between varieties. Most of this variation was caused by genetic differences between the cultivars, but there was also a significant effect of environment (18).

The colour of the carrot varieties discloses the composition of the different varieties. Yellow varieties contain the carotenoids lutein and β-carotene; orange varieties contain lutein, ζ-carotene, α-carotene and β-carotene; and red varieties contain lutein, ζ-carotene, β-carotene and lycopene (19). In Asia, black or purple carrot varieties have been grown and consumed for thousands of years but they are still not very well known in the western world. The reason for the dark colour is that they, different from western varieties, contain the phenolic compounds anthocyanins. In a screening between 15 different black carrot varieties, the total anthocyanin concentration ranged from 45 mg/kg dry matter in ‘Gujarat’ to 17,400 mg/kg dry matter in the variety ‘Germany’ (20). Santos and Simon (21) estimated heritability values for α-, β-, ζ, lycopene, phytoene and total carotenoids in carrot. The results showed that the main variation was caused by genetic composition, but the environmental conditions were also important.

In tomato, a significant positive correlation between the content of the carotenoid lycopene and the red colour is found, and the genotype explained 29-43 % of the variation of lycopene content (22).

In Brassica-vegetables, there is a large variation in the content and composition of glucosinolates. For aliphatic glucosinolates in broccoli (sinigrin, glucoraphanin and progoitrin), more than 60 % of the variation were genetic and only 4.5 % could be explained by climate and other environmental factors (23). For the indolyl glucosinolate glucobrassicin in Chinese cabbage, 61 % of the variation was genetic and 9 % of the variation could be explained by climatic factors (24). Glucoraphanin is one of the most abundant glucosinolates present in broccoli and its cognate isothiocyanate is sulforaphane, a potent inducer of mammalian detoxification enzyme activity and anti-cancer agent. Considerable environmental and genetic variation on the levels of glucoraphanin in broccoli heads are shown, and again, the effect of genotype was greater than that of environmental factors (25). Figure 1 shows the variation in the glucoraphanin concentration in 32 broccoli genotypes. From the figure we can see that the broccoli variety ‘Marathon’, one of the main varieties grown in Norway, has a relative high concentration of glucoraphanin. On the other hand the commercial variety ‘Everest’ has a very low concentration. ‘Marathon’ contains about 5
times more glucoraphanin than ‘Everest’. In a grocery store it is not possible to observe the difference between these broccoli varieties.

**Figur 1.** Glucoraphanin concentration (μmol/g dry weight) for broccoli genotypes grown in 1997 and 1998 (Farnham *et al.* 2004).

### Breeding for increased content of bioactive compounds in food plants

The various effects of bioactive compounds on the quality of both food and feed have created interest in their natural biosynthetic pathways, and in the possibility of manipulating the levels to produce new and improved varieties. Until now plant scientists have mostly been committed to describe the genetic variation in the different plant species and not to develop new varieties with enhanced concentration of bioactive substances.

At the Agricultural Research and Development Centre, Madison, Ohio, a breeding project aims to select parents for tomato F<sub>1</sub>-hybrids with improved lycopene content and colour (22). In Italy a strawberry breeding programme, in addition to breeding for agronomic and quality traits, also aims to select for total phenols content and total antioxidant capacity (26).

Potatoes with yellow flesh contain carotenoids, zexanthin and lutein, and there is a relationship between the intensity of yellow flesh and the concentration of carotenoids. The total carotenoid content of white cultivars and breeding lines
ranges from 0.5 to 1 mg/kg fresh weight. Yellow flesh cultivars may have carotenoid contents up to 2.7 mg/kg while more intensely yellow breeding clones will range up to 8 mg/kg (27).

Red skin of potatoes is due to a high concentration of anthocyanins in the epidermal layer. However, much higher levels of anthocyanins are present in clones that have pigmented flesh. The degree of pigmentation can vary from streaks or blotches of pigments to solid dark pigmentation. The concentration of anthocyanins can have a large range. However, as the skin is a small volume of the whole tuber a red-skinned white-fleshed potato has no more than 15 mg anthocyanins per kg fresh weight. Much higher levels of anthocyanins are present in clones that have pigmented flesh - ranging from 150 to nearly 400 mg/kg fresh weight (24).

At University of Idaho, new potato cultivars with increased level of carotenoids and anthocyanins are developed. The objective of the breeding is to select a potato with acceptable agronomic values (yield, storage ability) together with increased levels of carotenoids and anthocyanins (28).

The best example of deliberate breeding for high level of health promoting glucosinolates is probably the selection in broccoli for higher levels of glucoiberin and glucoraphanin, the precursors of the isothiocyanates iberin and sulphoraphane, respectively (29). This was achieved by crossing a standard broccoli cultivar with *B. villosa*, a wild relative of *B. oleracea* from Sicily which accumulates high levels of glucoiberin in flower buds. The F1 hybrids had high levels of both glucosinolates. By using a series of backcrosses, two regions of the *B. villosa* genome with QTLs for high glucosinolate content were introdused into a commercial agronomic broccoli background. These high glucosinolate broccoli cultivars have subsequently been used in human intervention trials in UK, and shown to deliver about four times the amount of sulphoraphane to the systemic circulation than standard cultivars (30). It is important to note that the isothiocyanates derived from these glucosinolates contribute little to flavour and it is practically possible to enhance their levels. However, increasing the levels of certain other glucosinolates that result in more pungent isothiocyanates, such as 2-propenyl or 3-butenyl, may not be desirable.
Environmental influence on the contents of bioactive compounds

Fertilization
Sulphur and nitrogen fertilization are shown to influence the pattern and levels of glucosinolates in broccoli (31). In most of the field experiments, it is shown that an increased sulphur fertilization gives a higher glucosinolate concentration in Brassica-vegetables (32). In contrast, 70 % reduction of the alkyl glucosinolates glucoraphanin and glucoiberin was observed in broccoli supplied with 200 kg N ha\(^{-1}\) in comparison with plants receiving no nitrogen fertilisation (33). Furthermore, enhanced nitrogen supply was found to lower the anthocyanins content in Merlot grapes (34). Anthocyanins in red cabbage also seem to be reduced by high nitrogen supply, but potassium and phosphorous have small influence (35). The highest lycopene concentrations and best colour in tomatoes were reached under the lowest nitrogen level, but the yield increased with higher nitrogen level. On the other hand, the lycopene concentration increased with increasing phosphorous and potassium supply (36). In carrot the carotene content seems to increase with increasing nitrogen supply (37).

King et al. (38) investigated the effects of nitrogen and phosphorus fertilization on total carotenoid content in spinach. Increases in nitrogen fertilization from 0 to 300 kg ha\(^{-1}\) had little effect on total carotenoid content, but the highest carotenoid contents were obtained with maximized phosphorous application (50 kg ha\(^{-1}\)).

Irradiation
Plants exposed to full sunlight have been demonstrated to contain more flavonoids than those grown in shade (39). Increasing irradiation combined with low mean temperature resulted in higher contents of the glucosinolates glucoraphanin and glucoiberin in broccoli and white cauliflower (39). On the other hand, the glucosinolate content in green cauliflower seem to be nearly unaffected by temperature and irradiation.

In contrast to the flavonoids and glucosinolates, irradiation seems not to influence the carotenogenesis and consequently the concentration of carotenoids in plants.

Water supply
Low rainfall during the vegetation period increased the total glucosinolate content in most of the Brassica vegetables (40-41). Generally, a reduced water supply leads to increased concentrations of bioactive compounds in plants (39).
**Temperature**

Generally, the glucosinolate content of broccoli and cauliflower is strongly influenced by temperature and to a lesser extent by irradiation during plant development. Broccoli grown at temperatures ≤12°C combined with increased radiation had the highest concentration of the alkyl glucosinolates glucoraphanin and glucoiberin. In contrast higher contents of the indole glucosinolate glucobrassicin were found if the broccoli were grown under higher temperatures and low radiation (42). In a field experiment in Spain, Rosa and Rodrigues (40) studied 11 broccoli varieties grown in the warm season (April–July) and the chilly season (August-January) and reported that the glucosinolate concentrations were generally higher in broccoli grown in the chilly season.

Regarding carotenoids, daily mean temperatures below 16.5°C were beneficial for the β-carotene synthesis in broccoli, whereas the highest concentration of β-carotene in carrot was acquired at 18°C (43). Beneficial temperatures for lycopene formation in tomato ranged from 16°C to 21°C (39).

**Conclusion**

To increase the intake of health related bioactive substances, an important strategy is to increase the concentration in food plants through production of new varieties with increased contents. Several investigations have shown that there is a large genetic variation for the concentration of these substances and that this variation can be exploited. At the same time, environmental factors like fertilization and climate also influence the concentration of bioactive substances.

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The importance of genetic modification for contents of bioactive compounds in feed and food plants

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Abstract

Plant breeding has been of immense importance to human beings throughout history. The present human population could not have been fed without the many fold increases in crop yields that plant breeding has provided. Over the last decade, genetically modified crops have become accepted by many countries, provided that they are subjected to thorough safety assessment and authorisation on a case by case basis. There are risks associated with plant breeding. The novel trait found in any new variety, independent of the breeding technique involved, will have its basis in genetic alternations. In addition to these traits specific genetic alternations, there may be additional genetic alternations. Some of those alterations, which may not be related to the new trait of the breed, may cause adverse effects such as production of bioactive compounds with toxic effects. Some adverse effects may be discovered while others remain unknown. The risks associated with genetic modification may be higher than for some traditional breeding technologies but at the same time risks may be lower than that of some other traditional breeding technologies. Despite this, GMOs are often understood as inherently risky until their safety is proven through a rigorous safety assessment and authorisation procedure, while at the same time crops generated using traditional technologies are considered safe and no documentation or authorisation is needed. While the first GMOs were developed with agronomical traits, such as pest resistance and pesticide tolerance, new generations enhance the level of nutrients or reduce the level of toxicants naturally found in plants. Genetic modification represents a new and
powerful tool for plant breeding. The global community has decided to progress with precaution to limit the risk of adverse effects while at the same time harvest the benefits.

**Introduction**

Plant breeding has had, and still has, an immense importance to human well-being. The present human world population could not have been fed without the many fold increases in crop yields that plant breeding has provided. However, new breeding techniques, such as genetic modification, may provide a more effective way of breeding but are at the same time associated with risks.

Here, I will focus on the following:
1) Comparison between traditional plant breeding and genetic modification.
2) Genetic modification may constitute a risk for increased content of harmful bioactive compounds
3) Genetic modification may remove harmful bioactive compounds from food and feed
4) Genetic modification may add beneficial bioactive compounds in food and feed

**Comparison between traditional plant breeding and genetic modification**

GMO crops were introduced to the market in 1996. Since then the global area planted with GMO crops has increased rapidly (see Figure 1). In the USA, there have been issued in the order 13 000 permits for field trials, and in the order of 100 GMOs have been deregulated for commercialisation.

Since the beginning of the last century, plant breeders have hybridised different plant materials in order to create new variation on which selection can be imposed. Crossing can be done within a species or between more or less related species. Some species hybridise in nature, while others can only be crossed using special techniques. Embryo rescue was used to obtain a cross between different species as early as in the 1920-ies. The protoplast fusion technique was developed from the 1970-ies. Crossing of a crop species with a related, non-domesticated species is done in order to introduce genes/alleles not present in the gene pool of the crop species.
Figure 1. Global area of GMO crops. Reprinted with permission from Global Status of Commercialized Biotech/GM Crops. International Service for the Acquisition of Agri-biotech Applications (1).

By exposing plant material to chemical mutagens or ionising radiation, the mutation rate can be increased dramatically above the natural background mutation rate. Proof of mendelian inheritance of changes induced by X-rays was presented in the late 1920-ies, but induced mutagenesis did not come into practical plant breeding until the 1950-ies. Induced mutations include both mutations in genes and chromosomal rearrangements. In the IAEA Mutant Variety Database (http://www-mvd.iaea.org, accessed 12.01.09) 2385 released varieties developed through mutagenesis are listed. Around 90 % of the varieties have been mutated using radiation. Recent analyses of induced mutant populations using the TILLING technique indicate a mutation rate of 5000-50 000 per cell (2).

A genetically modified organism (GMO) is an organism whose genetic material has been altered using genetic engineering techniques. These techniques, generally known as recombinant DNA technology, use DNA molecules from different sources, which are combined into a genetic cassette containing a new set of genes. This DNA cassette is then transferred into an organism, giving it a set of modified or novel genes. Transgenic organisms, a subset of GMOs, are organisms which have inserted DNA that originated in a different species.
Cisgenic GMOs do not contain genes from other species but have inserted a new genetic combination of their own genome.

All the techniques described above introduce genetic variability and new genetic materials. The current use of the term genetically modified organism (GMO) is therefore misleading. All new plant varieties are genetically altered through the breeding process. Genetic modification is one out of many techniques that introduced genetic alterations.

Independent of the specific technique used to introduce genetic variability the pool of variants go through a process of selection. In this process the variants with the superior genetic properties is selected. However, selection for one trait may lead to correlated effects on other traits such as bioactive compounds. This may occur when one gene affects several traits or it may occur due to effects of closely linked genes. Disease and pest resistance is often based on the production of certain enzymes or secondary metabolites that are toxic to plant pathogens or insects. These substances are sometimes toxic to human beings as well, in which case selection for resistance may lead to adverse effects to human health.

A number of genes closely linked to the genes encoding the desired trait are likely to be co-introduced into any new variety descending from crossing or hybridization. In most cases the identity and function of these genes will be completely unknown. Unlike introduction of new alleles/genes by crossing, introduction by transformation does not involve the linkage drag described above and thus there will be no unintended introduction of irrelevant genes linked to the gene of interest.

The food safety of plants genetically altered through plant breeding was not questioned until transgenic crop plants came into commercial production in the 1990-ies. A large number of food crop varieties cultivated during the last century were developed using induced mutagenesis or introgression of genes from related species. There are not many examples of food crops with an adverse effect on human health that is due to breeding and not to characteristics intrinsically present in the species. This may indicate that there is not a strong need for safety assessment of foods derived from crop varieties developed through these techniques. However, subtle or long-term adverse effects of genetically altered plants or traditional varieties may be difficult to detect.
Genetic modification may constitute a risk for increased content of harmful bioactive compounds

The novel trait found in any new variety, independent of the breeding technique involved, will have its basis in trait specific genetic alternations. Furthermore, in addition to these traits specific genetic alternations there may be more or less of additional genetic alternations. The unintended alternations may be discovered while others remain unknown. Some of the unintended traits may be unwanted due to adverse effects on the plant, the environment or human health.

All breeding material is evaluated throughout the breeding process, and individuals with obvious unfavourable traits are discarded. However, this evaluation has historically focused on the quality of the new plant and especially on the new traits that were the aims of the breeding process. Thus, unintended effects due to the breeding process have not been looked at. The screening is generally limited to easily observable traits, that is, morphological or agronomical traits with known adverse effects. Contents of toxic compounds, allergens and nutrients are sometimes measured in plants were they are known to occur (e.g. glycoalkaloids in potato). However, in general very little safety related research is conducted on non-GMO varieties.

Genetically modified (GM) varieties, which are derived through recombinant DNA technology, are by legislation required to be subject to extensive safety documentation, public safety assessment and regulatory authorisation before they are accepted for cultivation or food purposes. The safety assessment is based on European and international guidance documents developed by international bodies such as The European Food Safety Authority (EFSA) and the CODEX ALIMENTARIUS within the United Nations system. Several issues must be thoroughly documented. This includes; how the food crop was developed, the molecular biological data which characterizes the genetic change, composition of the novel food compared to non-modified counterpart foods, nutritional information compared to non-modified counterparts, potential for introducing new toxins, and the potential for causing allergic reactions. The compliance costs for regulatory approval, to ensure health and environmental safety, are 6 to 14 million USD (3).

There are not yet any well documented examples of adverse health effects associated with commercialized GMOs. Regarding non-GMOs there are a limited number of well documented incidents with adverse health effects associated with the breeding (4). One example of this is selection for pest resistance leading to increased levels of furanocoumarins in celery. Furanocoumarins can cause severe skin irritations in humans and are carcinogenic. Varieties bred for enhanced insect resistance in celery has caused
skin problems among workers that handle these plants. Another example is potato and tomato, where selection for disease resistance, or genetic drift, can lead to increased and toxic levels of glycoalkaloids.

There exist, as far as I now, only one report on the comparative safety of various breeding techniques. This is the National Academy of Sciences report from 2004 on approaches to assess unintended health effects in genetically engineered foods (5). Various breeding techniques are scored along the scale likelihood of unintended effects (Figure 2).

![Figure 2. Likelihood of unintended effects from different breeding techniques. The GMOs are made from different rDNA techniques associated with different levels of risk. Reprinted with permission from Safety of Genetically Engineered Foods: Approaches to Assessing Unintended Health Effects, 2004 © by the National Academy of Sciences, Washington, D.C.](image-url)
The main finding in this assessment is that there are risks associated with all breeding techniques and that there is no particular high risks associated with GMOs relative to some of the older and so called conventional breeding techniques. Thus, the belief that risks associated with foods derived from GMOs are qualitatively different from risks associated with foods from other crop breeding techniques are not supported by scientific inquiry so far. Indeed, most if not all of the biological mechanisms active when a GMO is generated, are also found to be active to a smaller or greater extent when the other breeding techniques are applied. This is supported by a workshop organised recently by EFSA which in relation to risk assessment recommend the following “The focus on only GM crops defies scientific evidence. In the longer term, risk assessors could develop an alternative approach on a scientific basis.”(6).

Genetic modification may remove harmful bioactive compounds from food and feed

There are at least 35 000 different secondary metabolites produced by plants (7). Many of these are toxic and play an evolutionary role in the protection against herbivore animals, insects and micro organisms. When considering consumption of food plants with toxicants the margin of safety is small in many cases (8). The presence of harmful bioactive compounds in plants is not only due to endogenous production but may be produced by micro organisms as well. It has been suggested that toxicological data, as well as structural similarity “should serve as a guide for the removal of the most toxic compound from plant foods” (9). Genetic modification provides, together with traditional breeding techniques, means to reduce the level of harmful bioactive compounds in food plants, both those caused by plant chemistry and those caused by micro organisms.

Cyanide
It is estimated that at least 2500 plant taxa produce cyanogenic glycosides which are toxic compounds (10). Among these we find crops such as cassava, sorghum, almond, lima beans and white clover. Bitterness in food from such crops is determined by the content of the toxin (11). Cassava is the most important root crop in the world. It is the second most important staple crop in Africa and is used extensively for starch production in South East Asia (12). A major drawback of the cassava crop is a high level of the cyanogenic glycosides, especially linamarin, which generate hydrogen cyanide when the plant tissue is disrupted. When cassava products are used as staple food the toxic compounds
are removed by careful processing (13). Unfortunately, careful processing reduces the nutritional value by the loss of proteins, vitamins, and minerals. Moreover, incomplete processing results in high cyanide exposure and diseases such as tropical ataxic neuropathy after chronic low level exposure and konzo after acute exposure (14).

Traditional plant breeding has generated cultivars with high and with low levels of cyanogenic glycosides but not been able to establish new varieties devoid of these compounds. Using genetic modification several cyanogen-depleted cassavas have now been developed. This has been achieved by RNA inhibition technology to knock down expression of enzymes involved in biosynthesis (12). Another approach has also proved successful. Here, an enzyme is overexpressed that provides for the volatilizations and removal of the toxin during food processing (15). Both approaches provide a safer food product.

**Glycoalkaloids**

Glycoalkaloids, such as solanine, is a family of steroidal toxic plant metabolites in potato and related species commonly used in potato breeding (16). The highest levels of glycoalkaloids are found in flowers and leaves, but the levels found in tubers may also be considerable and vary substantially between cultivars (16). A level of 200 mg glycoalkaloids/mg potato, which is generally accepted as the highest tolerable limit, has a zero safety margin (17). It is also known that the level of glycoalkaloids in potato tend to increase during storage and after exposure to light. There are cases of food poisoning from consumption of potato. Mild clinical symptoms include abdominal pains, vomiting and diarrhoea. Potato related adverse health effects have also been found in experimental studies on human volunteers but are not frequently reported, probably due to a serious underreporting since potato are consumed daily and gastrointestinal disturbances are very common (18). Deaths caused by potato are rare, but according to Smith *et al.* (19) “The narrow margin between toxicity and lethality is obviously of concern.”

Several approaches have been exploited by genetic modification to successfully reduce the level of glycoalkaloids in potato. One of those approaches is based on the overexpression of a methyltransferase from the soybean plant. This reduces the level of free cholesterol and glycoalkaloids in the tubers (20). Several other approaches have achieved reduced levels of toxic glycoalkaloids in the edible tuber by the reduced expression of enzymes involved in glycoalkaloid glycosylation (21). A recent study was conducted to study changes in the overall metabolite composition in both genetically modified and traditional breed potato tubers. Interestingly, few differences were found between the genetically
modified tubers and their parental lines, while different traditional cultivars showed large variations in the metabolite profiles (22).

**Caffeine**

Even though caffeine was isolated in the early 1820s, the main biosynthetic pathways were not fully established until recently (23). The physiological role of purine alkaloids, such as caffeine, has until recently largely been unknown but the main hypothesis is chemical defence. The identification of genes involved in biosynthesis opens the possibility to make naturally decaffeinated coffee or to introduce caffeine as a natural pesticide to protect against herbivores or pathogens in other plants.

Since caffeine may produce adverse effects, such as palpitations, increased blood pressure and sleep disruption, efforts have been made to produce naturally decaffeinated coffee plants. This has not yet been fully achieved but the caffeine level has been reduced up to 70% by introducing RNAi constructs repressing homologous N-methyltransferases (24).

Interestingly, caffeine may be used as a pesticide as well. Tobacco, which does not contain caffeine, has been genetically modified to synthesize caffeine. This has been achieved by introducing a multi-gene vector expressing three N-methyltransferase genes sufficient for caffeine production. This plant was less susceptible to caterpillars. The expression of caffeine was relatively low (<5 ppm) and the protective effect may partially be caused by activation of endogenous defence-related genes and the plant was more resistant to pathogenic bacteria and virus (25).

**Detoxification of zearalenone**

Maize is often contaminated by moulds, including the zearalenone producing *Fusarium graminearum*. Even though such contamination has been well known for decades, preventive measures are costly and inadequate. Even though zearalenone is a mycotoxin, it is a safety issue in relation to consumption of plants. Recently a GM-maize harbouring a detoxification system was developed. A bacterial gene encoding an alkaline lactonhydrolyase was cloned and used to transform maize. The GM-maize had a substantially lower level of zearalenone and the same approach may be used to remove other problematic mycotoxins (26).
Genetic modification may add beneficial bioactive compounds in food and feed

There are serious nutritional and environmental deficiencies associated with the current agriculture and food supply in certain parts of the world. Even though most people obtain sufficient energy from their food there are nutrient deficiencies in major staple crops such as rice and maize. Plant breeding, including genetically modified crops, may undoubtedly contribute to an increased food production as well as to ameliorate some of the nutritional and environmental deficiencies associated with food production today (27). All the different nutrients found in a food plant may be modified, including proteins and amino acids, oils and fatty acids, carbohydrates, vitamins and minerals, as well as the other thousands of different metabolites. Here, just two examples will be mentioned.

Vitamin A
Millions of people die or become victims to chronic diseases due to food deficient of nutrients every year. Vitamin A deficiency is a leading cause of preventable blindness and child mortality and is a public health problem in more than half of all countries. According to the WHO and CDC (Center for Disease Control, USA), 250 million preschool children are vitamin A deficient. Half a million are estimated to become blind every year, of which a majority (70%) dies within one year after becoming blind.

Rice varieties have been developed with sufficient levels of vitamin A to strongly reduce the problem of vitamin A deficiency. By the addition of only two genes, phytoene synthase and phytoene desaturase, the biochemical pathway responsible for β-carotene is reconstituted (28). New varieties could have been on the market within a few years if the increased pro-vitamin A content had been achieved with traditional breeding techniques such as mutagenesis or wide-hybridisation. However, since gene technology was a part of the breeding process the regulatory requirements and heavy documentation needs are still not passed.

Antioxidants
Anthocyanines, which is a class of antioxidant pigments produced by higher plants, have been linked to increased protection against different age related diseases, such as cancers and cardiovascular disease (29). The levels of anthocyanines vary substantially between different food plants. Attempts have therefore been made to increase the expression of such pigments in major fruit or vegetables.
One recent example is tomato genetically modified to express higher levels of anthocyanine (30). The plant has been gene modified by the introduction of two transcription factors from snapdragon. The anthocyanine level was found comparable to that of blackberries and blueberries. The antioxidant level increased threefold and both the peel and the flesh of the tomato had intense purple coloration. In a pilot study it was shown that the lifespan of cancer-susceptible mice was significantly extended when fed the new anthocyanine tomato compared to the unmodified tomato.

**Conclusion**

Over the last decade GMOs have become accepted by many countries, provided the GMOs are subjected to thorough safety assessment and safety management on a case by case basis. The risks associated with genetic modification may be higher than some, while at the same time smaller than some others, of the traditional breeding technologies. Despite this, GMOs are understood as risky until their safety is proven through a rigorous safety assessment and authorisation procedure, while food crops generated with other technologies are considered safe and no documentation or authorisation is needed.

While the first GMOs were developed with agronomical traits, such as pest resistance and pesticide tolerance, new generations enhance the level of nutrients or reduce the level of toxicants naturally found in plants.

Genetic modification represents a new and powerful tool for plant breeding. The global community has decided to progress with precaution to limit the risk of adverse effects while at the same time harvest the benefits.

**References**


Increased levels of bioactive compounds in organically grown food plants. Possible health effects?

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Abstract

Recent studies show that an organic cultivation system is more likely to produce food plants with higher levels of secondary metabolites, higher content of vitamin C, and higher content of dry matter (less water). Conventionally produced foods have higher levels of vitamin A and compounds containing nitrogen, such as proteins and nitrate. Yield is consistently lower in organic cultivation systems. Both environmental factors and agricultural management practices will determine the phytochemical composition of the food plant. Choice of cultivar is the most important factor in this respect, but also use of pesticides will be a decisive factor. To what extent the differences in the two agricultural systems will have beneficial effects on health is not yet sufficiently documented. However, in vitro studies on cell cultures and animal models points in this direction. More research on health effects is needed, using in vitro techniques on animal and human cells. Also observational studies and controlled interventions in humans will be of importance in the years to come.
Introduction

Organic agricultural production is according to the International Federation of Organic Agriculture Movements (IFOAM) defined as “a production system that sustains the health of soils, ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. …” (1). This definition is wide and in line with the concept of “sustainability” and implies a systems approach to agriculture. In further detail, the definition excludes the use of synthetic fertilizers and pesticides including herbicides. It also calls for production methods that promote healthy, fertile soil such as crop rotation and mixture of crops. Further processing of food crops should be done carefully and refinement of the foods should be limited.

Consumers’ perception of organic foods is that it is better for the environment, containing less pesticide residues and being more nutritious and thus healthier (2). However, up to 2003 there were fairly few studies that had compared the nutritional content of organic versus conventionally produced food. I did a review of the scientific literature at that time and found that the results were somewhat inconsistent, both because the studies were few and because most of them were not experimentally controlled studies (3). The conclusions drawn in this review paper was that organic food plants were more likely to contain less protein, less nitrate, higher vitamin C but lower vitamin A, compared to conventionally grown plants. They tended also to have higher nutrient density (i.e. lower water content). The most striking and consistent feature was that organic food plants usually had elevated content of secondary metabolites, estimated to be 10-50 % higher, provided that the same cultivars were compared in the two production systems. The secondary metabolites, of which many are antioxidants, are present in every plant. Estimates indicate that more than ten thousand different compounds in this category exist (4), of which only a small fraction have been identified.

Recent research findings on plant food composition

Since 2003 there has been an increase in number of research reports relying on improved and more controlled methods (5). There are numerous factors that may affect plant phytochemical composition, including both environmental variations and agricultural management practices, which need to be taken into account when comparing food plants from organic and conventional systems.
Many studies have been done on the tomato, representing the second most consumed vegetable in Europe next to the potato. Tomatoes are an important source of nutrients and secondary metabolites with antioxidative properties having health protective effects, such as vitamin C, β-carotene and lycopene, and flavonoids (6). One well conducted study worth mentioning in this respect is the 10-year LTRAS (Long Term Research on Agricultural Systems) project on tomatoes cultivation run by the University of Davis in California from 1994 to 2004 (7). The research design allowed for comparisons between outcomes from organic and conventional management systems without the usual confounders that have hampered many similar studies and make them difficult to interpret. In this study tomatoes were cultivated on adjacent plots by organic and conventional methods. The same cultivar was used in the two systems and irrigated according to need. The application of composted manure was gradually reduced in the organic system as the soil organic matter was built up to a near constant level. The tomatoes were harvested when they were 90 % ripe. Crop yields and total biomass were measured each year and analysed for total nitrogen (N) and carbon (C). Samples of the tomatoes were dried at 60 °C and stored at 20 °C in the dark, and the content of flavonoids were measured. The results showed that the organic tomatoes had significantly higher content of the flavonoids quercitin, naringenin, and kaempferol. For quercitin, naringenin and kaempferol the ten years mean differences were 79, 31, and 97 % higher, respectively. The levels of flavonoids were increasing over time in both cultivation systems; however the rate of increase in the organic system was higher. Interestingly, this increase in flavonoids, especially quercitin and kaempferol, could be related to the accumulation of soil organic matter and the corresponding reduction in manure application in the organic system. The reduction in composted manure went from 45 to 18 tons/ha but this did not affect the tomato yield. Comparisons of yields in the two systems showed great variations between years, especially in the conventional system. The flavonoid content in tomatoes was negatively associated to available N in the soil; the lower availability of N the more flavonoids were accumulated in the plant.

A recent review of the impact of management practices of the different cultivation systems is summarised in a report from 2008 (5). This examines the results of 97 peer reviewed studies from 1980 and onward. 236 matched pairs of measures from organic and conventional samples of a given food were identified. These included 135 study-crop combinations. For each crop the study quality was categorized as “high”, “acceptable” or “invalid”. 70 % were deemed acceptable or high quality, i.e. 94 out of 135 study-crop combinations. Those 94 valid combinations were screened for accuracy and reliability of analytical methods. 55 were deemed invalid for specific nutrient measurements. 17 criteria were used to select the most appropriate matched pairs in order to reflect food in its fresh form. The levels of 11 nutrients were compared in organic versus
conventional food samples. The number of matched pairs in which the organic samples were found to contain higher levels of the nutrients was compared to the number of pairs in which the conventional samples were found to have higher levels. The levels of antioxidants were found to be higher in a majority of the organic samples (44 out of 59 matched pairs). In fact, total antioxidant capacity was 88 % higher, total phenolics 72 % higher, quercetin 87 % higher and kaempferol 55 % higher in the organic samples. The precursors of vitamins A, C, and E were also found to have higher levels in the organic samples in 41 out of the 67 matched pairs. Nitrate and protein content was found to be higher in a majority of the conventional samples, in 15 out of 18, and 23 out of 27 matched pairs, respectively.

In the EU project *Quality Low Input Food* (QLIF) which was running from March 2004 to March 2009, large scale controlled studies were carried out in which organic agricultural practices were compared with conventional ones in regard to effect on food quality, including composition and quantities of nutrients and secondary metabolites. The studies were carried out on 3000 hectares of agricultural land and conventional and organic crops were cultivated on adjacent plots. The project was carried out by an international team of researchers, involving 34 different research centres, universities and commercial enterprises. Over a hundred peer reviewed publications have appeared in international journals and books on a variety of topics related to the research (some of them are being referred to in this article), but not all results are yet published. However, in a review published in 2007 by one of the participating scientists the findings on increased content of antioxidants in organic food plants were confirmed. Her calculations based on review of 15 studies showed the content of phenolic compounds in organic crops to be 119 % higher (range -57 to +734) than in their conventional counterparts (8). In a press release in 2007 the research director, Carlo Leifert, stated that their preliminary results revealed that organic fruit and vegetables had on average 40 % more antioxidants, and contained higher levels of vitamin C, iron, copper and zink (9). He also pointed out that even 20 % more antioxidants in fruit and vegetables would mean that an intake of four portions a day would correspond to the recommended five-a-day. A summary report is made from the project (10).

**Effect of environmental factors and agricultural management practices**

As is evident from the literature, research comparing contents and composition of bioactive compounds in food plants come up with very contradictory results.
As mentioned earlier, many studies have been badly designed and often comparing food plants that had not been subjected to controlled conditions (7). It is therefore of great importance to identify the factors that may affect the composition of nutrients and bioactive compounds in food plants. Table 1 illustrates these factors in relation to the different processes in the food chain. Environmental factors, such as latitude, location, climatic variations, altitude, and soil type affect the composition of nutrients and secondary metabolites. But of interest here are the agricultural production practices which also affect plant composition, such as choice of variety/cultivar, fertilizer regimen, pesticide application, soil preparation methods and different cultivation practices, such as crop rotation, cover crops, crop mixing, in addition, timing of harvest, and storage length.

The EU funded QLIF crop production studies have used a factorial approach to field experiments to identify the effects of the individual components of the food production system on food composition. The most important factor determining content of antioxidants and nutrients in the food plant is the genetic make-up, i.e. the type of variety used. There can be large differences between different varieties of the same plant species, and in regard to secondary metabolites the differences can be manifold. This has been shown for many fruit and vegetable cultivars and for food grains. Genetic differences can also render the different variety of a plant species more or less responsive to factors that may stimulate stress induced synthesis of secondary metabolites (see below).

<table>
<thead>
<tr>
<th>Environmental factors</th>
<th>Production system</th>
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<tbody>
<tr>
<td>Latitude</td>
<td>Plant variety/cultivars</td>
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<tr>
<td>Climatic variations</td>
<td>Fertilizer regimen</td>
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<tr>
<td>Altitude</td>
<td>Use of pesticides</td>
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<tr>
<td>Soil type</td>
<td>Soil preparation methods</td>
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<td></td>
<td>Other practices (crop rotation, cover crops, crop mix etc)</td>
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<td>Ripeness at harvest</td>
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<td>Storage length</td>
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Table 1. Sources of variation in nutrient contents between organic and conventional plant foods.
**Plant fertilization management**

Next to the genetic factor, plant fertilization management is the most influential factor in regard to affecting composition of plants, including the secondary metabolites. Organic cultivation involves use of several methods to develop and maintain soil quality, such as intercropping, N producing plants, crop rotation, as well as use of cover crops and composted animal manure or green manure. With such methods the release of soil nutrients to growing plants will occur more slowly. Synthetic fertilizers in contrast, are more easily soluble and will thus be more available to the plant and result in more effective uptake of nutrients (11).

The plant scientist, Nancy Stamp, has suggested a model for explaining the relationship between nutrient availability in the soil and the growth and differentiation in plants called the growth-differentiation balance (GBD) hypothesis (12). This hypothesis proposes that the plant will allocate nutritive resources to growth if available in ample supply, but if resources are constrained the plant will divert more resources to the synthesis of secondary metabolites associated with differentiation (see Figure 1). That means that if N is available, the plant diverts more input into N-containing metabolites, such as protein, vitamin A and nitrate, while more C containing compounds are produced when N is more restricted, such as vitamin C and many of the secondary metabolites. The figure suggests further that when resource availability is critically low both growth and secondary metabolite synthesis will be hampered. In organic agriculture the availability of soil nutrients such as N are lower than when commercial fertilizers are used. According to the model, this explains why organic produce usually have a higher content of secondary metabolites.

In field trials with wheat and barley, Brandt (13) showed that the amount of fertilizer application had effect on content of phenolic acids (secondary compounds working as natural pesticides). Increasing the level of fertilizer led to a lower content of phenolic acids in the plants. Furthermore, the level of phenols in the plants was negatively correlated to degree of fungi attacks (*Drechslera teres* and *Rhynchosporium secali*) in experiment with barley. Infection of fungi caused by *Fusarium* was found to be higher in conventionally cultivated wheat compared to organic cultivated wheat over two cultivation seasons. Brandt summarizes these experiments in the following way: Intensive agriculture (high input) give larger yields, variable concentration of secondary compounds at an average low level, and low resistance against diseases. While organic agriculture gives medium yields, more constant content of secondary phytochemicals in higher concentration, and good resistance against diseases.
Secondary metabolites are also involved in the development of a plant into maturity, which implies that the plant allocates less of its resources into growth and more to C containing antioxidants involved in development of flowers, fruits and seeds. High input of N affects the plant’s natural maturation cycle, from growth phase to differentiation phase, in a way that causes the plant to remain in the growth phase and never reach maturation. This will also affect the content of secondary metabolites which is part of the maturation and responsible for developing colour, taste and aroma (11, 14).

**Stress induced secondary metabolites and use of pesticides**
Another factor determining the production of secondary metabolites is crop protection by use of pesticides. The use of pesticides on conventionally cultivated plants to protect them from pathogens may explain why these plants have lower content of secondary metabolites. Salicylic acid is one such example.
This compound is a polyphenol synthesized in response to plant pathogens, and is present in low amounts in all plants. Organic food may have higher levels, as shown in a study in which the concentration of salicylic acid was found to be six fold higher in organic vegetable soups than in soups based conventionally produced vegetables (15). Salicylic acid is known for its anti-inflammatory and anti-coagulant effects and has preventive properties in regard to atherosclerosis, myocardial infarction and colon cancer. Vegetarians, who only eat plant foods, were found to have levels of salicylic acid concentrations in blood similar to patients on prophylactic doses of this compound to prevent thrombosis (16).

Resveratrol is another phytochemical that is triggered by pest attack. It is a phytoalexin (natural pesticide) present in grapes, ground nuts and other legumes, dark coloured fruits and berries, and vegetables. It is synthesised in response to attack of moulds. In an experiment the effect of fungicide application on the synthesis of resveratrol in different grape cultivars was studied. It was shown that those cultivars that had been treated with fungicides had lower resveratrol concentrations than the untreated ones. The resveratrol concentration was from negligible to almost five times higher in the plots that were not treated with fungicides, depending on the type of cultivar (17). It is worth noting that the different grape cultivars seemed to have different ability to respond to such stress. In this experiment the Noble variety was the cultivar being able to respond most adequately to fungal attack.

Resveratrol has been in focus for extensive research in regard to effects on human metabolism and health during recent years. The reason is the interest for the protective effect of the Mediterranean diet, in which modest daily consumption of wine is one constituent. Resveratrol is thought to have preventive effects in regard to cardiovascular disease, glucose intolerance and cancer (18).

**Plant toxins**

A range of bioactive compounds that plants produce for self-defence may be characterized as “toxins”. Most organic food plants show moderately higher median values, which do not represent any risk to man (14, 19). Humans have developed mechanisms to metabolise or excrete most of these compounds. Many plant toxins are phytoalexins (natural pesticides) that may accumulate at different degrees as a response to stress related factors. The concentration of such compounds in food plants may vary with a factor of hundred. The content of furanocoumarines from organically cultivated parsley and celery root or glycoalkaloids from potatoes are usually higher than in these vegetables.
cultivated conventionally. The content in organic potatoes was found to be on the average 20% higher. However, the content was still lower than what has been defined as safe levels (200 mg glykoalkaloids per kg). Organically produced potatoes were also found to be more resistant towards pest attacks (14). Brant and co-workers (14) argues that many plant toxins show biphasic effects (*hormesis*) in bioassays with cells, implying positive effects on cell proliferation at low doses and toxic effects at high (see figure 2). Research is ongoing to study health effects of plant toxins that may occur at higher concentrations in organically produced food plants. This concerns glucoraphanin, a glucosinolate, which is found in broccoli and in other plants from the *Brassicacea* family. It is a very well documented defence compound found to be 2-6 times higher in organic than in conventionally grown broccoli. Many beneficial health effects of this compound are documented, especially anti-cancer effects. Other compounds that show biphasic patterns are isothiocyanates (garlic) and tannins (fruits and berries) (14).

**Figure 2.** Hormesis: Dose-response curve for most bioactive compounds in plants. From Kirsten Brandt: COST 926 conference 2005. http://orgprints.org/6194/
Effect of ripening stage at harvest and food processing

Organic production applied in the context of ‘sustainability’ implies the use of local foods. Commercial enterprises depending on export, long transport mileage and storage, usually pick their produce when it is still green. This may lead to lower concentrations of sunlight induced phytochemicals related to maturation (20), such as flavonoids and polyphenols giving colour and taste to fruits. These compounds are known to have favourable effects on prevention of cardiovascular disease and cancer. Differences in the concentration of these compounds can be manifold. Halweil and co-workers (21) point to research showing that blackberries picked green contains 74 mg of anthocyanins compared to 317 mg in ripe ones, and that apples and apricots had no vitamin C when picked green.

Further processing of food may also lower the nutritional value in relation to the fresh state. A study on five organic and five conventional tomato pasta sauces compared content of flavonoids and carotenoids and vitamin C. The result indicated a possible reduction of vitamin C content due to storage time and addition of olive oil in the sauce. The content of flavonoids and carotenoids showed no differences between the organic and the conventional sauces, suggesting that processing and storage conditions and length may have reduced the original differences between the sauces of organic and conventional origin (22).

Different grain products are often milled and refined. Organic processing usually results in a lower degree of milling or refinement. For instance, refining wheat or rice may eliminate 50-96 % of fibres, vitamins and minerals from these crops (23).

Possible health effects

Health effects of organically produced foods can be studied in an indirect manner, by examining the nutritive quality of food products or directly through looking at effects of such foods on living organisms. The possible indirect health effects of certain compounds found to be higher in organic foods have been considered above. Most researchers will now agree that many of the secondary metabolites, such as antioxidants and polyphenols, are important compounds for the maintenance of good health in humans. The question is, however, if higher concentration of these compounds, which apparently seems to be the case in organic foods, would give extended health effects. The evidence gathered so far is mostly from in vitro studies on animal or human cells, or from using animal
models. Still there are hardly any sufficiently controlled human studies. Thus it is still too early to draw any conclusions.

In a Danish study on human subjects, an organically produced diet was compared with an identical one produced conventionally. The results showed a higher content of flavonoids and biomarkers of antioxidant defence in blood and urine among those receiving the organic diet (24). However, the study was flawed by the fact that the organic and conventional food stuffs in many cases were not of the same variety and thus differed genetically. No information was given as to where and how these foods were produced. The effect could thus be due to different varieties used as well as to differences in the production systems (25).

A problem in a range of studies is that the health parameters measured as outcomes have differed substantially. In earlier studies, rather vague parameters such as general well-being, resistance to diseases and fertility were used (26). More recent studies have employed more specific indicators of health which makes it easier to determine the mechanisms of possible effects. Relevant studies on cells, animal models or humans, seem to elicit some common traits in terms of the effects of organic foods that can have relevance for health. These effects concern the impact on elements of the immune system, cancer and obesity.

**Impact on immune parameters of organic food**

Finamore and co-workers (27) showed that the proliferation capacity of colon and spleen lymphocytes from rats with reduced immune defence (caused by severe malnutrition) were different in a group receiving organically produced feed to a group receiving identical but conventionally produced feed. The lymphocytes from the rats given the organic feed had better capacity for proliferation than those from the rats receiving the conventional feed.

Some earlier studies have shown fewer infections in test animals given organically produced feed (26). In a recent study, two generations of three chicken lines were compared with regard to the effect of organic versus conventional feeds that otherwise were identical. The chickens on both types of feeds appeared just as healthy. However, further investigation showed that the organically fed chickens had lower body weight and higher immune reactivity (28).

Similar results were found by Lauridsen and co-workers (29). They fed three groups of rats over two generations identical diets, but based on three different production systems: Low fertilizer input without pesticide (L1minus P), Low
fertilizer input with pesticide (LIplus P) and high fertilizer input plus pesticide (HIplus P). The rats on low input diets (LIminusP and LIplusP) had higher serum immunoglobulin G levels and 14 % less adipose tissue than the rats on HIplusP diet. The LIminusP group had also a higher concentration of α-tocopherol than the two other groups.

A study on 17 nuns (the so-called “Cloister Study”) involved an eight-week dietary intervention. The test subjects received a 2+4+2 weeks of conventional/organic/conventional diet, respectively. The weekly menu was the same for all the eight weeks, except that fresh instead of frozen vegetables were served with the warm meals during the four weeks with organic food. During this period, the energy intake was found to be somewhat lower; the subjects ate less meat, more carbohydrate and fibre, but the intake of fat was the same as compared with the periods of conventional food. There was no change in body weight in the period on organic diet, but blood pressure and the concentration of T-helper cells were lower than in the weeks with the conventional diet (30). The dietary intervention was not blinded, and food choice and intake may thus have been affected by expectations of better health. However, this experiment is interesting since the results are somewhat similar to the findings in the in vitro and animal model studies described above.

**Effects on cancer development**

Many studies have shown promising effect of organically produced fruits and vegetables with regard to cancer and that it is the antioxidants and other secondary metabolites which have a crucial role in this prevention (31).

An in vitro study on human cancer cells from colon and breast examined the effect of extracts from organic and conventionally produced strawberries from five different varieties on cell proliferation. The organic strawberry extract, which had higher concentration of several antioxidants, including vitamin C, anthocyanins and phenolic acids, was shown to exert a higher inhibitory effect on growth of the cancer cells, both from breast and colon. The findings suggested that this effect was caused by vitamin C in synergy with the other secondary phytochemicals (32).

Falcarinol is a natural pesticide accumulating in carrots and other plants from the carrot family in response to mould attack, called liquorice rot. It is a toxin and can cause dermatitis by an allergic reaction. In vitro experiments with epithelial cells showed a biphasic reaction, with stimulating effect on cell proliferation at low concentrations and toxic effect at higher concentration (33). An in vivo experiment with rats using carrot extract containing falcarinol inhibited the development of cancer tumors in colon of rats (33).
**Effects on body weight**

Brandt and Mølgaard (4) have suggested that some secondary metabolites may have anti-nutritive effects. Many of these compounds have pharmacological properties and may have an inhibitory effect on body growth by reducing the absorption of nutrients from the intestine (such as phytates). They can thus give the same effect as calorie restriction by inhibiting uptake of protein, vitamins and minerals. It is known that lower protein intake and calorie restriction protects against many chronic diseases and delays the process of aging.

The above described studies by Lauridsen and co-workers in 2007 (29) and by Huber in 2004 (28) and 2007 (30) indicate a possible effect of organically produced food on weight gain. In all these studies the feeding experiments allowed for *ad libitum* eating. This implies that appetite regulating mechanisms can be involved, not only reduced absorption of nutrients (34). A new study has shown that the phytoalexines rutin and coumarin added to a high fat diet given to rats over eight weeks decreased body weight gain in the animals compared to a control group on similar diet without these compounds (35). Coumarin has been found to have an appetite reducing effect. There may also be other secondary compounds with such effects.

**Conclusions**

More well-controlled studies during recent years have given support to the notion that organic compared to conventionally produced foods have higher nutritive value and content of secondary metabolites that may be important to health. Both environmental factors and production methods have been shown to affect plant growth and composition including the content of secondary bioactive metabolites. In this article, focus has been on agricultural management practices. Table 2 summarises how the use of fertilizers and pesticides may affect the composition of secondary plant compounds.
Table 2. Effect of organic and conventional agricultural management practices on plant composition. Adjusted from Lundegaard and Mårtenson (11)

Large controlled studies with a more factorial approach to the effect of the different components involved in cultivation systems have shown that the organic production is more likely to favour the synthesis of secondary compounds in food plants. However, the most important factor is the choice of cultivar/variety. Conventional farmers often choose varieties based primarily on high yield, while the organic farmer may more thoroughly have to consider disease resistant properties as important in selecting variety. However, as has been shown, cautious fertility management, no use of pesticides and harvest at ripeness are also important factors that tend to favour higher synthesis of secondary compounds in organic management practices.

The question of whether a higher content of these secondary compounds, as tend to be the case with organic food plants, are more favourable in terms of health effects has not yet been answered satisfactorily. We know that consumption of fruits and vegetables are important for the prevention of cancer and cardiovascular diseases. It is assumed that it is the antioxidants in fruit and vegetables that are the most active compounds having the protective effect. Five portions a day (about 400-450 g) are recommended to obtain maximal protective effect (36). Thus, as argued by Leifert (9), even a modest increase of 20% in the content of antioxidants in organic fruit and vegetables would imply that four portions a day will be sufficient to obtain the same effect.
More research is however needed to identify the different secondary compounds and their effects. Phenomena, such as combined effects of different bioactive compounds and biphasic effects of plant toxins in dose-response trials, needs further investigation. Lastly cohort studies and controlled interventions investigating selected health related parameters in humans are needed to document if organically produced food in fact is healthier.

References


Scientific programme of the symposium:

Bioactive compounds in plants – benefits and risks for man and animals

DAY 1: Thursday 13 November

10:00 – 10:10: Harald Siem, The Norwegian Academy of Science and Letters: Welcome

10:10 – 10:30: Aksel Bernhoft, National Veterinary Institute, Oslo: Introduction, bioactive compounds in plants

10:30 – 13:00 Session 1: Poisonous and medicinal plants

Chair: Harald Siem

10:30 – 11:00: Berit Smestad Paulsen, University of Oslo: Highlights through the history of plant medicine

11:00 – 11:30: Kristian Ingebrigtsen, Norwegian School of Veterinary Science: Main plant poisonings in livestock in the Nordic countries

12:00 – 12:30: Barbro Spillum, Norwegian Directorate of Health: Human plant poisonings in the Nordic countries

12:30 – 13:00: Bernt Rognlien, Balderklinikken, Oslo: Use of plants in health treatment today

Lunch at the Academy

14:15 – 18:00 Session 2: Bioactive compounds in feed and food

Chair: Helle Margrete Meltzer

14:15 – 14:45: Rune Blomhoff, University of Oslo: Compounds responsible for reduced cancer risk related to consumption of vegetables, fruits and berries

14:45 – 15:15: Mette Svendsen, Ullevål University Hospital, Oslo: Compounds responsible for reduced risk for cardiovascular diseases related to consumption of vegetables, fruits and berries

15:15 – 15:45: Espen Bjertness, University of Oslo: Can plants slow the progression of cognitive impairment in old people or delay the onset of dementia?

16:15 – 17:00: Herman Adlercreutz, University of Helsinki: Phytoestrogens and human health

17:00 – 17:30: Andrzej Madej, Swedish University of Agricultural Sciences: Risk of adverse effects of phytoestrogens in animal feed

17:30 – 18:00: Discussion

Dinner at the Academy of Science and Letters for the presenters.
DAY 2: Friday 14 November

09:15 – 10:45 Session 3: Bioactive compounds in feed and food continuation
    Chair: Jan Alexander
09:15 – 0945: Hilmer Sørensen, University of Copenhagen: 
    *Glucosinolates in Brassica – health risks, but also benefits?*
09:45 – 10:15: Leon Brimer, University of Copenhagen:
    *Cyanogenic glucosides*
10:15 – 10:45: Christer Andersson, National Food Administration, Uppsala:
    *Bioactive plant compounds in social drinks – a risk for human reproduction?*

11:15 – 13:00 Session 4: Documentation needs
    Chair: Arne Flåøyen
11:15 – 11:45: Frans Verstraete, EU Commision, Brussels:
    *Management and regulation of bioactive compounds in current feed and food plants*
11:45 – 12:10: Knut Flatlandsmo, Norwegian Food Safety Authority:
    *Documentation of beneficial effects of bioactive plant compounds in food and feed*
12:10 – 12:35: Steinar Madsen, Norwegian Medicines Agency:
    *Does traditional use of herbal remedies guarantee safety?*
12:35 – 13:00: Helle Margrete Meltzer, Norwegian Institute of Public Health:
    *Bioactive compounds through food, nutraceuticals or pills?*

Lunch at the Academy

14:15 – 15:45 Session 5: Agricultural factors
    Chair: Aksel Bernhoft
14:15 – 14:45: Magnor Hansen, Norwegian University of Life Sciences:
    *The importance of plant cultivation and traditional breeding for contents of bioactive compounds in feed and food plants*
14:45 – 15:15: Knut Berdal, National Veterinary Institute, Oslo:
    *The importance of genetic modification for contents of bioactive compounds in feed and food plants*
15:15 – 15:45: Gerd Holmboe-Ottesen, University of Oslo:
    *Increased levels of bioactive compounds in organically grown plants?*

15:45 – 16:30 Final discussion and summing up
    Chair: Bal Ram Singh
Proceedings from previous symposia organised by
The Norwegian Academy of Science and Letters,
Committee for Information and Research in Geomedicine

1978  Geomedical aspects in present and future research
1984  Geomedical research in relation to geochemical registrations
1986  Geochemical consequences of chemical composition of freshwater
1987  Commercial fertilizers and geomedical problems
1988  Health problems in connection with radiation from radioactive matter in fertilizers, soils and rocks
1989  Excess and deficiency of trace elements in relation to human and animal health in Arctic and Subarctic regions
1990  Human and animal health in relation to circulation processes of selenium and cadmium
1992  Chemical climatology and geomedical problems
1993  Geomedical problems related to aluminium, iron and manganese
1995  Chemical data as a basis for geomedical investigations
1997  Some geomedical consequences for nitrogen circulation processes
1999  Geomedical problems in developing countries
2001  Natural ionizing radiation and health
2002  Osteoporosis: From mechanisms and risk factors to prevention
2003  Geomedical aspects of organic farming
2005  Endocrine disrupters
2008  Solar radiation and human health