Development and Validation of Analytical Methods for Mycotoxins in Food, Medicinal Herbs and Feed

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Para mi marido,
mis padres y hermanos,
y muy en especial
para Sandra.

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LIST OF ABREVIATIONS

AfB_1	Aflatoxin B_1
ANOVA	Analysis of variance
AOAC	Journal of Association of Official Analytical Chemists
CEN	European Committee of Standardization
CV	Coefficient of variance
DON	Deoxynivalenol
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
EFSA	European Food Safety Authority
EI	Electron ionisation
FAO	Food and Agriculture Organization
FL	Fluorescence
GC	Gas chromatography
GC/MS	Gas chromatography / Mass spectrometry
HPLC	High performance liquid chromatography
IAC	Immunoaffinity column
IARC	International Agency for Research on Cancer
ILSI	International Life Sciences Institute
IUPAC	International Union of Pure and Applied Chemistry
IRMM	Institute for Reference Materials and Measurements
JECFA	Joint FAO / WHO Expert Committee on Food Additives
KOBRA cell	Kok-bromination-apparatus
LC-FL	Reversed-phase high performance liquid chromatography with fluorescence detection

LC/MS	Liquid chromatography / mass spectrometry
LC/MS/MS	Liquid chromatography / tandem mass spectrometry
LLE	Liquid-liquid extraction
LSD	Lysergic acid diethylamide
NOAEL	No observed adverse effect levels
LOD	Limit of detection
LOQ	Limit of quantitation
PAT	Patulin
PBPB	Pyridinium hydrobromide perbromide
PBS	Phosphate buffered saline
PCD	Post-column derivatisation
PHRED	Photochemical derivatisation reaction
PMTDI	Provisional maximum tolerable daily intake
RASFF	Rapid Alert System for Food and Feed
RP	Reversed-phase
RSD_r	Relative standard deviation, calculated from results generated under repeatability conditions [%]
RSD_R	Relative standard deviation, calculated from results generated under reproducibility conditions [%]
SCOOP	Scientific Cooperation, European Commission, DG Health and Consumer Protection
S/n ratio	Signal-to-noise ratio
S_r	Standard deviation for repeatability
S_R	Standard deviation for reproducibility
SOP	Standard Operating Protocol
SPE	Solid-phase extraction
TFA	Trifluoroacetic acid

TDI	Tolerable daily intake
TLC	Thin layer chromatography
UV	Ultraviolet
ZON	Zearalenone
WHO	World Heath Organization

1. Introduction and literature review

1.1. Definitions of mycotoxins and major mycotoxins

Mycotoxins are natural contaminants defined as secondary toxic metabolites produced by fungi, and occur universally in food and feed (*1*). The definition of secondary toxic compounds is used to differentiate them from those compounds essential for all living organisms named primary metabolites, which are essential for the growth of the plant. Examples of primary metabolites are amino acids, nucleic acids and proteins.

The name mycotoxin combines the Greek word for fungus 'mykes' and the Latin word 'toxicum' meaning poison (*2-5*). Several hundred different mycotoxins have been discovered so far, exhibiting different structural diversity, with various chemical and physicochemical properties, but only a few present significant food safety challenges (*6*). Aflatoxins and ochratoxins (produced by *Aspergillus* sp.), fumonisins, trichothecenes and zearalenone (produced by *Fusarium* sp.), patulin (produced by *Penicillium* sp.) and ergot alkaloids (produced in the sclerotia of *Claviceps* sp.) are the most frequent occurring mycotoxins with the most severe effects in humans and animals (*7*).

Mycotoxins remain challenging to classify due to their diverse chemical structures, biosynthetic origins and their production by a wide number of fungal species. A first approach can be to classify them according to their differences in their fungal origin, chemical structure and biological activity. Also the classification can be done according to how frequently they occur and in what amounts. This is a more complicated task because mycotoxin contamination of food and feed depends on environmental and climatic conditions, harvesting techniques, storage conditions and some others factors. Typically, the classification schemes reflect the scientific background of the person doing the categorising. For clinicians the classification is done depending on the organ they affect: hepatotoxins, nephrotoxins, neurotoxins, immunotoxins, etc. For cell biologists the classification is done according to generic groups such as teratogens, mutagens, carcinogens and allergens. Organic chemists tend to classify mycotoxins according to their chemical structures e.g. lactones, coumarines, etc.; biochemists according to their biosynthetic origins e.g. polyketides, amino acid-derived, etc.; physicians by the illnesses they cause e.g. St.

Anthony's fire, stachybotrytoxicosis, etc.; and mycologists by the fungi that produce them e.g. *Aspergillus* toxins, *Penicillium* toxins, etc (*8*).

Table 1. Classification of mycotoxin producing fungi.

Major classes of mycotoxin-producing fungi	Fungi species	Mycotoxins
Aspergillus	*A. flavus,* *A. parasiticus,* *A. nomius*	Aflatoxin
	A. ochraceus	Ochratoxin
	A. clavatus *A. terreus*	Patulin
	A. flavus *A.versicolor*	Cyclopiazonic acid
Claviceps	*C. purpurea* *C. fusiformis* *C. paspali* *C. africana*	Ergot alkaloids: Clavines Lysergic acids Ergopeptines
Fusarium	*F. moniliforme* *F. proliferatum,*	Fumonisin
	F. graminearum, *F. culmorum* *F. crookwellense* *F. sporotrichioides* *F. poae,* *F. acuminatum* *F. sambucinum* *F. sporotrichioides*	Type A Trichothecenes T-2 toxin, HT-2 toxin Type B Trichothecenes Nivalenol, deoxynivalenol, fusarenon-X
	F. graminearum, *F. culmorum* *F. sporotrichioides*	Zearalenone
Penicillium	*P. verrucosum* *P. virridicatum*	Ochratoxin
	P. citrinum *P. verrucosum*	Citrin
	P. roqueforti	Roquefortine
	P. cyclopium *P. camemberti*	Cyclopiazonic acid
	P. expansum *P. claviforme* *P. roquefortii*	Patulin

A table with the main groups of mycotoxins and their corresponding producing fungi is presented in Tab. 1 showing that different species of fungi from the same genus can produce different mycotoxins.

1.2. Effects on humans and animal health

Mycotoxins, when present in food and feed in sufficient levels, can affect both human and animal health. But depending on a number of factors like the intake levels, duration of exposure, toxin species, mechanisms of action, metabolism, and defence mechanisms, the toxic effect will vary (*9*).

The toxic syndromes resulting from the intake of mycotoxins are known as mycotoxicoses (*10*). In case of animals, the exposure to mycotoxins occurs through the consumption of mouldy feedstuff while in case of humans, the exposure can be either direct due to the consumption of mouldy plant products or indirect, via the consumption of contaminated animal products (meat, milk and eggs), containing residual amounts of mycotoxins ingested by animals. Mycotoxicoses have been responsible for the major epidemics in men and animals in recent historic times. The most important mycotoxicosis has been ergotism, also known as St. Anthony's Fire. It is caused by the ingestion of grains contaminated by sclerotia of *Claviceps purpurea* and it has been known since, at least, 1750. After periodic outbreaks, the disease became epidemic in central Europe during the middle ages. A summary of the main mycotoxins, with description of their health effects and commodities affected is listed in Tab. 2.

Another example of mycotoxicosis is alimentary toxic aleukia. It was responsible for the death of thousands of people in Russia in 1940. It is known to cause fever, bleeding from the skin, nose, throat and gums, necrosis, and suppression of the immune system and mortality reaching 80% (*11*). Aflatoxins were discovered in 1960 following the deaths of 100,000 young turkeys in England (*12*).

The amount of mycotoxins needed to produce adverse health effects varies widely among toxins, as well as for each animal or person's immune system. Two concepts are needed to understand the negative effects of mycotoxins on human health:

- **Acute toxicity** is the rapid onset of an adverse effect from a single exposure.

- **Chronic toxicity**, the slow or delayed onset of an adverse effect, usually from multiple, long-term exposures.

Table 2. Most prevalent occurring mycotoxins with their possible health effects and the commodities affected.

Mycotoxin	Possible health effects	Commodities
Aflatoxins	Liver diseases (hepatotoxic, hepatocarcinogenic), carcinogenic and teratogenic effects, haemorrhages (intestinal tract, kidneys), immune suppression	Groundnuts and nuts, cereals (maize), milk, spices
Ochratoxins	Nephrotoxic, carcinogenic, immune suppression	Cereals (wheat, maize), wine, grape juice
Fumonisins	Pulmonary oedema, equine leukoencephalomalacia, nephro- and hepatotoxic, immune suppression	Maize
Trichothecenes	Digestive disorders (vomiting, diarrhoea), reduced weight gain, haemorrhages (stomach, heart, intestine, lung, bladder), oral lesions, dermatitis, infertility, degeneration of bone marrow, slow growth, immune suppression	Cereals (wheat, barley)
Zearalenone	Oestrogenic effects, oedema of vulva, prolapse of vagina, enlargement of uterus, atrophy of testicles, atrophy of ovaries, infertility, abortion	Maize, wheat
Ergot alkaloids	Gangrene, convulsions, hallucinations.	Rye
Patulin	Mutagenic, genotoxic, carcinogenic.	Fruit (apples, pears)

Mycotoxicoses can therefore be differentiated as acutely or chronically toxic, or both, depending on the kind of toxin and the dose. In animals, acute diseases include liver and kidney damage, attack on the central nervous system, skin disorders and hormonal effects. It is the long term toxicity which is of special concern because certain mycotoxins ingested in minor quantities with the daily diet for an extended period are known to be carcinogenic and to influence the immune response of a number of animal species, being a risk to human health. The International Agency for Research on Cancer (*13*) evaluated and classified the carcinogenicity of mycotoxins. Acording to this classification, both patulin (PAT) and zearalenone (ZON) are included into the Group 3: not carcinogenic to humans. Aflatoxin B_1 (AfB_1) is included in the Group 1: carcinogenic to humans (*14*).

In order to assess about the nature of the adverse effects of this contaminants, the Joint Food Agricultural Organization / World Health Organization Expert Committee on Food

Additives (*15*) evaluated their toxicological data. This evaluation is based on the determination of no observed adverse effect levels (NOAEL μg/kg of body weight/day). NOAEL is the greatest concentration of mycotoxin that does not cause detectable adverse effects in animals in toxicological studies.

Another assessment of the toxicity of a contaminant is made calculating the provisional maximum tolerable daily intake (PMTDI). When the PMTDI is used, an uncertainty factor is added in order to compensate for a deficiency in knowledge concerning the accuracy of test results and the difficulty in estimating the health effects in a different species and/or in different exposure conditions. For animal studies this factor is calculated dividing by 100 the lowest NOAEL, for humans dividing by 10. As regards PAT, the PMTDI is set at 0.4 μg/kg body weight and for ZON is established at 0.2 μg/kg body weight.

In the case that the contaminat is considered being genotoxic so as aflatoxins this hazard assessment approach does not apply and the maximum levels should be set as low as reasonably achievable.

1.3. Current situation of mycotoxins

The current situation in Europe of mycotoxin contamination can be monitored every week on the web site of the Rapid Alert System for Food and Feed - RASFF (*16*). Mycotoxins consistently are the hazard category with the highest number of notifications. Based on the most recent report an overview of the commodities where mycotoxins were found in 2006 is described in Tab. 3 and Fig. 1.

From a total of 877 notifications received in 2006 on mycotoxins, 800 concerned aflatoxins and most of these notifications concerned pistachio nuts (276) primarily originating from Iran (234). Aflatoxins are also frequently reported in peanut and derived products (257 notifications) originating from countries like: China, Argentina, Brazil, Ghana, and Egypt. Eighty-five notifications where received within the group of nuts and nut products, mainly originating from Turkey. Forty-three notifications concern edible almonds and derived products, primarily originating from the United States (37). This high number triggered the discussion within the European Union (EU) on imposing special conditions on the import of almonds from the United States to protect public health. Within the group of fruit and vegetables, 97 notifications concerned dried figs and derived products primarily originating

from Turkey (54) and 10 notifications concerned melon seeds primarily originating from Nigeria (6) and Ghana (3). Special attention should be paid to the sharp increase of notifications on aflatoxins in products originating from Turkey: 83 notifications in 2004, 118 notifications in 2005 and 163 notifications in 2006, showing that the number of notifications has doubled compared to 2004. Within the group of herbs and spices (41 notifications), primarily the following products (and derived products) were found to be contaminated with aflatoxins at levels above the EU-maximum level: chilli (18), kebab powder (7), paprika (4), nutmeg (3), ginger (2) and hot pepper powder (2). All notifications on kebab powder and hot pepper powder concerned products originating from Ghana (9), while notifications on chilli concerned products mainly originating from India (15). Other notifications concerned products originating from Ethiopia, Spain, Pakistan, Grenada, Egypt, Lebanon and Eritrea.

Table 3. Mycotoxin notifications reported in the RASFF during 2006 and classified by mycotoxin and group of food affected.

SUBSTANCE	Feed	Cereal products	Coffee and cocoa	Baby food	Fruit and vegetables	Herbs and spices	Fruit juices	Nuts and nuts products	TOTAL
Aflatoxins	4	5	2	-	69	37	-	684	**800**
Fumonisins	-	14	-	1	-	-	-	-	**15**
Ochratoxin A	-	11	12	-	27	4	-	-	**54**
Patulin	-	-	-	1	1	-	5	-	**7**
Zearalenone	1	-	-	-	-	-	-	-	**1**
TOTAL	**5**	**30**	**14**	**2**	**97**	**41**	**5**	**684**	**877**

The notifications concerning other mycotoxins than aflatoxins were for 2006: Ochratoxin A (54), fumonisins (15) and patulin (7). The ochratoxin A notifications concerned mainly dried vine fruit (22), cereals and cereal products (11), green coffee (6), instant coffee (5), dried figs (5), spices (4) and one sample of roasted coffee. As regards fumonisins, nine notifications concerned maize products originating from Italy. Four notifications on patulin concerned concentrated apple juice originating from Iran.

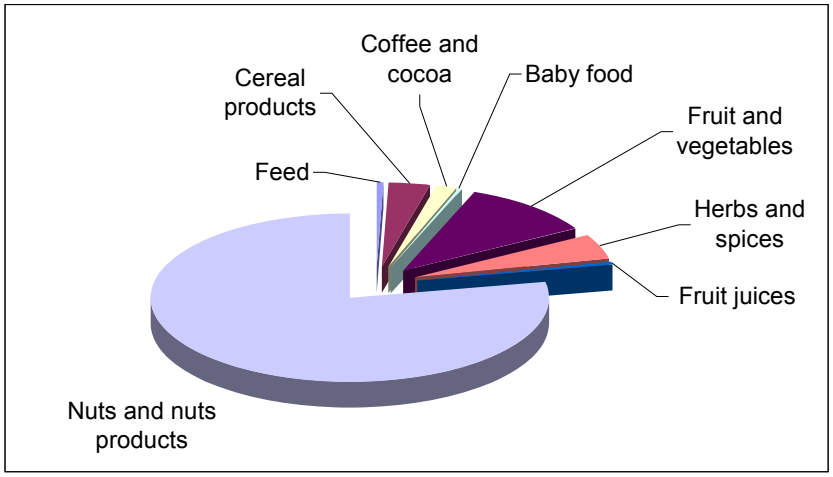


Figure 1. Distribution by group of food of the notifications in mycotoxins received in the RASFF in 2006

Mycotoxin exposure is more likely to occur in parts of the world where malnutrition is a problem, since in these countries little regulation exists to protect exposed populations and in addition, poor methods of food handling and storage are common. Furthermore it is known that malnutrition increases disease prevalence and reduces the ability of the human body to cope with mycotoxin exposure. Aflatoxin exposure has been suggested as a causal or aggravating factor for Kwashiorkor (a type of malnutrition, commonly believed to be caused by insufficient protein intake) in African children (*17*).

1.4. Major mycotoxins

1.4.1. Ergot and ergotism

Ergot is the alkaloid-containing product of a fungus, *Claviceps purpurea*, (Fig. 2) which grows on cereals, especially rye (*18, 19*).

Ingestion of the sclerotia is poisonous and causes a disease called ergotism. Ergotism has two main manifestations: gangrene (referred to as chronic ergotism) and convulsions (acute ergotism). Also known as Holy Fire or St Anthony's Fire (*20, 21*) and is characterised by intense burning pain and gangrene of feet, hands, and whole limbs, due to the vasoconstrictive properties of ergot. In severe cases, affected tissues became dry and black, and mummified limbs dropped off without loss of blood. Spontaneous abortion frequently occurred. Convulsive ergotism was often accompanied by manic episode and hallucinations.

These symptoms were due to serotonin antagonism by various components of ergot related to lysergic acid diethylamide. The gangrenous and convulsive forms of ergotism could occur concurrently (*5*, *12*).

Figure 2. Ergot on rye.

Source: Hans van Egmond, National Institute of Public Health and the Environment-Bilthoven, The Netherlands.

The first mention of a plague of gangrenous ergotism in Europe happened in Germany in 857 and is the oldest known mycotoxicoses in man and animals (*22*). It killed thousands of people. Humans acquire the disease by eating bread made from contaminated flour. Numerous epidemics of ergotism followed with thousands of people dying with the most susceptible victims often being children.

Nowadays, effective cleaning techniques at the mill make it possible to remove a large portion of ergotised grains (*23*). Therefore today, ergotism has been eliminated as a human disease, but it remains as a veterinary problem in cattle, horses, sheep, pigs and chicken (*24*). The existence of ergot strains free of alkaloids has also been reported (*23*).

Several medicinal products have been extracted from ergot. Ergometrine, although now declining in use, has been the most important drug for prevention and treatment of postpartum haemorrhage. Another example includes ergotamine, which is prescribed for various causes of headaches, including migraines. During the 20th century, the famous hallucinogen lysergic acid diethylamide (LSD) was discovered by Hofmann and originally was prescribed for psychiatric disorders, but was eventually made illegal due to abuse. In addition to causing hallucinations, ergotism causes itching, numbness, muscle cramping, burning and convulsions (*7*).

A review on methods for detection and determination of ergot alkaloids in grains has been published recently (*25*).

1.4.2. Aflatoxins

Aflatoxins are produced by many strains of *Aspergillus flavus* and *A. parasiticus*. There are four major aflatoxins B_1, B_2, G_1, and G_2. Their chemical structures are detailed in Fig. 3. AfB_1 being the most abundant of the four, the toxicity decreases from $B_1 \rightarrow G_1 \rightarrow B_2 \rightarrow G_2$. A long list of commodities are potential substrates for *Aspergillus ssp* growth. The ones with higher risk of contamination include corn, peanut, cottonseed, Brazil nuts, pistachio nuts. The ones with lower risk of contamination include figs, almonds, pecans, walnuts and grapes.

The name aflatoxin derives from the "a" from *Aspergillus* and the "fla" from flavus. The B designation of aflatoxins B_1 and B_2 resulted from the exhibition of blue fluorescence under ultraviolet (UV) light, while the G designation refers to the yellow-green fluorescence of the relevant structures under UV light. In addition, two metabolic products, aflatoxin M_1 and M_2, were isolated from milk of lactating animals fed with aflatoxin preparations; hence, the M designation. The acute toxicity of aflatoxin M_1 has been reviewed and it has been concluded that its toxicity is similar to or slightly lower than that of AfB_1 in rats and ducklings, and the carcinogenicity of aflatoxin M_1 is probably one to two orders of magnitude lower than that of AfB_1 (*26*).

The diseases caused by aflatoxin are called aflatoxicosis. Historically, they were discovered in England as a consequence to the death of 100.000 turkeys for which reason it was called 'Turkey X disease'.

Acute aflatoxicosis results in death and chronic aflatoxicosis result in cancer, immune suppression and other pathological symptoms (*27*). It has been documented that the severe malnutrition known as Kwashiorkor may be a form of paediatric aflatoxicosis (*28*).

Others speculate that aflatoxin might be involved in Reye's syndrome, an encephalopathy, and fatty degeneration of the viscera in children and adolescents (*29, 30*).

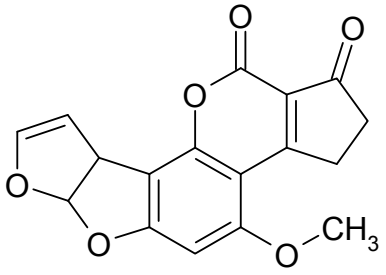
Chemical and physical properties of aflatoxins

Aflatoxins in dry state are very stable to heat, up to the melting point. A summary about main chemical and physical properties is presented in Tab. 4. In alkali solution hydrolysis of the lactone moiety occurs. Many oxidising agents, such as sodium hypochlorite, potassium permanganate, chlorine, hydrogen peroxide, ozone and sodium perborate react with aflatoxin and change the aflatoxin molecule in some way as indicated by the loss of fluorescence. The mechanisms of these reactions are uncertain and the reaction products remain unidentified in most cases. The hydrogenation of AfB_1 and G_1 yields to aflatoxin B_2 and G_2 respectively.

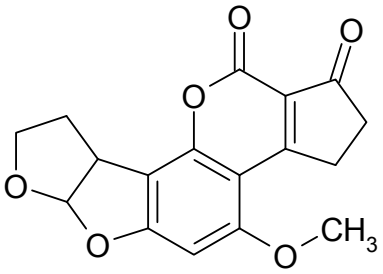
A number of analytical methods have been developed for the determination of aflatoxins in food and feed with liquid chromatography in combination with fluorescent detection, due to their natural fluorescence resulting in methods with simplicity, robustness and enough analytical performance at the contamination levels according to legislative limits. AfB_1, AfB_2, AfG_1 and AfG_2 can be determined simultaneously. Additional chemical enhancement techniques are necessary due to the quenching of fluorescence of AfB_1 and AfG_1 in aqueous solvents. The methods have in common the extraction with organic solvents followed by a clean-up step before separation. For clean-up, the use of solid-phase extraction with reversed-phase (RP-18) columns, immunoaffinity columns and MycoSep® columns are well established. Identification with mass spectroscopy is becoming increasingly popular. Methods feature high specificity, good reliability and possibility of automation.

Table 4. Chemical and physical properties of aflatoxins.

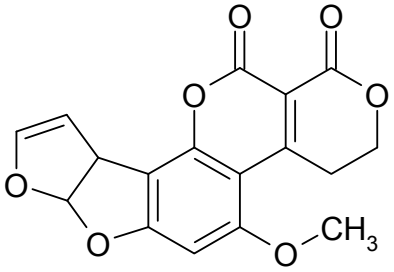
Aflatoxin	Molecular formula	Molecular weight [g/mol]	Melting point [°C]
B_1	$C_{17}H_{12}O_6$	312.28	268-269
B_2	$C_{17}H_{14}O_6$	314.30	286-289
G_1	$C_{17}H_{12}O_7$	328.28	244-246
G_2	$C_{17}H_{14}O_7$	330.30	237-240
M_1	$C_{17}H_{12}O_7$	328.28	299
M_2	$C_{17}H_{14}O_7$	330.30	293
B_{2A}	$C_{17}H_{14}O_7$	330.30	240



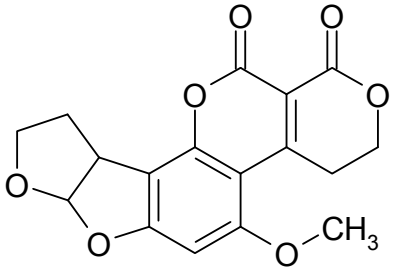
Aflatoxin B_1



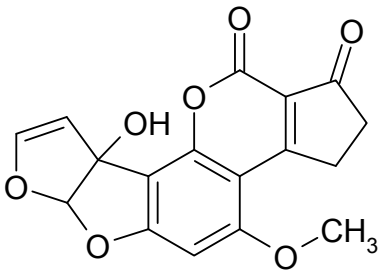
Aflatoxin B_2



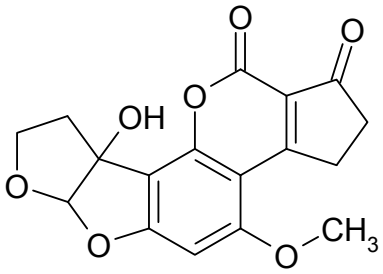
Aflatoxin G_1



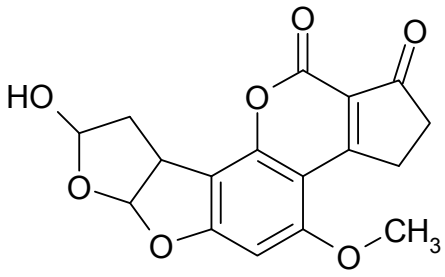
Aflatoxin G_2



Aflatoxin M_1



Aflatoxin M_2



Aflatoxin B_{2a}

Figure 3. Chemical structures of aflatoxins B_1, B_2, G_1, G_2, M_1, M_2, and B_{2a}.

1.4.3. Zearalenone

Zearalenone (ZON) is a mycotoxin described chemically as a phenolic resorcylic acid lactone in Fig. 4 (*7*).

This secondary fungal metabolite is produced by several species of *Fusarium* fungi, mainly *F. graminearum* and *F. culmorum*, which are species known to invade maize, barley, oats, wheat, rice and sorghum (*31*) (Tab. 1 and 2).

Co-occurrence with other *Fusarium* toxins like deoxynivalenol, nivalenol and fumonisin is often observed and depends on several factors like genotype, climatic condition, harvest season and storage condition. Nevertheless, available data indicate that maize has the highest risk of contamination while wheat, oats and soybean have been found to be contaminated occasionally (*32*, *33*).

ZON has important effects on the reproductive system because it binds to oestrogen receptors. Animal studies show that after oral exposure, it is metabolised mainly in liver to α- and ß-zearalenol, which are afterwards conjugated with glucuronic acid. Swine has been found to be the most sensitive domestic animal to ZON, showing symptoms of hyperestrogenism. Calves have been reported to show earlier sexual maturity, while cows are reported to suffer from vaginitis, prolonged oestrus and/or infertility when intoxicated with ZON (*33*).

Concerning humans, the carcinogenic potential of ZON has been evaluated by the International Agency for Research on Cancer (*13*). ZON was suspected to be the causative agent in an epidemic of precocious pubertal changes in young children in Puerto Rico between 1978 and 1981(*34*).

In 2003, an assessment about the dietary intake of ZON by the EU member states was carried out by the EU Scientific Co-operation on Questions relating to Food (SCOOP task) (*35*). Thirteen countries were asked to provide information on the exposure of the population to ZON in their country. It was concluded that the average daily intake of ZON in the adult population is less than the temporary tolerable daily intake additionally; harmonisation in the analytical methodology was advised.

Chemical and physical properties of zearalenone

ZON ($C_{18}H_{22}O_5$) molecular weight 318.37 and CAS N° 17924-92-4 is a beta-resorcyclic acid lactone with the structure described in Fig. 4. The IUPAC name is (*4S,12E*)-16,18-dihydroxy-4-methyl-3-oxabicyclo[12.4.0]octadeca-1(18),12,14,16-tetraene-2,8-dione. It is a white, odorless, crystalline substance. Its melting range lies between 161 and 164 °C. It is practically insoluble in pure water and tetrachloromethane and soluble in diethyl ether, chloroform, dichloromethane, ethyl acetate, alcohols and aqueous alkali. ZON standards are usually prepared in acetonitrile because of stability problems in methanol (*36*).

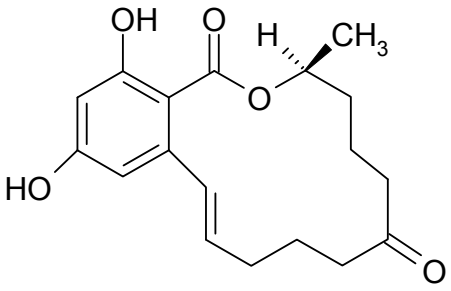


Figure 4. Chemical structure of zearalenone.

Analytical methods for the detection of ZON by gas chromatography (GC) are available requiring a derivatisation step (*37*). Often the aim of using GC methods is the simultaneous determination of several co-occurring mycotoxins. Consistent with the fluorescent properties of ZON, high performance liquid chromatography (HPLC) in combination with fluorescence detection is the method of choice nowadays. ZON exhibits greenish blue fluorescent light after excitation with UV light at 274 nm. Clean-up with immunoaffinity columns offers a higher specificity and cleaner extracts with a minimum level of interfering matrix components. Liquid chromatography / mass spectrometry (LC/MS) appears to be a promising technique with the major advantage that simultaneous determination of different classes of mycotoxins is possible.

1.4.4. Patulin

Patulin (PAT) is a toxic secondary metabolite produced by approximately 60 species of moulds belonging to 30 fungal genera like *Penicillium*, *Aspergillus* and *Byssochlamys*, which grow on fruit, including apples, pears, grapes (*38*) (Tab. 1 and 2). It has also been

reported to grow in vegetables, cereal grains and silage. *P. expansum* seems to be the mould responsible for PAT in apple juice (*39*).

The conditions in which *P. expansum* develops and produce patulin in apples have been the aim of several studies; as example Morales *et al*. (*40*) investigated this topic with a postharvest fungicide treatment, storage at low temperatures and controlled atmosphere storage. It was concluded that none of the factor tested avoided the fruit spoilage. Additionally, when ripe apples where stored at warmer temperature, a rapid increase (40%) of the lesion diameter was observed. Nevertheless, no patulin accumulation was reported after cold storage. This conclusion confirms the previously finding of Fallik *et al.* (*41*).

Apparently the removal of decayed tissue or the washing before processing reduces levels of PAT in final products (*42*). However, some studies demonstrated that PAT is also found in healthy tissues (*43*). Therefore, it appears that the prevention of PAT contamination rather than trying to remove it is the best way to avoid further contamination.

Alcoholic fermentation of fruit juices destroys PAT. Therefore, fermented products such as cider or vinegars will not contain PAT as long as apple juice was not used as an additive post-fermentation (*44*).

PAT was originally isolated because of its wide-spectrum antibiotic properties and was tested in humans to evaluate its ability to treat common cold. But it was found to be carcinogenic, teratogenic and mutagenic. Therefore, it was recommended that the level of PAT in the food should be reduced to the lowest level technically achievable. By far the most important source of PAT for humans are apples and apple juices (Tab. 3) and concern is expressed regarding the effect of PAT in the diet of young children.

PAT exposure of children consuming organic, handcrafted, or conventional apple juice was investigated using a probabilistic approach to evaluate the effectiveness of several risk management options aiming at reducing the risk of PAT exposure in children. It was observed that children consuming organic apple juice have a higher probability of exceeding the PMTDI in comparison to children consuming conventional and handcrafted apple juice. In order to reduce the probability to exceed the PMTDI it is necessary to lower the contamination of apple juice to concentrations lower that 25 µg/kg (*45*).

Chemical and physical properties of patulin

PAT ($C_7H_6O_4$) molecular weight 154.12 and CAS N° 149-29-1 is an unsaturated heterocyclic lactone with the structure described in Fig. 5. Its IUPAC name is 4-hydroxy-4,6-dihydrofuro[3,2-c]pyran-2-one.

PAT is a colourless, crystalline compound with a melting point of 110 °C. It is stable in acidic environment (pH 3.3-6.3) but unstable in an alkaline solution due to hydrolysis of the lactone ring. *P. expasum* is capable of producing organic acids during sugar metabolism, by which the stability of PAT is improved. PAT is relatively stable to thermal degradation in the pH range of 3.5 to 5.5, with a lower pH leading to greater stability. A heat treatment of apple juice containing PAT at 90 and 100 °C during 5 min results in a limited reduction of 6 and 13 % respectively. Maximum UV absorption is at 276 nm.

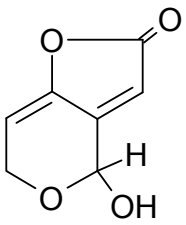


Figure 5. Chemical structure of patulin.

Due to its electrophilic character, PAT is able to bind with thiol and amino groups of glutathione, cysteine, thioglycolate and proteins for example. The low levels of sulfhydryl groups in apple juice compared to other fruit juices like orange juice explain the stability of PAT in apple juices. Presence of ascorbic acid or ascorbate causes a reduction of PAT in apple juice in a concentration-dependent manner. PAT is decomposed by free-radicals that are generated by the reaction of ascorbic acid to dehydroascorbic acid (*46*). Treatments with sulfhydryl groups or sulfite end with a degradation of patulin. At acidic pH a reversible binding of sulfite to patulin occurs. The resulting conjugate is toxic (*47*). Additionally the use of sulfite in apple juice, which is consumed by infants and young children is not recommended due to its allergic potencial.

One- and two-dimensional thin-layer chromatography (TLC) (*48*) was among the first and most popular methods as a cost-effective and easy to use method for PAT analysis. Nowadays HPLC coupled with UV detection is the method of choice in routine analysis, since the toxin is relatively polar and exhibits a strong absorption spectrum. Extractions with

ethyl acetate have been widely used. Purifications by solid phase extraction have been successfully used in recent years, since PAT is one of the few regulated mycotoxins for which no antibodies are available. The presence of UV absorbing compounds like 5-hydroxymethylfurfural can pose a problem for HPLC-UV methods. A number of GC methods are also available employing a derivatisation step like trimethylsilylation (*44*) or acetylation. The derivatisation of patulin with heptafluorobutyrylimidazole and detection by electron capture is also a good alternative (*49*). The derivatives obtained present good chromatographic properties to obtain reliable patulin detection.

The commercialisation of isotope-labelled patulin as internal standard with the use of a mass spectrometer as a detector make GC methods very reliable for patulin detection. A very recent study (*50*) used isotopically labelled ^{13}C patulin added into the samples before extraction and trimethylsilylation as derivatisation step in combination with GC/MS achieved excellent selectivity with big improvenment of precision and good recoveries and repeatability.

Therefore it can be said that mass spectrometry presents a good opportunity for the determination of PAT since apparently they offer a high selectivity although some matrix problems are possible to occur.

1.5. Prevention and control of factors influencing the mycotoxin content of feed and food

According to the Food and Agriculture Organization (FAO) an estimation of 25 % of the world's agricultural commodities are contaminated with mycotoxins, a fact leading to significant economic losses (*51*).

Contamination with mycotoxins in the field is very difficult to control because it is influenced by several factors including climatic conditions like relative humidity and temperature. Also other factors like soil moisture, stress, insect damage and mineral nutrition deficiencies (*52*) contribute to contamination.

Prevention of mycotoxin formation is the best way to protect consumers. But it is not always possible, for which reason a decontamination process may be necessary afterwards (*53*).

Both prevention and control of mycotoxin depend mainly on the commodity and fungus of concern; in addition several approaches may be used before harvest, immediately after harvest or during storage (*54*). The main approaches for pre-harvest prevention of mycotoxin formation include appropriate agricultural practices, and plant breeding for resistance to the fungus. The main post-harvest strategy involves drying of commodities and also cleaning the grains. During storage a variety of approaches to control are possible, including antifungal chemicals, gamma irradiation, physical approaches like aeration, cooling, and hermetic storage.

Once the commodities are affected, detoxification strategies are necessary. Detoxification consists in removing, destroying or reducing the toxic effects of mycotoxins and can be classified based on whether they use chemical, physical or microbiological processes. Unfortunately, the treatments have some limitations; one of them is that none of the currently available methods is suitable for all foods and animal feeds. Therefore, the effectiveness of a decontamination method depends on several factors like the nature of the food, environmental conditions and the type of mycotoxin. Chemical compounds such as ammonia and hydrochloric acid, seem to have a great potential as decontamination agents but the nutritional value of the foods decreases tremendously. Therefore, their use is limited and chemical treatment is not allowed in the EC (*54*).

1.6. International regulations and harmonisation activities

Knowing the adverse effects of mycotoxins on men and animals, many countries have established legislation that limits their presence in food and feed. Up to now more than 100 countries in the world are known to have specific limits for mycotoxins in foodstuffs and feedstuffs. Until the ‘90ies these regulations were depending on national authorities. But gradually several economic communities e.g. EU, Mercosur (Mercado Común del Sur), have taken precedence over the national regulations (*55*).

Organisations like the Joint Expert Committee on Food Additives act as scientific advisory body of the World Health Organization and the Food and Agriculture Organization. This Expert Committee provides assessment for the toxicity of additives, veterinary drug residues and contaminants. The hazard of mycotoxins has been evaluated in several sessions (*56*).

Table 5. Maximum levels of aflatoxin according to the Commission regulation (EC) No. 1881/2006 (*57*).

	FOODSTUFFS	Maximum levels [µg/kg]		
		AfB_1	**Sum of AfB_1, B_2, G_1 and G_2**	**AfM_1**
1	Groundnuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	**8.0**	**15.0**	**-**
2	Nuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	**5.0**	**10.0**	**-**
3	Groundnuts and nuts and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	**2.0**	**4.0**	**-**
4	Dried fruit to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	**5.0**	**10.0**	**-**
5	Dried fruit and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	**2.0**	**4.0**	**-**
6	All cereals and all products derived from cereals, including processed cereal products, with the exception of foodstuffs listed in 7, 10 and 12	**2.0**	**4.0**	**-**
7	Maize to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	**5.0**	**10.0**	**-**
8	Raw milk, heat-treated milk and milk for the manufacture of milk-based products	**-**	**-**	**0.050**
9	Following species of spices: Capsicum spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika) Piper spp. (fruits thereof, including white and black pepper) *Myristica fragrans* (nutmeg) *Zingiber officinale* (ginger) *Curcuma longa* (turmeric)	**5.0**	**10.0**	**-**
10	Processed cereal-based foods and baby foods for infants and young children	**0.10**	**-**	**-**
11	Infant formulae and follow-on formulae, including infant milk and follow-on milk	**-**	**-**	**0.025**
12	Dietary foods for special medicinal purposes intended for infant	**0.10**	**-**	**0.025**

Table 6. Maximum levels of ZON according to the Commission Regulation (EC) No. 1126/2007 (*58*).

	FOODSTUFFS	Maximum levels [µg/kg]
1	Unprocessed cereals other than maize.	**100**
2	Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling	**350**
3	Cereals intended for direct human consumption, cereal flour, bran as end product marketed for direct human consumption and germ, with the exception of foodstuffs listed in 4, 7 and 8.	**75**
4	Maize intended for direct human consumption, maize flour, maize meal, maize grits, maize germ and refined maize oil.	**100**
5	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize snacks and maize based breakfast cereals.	**50**
6	Maize snacks and maize based breakfast cereals.	**50**
7	Processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children.	**20**
8	Processed maize-based foods for infants and young children.	**20**

Table 7. Maximum levels of PAT according to the Commission regulation (EC) No. 1881/2006 (*57*).

	FOODSTUFFS	Maximum levels [µg/kg]
1	Fruit juices, concentrated fruit juices as reconstituted and fruit nectars	**50**
2	Spirit drinks, ciders and other fermented drinks derived from apples or containing apple juice	**50**
3	Solid apple products, including apple compote, apple puree intended for direct consumption with the exception of 4 and 5	**25**
4	Apple juice and solid apple products, including apple compote and apple puree for infants and young children and labelled and sold as such	**10**
5	Baby foods other than processed cereal-based for infants and children	**10**

In Europe, there are several organisations that take part in the risk assessment of mycotoxins. For example, until 2002 the Scientific Committee on Food and the Scientific Committee on Animal Nutrition of the European Commission expressed regularly their opinions about risks associated with the occurrence of mycotoxins in food or animal feed, respectively. In 2002, the European Food Safety Authority was established with the idea to offer risk assessment regarding food and feed safety and among other issues mycotoxins are discussed (*59*).

Actual maximum levels within the EU of aflatoxins, zearalenone and patulin are shown in Tab. 5 - 7.

An important activity within the EU is carried out by the Scientific Cooperation Task (SCOOP) on questions relating to food provided the scientific basis for the evaluation and management of risk to public health arising from dietary exposure to mycotoxins. In the 1990ies, these activities resulted in a report assessing exposure of *Fusarium* toxins, PAT and Ochratoxin A (*35, 60, 61*).

The International Life Sciences Institute (ILSI) is a non-profit-making, worldwide foundation established to advance the understanding of scientific issues relating to nutrition, food safety, toxicology, risk assessment and the environment, has a working group on natural toxins that organises international symposia on mycotoxins of European concern (*62*).

In order to assure a high level of protection of human life and health within the EU, a quick information-exchange called Rapid Alert System for Food and Feed (RASFF) was created (*16*). Further on, in 2002 with the introduction of the General Food Law Regulation (EC) No 178/2002 the system was further developed; the classification of the information started to be done under different headings - alert notifications, information notifications and news notification - depending on the extent of the risk and the need of direct action (*63*).

In July 2006, the Institute for Reference Materials and Measurements (IRMM) from the European Commission's Joint Research Centre was nominated as the community reference laboratory (CRL) for mycotoxins (*64*). This Community reference laboratory aims to facilitate the implementation of European legislation related to monitoring of mycotoxins in food of plant origin and animal feed and works together with appointed national reference laboratories of the EU Member States.

Additionally, certified reference materials are commercially available from IRMM. They can be classified as pure substances (standards), standard solutions (calibrators) or matrix materials (spiked or naturally contaminated) and include reference materials for i) aflatoxins in peanut, compound feed, and milk powder; ii) ochratoxin A in wheat; iii) deoxynivalenol in maize and wheat flour and iv) ZON in maize. IRMM also provides standard solutions for calibration purposes (calibrators) of AfB_1, AfB_2, AfG_1, deoxynivalenol and nivalenol in

acetonitrile and AfM_1 in chloroform. The certified reference materials are an important tool to assess about the quality of the measurement.

The availability of analytical methods is crucial for the establishment of regulatory limits for mycotoxins. In addition, analytical methods have to be validated at national and/or international level in which perfomance characteristics such as accuracy, precision, specificity, are checked. Afterwards they may be adopted as official methods (*65*).

Several international organisations are involved in the validation of the methods, including AOAC International, the International Union of Pure and Applied Chemistry (IUPAC) and the International Organization for Standardization at the global level, and the European Committee of Standardization (CEN) in Europe.

Methods of analysis accepted by these organisations must be validated by a collaborative study. Several protocols and guidelines for method validation and for the conduct of collaborative studies have been published (*66, 67*).

Some laboratories are accredited as part of their quality system. Accreditation demonstrates that the laboratories can produce accurate, high-quality results on a consistent basis, and an accreditation by a recognised body ensures that they are applying analytical quality assurance. Part of this analytical quality assurance needs the use of certified reference materials as well as the regular participation in interlaboratory comparison studies.

As part of a good analytical quality assurance system and complementary to the information about which analytical method has been used, it is recommended to provide the following information:

a) Surveillance data must be accompanied by a clear description of the analytical method used and an indication whether the method has been formally validated.

b) Limits of detection and quantification should be provided.

c) Recoveries determined by use of spiked samples or reference material should complement the analytical results and it should specify which spiking levels were applied and if the analytical data reported was corrected for recovery.

d) An estimate of the measurement uncertainty should be given.

e) The source of the calibrant should be provided and the method of preparation of the calibration solutions should be given.

f) An indication about the laboratory that reported the results should be given mentioning if it is accredited.

g) It should be indicated if the laboratory that reported the results takes part in inter-laboratory comparison studies and if so, for which analyte-matrix combinations.

1.7. Analysis of mycotoxins

Since the discovery of mycotoxins, several methodologies for their determination have been developed. Methods routinely used are mainly based on thin-layer chromatography, gas chromatography, or high performance liquid chromatography (*65*). Within the last years, liquid chromatography coupled with mass spectrometry has become more and more popular due to the trend of simultaneous determination of different classes of mycotoxins. As fast screening methods, immunochemical techniques have the advantages not to require any clean-up step and to be commercially available for most of the major mycotoxins.

Other emerging choices are the immunosensors, which offer a cost-effective alternative to the use of immunochemical techniques (*24*).

Biosensor arrays for the analysis of the expression levels of the genes involved the biosynthesis of the most important mycotoxins have been developed (*68*).

1.7.1. Sampling strategy

Except in case of liquids such as milk or highly processed and blended food, such as peanut butter, the lack of homogeneity in the agricultural commodity or food susceptible to the growth of toxigenic moulds presents a difficulty in determinating the true concentration of a mycotoxin in the lot to be analysed.

Characteristics of the analyte, the source or time of contamination, and the level of contamination are some of the most important factors that must be considered in determinating what constitutes an appropriate sample.

The first step to obtain a representative sample includes an adequate sampling procedure, which means that within a lot every single item should have an equal chance of being randomly selected. However, a lot is rarely homogeneous, and the distribution of mycotoxins within it is uneven. This means that concentrations can vary greatly within a lot.

Therefore, in order to get a uniform distribution proper blending within a lot is necessary. A vertical cutter mixer seems to be quite effective. Besides this, since the contaminated particles may not be distributed uniformly, the sample should be taken from several different locations.

Due to the crucial part that sampling plays in the precision of the determination of the levels of mycotoxins because of their heterogeneous distribution, methods of sampling for mycotoxins in agricultural commodities are defined in Commission Regulation (EC) No 401/2006 of 23 February 2006 (*69*).

Once the test sample is taken, a milling step will follow. This process will depend on the size of the test sample and type of commodity. The aim is to get sufficiently small particles for accurate analysis.

1.7.2. Analytical procedures

1.7.2.1. Extraction

Most analytical methods require the extraction of mycotoxins from solid food into a liquid phase, for this typically organic solvents or mixtures of solvents and water are used. Examples of solvents are chloroform, ethyl acetate, methanol, acetone and acetonitrile.

Sample extraction and preparation are the most time-consuming steps in the analytical process. In the last years, mycotoxin analysis has undergone significant improvements.

Chlorinated solvents have been used and are very efficient but due to safety considerations and the waste disposal problem they are rarely used.

Liquid-liquid extraction is based on the different solubility of the analyte in two inmiscible solvents. When an equilibrium between the two phases is reached, the amount of solvent has to be reduced and the analyte needs to be concentrated e.g. by rotary evaporation.

In general, this method is simple and easy to perform with standard laboratory equipment. However, it is in decline since, compared to new techniques, it is labour intensive because multiple extractions are necessary and large volumes of organic solvents (typically hexane

and cyclohexane) are required. In addition, due to adsorptions to glassware, some analyte losses can happen.

Typical examples of the use of this technique have been the analysis of PAT in fruit juices or aflatoxin M_1 in milk.

Liquid-solid extraction is the basic operation in the mycotoxin analysis. Solvent and sample need to be mixed. The two most commonly used techniques are blending and shaking. Special care must be taken to ensure that the entire sample is in intimate contact with the extraction solvent.

1.7.2.2. Clean-up procedures

Recently this process has resulted in considerable improvements with the development of immunoaffinity columns and solid-phase extraction cartridges. These procedures offer the possibility of automatisation and lower consumption of solvent.

Immunoaffinity columns (IAC) contain antibodies that are attached to an inert support material and are used to specifically bind the analyte while sample impurities pass through. These columns have simplified the mycotoxin analysis and since they are highly selective, very pure final solutions are achieved. In addition, these columns consume much less solvent than traditional methods and can be automated. Several commercial immunoaffinity columns are available for aflatoxins, fumonisins, ochratoxins, DON, type A trichothecenes (T-2 toxin) and ZON. These columns include a solid phase (e.g. agarose bed) to which antibodies targeting mycotoxin are covalently coupled. The toxin in the sample is bound selectively to the corresponding immobilised antibody. The following steps involve the removal of the other matrix components that are not bound, and the elution of the toxin including a possible concentration step before the actual determination step. An additional advantage is that the recoveries are higher than for liquid-liquid partitioning techniques. The disadvantages of the immunoaffinity columns are their limited capacity, their operability being limited to a specific range of pH, their single use and their high price.

Solid-phase extraction cartridges contain special packings with various surface chemistries that allow a more rapid clean-up and consume less solvents. Silica gel and RP-18 bonded silica columns are used frequently as they are pressure resistant and give reproducible results.

When the analyte is an ion, the other alternative is the "ion exchange mechanism". In this case, the separation is based on electrostatic interactions between the analyte of interest and the charged groups on the stationary phase. For ion exchange to occur, both the stationary phase and sample must be at a pH where both are charged.

Commercially available MycoSep® cleanup columns have a high specificity packing material consisting of adsorbents that retain almost all interfering substances, while the analyte does not show any affinity to this packing material. These columns are only available for a single analyte and are available for a range of mycotoxins such as deoxynivalenol and PAT.

1.7.2.3. Determination

The final step in the analysis should be simple, rapid, accurate, precise, sensitive, selective, of low cost and automated. Methods are broadly classified in two groups; those for which the presence of the toxin is indicated but the amount presents is less rigorously defined and those for which the amount of toxin can be quantified. The first type are known as rapid methods (screening) and the second as reference methods.

Rapid methods include enzyme linked immunosorbent assays (ELISAs) and biosensors using specific antibodies. Established chromatographic methods are necessary to confirm the results.

In ELISAs, the clean-up procedure is not as intensive as in the other analytical techniques and a sample homogenate containing the toxin is either directly quantified using a standard microtitre plate or a tube format enzyme-linked immunosorbent assay or a membrane-based format enzyme-linked immunosorbent assay. Currently, most of the commercially available ELISA test kits for mycotoxins are based on a competitive assay format that uses either a conjugate of an enzyme-coupled mycotoxin or a primary antibody specific for the toxin analyte. ELISAs are generally used to screen absence or presence above a certain level and for confirmation the classical analytical techniques can be performed.

Reference methods have several purposes, one is to confirm the result for samples that have been determined to contain mycotoxins based on a screening test and the second one is to more accurately quantify the amount of the toxin present.

Reference methods for mycotoxins generally involve chromatographic techniques such as HPLC, GC or TLC.

High performance liquid chromatography

HPLC methods, as the most frequently and widely used methods, are quite sensitive, have a reasonable low level of detection and have been developed for almost all major mycotoxins in different agricultural commodities except for the case of trichothecenes in which the use of gas chromatography is more widespread. HPLC methods are known to have a superior performance and to be more reliable than thin layer chromatography.

HPLC separates the mixture of compounds present in an extract of a sample by relative retention of the compounds to a stationary phase and mixtures of polar solvents as mobile phase. Subsequently, the compounds pass through a detector normally ultraviolet or fluorescence.

Gas chromatography

Although HPLC and thin-layer chromatography are the most common used techniques until now for the determination of mycotoxins, gas chromatography (GC) methods have been published for numerous mycotoxins, especially concerning the determination of A- and B-trichothecenes.

Since most of the mycotoxins are not volatile, a derivatisation step is necessary. Trimethylsilyl ethers are the most common derivatives because the derivatives are quite stable and can be detected by any of the detection techniques. Heptafluorobutyrate derivatives are formed by reaction with heptafluorobutyryl anhydride and are very sensitive to measurement with electron capture detector and also by the mass spectrometer (MS). Trifluoroacetyl esters are usually formed through reaction with trifluoroacetic acid anhydride. These derivates present good volatility but are relatively unstable.

For mycotoxin analysis flame ionisation detectors, electron capture detector and MS are the most often types of detectors. GC/MS is a powerful analytical tool, particulary for the analysis of trichothecenes offering good selectivity and detectability that compensate the labour and time spent on the derivatisation process.

Thin-layer chromatography

This technique is simple and fast, and although it might be used as a reference method it is often used as a mycotoxin screening assay. It is a powerful tool to determinate the presence of one or more mycotoxins in a sample involving simple equipment but on the other hand it requires some skills in its operation and interpretation. It has been widely used for the determination of mycotoxins but nowadays it has been replaced by other chromatographic methods because of their superior analytical performance.

The principle of TLC is based on a stationary phase attached to a glass or plastic plate and mixtures of solvents as mobile phase. The sample dissolved in a volatile solvent is deposited as a spot on the stationary phase. A standard will run simultaneously with the samples. The plate is immersed in a solvent reservoir and the solvent moves up the plate by capillary action. When the solvent front reaches the other edge of the stationary phase, the plate is removed from the solvent reservoir. Different components in the sample move up at different rates due to their different chromatographic behaviour between the mobile liquid phase and the stationary phase. Typical stationary phases for normal phase TLC include silica gel, aluminium oxide and cellulose. The components of the mobile phase are usually chlorinated solvents such as chloroform or dichloromethane, also other relatively non-polar solvents can be used in order to produce the selectivity necessary for the separation. These solvents are combined with polar solvents including acetone, alcohols, water and modifiers. The majority of TLC analyses of mycotoxins are performed on normal-phase silica gel plates, with the use of two different mobile phases and the development of plates in two different directions giving the greatest selectivity.

In case that the compounds of interest are not naturally fluorescent or don't absorb UV light, the application of a detection reagent by spraying or dipping is necessary in order to produce colour or fluorescence.

A further development is the use of high performance thin-layer chromatography that involves a reduction of layer thickness down to 100 μm and particle size down to 5 μm. This leads to an improved separation within a shorter time but it has a very small sample capacity. Automation is possible with the use of commercially available spotters and plate scanner.

TLC methods for a large number of mycotoxins (e.g. aflatoxins, PAT and ZON) are available via literature (*48*). Detection and identification procedures have been specifically developed for each single mycotoxin making use of molecular properties or reactions with spray reagents that are essential to be able to quantify the mycotoxin. On the other hand they normally use very toxic reagents and therefore they are outdated. An important drawback of TLC methods is that the limits of detection they offer are sometimes out of the actual legislative limits.

Chromatographic methods with mass spectrometric detection

Although LC/MS methods are fairly recent developments in mycotoxin determination they have become the state-of-art in the mycotoxin identification and quantification despite high costs and the need for experienced staff.

In addition, limitations of conventional HPLC methods, such as the need to derivatise samples before analyses have led to a more common use of LC/MS methods.

A mass spectrometer is an instrument that separates charged gas phase species according to their molecular mass and charge. An appropriate inlet, ionisation mode and ion analyser must be selected. Examples of inlet methods are gas chromatography, capillary electrophoresis, liquid chromatography, etc. Ionisation is provided by several techniques and the two most widespread for HPLC are atmospheric pressure chemical ionisation and electrospray ionisation. In the case of GC/MS, electron impact ionisation can be considered as 'hard ionisation' process and can produce many fragmented ions and possibly a poor abundance of the molecular ion. At the opposite, a 'soft ionisation' process as electrospray produces few fragmented ions with abundant molecular ion species.

For atmospheric pressure chemical ionisation, a nebulizer sprays the eluate and the resulting droplets are pre-dried in a heated capillary before being ionised through a corona discharge needle. The corona needle inserts electrons into the haze causing an ionisation of the mobile phase. A secondary reaction follows with the ionisation of the analyte molecules. For the electrospray ionisation charged droplets are produced by forcing the analyte solution through a charged orifice. A potential is used to disperse the emerging solution into a very fine spray of charged droplets. Thereafter the solvent evaporates and the droplets shrink with an increase of the charge concentration at the droplet surface. Finally the droplet surface

tension reaches a point that explodes and ends in smaller and lower charged droplets. This process is repeated until individually charged analyte ions are formed.

Several types of ion analysers are available such as ion trap, quadrupole or time-of-flight mass spectrometers.

Ion trap instruments are generally better suited for identification than triple quadrupole instruments because they allow successive series of trapping and fragmentation, but triple quadrupole instruments provide better selectivity in quatitative analysis. Hybrid instruments also exist and they provide the best of both setups with a linear ion trap in a triple quad instrument.

A significant advantage over conventional techniques is the development of multi-mycotoxin determination methods. Within a single run several mycotoxins belonging to different chemical families are detected. This very interesting approach is detailed further on in this chapter.

1.7.2.4. Analytical methods for the determination of aflatoxins

Numerous TLC methods have been developed for the determination of aflatoxins (*48*). Silica plates are most frequently used with a number of solvent mixtures. Most of these methods use chlorinated solvents such as dichloromethane or chloroform in the mobile phase, as extraction solvent or for sample clean up (*70*). These methods allow the identification of aflatoxins due to their strong bluish (AfB_1+AfB_2) and greenish (AfG_1+AfG_2) fluorescence. Under long wave ultraviolet light approximately 0.5 ng per spot can be routinely detected either visually or by densitometry. Some other developed methods have reduced the use of chlorinated or other toxic solvents (*71*).

The extraction with solid-phase extraction (SPE) cartridges or immunoaffinity columns is an essential part for the aflatoxin determination. MycoSep® SPE columns, which remove matrix components efficiently and can produce a purified extract within a very short time, are the most common examples for the use of SPE. Conventional SPE columns with silica, aluminia or RP-18 material have also been used.

Most of the recently developed chromatographic methods are based on reversed-phase HPLC with fluorescence detection after post-column derivatisation (*72*). The natural

fluorescence of aflatoxins is due to their planar condensed hetrerocyclic structure (Fig. 3). The different fluoresecence intensity is linked to the differences of their structural variation. This variation also plays a role in the toxicity properties of aflatoxins indicating that the double bond at the 8,9-position at the terminal furan ring is a crucial factor.

One of the most used analytical method for determination of aflatoxins in food is based on immuno-affinity column clean up followed by HPLC with post-column derivatisation (PCD) and fluorescence detection (*73*).

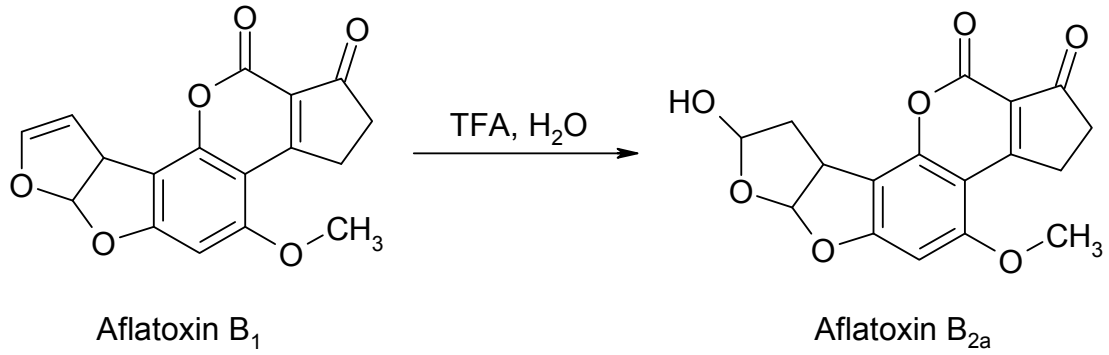


Figure 6. Conversion of AfB_1 to the hemiacetal AfB_{2a}.

Due to their quenching when present in aqueous solvents (*74*), aflatoxins have to undergo a derivatisation reaction before fluorescence detection. Because of the high toxicity of AfB_1 and because the determination of aflatoxins is often done with aqueous mobile phases, the quenching effect on this aflatoxin has been widely studied and several methods have been proposed. The basic principle of these approaches is based on the saturation of the terminal furan ring. This saturation of the double bond can be achieved with pre-column and post-column derivatisation. Another possibility of decreasing the quenching of fluorescence of AfB_1 includes the addition of cyclodextrins to the mobile phase (*75*). Cyclodextrins are molecules with a configuration capable of enclosing organic and inorganic species in their cavity. This method is not used normally for routine analysis since it is costly and offers no advantages to the other techniques discussed further on.

Pre-column derivatisation techniques are based on the transformation of the AfB_1 to their hemiacetal with trifluoroacetic acid (TFA) (*76*), this technique (Fig. 6) is less demanding in instrumentation than the others but has several drawbacks compared to the post-column derivatisation ones as for example the need of an additional evaporation step makes automatisation of the process difficult and the fact that the formed derivatives have a higher

polarity. This results in shorter retention times and can lead to an elution in the region of matrix interferences. Due to all these reasons, post-column derivatisation procedures are more common. Another reaction that leads to fluorescent hemiacetals is achieved with a post-column derivatisation procedure and ultraviolet radiation. This reaction happens in a transparent coil (*77*). The AfB_1 and AfG_1 hemiacetals are similar to those obtained by derivatisation with TFA.

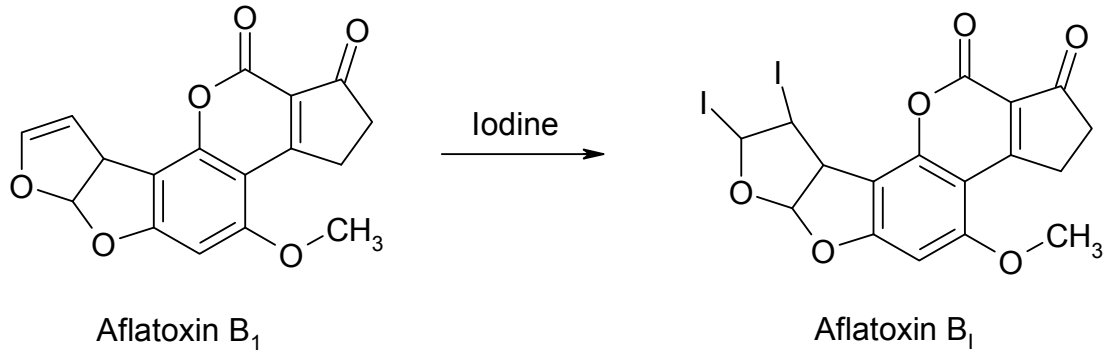


Figure 7. The iodine derivative formation of AfB_1.

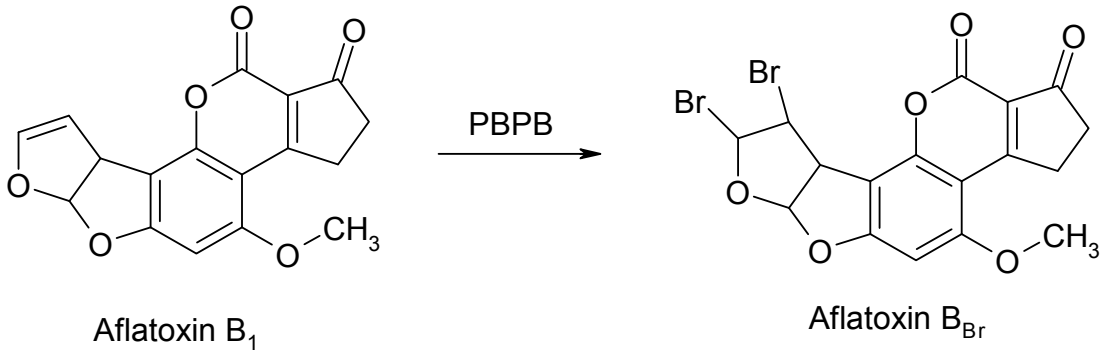


Figure 8. The bromine derivative formation of AfB_1 with pyridinium hydrobromide perbromide (PBPB).

Another common reaction is the one with halogens like iodine and bromine. Iodination (Fig. 7) requires harder conditions regarding temperature and time (*78*). Bromination (Fig. 8) can be achieved by the addition of a bromination agent like pyridinium hydrobromide perbromide (*79*) or by electrochemically generated bromine. This is achieved with the help of a special generator cell called 'KOBRA' (for Kok-bromination-apparatus) (*80*), in homage of his inventor Dr. W. Th. Kok who first described its use (*81*). The method includes the addition of potassium bromide and nitric acid to the mobile phase, once reached the KOBRA cell (Fig. 9), a phenomenon of electrolysis occurs and bromine is released.

Bromine reacts with AfB_1 and gives derivatives that fluoresce in the reversed-phase solvents.

Due to the fact that aflatoxin contamination is still an on-going problem, identification with newer techniques like mass spectroscopy (*82*) is becoming increasingly popular and with the new generations of tandem mass spectroscopy instruments, detection limits are comparable to the ones obtained with fluorescence detectors. Moreover the specificity compared to other techniques increases, but still some problems due to matrix effect are encountered (*83*).

A comparison between electrospray ionisation and atmospheric pressure chemical ionisation technique for the liquid chromatography / mass spectrometric determination of aflatoxin B_1, B_2, G_1 and G_2 in food is described by Takino *et al.* (*84*). Chemical noise and signal suppression were compared for both electrospray ionisation and atmospheric pressure chemical ionisation. Being atmospheric pressure chemical ionisation the best way to determine aflatoxins in food samples because of its lower chemical noise and modest signal suppression observed.

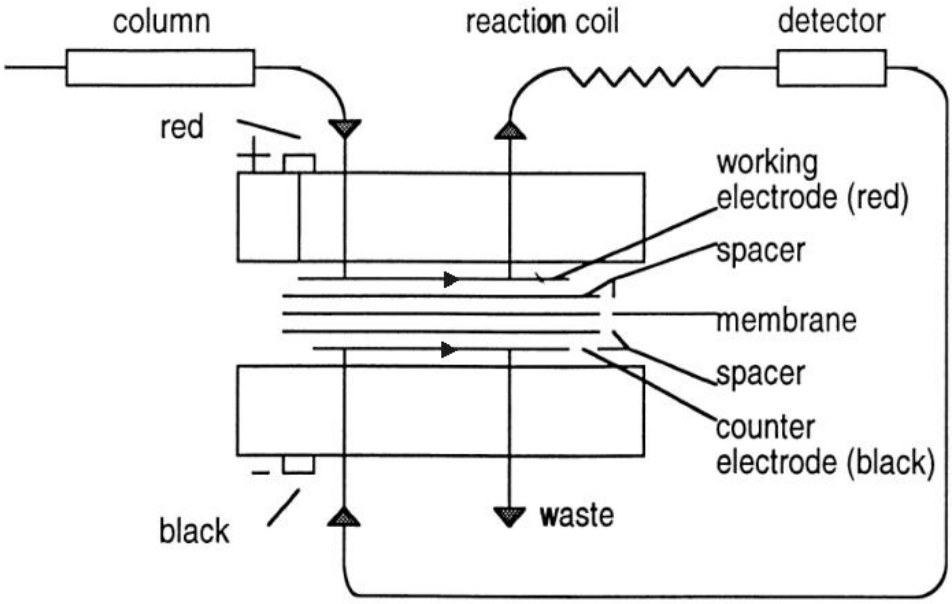


Figure 9. Layout of KOBRA-cell.

Source: doctoral thesis J. Stroka (2000) Determination of aflatoxins in food and feed with simple and optimised methods. University of Wuppertal (Germany).

The determination of aflatoxins is not easy in certain matrices like medicinal herbs, since they contain soluble low molecular weight substances that can interfere in the chromatographic determination and only few studies are available. Due to their increasing

use in our daily diet and their important role in the economy a special section (3.1) is dedicated in this manuscript.

1.7.2.5. Analytical methods for the determination of zearalenone

The extraction of ZON is usually performed with mixtures of organic solvents, commonly acetonitrile-water, but also methanol, ethyl acetate or chloroform. In complex matrices like animal feed an additional dilution and filtration step may be necessary before clean-up (*85*).

In addition to these conventional extraction procedures, several groups investigated microwave assisted extraction (*86, 87*) and pressurised liquid extraction (*88-90*) as robust and time-saving alternatives that seem to have the potential to enable automated sample handling. Though special instrumentation is needed, both techniques provided reliable results in grains when used in combination with LC/MS detection without any further sample clean up.

Conventional methods consist of successive liquid-liquid partitioning with chloroform-aqueous sodium hydroxide, but since the lactone ring can be hydrolyzed under alkaline conditions, the exposure of ZON to aqueous sodium hydroxide should be minimized. Other procedures based on antibody-based immnunoaffinity columns are also available. These immunoaffinity columns are marketed in the case of the company R-Biopharm Rhone as Easi-Extract®. The analysis of ZON using these columns is simple and robust.

Another alternative was introduced by the use of MycoSep® columns (section 1.7.2.2). The efficiency of modern sample preparation techniques for the analysis of the *Fusarium* toxins, deoxynivalenol and ZON in corn has been compared. Both MycoSep® and immunoaffinity columns were found to be quick and reliable as clean up methods for the determination of these mycotoxins. They also have the advantages over the usual analytical methods that they save time and have better precision in quantification. Furthermore, less experience in performing the analysis is needed compared to the usual analytical methods. For the particular case of ZON, it was found out that immunoaffinity columns are well suited for the determination of ZON in corn. Although their cost per analysis is a higher than for conventional methods, they give better precision parameters (*85*).

Thin layer chromatography has been widely used. The use of silica plates and mobile phases mostly composed of chloroform and mixtures of methanol, *n*-hexane and acetone have been

found to be effective. Toluene/ethyl acetate mixtures are also employed. Although ZON exhibits greenish blue fluorescent light after excitation with UV light at 254 nm, to further increase the fluorescent signal TLC plates are sprayed with a solution of 1% aqueous $K_3Fe(CN)_6$ and $FeCl_3$, followed by 2M HCl. TLC detection limits for ZON in grain or feed are in the range of 100 µg/kg (*91*).

Although methods for the determination by GC/MS are available (*92*), due to the strong native fluorescence of ZON, HPLC in combination with direct fluorescence detection (274/450 nm) and IAC clean up is the most commonly used method.

Up to now, two analytical methods have been validated in collaborative trials, one for a variety of cereals and cereal-based products (*93*) and another one for animal feed (*94*), both for levels above 100 µg/kg. These methods include an acetonitrile-water extraction with IAC clean-up.

A milestone towards the improvement of the comparability of measurement results among laboratories was achieved with the development of "tool-boxes", which in the case of ZON contains a calibration solution, a blank matrix (maize flour) and naturally contaminated maize flour, all with certified values (*95*).

Many methods have been developed in the last five years based on liquid chromatography/mass spectrometry techniques. One of the first methods based on atmospheric pressure chemical ionisation was proposed in 1998 by Rosenberg *et al.* (*96*). The quantification of ZON in cereal samples at lower levels was successfully demonstrated and a limit of detection of 0.12 µg/kg corn was obtained.

Other ionisation techniques like electrospray and ionspray have also been proposed, offering enhanced performance and increased selectivity and sensitivity. Zöllner *et al.* (*97*) introduced a tandem mass spectrometry technique, which was later on validated by an interlaboratory test on certified grain material. Both positive and negative ionisation modes were evaluated and the best results were achieved with the latter obtaining a limit of detection of 0.5 µg/kg. The method was afterwards applied to the analysis of ZON and its metabolites in beer samples, with quantification in the negative ion mode. Nevertheless, some limitations have been identified when similar compounds have to be analysed in the area of the selected ions. Furthermore, the scope of the proposed method was extended to the analysis of urine, muscle, and liver samples of pigs fed with contaminated oats (*98*), in

order to investigate the fate of ingested ZON and the incorporation of its metabolites in animal tissues, which are intended to be used for human nutrition.

1.7.2.6. Analytical methods for the determination of patulin

Early methods to determine PAT in apple juice employed TLC with ethyl acetate extraction and clean-up on a silica gel column. Detection is achieved by spraying the plate with 3-methyl-2-benzothiazoline hydrazone with a detection limit of 20 µg/L (*48*). Reversed-phase TLC plates have been investigated with different mobile phases. Mixtures of ethanol-water, benzene-methanol-acetic acid, and chloroform-methanol have been probed to be effective. Gas chromatography based methods for PAT need a derivatisation step, generally involving the formation of trimethylsilyl ether derivatives (*99*). Additionally isotopically labelled PAT as internal standard is required. Derivatisation of patulin to the trifluoroacetate or heptafluorobutyrate with trifluoroacetic acid anhydride and heptafluorobutyric acid anhydride doesn't seem to be so satisfactory as the preparation of heptafluorobutyrate derivatives from heptafluorobutyrylimidazole (*100*).

HPLC coupled with UV detection is the most common detection method for the determination of PAT since the toxin is relatively polar and exhibits a strong ultraviolet absorption.

Clean-up methods are based on repetitive liquid-liquid extraction or on solid-phase extraction. A lot of care must be taken in order to obtain interference-free chromatograms when solid-phase extraction is used. One of the most popular methods for the determination of PAT includes repeated extraction with ethyl acetate and clean-up by liquid-liquid partitioning with a sodium carbonate solution. The ethyl acetate extract is dried with anhydrous sodium sulfhate. After evaporation of ethyl acetate, PAT is quantitatively determinated by HPLC with UV detection. In case of purees, a prior treatment with pectinase enzymes is necessary. It has been shown in a collaborative trial that this method is able to quantify PAT at a level of 25 µg/kg; it has been adopted as official method by AOAC (*101*).

One drawback of this method is that it is time and labour consuming. Other simple and rapid methods use a single extraction with a so-called hydrophilic-lipophilic balanced polymer

(*44*). Others use MycoSep® columns for clean-up or combinations thereof with common reversed-phase columns (*102*).

A fast, practical and simple approach is based on a single extraction with ethyl acetate: hexane and sodium carbonate partitioning; the limit of quantification achieved was 7 µg/kg (*103*).

Two methods have been developed by Rychlik *et al.* (*104, 105*) to quantify PAT by stable isotope dilution assays using ^{13}C-labelled PAT as internal standard. One method used LC/MS in negative electrospray ionisation mode without derivatisation, and the other method utilised high-resolution gas chromatography/high resolution mass spectrometry after trimethylsilylation of the isotopomers. The LC/MS method showed a 100 times lower detection limit and better repeatability compared with the standard HPLC-UV.

A comparative study between atmospheric pressure chemical ionisation and atmospheric pressure photoionisation was performed by Takino *et al.* (*106*). Results were very similar in both cases with limits of detection of 0.13 and 0.20 µg/kg in standard solutions, respectively. Being the only difference, a cleaner chromatogram was obtained with atmospheric pressure photoionisation.

1.7.2.7. Analytical methods for the simultaneous determination of mycotoxins

Since the discovery of co-occurrence of different toxins and related synergistic toxic effects (*107, 108*), a big effort has been placed on developing analytical methods for the simultaneous determination of different classes of mycotoxins using LC/MS/MS.

These multi-analyte methods are extremely costly in terms of investment, but are simple and labour effective. In addition, some compromises have to be taken in the choice of extraction solvent and mobile phase due to the wide chemical diversity of mycotoxins (*24*).

Early attempts with mass spectrometry focussed on the identification of mould species according to their metabolite profile (*109*). Some other attempts were done for the simultaneous quantitation of *Aspergillus* and *Penicillium* mycotoxins in crude building materials (*110*) and in an artificial food matrix (*111*). The latter method had low recoveries of some analytes, but excellent accuracy and precision were obtained, so after a slight

modification of the extraction solvent the method was later on applied for the simultaneous determination of aflatoxins, ochratoxin A, mycophenolic acid, penicillic acid and roquefortine C (*112*).

Afterwards, a method for the quantitative analysis of deoxynivalenol, fumonisin B_1 and ZON in maize was developed based on a two-step SPE procedure including accelerated solvent extraction and an internal standard for each analyte (*90*). The limits of detection were below the maximum concentration levels permitted in the EU, and the only problem was the low recovery for ZON.

Since the commercialisation of the MycoSep® columns for clean-up of the raw extracts a new generation of methods started. One example is the method developed for the simultaneous determination of type A- and B-trichothecenes as well as ZON by Berthiller *et al.* (*113*) which used zearalanone as internal standard for ZON. Another example (*114*) was developed to determine Fusarium toxins simultaneously in cereals and cereals based products by LC/MS. In this method it was noticed that for some matrices strong signal suppression even with MycoSep® clean-up occurred, as MycoSep® columns are less specific to the target analyte or analyte group in comparison to immunoaffinity clean-up procedures. Therefore a further dilution of sample extracts or the use of matrix matched calibration was performed in order to overcome or control the matrix effects.

The matrix matched calibrant series are prepared from sample extracts that are free of the target analyte so called blank material, but contain the same quality and quantity of background interference substances that are assumed to cause interferences such as matrix suppression in LC/MS. These interference substances are found in the sample extracts themselves. The stage at which an exchange from neat solvent to matrix containing solvent will be done, or at which pure neat solvent calibrants are added to blank sample extracts, depends on the method. It can be done at the very end of the sample extraction/clean-up procedure e.g. by adding the analyte to blank cleaned-up sample extracts just before evaporation or by addition of the analyte to the crude extract. In the latter case, this matrix matched calibrant is also subject to the same procedure during extraction, thus the procedure covers effects that are introduced by the sample clean-up. However this is not the most common way and normally the preparation of solutions containing the matrix is done just before injection, as the matrix matched solution must have been treated the same way as the samples.

A method for the simultaneous determination of *Fusarium*, *Aspergillus* and *Penicillium* toxins was reported by Sørensen and Elbæk (*115*).

The most recent methods include a direct clean-up of the raw extract with a clean up based on solid phase extraction and introducing instrumental improvements for the simultaneous determination of multi-component mycotoxin contaminants in foods and feeds by ultra-performance liquid chromatography - tandem mass spectrometry (*116, 117*). A significantly decreased time of the analysis was achieved. In both cases, recoveries of around 70% were obtained for all analytes and no significant matrix effect was reported. Nonetheless the disadvantage is that these methods are not reliable for all mycotoxins of interest, and for example fumonisins and ergot alkaloids cannot be determined with these methods because the clean-up and /or extraction conditions are incompatible.

In order to solve this problem, some methods prescribe to inject raw extracts into the LC/MS, i.e. omitting clean-up of the sample. By this approach 22 mycotoxins including the ergot alkaloid ergotamine were determined in different food matrices (*118*). Samples were extracted with an acetonitrile/water mixture and diluted with water prior to injection. Matrix effects were investigated for every analyte/matrix combination and validation data obtained suggested that the analysis of diluted raw extracts is indeed feasible and at the same time sensitive enough for determining most mycotoxin levels set in the legislation.

More recently, a simultaneous determination of 87 mycotoxins was achieved by Sulyok *et al.* (*119*) based on the extension of a previously published method based on liquid chromatography/electrospray ionisation tandem mass spectrometry.

1.7.3. Challenges in mycotoxin analysis

The number of mycotoxins that are regulated in food in the EU increased within the last decade from 1 to 7 different mycotoxins, respectively groups of closely related mycotoxins, such as type A trichothecenes, fumonisins B_1, B_2 and AfB_1, B_2, G_1 and G_2.

Among these newly regulated mycotoxins are PAT and ZON for which legislative limits have been established recently in baby food. In addition, recommended limits for animal feed have been established, too.

In addition to the increased number of regulated mycotoxins, also the number of products for each regulated mycotoxin has increased. One driver for such an increase in regulations was to provide safeguard for persons who require special protection; namely infants and small children. This puts a lot of burden to the parties involved in the control of relevant products. With the same intention regulatory limits for mycotoxins in medicinal herbs are currently discussed at the European Pharmacopoeia level.

Proper enforcement of these regulated limits requires a sufficient repertoire of analytical methods with known and sufficiently reliable performance characteristics. Furthermore sufficiently reliable methods should be user friendly, fast, cost effective and take other side regulations into account e.g. Montreal Protocol (*120*).

As a result one important challenge in safety control of foodstuffs and medicinal herbs is the availability of analytical methods that are mutually accepted by all parties. This can be achieved by the development of such user friendly, fast and cost effective methods that have been evaluated in collaboratively trials following generally accepted protocols, such as the "IUPAC/AOAC Harmonized Protocol for the Conduct and the Evaluation of Analytical Methods".

In this work several methods have been developed and validated for two mycotoxins that, next to their foreseen regulation at that time, were of interest because of their pharmacological properties, namely PAT, which is an antibiotic, and ZON, which is an endocrine disruptor, thus has hormonal properties. The other part of this work concerns AfB_1 which main interest is related to its carcinogenic propierties.

2. Objectives

This thesis concerns the development and validation of analytical techniques applied to the determination of naturally occurring toxins known as mycotoxins.

The main purpose of this research is the development and validation of analytical methodology for the efficient implementation of EU food legislation in the field of mycotoxins. First of all this includes the need of development and afterwards the validation of these analytical methods. Validation includes the organisation of inter-laboratory tests to demonstrate the transferability of the testing protocol to various laboratories and to determine the precision parameters of the method. AfB_1, PAT and ZON were chosen for this study as they represent three of the most important mycotoxins concerned in EU legislation.

The structure of this work is divided in three main parts, from which independent conclusions are drawn.

First objective was the optimisation of a method for the determination of AfB_1 in two different matrixes, medicinal herbs and tiger-nuts, with the aim of providing the analytical basis for setting legal limits for AfB_1 in both matrices.

Secondly, to develop and validate by collaborative study a method for the determination of ZON in animal feed and baby food in order to allow the enforcement of the existing legislative limits.

Third objective covers the determination of PAT applying different methodologies but taking into account the requested legislative limit. Two methods were validated in a collaborative trial. Another part of this objective includes the development of a liquid chromatography/mass spectrometry for a survey of more than 200 samples.

3. Determination of aflatoxins

3.1. Determination of aflatoxin B_1 in medicinal herbs

3.1.1. Introduction and scope of the work

As mentioned in Chap. 1, aflatoxins pose a potential health risk for humans and animals. In particular, aflatoxin B_1 (AfB_1) is considered as the strongest natural occurring carcinogen for rats. Legal limits are established in more than 100 countries worldwide (*121*) for a considerable number of commodities, mainly those with the highest risk of contamination such as groundnuts, nuts and dried fruit, cereals and milk. In order to enforce regulatory limits, a large amount of analytical methods have been developed for their determination in food and feed. Additionally, several methods have been validated in collaborative trials for most of the relevant food and feed matrixes (*71, 122, 123*). For the particular case of medicinal herbs and plant root material, although their contamination with aflatoxins has been confirmed (*124*), their regulatory limits in EU were still being discussed at the moment of this thesis work.

Considering the drastic increase of the use of medicinal herbs in the last years (reaching 89% of people in developing countries) together with the fact that in many countries they are not registered as medicines but as dietary health supplement (*125*), the need to develop a suitable method capable to detect their contamination by aflatoxins became apparent.

Therefore, in agreement with the European Pharmacopoeia it was decided to identify the most appropriate method for determination of AfB_1 at a level of 2 μg/kg in order of being able to set a legislative limit. For cereals and nuts there is already a limit in force for AfB_1. The medicinal herbs selected were senna pods *(Cassia angustifolia)*, ginger root *(Zingiber officinale)* and devil's claw root *(Harpagophytum procumbens).*

Although there are more than 20.000 medicinal herbs available in the market very little research has been done regarding mycotoxin analysis in these matrices. In 1991, the fungal flora of six Asian medicinal plants was determined and eight species of fungi were identified, among them *Aspergillus flavus* and *A. niger* appeared very frequently. Levels of

AfB_1 and AfG_1 varied depending to the species and plant from which *Aspergillus* was isolated (*126*). In recent years, other studies have confirmed that aflatoxins are able to contaminate medicinal plants, aromatic herbs and herbal infusions (*127*).

As described in Chap. 1, the most common analytical methods for aflatoxins include immunoaffinity clean-up and reversed-phase liquid chromatography with post-column bromination with electrochemical reaction cell, or ultra violet irradiation with a photochemical derivatisation cell before fluorescence detection (LC-FL) (*128*), and methods based on solid-phase extraction clean-up in combination with LC/MS (*129*).

Medicinal herbs are complex matrices that contain soluble low molecular weight substances like pigments and polyphenols that can interfere with the chromatographic determination of aflatoxins. For clean-up immunoaffinity columns were used since they are rather powerful and together with LC-FL formes a specific and widely available detection system.

The procedure described by Reif and Metzger (*130*) was chosen as a candidate method, since it was proposed by one of the participants of the trial and it had been previously applied to various plant materials with high reproducibily and good recoveries. This method achieves a detection limit of 50 µg/kg. Since the EU maximum level at the time of these experiments for AfB_1 was 2 µg/kg, this maximum level was also set as target for this experiment in agreement with the scientific opinion of the European Pharmacopoeia. In order to achieve this level some modifications to the before mentioned method were necessary.

The changes were focussed on the use of different solvent/matix ratios, with other dilution proportions and a different elution sequence.

Additionally, it was decided to perform a second method based on the extraction with acetone-water and compare it with the previous one.

In order to avoid the fluorescence - quenching of AfB_1 in aqueous solvents (*74*) three different post-column derivatisation systems were compared that transform AfB_1 into a non-quenching derivative. One mandatory that was used as reference based on bromination by electrochemically generated bromine (KOBRA cell) and two optional systems (photochemical reaction or bromination by pyridinium hydrobromide perbromide). This was

achieved by re-injection of the purified HPLC extracts after the mandatory analysis sequence and re-analysis using one of the optional post-column derivatisation systems.

Since any method which is intended to be proposed for standardisation must be validated in a collaborative trial to determine the method performance characteristics, this study was designed according to internationally accepted standards (*131*) even though only four laboratories participated in the trial; normally eight is the minimun number required. Being this issue agreed beforehand with the European Pharmacopoeia (further comments are detailed in section 3.14).

Results were reported based on both peak height and peak area, in order to evaluate possible differences in calculation modes.

3.1.2. Test materials for the collaborative study

The test materials of senna pods, devil's claw root and ginger roots were supplied by PhytoLab (*132*). All materials were received as ground powder.

3.1.3. Homogeneity of the test materials

Due to the fact that a limited amount of test material was provided and a time frame was set for this study it was agreed that as PhytoLab (*132*) supplied the three powdered matrices, the remaining three laboratories performed the homogeneity test. Therefore each of the three laboratories remaining analysed a different matrix. From each matrix two batches, one blank and one naturally contaminated were analysed. This homogeneity testing was done prior to the trial.

The three materials were tested by replicate analysis (n = 5) on a five grams basis. The material to be analysed was randomly chosen. The method followed is described in Annex 1, based on methanol-water extraction. Immnunoaffinity columns from R-Biopharm (*133*) were mandatory. Three different post-column derivatisation systems were allowed: bromination by electrochemically generated bromine, photochemical reaction or bromination by pyridinium hydrobromide perbromide (PBPB).

The statistical test to evaluate the homogeneity of these materials was based on the calculation of the coefficients of variation (CV).

For all materials, the CV ranged from 10.2 % to 15.8 % (Tab. 8) with the exception of devil's claw root (CV: 42.8 %). The latter result indicated that the material most likely was not homogeneous at five gram portions. This led to the replacement of the naturally contaminated devil's claw root material in the collaborative trial by an AfB_1–free (blank) devil's claw root fortified at 1 µg/kg. For senna pods and ginger root, the material was considered as sufficient homogeneous for the purpose of this study.

Table 8. Homogeneity test of medicinal herbs composition.

Matrix	Level	CV [%]
Senna pods	low	15.8 %
Senna pods	high	14.5 %
Ginger root	high	10.2 %
Devil's claw root	high	42.8 %

3.1.4. Organisation of the Collaborative Study

During the collaborative trial two extraction methods were validated:

- In the mandatory extraction procedure the test portion was extracted with a methanol-water solution.
- In the optional extraction procedure acetone-water was used as extractant in combination with an adapted immunoaffinity clean up procedure.

All four laboratories conducted a common mandatory extraction and three the optional.

Electrochemically generated bromination was the mandatory post-column derivatisation method for all participants. Additionally, two participants tested photochemical reaction and one bromination by pyridinium hydrobromide perbromide. After this mandatory analysis sequence, a second sequence was carried out by switching the post-column derivatisation and injection of a second aliquote from the same purified HPLC extracts.

As well, two calculation modes (peak height *vs*. peak area integration) were investigated.

3.1.5. Determination of method performance parameters

The limit of detection and limit of quantification were obtained from the 95% confidence interval of the calibration graph, calculated by the Software MVA (*134*). The calibration points were 50, 100, 200, 300 and 400 ng/mL. The precision parameters RSD_r, RSD_R and Horwitz values were calculated according to the IUPAC/AOAC Harmonized Protocol (*135*) using an Excel® template (*136*). Horwitz values compare the between-laboratory variability (RSD_R) at the different levels in the three matrixes with the values predicted from collaborative trial studies taken from published literature. Generally Horwitz values up to 1.0 indicate satisfactory inter-laboratory precision but values up to 2.0 are generally considered as acceptable.

3.1.6. Experimental

The method of analysis that was used in this study, the materials and instructions can be found in Annex 1. A schematic of the methodology is displayed in the figures of Annex 2.

3.1.7. Results and Discussion

The routinely applied methods of analysis for the determination of aflatoxins have common principles. They involve as first step the extraction with an aqueous organic solvent, containing methanol, acetonitrile or acetone. Afterwards a filtration of the test sample and dilution with water or phosphate buffered saline are realised. Then a diluted portion of the extract is purified over an immunoaffinity clean-up, as this eases the isolation from interfering matrix components. Finally, the toxin is removed from the IAC and determined.

The choice of organic solvent in the extract, its concentration and the sample-to-extract ratio differ for most published methods for aflatoxins in general and in particular on the matrix investigated as well as the target level analysed (*74, 77*).

Results were reported based on both peak height and peak area, in order to evaluate possible differences in the robustness of calculation modes. The background for this investigation was related to the availability of modern calculation software tools for peak evaluation. Indeed signals are very often integrated automatically and reported by area. In the past it has been shown that calculation is a crucial element for robust and precise analysis (*137*). This is

especially true for trace analysis, as signal evaluation by peak height can be valuable for small peaks with small signal-to-noise ratio, as differences in baseline setting tend to influence peak area more than peak height measurements.

The analytical results obtained in this study are listed in Annex 3 (Tab. 1 - 6). The resulting method performance parameters as obtained by the different optional procedures applied were compared and evaluated for suitability according to criteria for method performance as given in the CEN Technical Report 13505 (*138*). This report defines minimum performance criteria such as repeatability, reproducibility and recovery for analytical methods in the field of mycotoxin analysis for official food control purposes. Criteria in this report have been selected on the basis of experience of what can be regarded as sufficiently precise for "official" use, both from individual expert's opinion as well as from interlaboratory studies. According to this report, the recovery shall be between 50 – 100 %, the repeatability (RSD_r) shall not exceed 40 % and the reproducibility (RSD_R) not 60 % for AfB_1 measurements at levels below 1µg/kg in a collaborative trial. For AfB_1 levels between 1 – 10 µg/kg these values are: 70 – 110 % for recovery, 20 % for RSD_r and 30 % for RSD_R.

3.1.7.1. Extraction efficiency

Since immunoaffinity columns contain a solid phase with immobilised antibodies, special attention was paid to the sensitivity of the immunoaffinity columns towards organic solvents. Taking into account that immunoaffinity columns are quite robust to methanol, solutions up to 16% methanol were tested without showing a negative impact on antibody binding efficiency. In the case of acetone and acetonitrile concentrations of 5% can already damage the antibodies and losses of aflatoxins B_2, G_1 and G_2 have been reported (*139*). The two extraction modes selected were methanol-water (70+30, v/v) and acetone-water (85+15, v/v). Acetonitrile was not even tried since an extensive investigation about how different matrices contaminated with aflatoxins interact with several mixtures of organic solvents (acetonitrile, acetone and methanol) was already performed by Stroka *et al.* (*140*). It was concluded that methanol or acetone are more recommended than acetonitrile as extraction solvents. Especially in cases like dry matrixes such as powders and matrices with high content of water-soluble constituents, where phase separation or water absorption may occur.

The sample-to-extract ratio is an important parameter for several reasons. A higher ratio, which means more sample per solvent results in more concentrated extracts, this also means that larger amounts of test portion are taken for analysis; therefore the limit of quantification is decreased. Furthermore, with larger amounts during the extraction the risk that the test portion would not be representative is reduced. This is important considering how inhomogeneously aflatoxins are distributed in some plant products (*141*). Nonetheless, with larger sample-to-extract ratios low recovery rates might occur. This was observed when senna pods were extracted and the recoveries achieved were lower than 70%. This low recoveries were more drastic when methanol based extracts were used. In that case, only 5 g of senna pods could be extracted with 100 mL of methanol-water (70+30, v/v) in order to recover more than 70%. When acetone-water (85+15, v/v) was used, similar recoveries were achieved with 10 g of senna pods material.

3.1.7.2. Post-column derivatisation techniques

All four laboratories tested samples extracted with methanol-water with KOBRA post-column derivatisation, while three laboratories also tested samples extracted with acetone-water. These three laboratories performed the optional method by injecting sample extracts using a second post-column derivatisation method. This was in two cases a photochemical reaction system and one participant used derivatisation by pyridinium hydrobromide perbromide. The calibration curves obtained by the four laboratories participating in the collaborative trial were used to estimate the limit of detection (LOD), the limit of quantification (LOQ) and the residual coefficients of variation (CV) (Tab. 9).

It could be shown that this way of estimating LOD, LOQ and residual CVs results in comparable figures for all four laboratories, indicating that this is a robust way to obtain these parameters even from different laboratories. Figures are generally higher than those estimated by the signal-to-noise ratio. This can be explained by the fact that all analytical procedures in the preparation of calibrant solutions are considered. An important pre-requisite for getting realistic figures with this procedure is however that the calibration range chosen for estimation is near the expected LOD and LOQ.

No significant difference in terms of interferences in the chromatograms was observed with any of the derivatisation principles, which indicates that the use of the different post-column

derivatisation systems is a robust way for the derivatisation of AfB_1. As a result, laboratories might choose any of the post-column derivatisation systems depending on the available equipment, while maintaining comparability of results.

Table 9. Limit of detection (LOD), limit of quantification (LOQ), and residual coefficients of variation from the calibration curves obtained by four laboratories[a].

	LOD [µg/kg]				LOQ [µg/kg]				Residual CV [%]			
	KOBRA[b]		optional[c]		KOBRA		optional		KOBRA		optional	
Lab	A[d]	h[e]	A	h	A	h	A	h	A	h	A	h
1	0.40	0.29	0.30	0.30	0.60	0.43	0.49	0.44	2.2	1.6	1.8	1.6
2	0.26	0.19	0.60	0.60	0.40	0.29	0.89	0.90	1.4	1.3	3.3	3.3
3	0.55	0.46	-[f]	-	0.83	0.68	-	-	3.6	2.4	-	-
4	0.41	0.40	0.39	0.42	0.63	0.60	0.58	0.62	2.3	2.3	2.1	2.4

[a] Mean values of at least 5 calibration curves from each laboratory.
[b] KOBRA = electrochemical derivatisation.
[c] optional = photochemical derivatisation (laboratories 1 and 4) and addition of pyridinium hydrobromide perbromide (laboratory 2).
[d] A = integration by area.
[e] h = calculation by height.
[f] - = laboratory 3 did not perform an optional method.

3.1.7.3. Method variability

The detailed analytical results of the four laboratories for the three matrices are given in Annex 3 sorted by extraction method, post-column derivatisation mode and calculation category. Tab. 10 summarises for the three matrices (i.e. senna pods - SP, ginger root - GR and devil's claw root - DC) the performance parameters of the mandatory and optional extraction methods in combination with the electrochemical post-column derivatisation (KOBRA cell) based on calculation by area.

This collaborative trial study has been carried out with four laboratories, which is a smaller number than the one usually required by the IUPAC Harmonized Protocol (*135*). At the time the study was conducted it was however not possible to extend the pool of participating laboratories due to financial and time restrictions. It was also not considered by the European Pharmacopoeia to be essential to increase the number of participating laboratories for such a method validation. As a result, the study was carried out with a limited number of laboratories, which is also accepted by the IUPAC Harmonized Protocol in such cases. A higher uncertainty in the calculated precision parameters must however be taken into account.

It can be concluded that the overall recovery for the optional acetone-water extractions was higher. The precision characteristics of the method were evaluated by calculating and comparing the Horwitz values. Both RSD_R and the resulting Horwitz ratios were found to be sufficient for ginger root and devil's claw root materials independently of the extracts used, while for naturally contaminated senna pods the optional extraction with acetone-water showed higher RSD_R values than those recommended by CEN (*138*). However, Horwitz ratios were in all cases not exceeding a value of 1, which indicates that these methods perform satisfactory.

3.1.7.4. Signal evaluation - peak area vs. peak height

It could be shown that the method was very robust regarding the different modes of calculation (height *vs*. area). Despite low concentration levels tested, signals seemed to be sufficiently large so that the peak calculation mode had no influence on the repeatability (RSD_r). A typical chromatogram of a ginger root sample containing ca. 3 µg/kg AfB_1 is shown in Fig. 10. The chromatogram was obtained using methanol-water extraction and electrochemical post-column derivatisation. For all other options, chromatograms looked similar and were free of interferences.

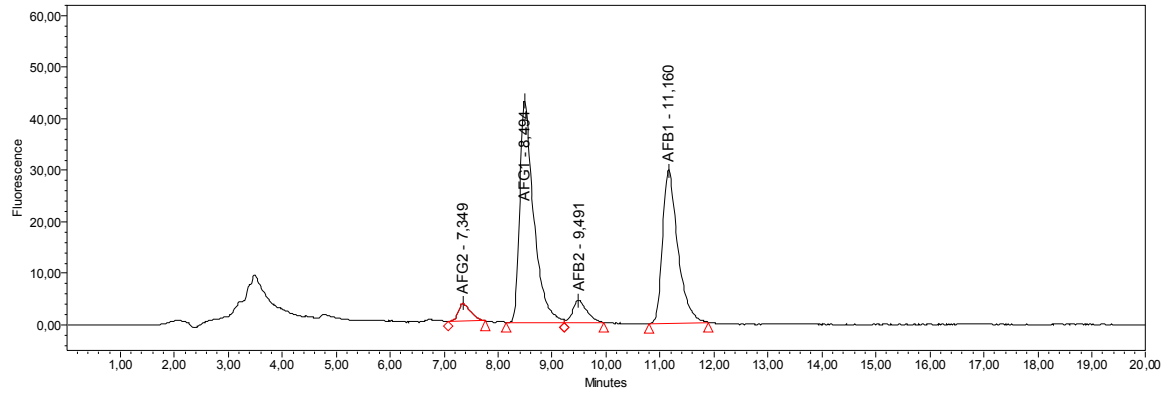


Figure 10. Chromatogram of a contaminated sample of ginger root containing aflatoxins G_2, G_1, B_2 and B_1 as four well visible and integrated peaks. The level of aflatoxin B_1 in the sample is estimated as 3 µg/kg.

Table 10. Performance parameters of two methods to determine AfB_1 in senna pods (SP), ginger roots (GR) and devil's claw root (DC).

Matrix[a]	SP1 $_{nc}$		SP2 $_{nc}$		SP $_{rec}$		GR2 $_{nc}$		GR $_{rec}$		DC $_{f}$		DC $_{rec}$	
Parameter / Method[b]	MEP	OEP	MEP	OEP	MEP	OEP	MEP	OEP	MEP	OEP	MEP	OEP	MEP	OEP
Mean [µg/kg]	2.0	2.2	14.9	17.9	0.78	0.98	2.1	2.9	0.91	1.03	0.92	1.06	0.83	1.00
RSD_r [%]	5.5	24.1	5.1	3.2	22.0	15.3	10.0	17.2	4.2	1.8	5.5	6.3	7.8	3.3
RSD_R [%]	27.0	41.4	18.1	21.2	35.2	15.3	30.2	17.2	5.8	7.5	7.6	7.4	10.5	4.6
Horwitz ratios	0.7	1.0	0.6	0.7	0.8	0.3	0.8	0.4	0.1	0.2	0.2	0.2	0.2	0.1
Recovery [%]	-	-	-	-	78	98	-	-	91	103	92	106	83	100

Values out of acceptance range proposed by CEN.
[a] nc: naturally contaminated; rec: recovery based on a fortification level of 1µg/kg for all matrixes;
f: fortified material at 1 µg/kg.
Recovery was calculated substracting the naturally contaminated level from the level found.
[b] MEP=mandatory or OEP=optional extraction procedures combined with the electrochemical post-column derivatisation (KOBRA cell) based on an integration by area.

3.1.8. Conclusions

The IUPAC harmonised guidelines for validation of methods requests that the minimum number of material to be analysed should be five. In case of a single level specification for a single matrix this number of material may be reduced to three. The minimum number of laboratories must be eight, only in special cases where a lower number can be justified, e.g. high costs for such a collaborative study, limited availability of suitable laboratories, etc. the number of valid datasets might be reduced to a minimum of five.

In this collaborative study, only a limited amount of suitable test material was available and the allocation of further suitable and well characterised material was difficult and costly. Thus it was decided to run the collaborative study with three materials and only a reduced number of laboratories were able to participate (n=4).

Reif and Metzger (*130*) published in 1995 a method achieving recoveries ranging from 66.4% to 99.4 % depending on the sample matrix and a precision of 5.15 % RSD (n=6) for a fortified capsicum material (fortification level was 1.78 µg/kg). The method was validated in a single laboratory validation for specificity by retention time as well as excitation and emission spectra, precision by multipe analysis (n=6), accuracy by means of recovery experiments for aflatoxins B_1, B_2, G_1 and G_2, and linearity up to 10 ng/mL (equivalent to 80 µg/kg) according to the guideline on Validation of Analytical Procedures of the International Conference on Harmonization of Technical Requirements for Registration of

Pharmaceuticals for Human Use (*142*). In the majority of test scenarios (level-matrix) the method performance values obtained in the collaborative trial compared well with those published by Reif and Metzger (*130*). It must however be noted that for one senna pod (SP_{rec}) and one ginger root ($GR2_{nc}$) sample increased RSD_r values of 22.0 % and 10.0 % respectively were calculated for the mandatory extraction procedure, while for the other levels of the same matrices, lower RSD_r values in the range of 5 % were obtained. This, on one hand shows that the method in general is capable to perform at a similar precision level for senna pods, ginger root and devil's claw root as published by Reif and Metzger (*130*) for fortified capsicum.

The increased precision parameters obtained in this medicinal herbs collaborative trial can be attributed to the fact that the single analytical values of the evaluation set were not recognised as outliers due to the low number of data participants per scenario. As illustration, one participant reported 73 % and 26 % as recovery values for a duplicate analysis (Tab. 1 of Annex 3). Values clearly indicating that the latter one is doubtful. However, this data set was not identified by the statistical evaluation procedure of the IUPAC Harmonized Protocol (*135*) as outlier set and thus were not removed - this anyhow would further cut down the already small number of analytical values for the calculation of precision estimates.

The method performance parameters derived from the collaborative trial were compared with the requirements of CEN TR 13505 (*138*), as this document is well accepted for the evaluation of method performance parameters in the field of food, while no criteria were established for medicinal herbs at that time of these experiments. In addition, some of the medicinal herbs used in this study are also used as food (e.g. ginger). As conclusion, it can be said that the proposed method performed satisfactorily in the collaborative trial, showing that the method fulfils the requirements on method performance according to CEN TR 13505 (*138*).

Finally the legislative level proposed was achieved with a suitable method enforcing the maximum level for certain spices as established in the regulation (*57*).

3.2. Determination of aflatoxin B_1 in tiger nuts

3.2.1. Introduction and scope of the work

Tiger nut or chufa exists in two varieties with different applications: *Cyperus esculentus* L. var. *esculentus* and *Cyperus esculentus* L. var. *sativus.* The latter is cultivated for human consumption and the rhizomes are used to obtain the tiger nuts (*143*). In Europe, the cultivation of tiger nuts is widely spread through the eastern part of Spain (Region of Valencia), which is also where most of the European consumption takes place. Tiger nuts are also grown in Central Africa and in the southern part of the United States (Florida). The cultivation of tiger nuts in Valencia has a long tradition. The result is a product with exceptional quality characteristics that has been designated as Denomination of Origin "Chufa de Valencia" and a Regulating Council was created in order of taking care of the production so that a high quality product reaches the consumer (*144*).

The Spanish annual consumption of tiger nuts is about 5-7 million kg/year, and around 3 million kg were imported during 2004. Exporting countries are Nigeria, Niger, Burkina Faso, Benin, Mali and Ghana. The main use of the tiger nuts in Spain is the production of horchata in a quantity of 40-55 million litres/year. Horchata is a soft drink consumed by a large part of the population, especially children.

Horchata is considered mainly as a refreshing drink, but it is also nutritive and rich in minerals and vitamins. After harvesting, tiger nuts are washed and sorted in order to separate the product from soil, insect or damaged nuts. This is an important step for minimizing possible aflatoxin contamination present in the raw material and it is nowadays performed with the help of industrial machines. In the next step the tiger nuts are dried to reduce the water content from 50 to 11 %, this is the last step in the processing and it should be performed carefully since mycotoxin producing micro-organisms can still start to grow at this moment on insufficiently dried tiger nuts.

For the preparation of one litre of horchata approx. 200 g of tiger nuts and 150 g of sugar are used. This product must be distinguished from another different type of horchata that is based on rice and vanilla, and produced in Central and South America (*145*).

Tiger nuts are also used for the production of oil for human consumption and they are an important animal feed ingredient in the southern United States (*146*).

In 1996, Bankole and Eseigbe (*147*) recognised tiger nut as one of the commodities susceptible to aflatoxin contamination and detected aflatoxins in 35% of tiger nut samples collected from Nigeria with concentrations ranging from 10 to 120 µg/kg. Adebajo (*148*) also reported the presence of aflatoxin in tiger nut at toxicological unsafe levels.

EU maximum levels at the time of these experiments (2005) for AfB_1 and total aflatoxins were 2 µg/kg and 4 µg/kg, respectively for most of the commodities regulated (*149*) but no legislative limit was set for tiger nuts or products thereof. Since tiger nut is a product commonly consumed by children, their total daily intake (ng/kg/body weight/day) should be monitored. Indeed due to their low body weight in case of contamination they are the most affected. According to the Rapid Alert System for Food and Feed (*16*), three notifications were made about contamination levels of aflatoxin in EU imported tiger nuts in 2002. Moreover, in April 2004, one notification was made about tiger nuts imported from Ivory Coast with a contamination level of 300 µg/kg AfB_1 (*150*).

These alerts and the fact that no method was reported for the analysis of AfB_1 in horchata raised the interest for this topic. The method described here has been developed on the basis of a previously described one by Stroka and Anklam (*73*). A small survey on commercially available horchata, from Spanish and Belgian supermarkets was conducted after method development.

3.2.2. Test materials

The survey was carried out on horchata, from supermarkets in Spain and Belgium (imported product from Spain) with production dates from 2004. The method has been tested for eight brands and different batches of some of these brands with a total of 22 samples.

3.2.3. Determination of method performance

The limit of detection and limit of quantification were obtained from the 95% confidence interval of the calibration graph, calculated by the Software MVA (*134*).

3.2.4. Experimental

The method of analysis that was used in this study, the materials and instructions can be found in Annex 4.

3.2.5. Results and discussion

The method is based on an immunoaffinity clean-up, followed by a HPLC separation electrochemical post-column derivatisation and fluorescence detection.

It could be shown, that the clean-up procedure by immunoaffinity chromatography after dilution of the sample with phosphate buffered saline and glass micro fibre filtration is fast, simple and reliable despite a solid residue content of the product of a required minimum of 12% and a fat content of around 2% in horchata drinks. Chromatograms were free of interferences in the region of the AfB_1 peak (Fig. 11).

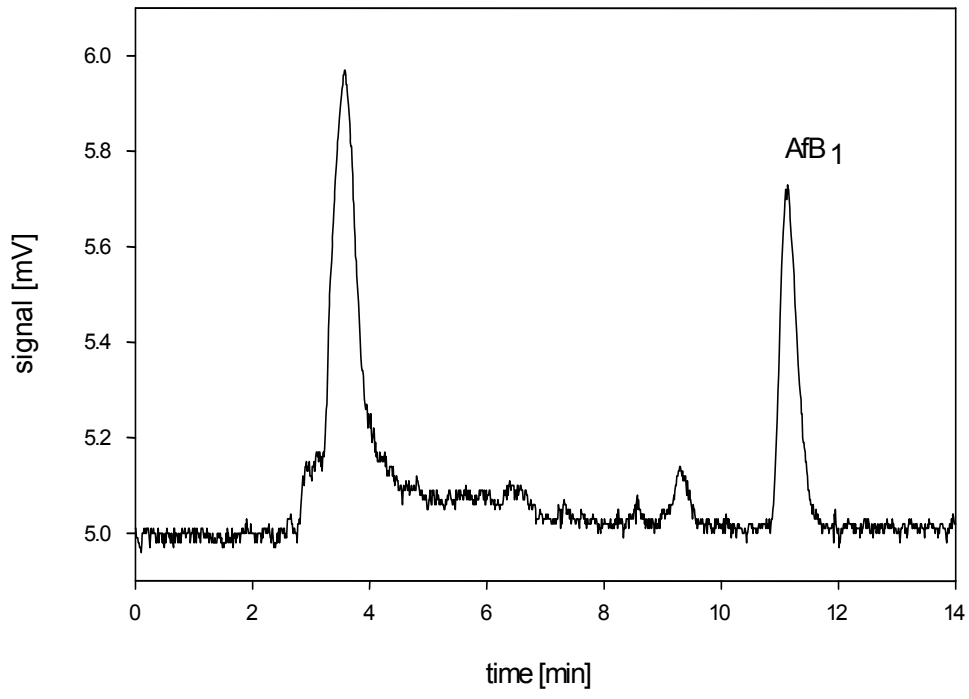


Figure 11. Chromatogram from a horchata sample containing AfB_1 at the estimated limit of quantification (0.06 µg/kg).

The mean recovery of the method was 88% (n = 6). These recovery experiments were performed using blank samples spiked at a level of 2 µg/kg of AfB_1 in the product. This

shows that the method procedure is suitable to purify and concentrate the analyte by immunoaffinity from the matrix and that the matrix has no significant effect on the recovery during clean up. Additional recovery experiments at lower levels were not carried out as results from collaborative studies (*73, 123, 151*) and own experience showed that recovery figures for the used methodology are rather stable in terms of fortification levels. Being more influenced by the matrix analysed.

The resulting relative standard deviation was 9% under repeatability conditions. On the basis of the assumption that the internal reproducibility is linked by a ratio of 3:2 with the internal repeatability, the estimate for the internal reproducibility is 14%.

Based on a "fitness for purpose approach" which is characterised by the determination of an acceptable standard uncertainty that is derived from the function given below, the calculated reproducibility of 14% from this study was compared to that parameter. This approach (*69*) can be used for those cases where no fully validated method precision data is available.

$$Uf = \sqrt{(LOD/2)^2 + (\alpha \times C)^2}$$

In this formula *Uf* is the maximum acceptable standard uncertainty in µg/kg, LOD is the limit of detection expressed in µg/kg, α is a factor (0.1 - 0.2) that depends on the concentration of interest (*C* in µg/kg). For a concentration below 50 µg/kg, α is 0.2. The resulting maximum acceptable standard uncertainty for a target level of 2 µg/kg and a limit of detection of 0.02 µg/kg is 0.40 µg/kg. Expressed as relative standard uncertainty this figure is 20%.

As the internal reproducibility is accepted as standard uncertainty, the calculated figure of 14% shows that the method can be considered as "fit for purpose" according to this scheme. In most of the 22 tested samples AfB_1 could not be quantified. Only in one case the presence of AfB_1 could be detected at the level of the estimated limit of quantification of 0.06 µg/kg (Fig. 11).

3.2.6. Conclusions

A rather simple and fast method for the determination of AfB_1 in horchata was developed and validated under single laboratory conditions. It could be demonstrated that the described

method shows good performance characteristics when compared with minimum requirement data calculated from a "fitness for purpose" function that is used in EU legislation on mycotoxin methodology for official food control. The described method therefore is a good candidate for such use.

Based on this small survey, no indication was found for a consumer risk from the tiger nut based soft drink as sold on the Spanish and Belgian markets in 2004. However, a survey with more samples would be necessary to obtain a full picture.

4. Determination of zearalenone

4.1. Introduction and scope of the work

As mentioned in the Chap. 1, zearalenone (ZON) has important effects on the reproductive system because it binds to oestrogen receptors (*33*). The potential carcinogenicity of ZON has been evaluated by the International Agency for Research on Cancer (IARC) and it was concluded that it is not classifiable as being carcinogenic to humans (Group 3) (*13*) although ZON was suspected to be the causative agent in an epidemic of precocious pubertal changes in young children in Puerto Rico between 1978 and 1981 (*34*).

Nevertheless a provisional maximum tolerable daily intake of 0.2 µg/kg body weight / day has been set by the Scientific Committee on Food (*15*).

In 2003 the scientific co-operation on questions relating to food (SCOOP) collected and evaluated data of the dietary intake of ZON in food by the population of EU Member States. They concluded that the mean daily intake was not exceeded (*35*).

Legislative limits were amended in the moment of these experiments according to the Commission Regulation (EC) No 856/2005 (*152*) as follows:

- 200 µg/kg for unprocessed maize.
- 200 µg/kg for maize intended for direct human consumption, maize flour, maize meal, maize grits, maize germs and refined maize oil.
- 50 µg/kg for maize snacks and maize based breakfast cereals.
- 20 µg/kg for processed cereal-based foods intended for infants and young children.

Therefore, the aim of this work was the development and validation of a reliable method that helped to enforce these legislative limits. The legislative limits are summarised in Tab. 6 of section 1.6.

Currently, extraction of ZON is achieved with several mixtures of solvents like ethyl acetate, methanol, chloroform, acetonitrile and water. For the purification and preconcentration of ZON the preferable techniques are based on immunoaffinity columns for which several suppliers are available. SPE columns are also available.

For the detection step, wide ranges of analytical methods have been described in section 1.7.2.2. ELISA seems to be the most useful tool for screening raw materials and the TLC method is mentioned as an interesting alternative. GC has also been described. As a result LC-FL detection is the most commonly used method, due to the strong native fluorescence of ZON. Nevertheless because of the universal, selective and sensitive detection by mass spectrometry, this detection method is gaining popularity in combination with HPLC systems (*153*).

After method development, the final method was based on IAC clean-up followed by LC-FL with an excitation wavelength of 274 nm and an emission wavelength of 446 nm. The test portion of the sample is extracted with methanol-water (75+25, v/v). The sample extract is filtered, diluted and passed over an immunoaffinity column. ZON is eluted with methanol.

Afterwards, a collaborative trial of the method for the determination of ZON in baby food and animal feed was conducted. The study involved 39 participants in 16 EU member states, Turkey, Uruguay and China representing a cross section of industry, official food control and research institutes.

4.2. Test materials for the collaborative study

For this inter-laboratory comparison exercise the following products were purchased from local food supermarkets and animal feed warehouses. Various brands of dry cereal based baby food and various kinds of animal feed so as pig and cattle feed. Blank oat materials have been kindly provided by Mr. Hans de Keijzer, European Flour Milling Association (*154*).

For the collaborative trial, suitable baby food and animal feed mixtures were produced from the materials listed in Tab. 11 and 12. In the case of animal feed several different types of feed were blended, after they were confirmed to be free of ZON (<20 µg/kg). All commercial baby food samples tested were found to be free from ZON. Therefore for the ZON-free blank baby food a mixture of the different baby food materials containing rice, corn and wheat was prepared.

To mimic naturally contaminated baby food test materials, a blend of different types of the blank baby food was further blended with highly contaminated wheat to achieve the desired

levels. For animal feed test materials, blank animal feed ingredients, low and medium contaminated feed compound (100 – 500 µg/kg ZON), corn and highly contaminated wheat were blended to achieve the desired levels. The composition of the test materials is given in Tab. 11 and 12.

Table 11. Composition of baby food test materials for determination of ZON.

Levels of ZON in test material	Matrix[1]	Amount [kg]	Composition[2]
Blank	Cornflakes	3.1	Corn 98%, sugar, barley
	Biscuit cereal	3.1	Milk powder, vitamins, flours (wheat, rice, oat, corn, rye, barley), biscuit 16%
	Muesli mixture	2.2	Oat, wheat, fruits 21% (apple, banana), rye, sorghum, barley
	Milk puree	1.6	Milk powder, vitamins, flours (wheat, rice, oat, corn, rye, barley)
Low	Biscuit cereal	2.0	Milk powder, vitamins, flours (wheat, rice, oat, corn, rye, barley), biscuit 16%
	7 grains	2.0	Wheat 88.2%, oat 2.9%, rice 1.9%, sorghum 1.7%, barley 1.7%, corn 1.7%, rye 1.7%, vitamins
	Wheat	0.25	Wheat
Medium	Cereals mixture	1.0	Cereals 56.2% (rice, corn flour), skimmed milk, milk-fat, corn-oil, vitamins, vanilla
	Milk puree	1.0	Milk powder, plant-oil, vitamins, wheat, sugar
	Muesli mixture	1.0	Oat, wheat, fruits 21% (apple, banana), rye, sorghum, barley
	Low	1.0	(See "Low")
	Cornflakes	0.5	Corn 98%, sugar, barley
	Oatflakes	0.5	Oat 65%, malt, sugar, wheat, corn-starch
High	Medium	2.0	(See "Medium")
	Biscuit cereal	2.0	Milk powder, vitamins, flours (wheat, rice, oat, corn, rye, barley), biscuit 16%

[1]baby food material for the blank were all free of ZON

[2]in decreasing amounts

Table 12. Composition of animal feed test materials for determination of ZON.

Levels of ZON in test material	Matrix	Amount [kg]	Composition[1]
Blank	Compound pig feed	4.4	Peas, soy, wheat, barley, tapioca, cabbage seeds, animal fat, corn, calcium carbonate
	Compound rabbit feed	2.2	Wheat, alfalfa, sunflower seeds, cabbage seeds, straw molasses, barley, roasted soy
	Compound horse feed	2.2	Barley flakes, oat, cornflakes, oil, alfalfa (luzerne), grass fibre pellets
	Compound chicken feed	2.2	Barley, wheat, oat, several cereals, oil corn
	Alfalfa	1.0	Alfalfa
Low	Compound pig feed	3.0	Peas, soy, wheat, barley, tapioca, cabbage seeds, animal fat, corn, calcium carbonate
	Corn	2.6	Corn, blank
	Compound rabbit feed	1.5	Wheat, alfalfa, sunflower seeds, cabbage seeds, straw molasses, barley, roasted soy
	Corn	0.6	Corn, contaminated
	Wheat	0.2	Wheat, contaminated
Medium	Corn	2.5	Corn, blank
	Compound pig feed	1.5	Peas, soy, wheat, barley, tapioca, cabbage seeds, animal fat, corn, calcium carbonate
	Compound rabbit feed	1.5	Wheat, alfalfa, sunflower seeds, cabbage seeds, straw molasses, barley, roasted soy
	Compound horse muesli	0.5	Oat, barley flakes, cornflakes, peas, molasses, plant oil
	Compound dog feed	0.5	Meat 14%, cereals 14%, sugar, oil, minerals, carrots 4%
	Alfalfa	0.5	Alfalfa
	Corn	0.5	Corn, contaminated
High	Medium	2.0	(See "Medium")
	Compound pig feed	2.0	Peas, soy, wheat, barley, tapioca, cabbage seeds, animal fat, corn, calcium carbonate

[1] in decreasing amounts.

Whole grain kernels were first milled with a Romer RAS® mill (*155*) prior to blending. All other materials were blended directly in a modified concrete mixer for 30 min. After blending, the test material was milled with a Retsch (*156*) centrifugal mill (Model ZM 100) with a sieve of 3 mm. This ground material was mixed again in the concrete mixer for 30 min and milled for a second time, down to a particle size of < 1 mm in the centrifugal mill. Finally, the twice mixed and milled material was mixed again in the concrete mixer for 2 - 3 h and then milled a final time to achieve a particle size of < 0.5 mm. The materials were subsequently filled into 50 mL containers (approx. 30 g each container) and frozen at -18°C. The filled containers were kept at this temperature until analysis for homogeneity or dispatched for collaborative trial testing.

4.3. Homogeneity of the test materials

For homogeneity testing every 10th sample was taken from the sequence during packing. These selected test materials were analysed with HPLC-FL detection for the homogeneity study.

The number of the first container from which the sampling started was randomly selected from the first ten samples for each material, after which the following samples could be collected as described above. As an example, baby food material was checked by taking the third, the 13th, the 23rd… sample.

The obtained batches for analysis were split with the RIVM (*157*) for homogeneity. The homogeneity test showed that in all cases sufficiently homogeneous material was achieved based on the resulting CV (Tab. 13 and 14).

Table 13. Homogeneity of baby food test material.

Levels of ZON in test material	Target content [µg/kg]	n	Average content from the homogeneity test [µg/kg]	CV [%]
Blank	0	10	n.d.	n.d.
Low	10	12	10.1	9.4
Medium	20	10	17.5	5.2
High	50	10	47.3	6.7

CV: coefficient of variance.

Table 14. Homogeneity of animal feed test material.

Levels of ZON in test material	Target content [µg/kg]	n	Average content from the homogeneity test [µg/kg]	CV [%]
Blank	0	12	9.2	4.6
Low	40	14	37.5	4.2
Medium	75	12	80.2	7.8
High	390	12	330.1	9.1

CV: coefficient of variance.

4.4. Method development

Up to the moment of these experiments, many of the methods for the determination of ZON available in the literature use an immunoaffinity clean-up column after extraction of ZON with an aqueous organic solvent from the matrix, followed by LC-FL detection (*93*). Based on the extraction solvent used, these methods can be divided in two groups, which either use acetonitrile-water or methanol-water for extraction. The extraction of ZON with acetonitrile-water, as published by Visconti and Pascale (*158*), MacDonald *et al.* (*93*) and Campbell and Armstrong (*159*) is preferred by some analysts as this extraction procedure appears to yield higher apparent recoveries. Also a similar methodology has been used in a collaborative study for the determination of ZON in a variety of cereals and cereal-based products and for animal feed at levels above 100 µg/kg (*93*). Additionally, the current standard for the quantification of ZON in animal feed by the International Organization for Standardization feedstuffs method committee (ISO TC 34/SC) makes use of acetonitrile-water for extraction and offers a quantification limit of 50 µg//kg (ISO CD 17372) (*94*).

Other authors prefer the extraction with methanol-water (*70*). As one goal of this work was to develop a method for the determination of ZON in food intended for infants and small children, but as well the method should also work on animal feed matrixes. And due to the fact that so far all the published methods work on ranges above the one required to fulfill current EU legislation, which regulates food intended for infants and small children at the level of 20 µg/kg, and additionally have never been tested on more complex matrices than pure grains, additional method development and testing was necessary.

Starting point for this development was to compare the benefits and drawbacks of the currently most propagated extraction solvents acetonitrile-water and methanol-water and

when necessary also include other extraction solvents. Methanol is known to be not so harmful to the antibodies immobilised in immunoaffinity columns (IAC). This theoretically allows higher concentration of methanol and more volume of diluted extract onto the IAC, resulting in a higher sample loading. This could be of use for achieving lower working ranges with a smaller LOQ and/or avoiding an evaporation step prior to injection in the HPLC system as it is used by some authors (*159*). Another argument that appears according to the literature is that acetonitrile based extracts gives cleaner extracts which would be beneficial in case of animal feed matrices. Furthermore, to avoid the known phenomenon of solvent layer separation on the basis of salting out effects *(140)*, binary mixtures of water with acetonitrile were excluded and replaced by a mixture of acetonitrile-methanol as it has been proposed by Visconti *et al.* (*158*).

Due to this reasons it was decided to start with a method based on acetonitrile as solvent. Nevertheless the stability of the IAC towards the three solvents, methanol, acetone and acetonitrile was evaluated. Easi-Extract® columns were used for all experiments. The experiment was performed by diluting 1.0 mL of a standard solution of 10.0 ng/mL ad 50.0 mL. Three sets of solutions were prepared; the first one contained 5 different mixtures ranging from 4% till 20% of methanol-water, the second one contained mixtures ranging from 4% till 20% of acetone-water and the third one with mixtures ranging from 4% till 20% of acetonitrile-methanol (50+50, v/v)-water.

From the 50.0 mL mixtures, only 40.0 mL were applied onto the immunoaffinity column, and then the methodology was followed as just mentioned with washing and eluting steps. A total of 15 IAC purified extracts were compared in this way. Recoveries are presented in Tab. 15, ranging form 96-101% for the methanol mixtures.

Based on this experiment it can be concluded that this type of IAC is rather stable to all tested mixtures containing up to 20% organic solvent. As none of the alternatives that were tested for methanol-water extraction method offered any apparent benefit, the methanol-water based extraction was chosen as a starting point. A similar approach has also been described by Fazekas and Tar (*70*) and was used in an internal method description of a collaborating laboratory that has been found to be applicable for a wide variety of food matrices.

Table 15. Stability of immunoaffinity columns to methanol, acetone and methanol-acetonitrile mixtures.

Solvent-water mixture	Recoveries [%]
4% methanol-water	97
8% methanol-water	107
12% methanol-water	95
16% methanol-water	109
20% methanol-water	105
4% acetone-water	87
8% acetone-water	92
12% acetone-water	96
16% acetone-water	97
20% acetone-water	91
4% [methanol-acetonitrile (50+50, v/v)]-water	101
8% [methanol-acetonitrile (50+50, v/v)]-water	102
12% [methanol-acetonitrile (50+50, v/v)]-water	96
16% [methanol-acetonitrile (50+50, v/v)]-water	97
20% [methanol-acetonitrile (50+50, v/v)]-water	100

The authors of this method propose to extract 20.0 g of test material with 40.0 mL of a mixture of methanol-water (80+20, v/v) in the presence of sodium chloride. The extract is then shaken for 30 min, filtered and diluted with water (4.0 mL ad 100.0 mL). Afterwards, 50.0 mL of this diluted extract are purified on an immunoaffinity clean-up column. The IAC is washed with 20 mL of water and the purified ZON is eluted with 1.5 mL of methanol. After dilution of the eluate with water, ZON is determined by LC-FL from this solution.

Several modifications were studied in order to fulfill the required method performance characteristics by EU legislation *(57)*.

The prospective method in this study aimed at low LOQs. This can easily be achieved by the application of larger extract fractions that contain a lower content of organic solvent on the IAC. On the other hand the extractability and thus recovery of ZON can suffer from a too low content of organic solvent, as ZON is poorly soluble in water. At first instance, a comparison between methanol-water (50+50, v/v) and (75+25, v/v) as possible extraction solvents was performed in two matrixes, one baby food, and one animal feed. Additionally the use of Tween 20® was tested. Tween 20® or also known as Polysorbate 20 is used as a

non-ionic detergent and emulsifier and has already been shown to purify IAC that were used for clean-up of corn samples (*160*).

The resulting recoveries can be found in Tab. 16. It can be seen that for both matrixes, an addition of 100 µL Tween 20® resulted in lowered recovery rates.

Table 16. Comparison of recoveries with two extraction solutions methanol-water (50+50, v/v) and (75+25, v/v) and additionally with the addition of 100 µL Tween 20®.

	Recoveries in methanol-water mixtures [%]			
	(50+50, v/v)	**(50+50, v/v)+Tween 20®**	**(75+25, v/v)**	**(75+25, v/v)+Tween 20®**
Baby food	65	58	95	60
Pig feed	100	55	98	70

But for baby food methanol-water (75+25, v/v) provided higher recoveries, while for the animal feed analysed both extracts performed equally well. Therefore, additional experiments were performed with pig, chicken and dog feed in order to elucidate whether 50 or 75% of methanol provided higher recoveries. Recoveries were in general a bit higher for the (75+25, v/v) methanol-water mixture (Tab.17).

Table 17. Comparison of recoveries with methanol-water (50+50, v/v) *vs.* (75+25, v/v) in three animal feed samples.

	Recoveries in methanol-water mixtures [%]	
	(50+50, v/v)	**(75+25, v/v)**
Pig feed	95	110
Chicken feed	92	95
Dog feed	90	105

Studying different solvent-to-sample ratios was the next parameter; this ratio varies from method to method, while usually no information is given by authors for their choice of the ratio. It can however be assumed that smaller solvent-to-sample ratios can result in a saturation of the extraction solvent when samples with a large amount of solutes are extracted e.g. samples with a high content of sugars. A summary of some published solvent-to-sample ratio is given in Tab. 18.

Table 18. Solvent to test portion ratios of the methods available in the literature.

	Extract [mL]	Test portion [g]	Ratio
McDonald *et al.* *(93)*	100	25	**4**
ISO TC 34/SC *(94)*	150	50	**3**
Visconti and Pascale *(158)*	50	20	**2.5**

As mentioned, the prospective method in this study aimed to lower LOQs which can easily be achieved by the application of larger extract fractions on the IAC. Therefore it was concluded that 20.0 g were suitable for 150.0 mL of methanol-water (75+25, v/v). Recoveries of 90-105% with CV <10% and LOQ of 2 ng were achieved.

Taking into consideration the modifications proposed the main features of the method are as follows:

- extract 20.0 g of test material with 150.0 mL of methanol-water (75+25, v/v) in the presence of 2 g of NaCl,
- sonicate and shake for 15 min,
- filtrate using a folded filter paper Whatman No. 113V,
- from the filtrate, dilute 30.0 mL ad 150.0 mL with water ,
- filtrate with a glass microfiber filter paper, Whatman GF/A,
- 50.0 mL of the filtrated extract are purified on the IAC,
- IAC is washed with 20.0 mL water,
- elute two times with 0.75 mL of methanol,
- dilute to final volume of 3.0 mL with water,
- mobile phase was based on a mixture of methanol-water (75+25, v/v).

Since the method aimed to be applicable to most types of animal feed, including those that contained colour pigments, several animal feed matrices mainly dog feed, horse feed, pig feed, chicken and rabbit feed were analysed in order to check if the chromatographic determination was affected due to any of the components. The methodology used was the one above described. An example of a chromatogram of a natural contaminated sample of animal feed containing colour pigments can be seen in Fig.12.

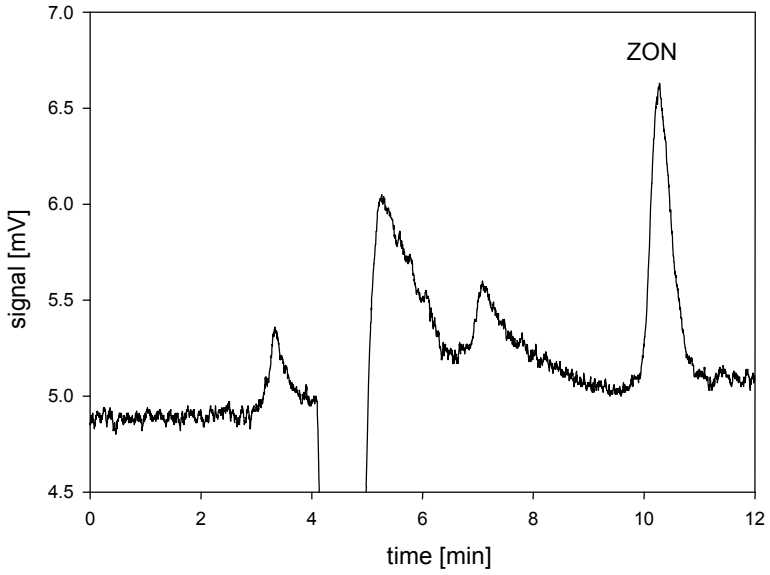


Figure 12. Chromatogram of a naturally contaminated dog feed sample containing colour pigmented ingredients. The zearalenone peak reflects a contamination level of 15 µg/kg ZON in the sample.

Further on, it was evaluated if the dilution prior to the IAC clean-up should be more appropriate with water or phosphate buffered saline (PBS). PBS is a buffer solution offering the additional advantage that helps to maintain a constant pH during immunoaffinity clean-up. This can be essential if the immobilised antibody is not stable towards small pH changes as they can occur when e.g. slightly acidic reacting samples are extracted. Dilution with PBS gave more consistent result in comparison to water and additionally, filtration with a glass microfiber filter resulted in clean chromatograms.

The main aim of the IAC is the almost complete purification of ZON prior to HPLC separation. This reduces the requirements on the chromatographic performance to separate ZON from possible interfering peaks. Despite the fact that IAC clean-up procedures are very selective, it is generally observed that purified extracts contain many other substances next to the target analyte.

In the case of ZON analysis it was observed that after dilution of the extract with PBS a precipitation occurred that required further filtering through a glass fiber filter. Nothing was known about how such diluted extracts behave when further diluted with water. This will be the case when IACs are washed with pure PBS or water, after a diluted extract has been applied and the residual volume of the diluted extract is still in the immunoaffinity gel of the column. In order to circumvent the possibility of such a precipitation that most likely would

be co-eluted with ZON when flushed with neat solvent, the washing procedure was adopted by applying a washing solution containing the same amount of organic solvent than the diluted extract. Two of such washing solvents were compared; one contained 15% methanol in PBS, another 15% of methanol in water. An animal feed sample was analysed with both modes in replicates. In this respect no difference was observed (Tab. 19). Therefore the method goes ahead with a first washing solvent of 5 mL (15% methanol in PBS) and subsequently a second wash with 15 mL of water.

Table 19. Comparison of two different washing solutions of the IAC, one with 15% methanol in PBS and another one with 15% PBS in water.

Material	Washing solution	n	Average content found [µg/kg]	CV [%]
Animal feed	15% MeOH in PBS	5	345	5.5
	15% MeOH in water	5	327	4.2

The performance of the proposed extraction with methanol-water (75+25, v/v) followed by an IAC clean-up procedure including a washing step with 15% methanol in PBS can be nicely demonstrated by comparison of two chromatograms of the same pig feed sample, which are illustrated in Fig. 13 and 14. The ZON peak appears at 13 min. (Fig. 13) as a single peak, reflecting a contamination level of 6 µg/kg ZON in the sample. In Fig. 14 the ZON peak appears with a nearby eluting peak in the region of 13-14 min. The chromatogram in Fig. 14 has been obtained with the ISO method for ZON for animal feed (*94*).

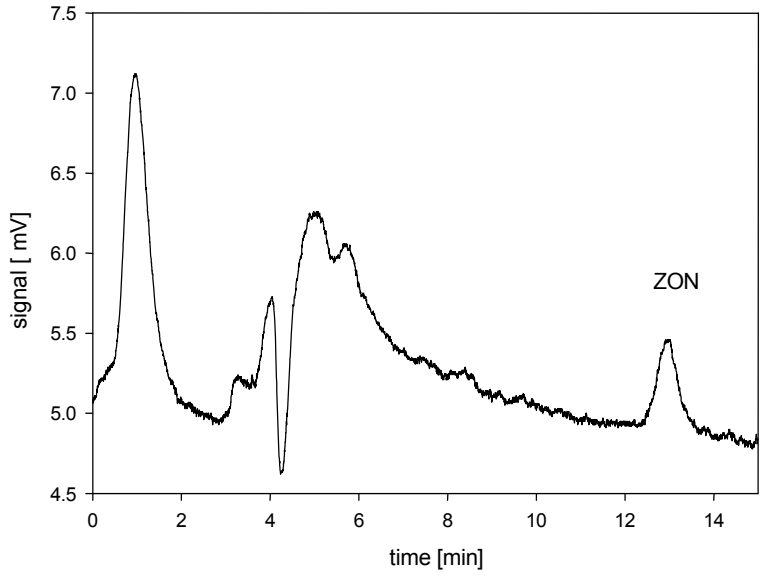


Figure 13. Chromatogram of animal feed sample based on the new method proposed.

The nature of this interfering peak was not further investigated, as the resulting chromatogram came not from the preferred extraction methodology. However, this experiment indicated the difficulties that can be encountered when extracting with acetonitrile-water. This most likely led to the extensive clean-up efforts described in the method protocol that was used during the collaborative trial of this method (*159*).

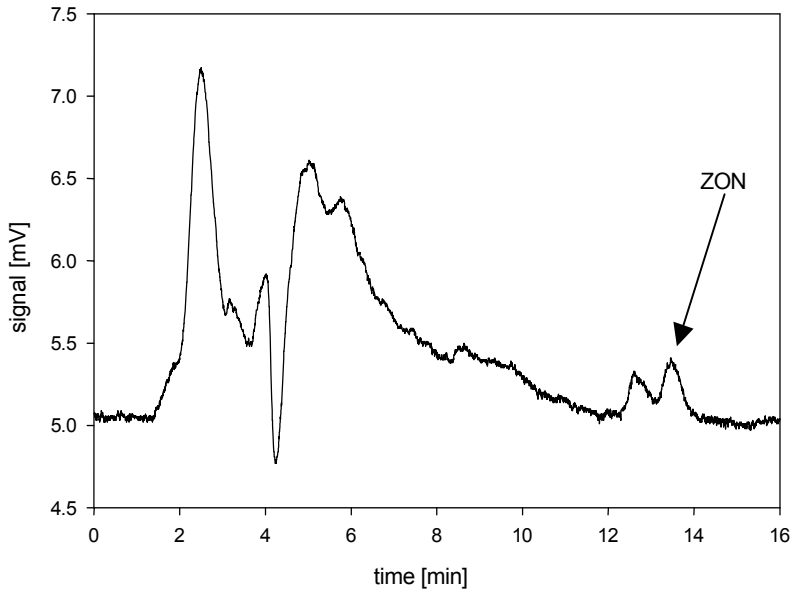


Figure 14. Chromatogram of animal feed sample based on acetonitrile extraction.

Concerning the extraction mode, shaking or ultrasonication of the material with the extraction solvent have been proposed and some authors compared different extraction modes (*161*). Unfortunately all these comparisons lack information on the particle size of the extracted material, which is important to know when comparing results from different extraction modes. Another aspect in the extraction of test samples for the determination of mycotoxins in general is that laboratories tend to avoid high speed blending as it bears the risk of cross contamination unless different extraction vessels are used. Blending or shaking with an appropiate solvent are commonly used to extract mycotoxins from the commodity.

Furthermore, the extraction of larger number of samples has to be carried out sequential, while shaking, as the simplest alternative, and sonication allow a parallel extraction of several samples, which is also more time efficient than blending. Usually extraction times are in the range of 30 min or more, which should be sufficient to reach distribution equilibrium, provided that the sample has been milled sufficiently to obtain a favorable particle size.

For the following experiments all samples have been produced by passing the raw test material through the high speed centrifugal mill (Model Retsch ZM100) at a mesh of 0.5 mm to obtain sufficiently fine test material. Two extraction modes were evaluated; 15 min of shaking followed by another 15 min of sonicating versus 1 h of shaking. For this purpose, a sample of animal feed and baby food were analysed in replicates with both extraction modes. Comparable results were achieved for recovery and precision (Tab. 20).

Table 20. Evaluation of two different extraction modes (15 min shake + 15 min sonication vs. 1 h shaking) in animal feed and baby food naturally contaminated.

Material	Extraction mode	Target content [µg/kg]	n	Average content found [µg/kg]	CV [%]
Animal feed	15 min shake + 15 min sonication	390	5	358	6.5
	1 h shake		5	361	6.7
Baby food	15 min shake + 15 min sonication	50	5	47	7.3
	1 h shake		5	48	6.2

As a result, it was considered to define both extraction modes as equivalent, thus leaving laboratories the choice to extract for one hour or to reduce the time of extraction by implementing a sonication step. A sonication of a longer period than 15 min was not considered, as samples got rather warm after 15 min of extraction in the ultrasound bath.

After all these modifications were implemented into a new method an organisation of a collaborative trial followed.

4.5. Organisation of collaborative study

The instructions for participants in the inter-laboratory comparison are given in Annex 5. The pool of interested participants for this study grow to an extent, that a single collaborative trial including all participants and covering both matrices (animal feed and baby food) would have resulted in an unnecessary large number of analyses and data. As a result the pool of participants was split into two groups, one analysed baby food and the other one animal feed.

A total of 39 collaborators from 19 different countries were invited to participate in the collaborative trial. These collaborators represented a cross-section of government, food control, university and food industry affiliations. One participant did not return results.

For the collaborative trial the participants received:

1. Eight coded sample containers with blind duplicates at four concentration levels plus four 'blank'-labelled ones per matrix (baby food or animal feed) for spiking.
2. One amber vial marked 'ZON Standard' containing ZON, which had to be employed as the calibrant ZON solution, as described in the method.
3. Eight vials marked 'Spike solution A, B, C and D' and 'Spike solution 1, 2, 3 and 4' to be used for spiking procedures.
4. Twenty six immunoaffinity columns with antibodies for ZON from the brand R-Biopharm (*133*).
5. A copy of the collaborative study method.
6. A copy of the spiking protocol.
7. Chromatograms of analysed materials containing ZON.
8. A 'Collaborative Study Materials Receipt' form.
9. Report forms.
10. A results questionnaire.

Each participant was required to prepare one extract from each container and perform the analysis by LC-FL. Additionally the participants of the baby food trial were required to spike the four materials indicated as 'Blank' using the 'Spike Solutions 1, 2, 3 and 4'. The participants of the animal feed trial were required to use the ones indicated as 'Spike Solutions A, B, C and D' for fortification.

4.6. Experimental

The method of analysis that was used in this study, the materials and instructions can be found in Annex 5.

4.7. Results and Discussion

4.7.1. Collaborative trial results

All data submitted for the study are presented in Tab. 21 and 22. The data are given as individual pairs of results for each laboratory identified with the laboratory ID codes that were used for reporting. Blank samples were spiked in duplicate for both matrices, with 20 µg/kg and 30 µg/kg of ZON (identified as sample '20' and '30') for baby food and with 100 µg/kg and 150 µg/kg of ZON (identified as sample '100' and '150') for animal feed (all levels unknown to participants). All other samples were blind duplicates of 'blank' (bl) and of naturally contaminated materials identified with the target level analogue to the spiked materials. The results are also presented as Youden plots in Annex 6 (Fig. 1 to 10). The Youden plot is a graphical data analysis technique for comparing both the repetibility and reproducibility. The Youden plots confirmed the validity of the identification of outlier laboratories shown in Tab. 21 and 22 and additionally showed some trends that may not otherwise be apparent from studying the tables alone. The Youden plots do not show any inconsistencies in the statistical handling of the data used to generate the precision data from the collaborative trial results. The results from laboratory 15 for baby food samples were excluded from the analysis due to the fact that they reported no detectable levels of ZON in any sample.

One laboratory (# 6) analysed the animal feed samples by GC/MS after agreement with IRMM, as LC-FL was not available during the time of the trial. This data set is listed in Tab. 22 for information purposes, but was not used for statistical analysis. It can be seen that in this particular case the results generated with GC/MS show systematically higher concentrations for all samples. The reason for this was not further elucidated, but will be subject for investigation in the future.

Table 21. Individual results of ZON in baby food determined using HPLC fluorescence detection as reported by the collaborative trial participants.

ZON concentration [µg/kg] (target values)												
Lab ID	**L[1]**	**L[1]**	**H[1]**	**H[1]**	**bl**	**bl**	**low[2]**	**low[2]**	**mid[2]**	**mid[2]**	**high[2]**	**high[2]**
1	22	22	24	24	0	0	10	9	18	18	46	46
2	18	19	27	28	0	0	8	9	17	18	41	43
3	20	20	29	28	0	0	10	10	18	18	47	46
4	38	39	61	56	0	0	10	9	18	18	48	46
5	19	18	27	26	0	0	9	9	18	18	42	41
6	14	19	24	29	0	0	7	8	17	17	45	46
7	17	17	25	24	0	3	8	7	15	15	38	41
8	18	17	26	24	0	0	9	9	15	17	39	39
9	21	20	31	29	<	<	10	10	20	20	45	46
10	19	19	29	30	0	0	9	10	18	18	49	49
11	17	19	26	26	<	<	8	7	17	16	34	46
12	16	22	24	25	1	0	12	11	19	14	43	44
13	18	18	25	27	0	0	9	8	16	16	41	39
14	20	19	30	29	0	0	9	9	18	18	45	46
15	<LOD	6	5	5	0	0	<LOD	<LOD	<LOD	<LOD	13	14
16	16	15	26	24	0	0	8	4	14	17	41	19
17	-	-	-	-	-	-	-	-	-	-	-	-
18	17	13	23	26	0	4	5	11	16	14	36	40
19	17	17	25	25	0	0	8	9	12	15	39	39
20	19	19	29	25	0	0	9	9	17	18	44	46
21	24	24	37	34	0	0	11	11	22	22	56	54

Invalid data that was removed prior statistical analysis. For detailed information see the comments of participants below.
Outliers identified by statistical analysis.
Missing data at the time of drafting the report.

[1] Fortified material at low (L, 20 µg/kg and high (H, 30 µg/kg) levels.

[2] Naturally contaminated material at three levels (low - 10 µg/kg, mid - 20 µg/kg and high - 50 µg/kg).

<LOD less than the limit of detection.

Table 22. Individual results of ZON in animal feed determined using HPLC-fluorescence detection as reported by the collaborative trial participants.

ZON concentration [µg/kg] (target values)												
Lab ID	**L[1]**	**L[1]**	**H[1]**	**H[1]**	**bl**	**bl**	**low[2]**	**low[2]**	**mid[2]**	**mid[2]**	**high[2]**	**high[2]**
1	86	89	122	128	8	0	24	26	60	62	162	272
2	39	57	81	81	10	16	22	24	52	71	278	278
3	88	87	117	134	7	8	27	28	75	78	359	378
4	101	100	134	150	22	24	24	34	44	46	284	249
5	87	78	141	119	10	10	33	36	65	68	284	307
6	130	130	230	240	30	50	40	90	190	210	410	630
7	89	84	125	130	7	11	33	33	69	71	338	348
8	80	82	114	154	19	20	34	38	63	107	252	261
9	81	106	124	105	10	10	26	28	66	72	286	309
10	7	111	172	-	16	17	38	4	11	12	56	405
11	75	84	113	99	12	10	40	40	66	66	317	340
12	92	94	136	139	11	13	35	36	86	89	380	389
13	83	88	114	117	20	21	35	36	86	78	313	314
14	102	95	142	141	12	12	36	54	80	80	331	315
15	47	44	244	63	7	73	0	535	7	13	217	312
16	138	121	177	169	56	62	147	131	60	72	295	317
17	62	73	104	101	6	6	27	28	63	68	288	309
18	88	105	130	138	11	11	31	37	77	81	279	373

Invalid data that was removed prior statistical analysis. For detailed information see below the comments of participants.
Outliers identified by statistical analysis.
Missing data at the time of drafting the report.
[1] Fortified material low (L, 100 µg/kg and high (H, 150 µg/kg) levels.
[2] Naturally contaminated material at three levels (low - 40 µg/kg, mid - 75 µg/kg and high - 390 µg/kg).
<LOD inferior to the limit of detection.

A better visual impression from the data of Tab. 21 and 22 are presented in Fig. 15 and 16, respectively as Box-and-Whisker Plots, which are a graphical way of showing the location, shape and width of the distribution of groups of numerical data, dividing it into four parts using the median and quartiles. The first quartile is the median of the lower part of the data. The second quartile is another name for the median of the entire set of data. The third quartile is the median of the upper part of the data.

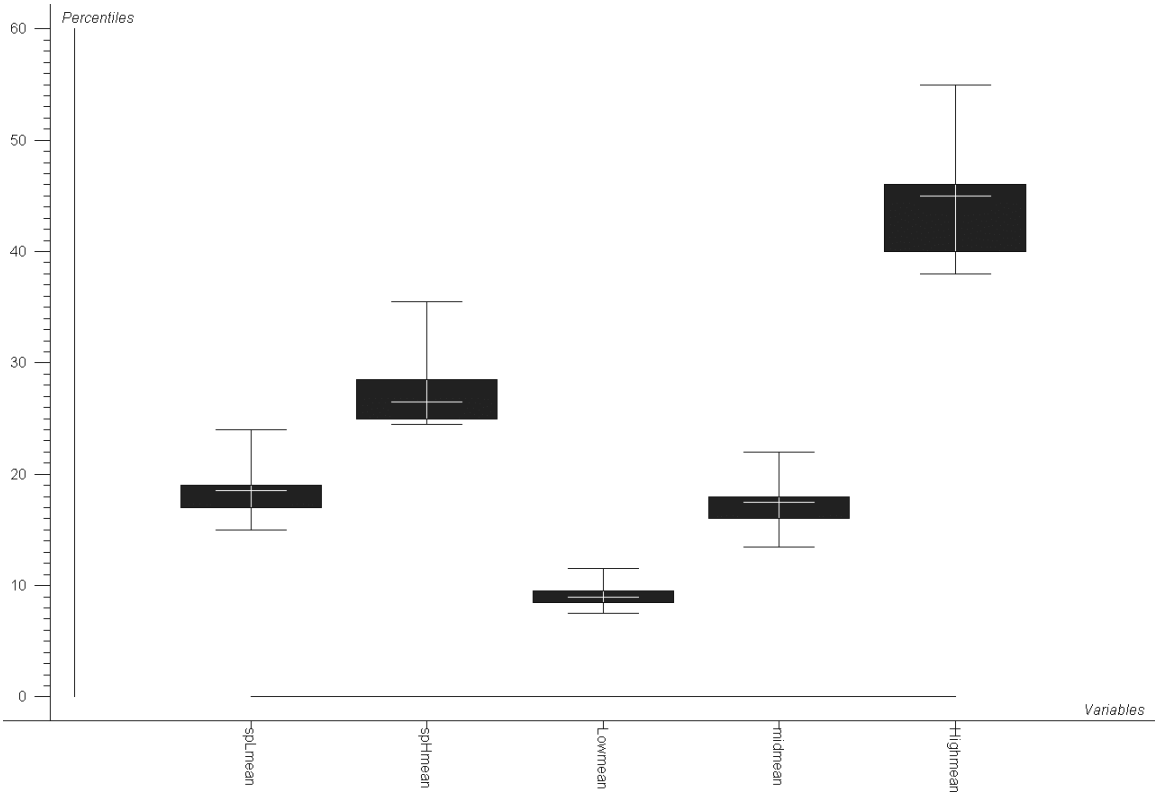


Figure 15. Box-and-Whisker plots for ZON in baby food.

Box-and-Whisker plots visualizing the single results for baby food after outlier removal.

- spLmean; average of the duplicates (spike low level - 20 µg/kg).
- spHmean; average of the duplicates (spike high level - 30 µg/kg).
- Lowmean; average of the duplicates (low level- naturally contaminated material -10 µg/kg).
- Midmean; average of the duplicates (medium level- naturally contaminated material - 20 µg/kg).
- Highmean; average of the duplicates (high level- naturally contaminated material - 50 µg/kg).

Plots created with CAMO Software (*162*).

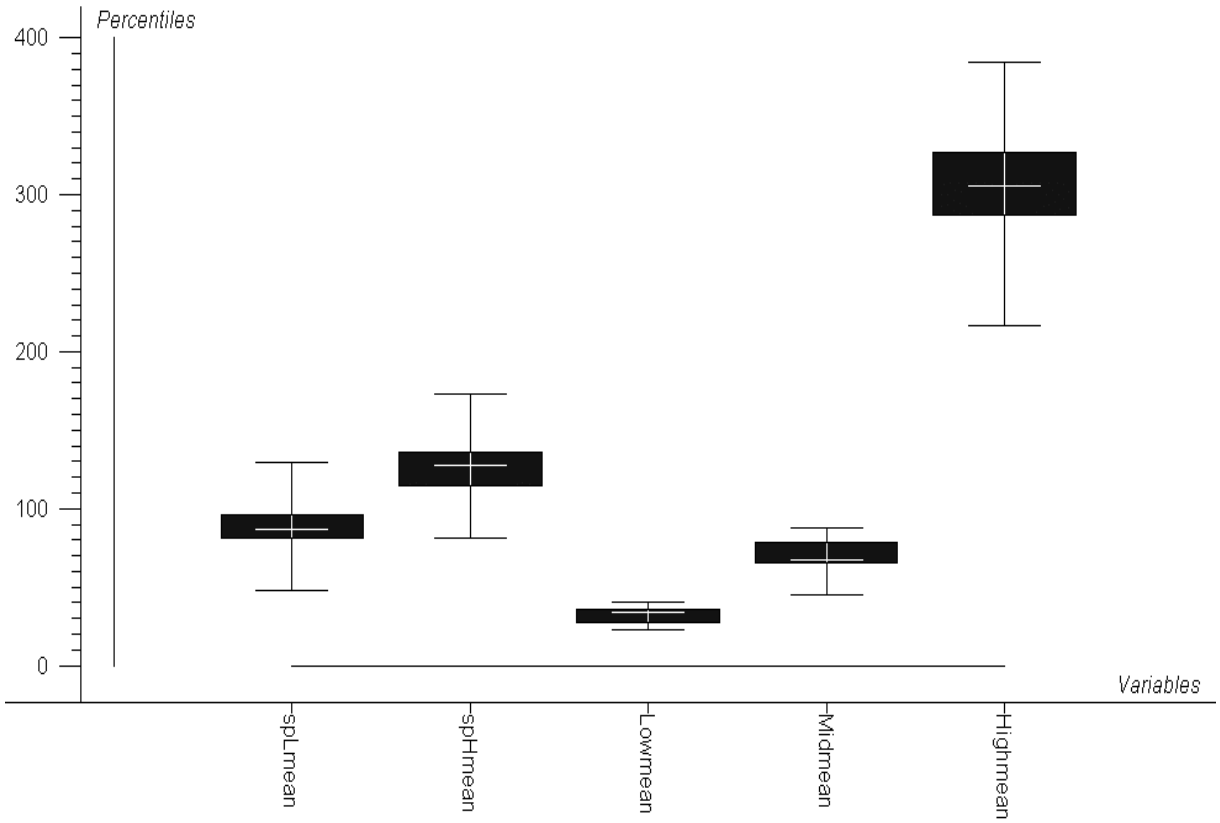


Figure 16. Box-and-Whisker plots for ZON in animal feed.

Box-and-Whisker plots visualizing the single results for animal feed after outlier removal.

- spLmean; average of the duplicates (spike low level - 100 µg/kg).
- spHmean; average of the duplicates (spike high level - 150 µg/kg).
- Lowmean; average of the duplicates (low level- naturally contaminated material - 40 µg/kg).
- Midmean; average of the duplicates (medium level- naturally contaminated material - 75 µg/kg).
- Highmean; average of the duplicates (high level- naturally contaminated material - 390 µg/kg).

Plots created with CAMO Software (*162*).

4.7.2. Comments of Participants

Generally it was remarked that the method was easy to use and well documented. Some participants reported that problems occurred with interferences especially for animal feed. Specific comments that led to the exclusion of participants as "non compliant" for statistical analysis were the following:

For the analysis of baby food, participant 1 stated that the spiking procedure used in this method is usually not performed in his/her laboratory and that they doubt the correctness of their data. The data for the spiking experiments was therefore removed. Participant 4 used different volumes of extraction solvent and not those required for this method. This resulted in recoveries approximately twice of what was expected. An "intermediate" re-calculation of

the values adjusted to the amount of solvent used, still showed results outside of the expected range, while the participant reported "in-house" recoveries of 88 - 95%. As these values did not match with the results calculated for the trial, the data were excluded. Participant 15 did not find ZON in any baby food sample in the expected range and a systematic problem seemed to be the origin of this phenomenon. Therefore all results from participant 15 were excluded.

For the analysis of animal feed, participant 2 reported that the immunoaffinity columns were frozen for a short period instead of stored at +4° C as required. The study director proposed to continue, as previous experience showed that immunoaffinity columns are often robust enough to survive single freezing events. As no outliers were observed, the data from the participant were considered as valid indicating also the robustness of the column used and of the method. Thus the data were accepted for statistical analysis. Participant 9 indicated significant interferences in the chromatogram for the low spiked samples and put doubt in their correctness. The data were therefore removed. Participant 10 mentioned that one spiking vial broke, thus no analysis was performed for one experiment. In addition, the participant mentioned no prior experience with this method, as usually ELISA is used for the analysis of ZON. As the calculated values revealed some rather doubtful results in this respect, the data from this participant were removed as non-compliant. Participant 15 also reported large interfering peaks in all samples, which made the quantification of ZON difficult and the results doubtful. As there was no time to solve this matter (e.g. another HPLC column) the results were removed as non-compliant prior to statistical analysis.

4.7.3. Statistical analysis of results

In some cases data were excluded from the statistical analysis. This was the case when statistical evaluation was impossible because values were reported as zero or below the detection limit or when the participants themselves identified data sets as doubtful.

Precision estimates were obtained using a one-way analysis of variance approach according to the IUPAC Harmonized Protocol (*135*). Details of the average analyte concentration, the standard deviations for repeatability (RSD_r) and reproducibility (RSD_R), the number of statistical outlier laboratories, the Horwitz values and the percentage recovery are presented in Tab. 23 and 24. The collaborative trial results were also examined for evidence of

systematic error ($p<0.025$) using Cochran's and Grubbs tests progressively (*135*). Cochran's and Grubbs tests are the classical statistical tests to identify the laboratories that present statistically non-coherent results also known as outlier detection. Pairs of results that were identified as outliers are indicated with shaded background in Tab. 21 and 22. The total number of outliers identified was four for baby food and five for animal feed.

It appeared that there was some residual ZON in the 'blank' samples for animal feed. Therefore for the calculation of the recovery of the spiked samples, the mean blank value – calculated as robust mean equal to 12.0 µg/kg - was first subtracted from valid results. The robust mean was calculated using 'RobStat' sofware (*163*).

Table 23. Performance parameters for the determination of ZON in baby food[1].

Added [µg/kg]	Labs$^{m(n)}$	Average [µg/kg]	S_r [µg/kg]	S_R [µg/kg]	RSD_r [%]	RSD_R [%]	r [µg/kg]	R [µg/kg]	Horwitz Value	Mean Recovery [%]
20	17 (0)	18.4	1.6	2.4	8.7	12.9	4.5	6.6	0.4	92
30	17 (0)	26.6	1.5	2.2	5.7	8.2	4.2	6.1	0.3	91
nc[2] (0)	19 (0)	< 2	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]
nc[2] (10)	17 (2)	9.1	0.5	1.2	5.9	13.0	1.5	3.3	0.4	n.a.
nc[2] (20)	18 (1)	17.1	0.9	2.2	5.3	13.0	2.5	6.2	0.4	n.a.[3]
nc[2] (50)	17 (2)	44.0	1.2	4.5	2.8	10.1	3.4	12.5	0.4	n.a.[3]

[1]number of laboratories, where m = number of labs retained after outliers removed and (n) = number of outliers.
S_R = Standard deviation for repeatability; S_r = Standard deviation for reproducibility.
R = reproducibility and r = repeatability are calculated according to the IUPAC Harmonized Protocol (*135*).
The parameters have been calculated using the excel template CLSTD.XLT (*136*).
nc [2] = naturally contaminated.
n.a.[3] = not applicable.

Table 24. Performance parameters for the determination of ZON in animal feed[1].

Added [µg/kg]	Labs$^{m(n)}$	Average [µg/kg]	S_r [µg/kg]	S_R [µg/kg]	RSD_r [%]	RSD_R [%]	r [µg/kg]	R [µg/kg]	Horwitz Value	Mean Recovery [%]
100	14 (0)	87.4	6.8	18.7	7.8	21.4	19.1	52.5	0.9	75
150	15 (0)	126.0	10.7	22.4	8.5	17.7	29.8	62.6	0.8	72
nc [2] (0)	15 (0)	<20	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]
nc [2] (40)	12 (3)	31.5	1.8	5.5	5.7	17.3	5.0	15.2	0.6	n.a.[3]
nc[2] (75)	14 (1)	69.7	5.0	11.4	7.1	16.3	13.8	31.8	0.7	n.a.[3]
nc [2] (390)	15 (0)	307	29.2	47.5	9.5	15.5	81.7	133	0.8	n.a.[3]

[1] number of laboratories, where m = number of labs retained after outliers removed and (n) = number of outliers.
S_R = Standard deviation for repeatability; S_r = Standard deviation for reproducibility.
R = reproducibility and r = repeatability are calculated according to the IUPAC Harmonized Protocol (*135*).
The parameters have been calculated using the excel template CLSTD.XLT (*136*).
nc[2] = naturally contaminated.
n.a[3] = not applicable.

These values were compared with the performance requirements listed in Tab. 25 according to the European legislation that at the moment of this research was the Commission

Directive 2005/38/EC (*164*). Nowadays performance requirements are set in Commission Regulation (EC) No 401/2006 (*69*) remaining the same values.

The previous tables are summarised in Tab. 26 with remarks on the qualification of each contamination level tested. As a result the method is qualified for baby food from a contamination level of 9.1 µg/kg and for animal feed from 31.5 µg/kg.

Table 25. Acceptance criteria for analytical methods for ZON according to Commission Directive 2005/38/EC (*164*).

Level [µg/kg]	ZON		
	RSD_r [%]	RSD_R [%]	Recovery [%]
≤ 50	≤ 40	≤ 50	60 – 120
> 50	≤ 25	≤ 40	70 – 120

Table 26. Method performance parameters obtained in the collaborative trial.

Method	Matrix	Level[1] [µg/kg]	Obtained parameter			Qualified YES/NO[2]
			RSD_r [%]	RSD_R [%]	Recovery [%]	
HPLC-FL	Baby Food	9.1	6.0	13.0	-[3]	YES
		17.1	5.3	13.0	-[3]	YES
		18.4	9.0	13.3	92	YES
		27.2	5.7	8.2	91	YES
		44.0	2.8	10.1	-[3]	YES
	Animal Feed	31.5	5.7	17.3	-[3]	YES
		69.7	7.1	16.3	-[3]	YES
		87.4	7.8	21.4	75	YES
		126.0	8.4	17.7	72	YES
		306.8	9.5	15.5	-[3]	YES

[1] Mean level as reported in the collaborative trial.
[2]The qualification required that the performance parameters obtained in the collaborative trial fulfilled the requirements set in Directive 2005/38/EC *(163).*
[3]Materials for which no recovery data is given (marked with '-') were naturally contaminated.

4.7.4. Precision characteristics of the method

Due to differences in reporting limits for not detectable amounts of ZON, the results for 'blank' materials were not analysed statistically. The results however clearly indicated that all participants could identify the blank pairs of samples for baby food as not containing detectable ZON or containing levels which were detectable but close to limits of

quantification. In case of animal feed the levels detected indicate that the method works to levels well below the target level and that marginally contaminated levels could clearly be identified.

Based on results for spiked samples (blind duplicates at two levels), as well as naturally contaminated samples (blind duplicates at three levels), the relative standard deviation for repeatability (RSD_r) in baby food ranged between 2.8 - 9.0 % and in animal feed between 5.7 - 9.5 %. The relative standard deviation for reproducibility (RSD_R) in baby food ranged between 8.2 - 13.3 % and between 15.5 - 21.4 % for animal feed.

The recovery values for ZON in baby food derived from the spiked samples were found to range between 91 - 92 % for baby food and between 72 - 75 % for animal feed.

4.8. Conclusions

The acceptability of the precision characteristics of the method were assessed on the basis of the European legislation valid at that moment (*164*), which compares the RSD_r and RSD_R at the various levels with those values given in Tab. 26.

The results of this inter-laboratory validation trial show precision characteristics which fulfil the criteria (RSD_r, RSD_R and recovery) at the levels of determination stipulated by the European legislation (*164*). The enforcement of the new legislative limits for various food products was possible and was laid down as detailed in Regulation 1881/2006 (*57*).

The method fills the gap allowing the determination of ZON in both matrices (baby food and animal feed) at sufficiently low levels compared to previous studies that were all validated at higher levels (*93, 94, 159*). Additionally, the fact that the method is based on immunoaffinity clean-up with methanol-water extraction shows interference free chromatograms without the need of special filtration conditions. Further careful aliquotation procedures allow skipping the need for any evaporation thus also allowing automatic sample clean-up.

5. Determination of patulin

5.1. Method development for a liquid chromatographic method

5.1.1. Introduction and scope of the work

The method of choice for determination of patulin (PAT) usually consists of high-performance liquid chromatography (HPLC) coupled with ultra-violet (UV) detection (*50*) and has already been applied to different matrices, with the clean-up being a critical step in the determination of low levels. Several methods have already been evaluated for the determination of PAT. One method, which includes a repetitive liquid-liquid extraction (LLE), has been validated with a quantification limit at 25 µg/kg and has been adopted by AOAC International (*101*). Another simple and rapid method uses a single extraction with a so-called hydrophilic-lipophilic balanced polymer (*44*). MycoSep® columns are also used for clean-up, either alone or in combination with common reversed-phase columns (*102*). Other methods involve extraction with Extrelute® or purification with Sep-Pak® Florisil cartridges (*165*). Up to now, no method has been collaboratively tested, to our knowledge, under the conditions required for acceptance for future CEN adoption (*138*).

Legislative limits in the moment of these experiments were set for PAT according to European Regulations (*166*) as follows:

- Fruit juices, fruit nectar and concentrated fruit juices at 50 µg/kg.
- Spirit drinks, cider and other fermented drinks derived from apples or containing apple juices at 50 µg/kg.
- Solid apple products (compote, puree) at 25 µg/kg.

In the case of foodstuffs intended for infants, the limit was set at 25 µg/kg, but at the same time a level of 10 µg/kg was envisaged for these products depending on the availability of a suitable method for the precise determination of PAT (*167*). Therefore extensive method development was carried out within the framework of this thesis until promising method performance criteria at a level of 10 µg/kg was achieved with a method involving extraction of a portion of apple juice or puree with ethyl acetate-*n*-hexane (6+4, v/v). The sample extract is then centrifuged, passed over a silicagel column for clean-up and evaporated. The

residue is finally re-dissolved in 0.1 % acetic acid solution. The separation and determination of the PAT was performed by reversed-phase high performance liquid chromatography (HPLC) and detection by ultraviolet (UV) absorption at 278 nm. For details see Annex 7 - Solid Phase Extraction Method A. As a result, with this method A, a rapid and simple extraction and clean-up was obtained, which still allows the determination of PAT at the target level of 10 µg/kg with a sufficient precision ($RSD_r \leq 30\%$, $RSD_R \leq 40\%$, Recovery 50 - 120%).

Additionally, due to the large number of laboratories that intended to participate in the validation process, the participants were split into two groups and an additional method B was validated. This method B is a slightly modified version with the same principle as the one previously published (*101*). The main modifications related to the aliquotation. The PAT is extracted three times from the juice or the de-pectinated puree with neat ethyl acetate. The combined ethyl acetate phases were re-extracted with sodium carbonate solution and evaporated. The residue was then re-dissolved in 0.1 % acetic acid solution and separated by HPLC as in method A. For details see Annex 7 - Liquid Liquid Extraction Method B. Both methods were tested for the determination of PAT in apple juice and fruit puree at the proposed European regulatory limit of 10 µg/kg.

5.1.2. Test materials for collaborative study

The following products were purchased from local Belgian food supermarkets or were received free of charge from suppliers: various brands of apple juice, blueberries, fruit puree for infants, and fresh apples for compote. Contaminated blueberries and *P. expansum* inoculated apple pieces were kindly provided by Dr. Monica Olsen (National Food Administration, Sweden).

For the collaborative trial, apple juice (juice) and fruit puree (puree) was produced from the above-listed materials. In the case of juice several commercial brands of juice were blended, after they were confirmed to be free of PAT (<2 µg/kg).

For the production of PAT-free puree, single batches were prepared from 4 kg of freshly prepared apple puree from fresh mould-free apples, 0.5 kg of PAT-free blueberries and 3 kg of a commercially available fruit puree for infants, containing in decreasing amounts: apple,

banana, lemon, wheat biscuits, wheat syrup, whole milk and vegetable oils. The quantitative composition of this preparation was not given on the label of the material.

To obtain naturally contaminated material, a highly contaminated apple juice was prepared by inoculation of two litres of apple juice with *P. expansum* isolated from inoculated apple pieces. The inoculated juice was placed at 25 °C for a period of five days and resulted in a level of 130 mg/kg PAT. This contaminated juice was filtered over membrane filters of 0.45 µm and 0.2 µm in order to separate the juice from any spores, thereby minimising the risk of further mould growth. After a dilution of 1/1000 of this highly contaminated juice with PAT-free juice this diluted juice (130 µg/kg PAT) was used for blending with the test matrices in order to obtain the desired target concentrations. Blending of the contaminated juice with the test sample materials was carried out with a 750-Watt mixer at high speed for 15 min. The materials were subsequently filled into 50 mL containers and frozen at -18 °C. The filled containers were kept at this temperature until analysis for homogeneity or dispatch for collaborative trial testing. An amount of 7.5 kg for each contamination level and 20 kg for blank material was prepared for each matrix.

5.1.3. Homogeneity testing of the collaborative study materials

According to generally accepted procedures for homogeneity testing, every 10^{th} sample had been taken from the sequence and was subjected to analysis. The number of the first glass from which the sampling started was randomly selected for each material. As an example, puree with the target content of 10 µg/kg PAT was checked by taking the third, the 13th, the 23rd … sample. The content of each single container was split into two equal parts after mixing and then analysed. This resulted in a duplicate set of values for each container (1st and 2nd analysis). For the homogeneity study the test materials were analysed with method A (Annex 7 for details).

Unlike in other studies, no bulk homogeneity was determined, as the puree could not be sterilised without altering the composition by formation of large amounts of 5-hydroxymethylfurfural. Thus, once prepared, it had to be instantly processed by blending and packing. However this did not pose any problems, since the homogenisation of soft/wet sample materials (i.e. puree) is easier than dry-milled samples.

The individual results from the homogeneity testing are shown in Tab. 27 and 28.

Table 27. Individual results from the analysis of the randomly selected containers of apple juice.

Level	Juice1 [µg/kg]		Juice2 [µg/kg]		Juice3 [µg/kg]	
Sample	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**
1	3.47	3.80	7.33	7.78	12.87	11.39
2	3.65	3.61	7.78	7.67	13.18	12.84
3	3.91	3.61	7.11	9.48	13.41	11.81
4	3.36	3.69	10.37	7.81	13.59	-
5	4.38	4.09	8.00	7.41	13.18	10.82
6	3.65	3.40	7.78	7.59	13.59	12.27
7	4.09	3.43	7.93	7.74	12.91	10.75
8	3.40	3.61	7.89	7.67	12.84	10.29
9	3.61	3.29	9.07	7.93	13.14	-
10	2.96	3.51	10.56	7.89	9.11	11.58

Data sets in shaded fields were not taken for statistical analysis (ANOVA).

Table 28. Individual results from the analysis of the randomly selected containers of puree.

Level	Puree1 [µg/kg]		Puree2 [µg/kg]		Puree3 [µg/kg]	
Sample	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**
1	2.49	2.99	6.57	6.38	9.74	10.01
2	3.03	2.83	6.69	6.92	10.05	10.20
3	3.03	2.64	6.22	6.53	14.47	10.71
4	2.80	3.06	5.37	6.34	10.40	10.98
5	2.72	2.80	6.57	6.65	12.45	7.76
6	-	-	6.88	6.49	8.30	-
7	-	-	6.65	6.92	10.47	10.44
8	-	-	7.11	7.61	10.98	9.50
9	-	-	6.80	4.72	10.98	11.21
10	-	-	6.69	5.30	10.78	-

Data sets in shaded fields were not taken for statistical analysis (ANOVA).

Data analysis for testing the homogeneity was performed by an analysis of variance (ANOVA), allowing the partitioning of the whole variance into the individual components of variability. In this study 10 samples were taken for analysis in duplicate. In cases where interferences were observed in the chromatogram, this led to the exclusion of the corresponding test set. In the case of the low-contaminated puree material only 5 duplicates were analysed, as problems occurred during clean-up that led to unsuitable analytical results

in the last five sets. Nevertheless this did not pose any problems, as ANOVA can also be carried out with a smaller number of test sets.

The following variances and standard deviations can be calculated:

- Overall variance: $\sigma^2_{Total} = \sigma^2_{between} + \sigma^2_{within}$
- Within-container standard deviation $= \sqrt{Within-Group-Variance} = \sqrt{\sigma^2_{within}}$

Since the homogeneity within the containers can be considered as negligible this component reflects method precision.

- Between-container standard deviation $= \sqrt{\frac{(\sigma^2_{between} - \sigma^2_{within})}{2}} = \sigma_s$

This component reflects the heterogeneity of the material and can only be calculated if the F-test of the ANOVA indicates a significant difference of $\sigma^2_{between}$ and σ^2_{within}.

A batch is regarded as homogenous if the calculated F-value with a confident level of 95 % (Tab. 29 and 30, 2nd column) is below the tabulated percentile point for the F-distribution and when those coefficients of variance (CVs) are not significantly ($F_{95\%}$-test) different to the normal method RSD. According to the results from the F-test, all materials can be considered as homogeneous (Tab. 27 and 28).

Formulas and definitions from this section have been taken from (*168*).

Table 29. ANOVA on the patulin content in selected containers of apple juice prepared for the homogeneity test.

Target level [µg/kg]	F	F crit. (P = 5 %)	CV [%]
5	2.09	3.02	8.8
10	0.70	3.02	11.7
15	0.81	3.50	10.6

Table 30. ANOVA on the patulin content in selected containers of fruit puree prepared for a homogeneity test.

Target level [µg/kg]	F	F crit. (P = 5 %)	CV [%]
5	0.33	5.19	6.7
10	1.24	3.02	10.2
15	0.63	3.50	13.3

An acceptable analytical coefficient of variance for the establishment of the in-house performance characteristics was regarded to be ≤ 15 %, on the basis that this value is sufficiently below the minimum required repeatability (RSD_r) from the stipulated legislation (Tab. 41).

The mean content of PAT for the juice and the puree materials calculated from the homogeneity study are shown in Tab. 31 and 32.

Table 31. Mean content of the juice materials using method A.

Material	Target content [µg/kg]	Average content from the homogeneity test [µg/kg]
Juice1	5	3.6
Juice2	10	8.1
Juice3	15	12.2

Table 32. Mean content of the puree materials using method A.

Material	Target content [µg/kg]	Average content from the homogeneity test [µg/kg]
Puree1	5	2.8
Puree2	10	6.5
Puree3	15	10.5

Since the analysis of the highly contaminated apple juice (130 mg/kg PAT), which was used for adjusting the PAT content in the various test materials could be performed by direct injection of the diluted juice without any sample clean-up, it could be assumed that the obtained value was the best estimate of the real value. Neglecting aliquotation errors during the blending process, the recovery values for the method used could be directly obtained from the target value and the mean value from the homogeneity test (Tab. 31 and 32) and show that the method also fulfilled this criterion stipulated by European legislation (*69*) in the in-house experiments.

5.1.4. Method development

5.1.4.1. Inventory of existing methods

Several methods have been evaluated as candidates for the determination of PAT at the targeted concentration of 10 µg/kg. At present only one method has shown in a previous collaborative trial to be able to quantify PAT at 25 µg/kg (*101*). The principle of this method is based on a repetitive liquid-liquid extraction (LLE) followed by liquid chromatography (HPLC). One drawback of this method is that it is time and labour consuming. However

with some changes, it is very likely possible to determine PAT even below the concentrations previously tested in the collaborative trial with satisfactory results. As a matter of fact the sample clean-up procedure of this candidate method includes a concentration step that increases the PAT concentration 20 fold at injection for HPLC compared to the original juice. This is a concentration that should easily allow the determination of PAT at the aimed target level. However, other method-related factors must have contributed to the relatively high variance in results as they were obtained in the previously conducted collaborative trial.

Other candidate methods involve solid-phase extraction (SPE) followed by HPLC. Recently several approaches have been published with promising results. One very simple and rapid method uses a single extraction with a so-called hydrophilic-lipophilic balanced polymer (*44*). Others use MycoSep® columns for clean-up or combinations thereof with common reversed-phase columns (*102*). At present, none of these methods have been collaboratively tested to our knowledge under conditions as required for acceptance by CEN (*169*).

One main difference between SPE and liquid-liquid extraction (LLE) methods is that during LLE the main interferences are removed by re-extraction of polyphenols and phenolic acids under alkaline conditions. On the other hand this is a critical step as the lactone ring of PAT is subject to degradation under alkaline conditions. When SPE is used, the interferences are not removed in such a powerful way therefore more care has to be paid in order to get interference free chromatograms.

5.1.4.2. In-house testing

The previously collaboratively tested method had resulted in method performance indicating that the method - if used as is - is unlikely to give suitable results for contamination level of 10 µg/kg especially in the requested matrices such as purees. Strategies to decrease the reported RSD_R values obtained in the previous study are necessary to be discussed to further evaluate the suitability of LLE based methods. It can be assumed that the LLE step and the dissolution of the residue after evaporation might be likely the sources of errors. However no data on this is available yet. One problem that occurs frequently when products other than juice are analysed is that no clear separation of the liquid layers is obtained during extraction and a centrifugation step is necessary. Centrifugation however is difficult when repetitive LLE is performed and was therefore not considered as a strategy.

The most recent and initially most promising SPE method (*170*) has been tested in-house according to the procedure given in the literature. Despite its appealing simplicity, it was not possible to achieve interference free chromatograms. Another method that is comparably simple and fast (*102*), which uses MycoSep® column clean-up in combination with common reversed-phase columns, has been tested also in our laboratory. Similar to the results above no single clear PAT peak could be obtained with this method.

These facts lead to the initiative to start with new strategies. One strategy was the use of polyphenol binding polymers such as polyvinylpyrrolidone, polyvinylpolypyrrolidone or polyamide to remove interfering compounds such as polyphenols from juices. Indeed such polymers have been reported previously to have no effect on the level of PAT (*38, 171*). Despite these different approaches to purify the juice, no useful result was obtained. Either not enough interferences were removed, or losses of PAT occurred, indicating that this procedure might be useful for technological uses but not for analytical procedures.

Another strategy was to use a modified single LLE procedure, followed by a clean-up with a suitable SPE. Therefore a LLE was used, with the addition of water free sodium sulfate to the extraction mix. Sodium sulfate binds water in its crystal structure and has been reported previously for such use in pesticide extraction. It excludes the water from the extraction, forcing the PAT transfer to the ethyl acetate phase.

As the MycoSep® columns are not suitable for the clean-up procedures with ethyl acetate, these columns could not be tested. This led to test other column types filled with strong-anion-exchange resin, aluminium oxide, polyvinylpolypyrrolidone, polyamide, silicagel and in-house made columns.

Strong-anion-exchange, polyvinylpolypyrrolidone and polyamide clean-up procedures have been found unsuitable. More promising results were obtained with self-made neutral and basic aluminium oxide columns; no significant difference was observed between neutral or basic aluminiumoxide.

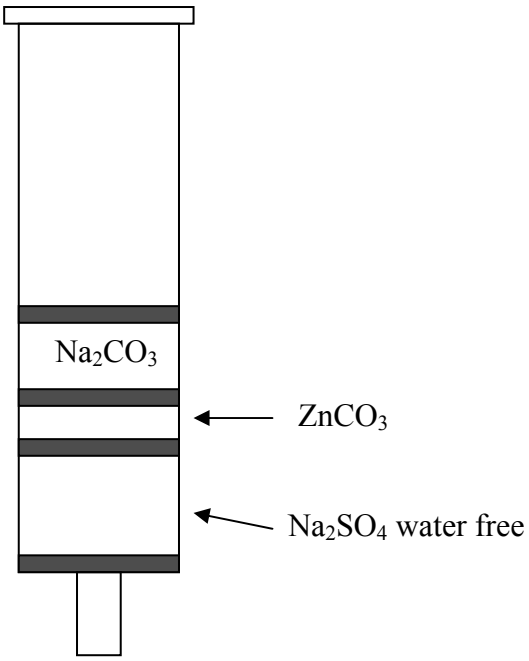


Figure 17. Design of the self-made clean-up column for extraction of PAT from apple products. Each layer is separated by a frit.

Extensive testing has been made with a multi-layer column (Fig. 17) containing a layer of sodium carbonate, the substance used for the re-extraction of interferences in LLE, basic zinc carbonate and water free sodium sulfate. In fact sodium carbonate columns alone already showed a significant clean-up capacity, while the zinc carbonate had additional benefits removing more interferences. It is assumed that the zinc carbonate, which is in fact a mixture of zinc hydroxide with approx. 60% zinc carbonate has the ability to bind interferences stronger than sodium carbonate alone. The sodium sulfate layer was included to remove any water residues from the extract and to avoid the transfer of any alkaline substances that can cause problems in the evaporation step that follows this clean-up procedure. For evaporation as a matter of fact it is required to change from ethyl acetate to 0.01% acetic acid and to concentrate the extract.

Satisfactory results were obtained with this column for a wide range of products, even those containing vitamin mixes and a variety of juice-mixes e.g. multivitamin and multifruit juices. An example of a chromatogram is displayed in Fig. 18. However, it was found that the performance of the clean-up procedure was strongly dependent on the way the column was produced. This became evident when additional laboratory technicians had to prepare these SPE columns according to a detailed instruction and to analyse extracts with these

columns. Therefore it would have been very critical when participants would have to prepare their own columns, because no supplier for such columns exists at present.

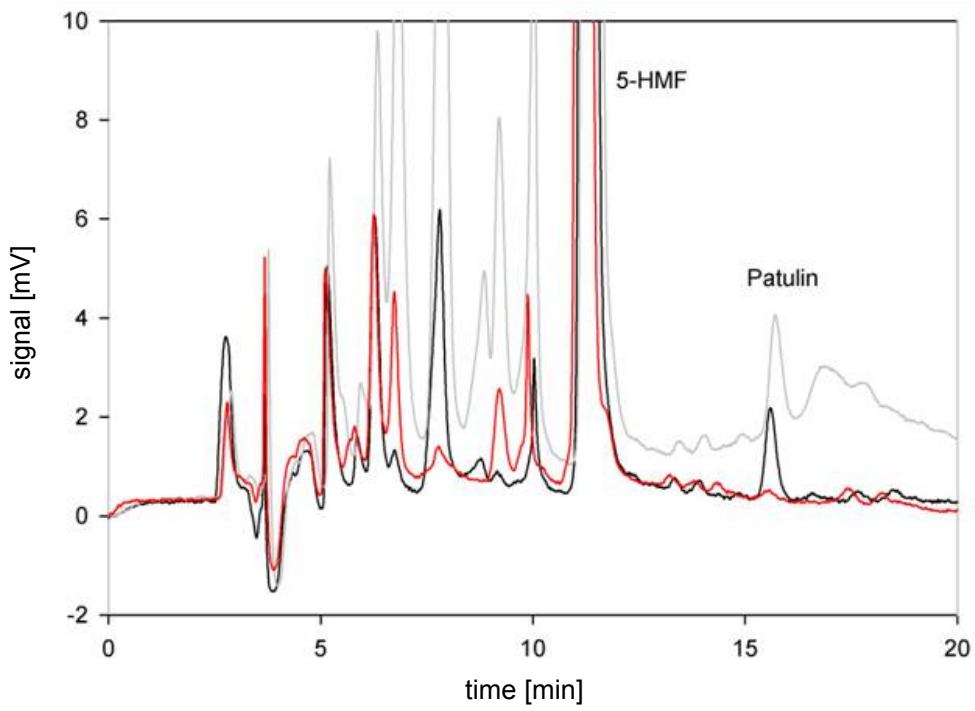


Figure 18. Three chromatograms of a blank apple juice (red line), and the same juice spiked at 10 µg/kg with PAT (grey and black). In case of the blank (red) and one spike (black) a combined Na_2CO_3/$ZnCO_3$ clean-up column was used. An additional clean-up (grey) was performed only with the use of the Na_2CO_3 layer.

Silicagel SPE columns were the following alternative for their suitability to purify PAT-containing extracts, as this column type is commercially available. Such a procedure has been described more than three decades ago (*172*) and was even tested in a collaborative trial in 1988 (*173*). Promising results were achieved in combination with the selected extraction solvent, ethyl acetate-*n*-hexane (60+40, v/v).

The analytical method consists of a sample preparation and a chromatographic step, and both must be seen as interacting combinations. Thus, a very powerful sample clean-up can ease the demands on the chromatographic system as no interference might occur. On the other hand a good chromatographic separation might ease the demands on the sample clean-up, as interfering compounds might be separated from the PAT peak in the chromatogram.

In combination to the clean-up procedures mentioned above, several HPLC separation principles have been tested. The first approach was to test different mobile phases in combination with RP-18 reversed-phase columns. As PAT is a very polar compound, the

separation on RP-18 columns is not very easy. This has been already described in the literature. However due to the wide availability of this column type it is favoured for HPLC mycotoxin analysis.

Different mixes of acetonitrile and tetrahydrofuran with water (with and without acids to stabilise the pH) were tested with a special RP-18 column that is designed to achieve good separations in mobile phases with high water content (>95%). Such high water contents are required, as an increase of acetonitrile or any other organic solvent would not allow a sufficient retention of PAT for separation.

The concentrations tested for acetonitrile ranged from 0.5 % to 5% and for tetrahydrofuran from 0.2% to 0.8 %. Acids tested as modifiers were perchloric acid, acetic acid and boric acid. Also sodium tetraborate was tested, as it is known to form complexes with various organic compounds containing hydroxyl groups.

In some cases suitable chromatograms were obtained from apple juices and purees with the already described mobile phases in the methods available in the literature. Some apple products caused interferences in the chromatogram that did not allow any quantification of PAT at the intended level. This problem could also not be solved by changing the mobile phase.

Nevertheless the influence of such interferences cannot only be seen from the view of chromatographic separation as they had different magnitudes, depending on the clean-up procedure used.

After extensive testing with the above mentioned combinations of procedures no major improvement was achieved concerning separations on RP-18 HPLC columns. Therefore it was decided to stop the investigations. This is because chromatographic separations are in fact different from laboratory to laboratory as a matter of different instruments, age of the column used, etc. and therefore it might not be possible to translate small improvements form one laboratory to another.

Several problems have occurred during chromatography, which could not be investigated due to the time frame given. One main problem was the occurrence of a ‘saw-tooth’ shaped peak in the retention time window close to PAT, which occurred randomly in samples and standards (Fig. 19). The reason for this could not be traced but is believed to be a system

intrinsic phenomenon as it also occurred when samples were analysed with already established procedures, while such type of interference had never been reported before.

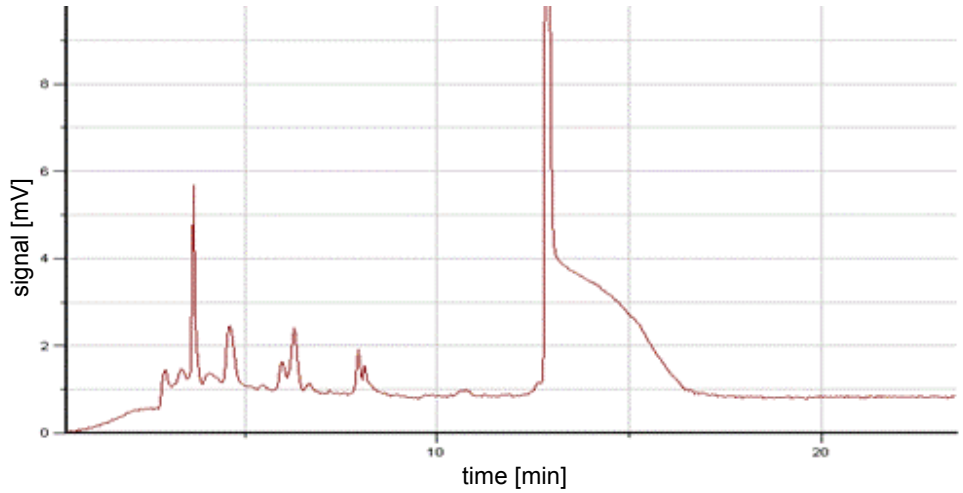


Figure 19. Example of chromatogram with 'saw-tooth' peak corresponding to a sample of patulin.

A totally different approach was to switch from RP-18 reversed-phase separation to another more favourable separation principle. As mentioned above PAT is a very polar compound, which results in little retention on RP-18 columns, even in 100% aqueous mobile phases. This led to tests with a ZIC®-HILIC column (Merck SeQuant AB). This column has a stationary phase attached to porous silica and normally results in good retention behaviour for polar or ionic substances, even when mobile phases with a high content of organic solvents are used. Initial tests with pure PAT standards and different mobile phases gave no suitable result (no peak obtained). Despite the fact that this or other chromatographic systems, such as the use of cyano- (CN) or amino- (NH2) phases, might have been promising, this strategy was not further followed. This was due to the time restrictions of the project and the fact that only one HPLC system was available for this project at that time.

5.1.5. Organisation of the collaborative study

A total of 43 collaborators from 17 different countries (Europe, Japan and Brazil) were invited to participate in the collaborative trial. These collaborators represented a cross-section of government, food control, university and food industry affiliations.

This group was split into two sections of 20 and 23 to test method A and method B respectively. Six participants did not return results and were deleted from the list. From the

remaining 37 participants, 34 reported results that included the documentation that was requested (17 for each method).

Despite the fact that the developed method A turned out to be faster and simpler than previously validated methods and performed well in-house, it was decided to validate the two different methods A and B in parallel. This strategy was supported by the following facts:

- This number of invited laboratories exceeded, by more than the double, the number that was necessary to carry out a method validation. Therefore a single large study with more than 40 participants did not promise any added value.
- Method validation normally requires that participants have sufficient experience in the method to be tested, as a new 'unknown' method might result in higher and unacceptable precision data. Unfortunately a large proportion of the laboratories had, mainly due to the tight time frame for this project, only very little time to gain experience with method A, while experience with method B already existed in most cases.
- Prior to the trial, each participant received a questionnaire and a short description of method A. Participants were asked for their previous experience in PAT analysis, current detection performance parameters, to report results and experience with method A and also to indicate which kind of method principle they preferred. Most participants answered, even though it was not obligatory in order to participate in the final collaborative trial.

For the collaborative trial study each participant received:

1. Eight coded sample containers with blind duplicates at four concentration levels plus four 'blank'-labelled ones per matrix (juice and puree) for spiking.
2. One amber vial marked 'PAT Standard' containing PAT, which was to be employed as the calibrant PAT solution described in the method.
3. Eight amber vials marked 'Spike solution A, B, C and D' and 'Spike solution 1, 2, 3 and 4' to be used for spiking procedures.
4. A copy of the method.
5. A spiking protocol.
6. A technical note concerning the chromatography of PAT.
7. A 'Collaborative Study Materials Receipt' form.
8. A unique identification code for on-line reporting.
9. Method A only one single unit of 24 SPE silica gel columns.

Each participant was required to prepare one extract from each container and perform the analysis by HPLC. Additionally each participant was required to spike the four indicated 'blank' materials using the provided 'Spike solutions A, B, C and D' for juice fortification and 'Spike solutions 1, 2, 3 and 4' for puree fortification.

5.1.6. Experimental

The analytical methods that have been used in this study, the materials and the instructions that were given to the participants can be found in Annex 7.

5.1.7. Results and Discussion

5.1.7.1. Collaborative trial results

All data submitted for the study are presented in Tab. 33 - 36.

The data are given as individual pairs of results for each laboratory identified with the login codes that were used for the on-line reporting. Blank samples were spiked with 10 µg/kg and 25 µg/kg of PAT identified as sample '10' and '25'. Samples 'bl', '0.5', '1.0' and '1.5' were blind duplicates of 'blank' and of naturally contaminated material with approx. 0.5x, 1.0x and 1.5x of the target level of 10 µg/kg PAT. The results are also presented as Youden plots in Fig. 1 - 20 (Annex 8). The Youden plots confirmed the validity of the identification of outlier laboratories shown in Tab. 33 - 36 and additionally show some trends that may not otherwise be apparent from studying these tables alone. The Youden plots did not show any inconsistencies in the statistical handling of the data used to generate the precision data from the collaborative trial results.

Table 33. Individual results of patulin concentration in the fruit juices analysed with method A.

	PAT concentration [µg/kg] (target values)											
Lab ID	**10[1]**	**10[1]**	**25[1]**	**25[1]**	**bl**	**bl**	**5[2]**	**5[2]**	**10[2]**	**10[2]**	**15[2]**	**15[2]**
1752003	8.65	8.73	20.22	20.66	<4.80	<4.8	<4.8	<4.80	6.10	5.70	9.77	10.8
1862003	7.30	6.90	17.70	18.20	0.40	0.50	2.00	1.50	4.90	4.80	5.20	7.30
1712003	7.68	8.05	16.81	18.00	1.31	1.30	2.53	2.85	6.20	6.04	10.81	10.8
1622003	9.85	9.34	6.20	11.32	<2.40	<2.4	3.75	3.69	8.12	6.12	15.06	13.8
1872003	6.55	5.95	13.73	12.15	<1.00	<1.0	2.84	2.74	6.16	5.46	9.02	8.58
1572003	12.89	11.67	22.35	229	302	32.72	6.36	5.15	1.01	32.72	17.92	13.8
1832003	6.67	6.46	17.08	15.72	0.71	0.56	2.50	2.47	6.08	5.40	9.07	9.25
1822003	7.64	7.94	17.86	17.09	0.75	0.66	2.62	2.54	6.42	5.52	9.73	11.9
1792003	9.02	7.16	5.14	8.87	8.98	10.07	12.73	12.45	15.31	14.03	11.64	13.0
1772003	1.90	2.70	13.40	12.20	<1.00	<1.0	1.70	1.00	<1.0	<1.0	10.40	7.80
1742003	-4.80	7.35	16.47	18.63	<4.80	<4.8	5.03	<4.80	6.06	<4.8	12.42	10.8
1702003	6.70	6.40	12.70	16.10	0.00	0.00	3.00	2.70	4.50	5.70	8.30	10.1
1662003	3.57	4.33	17.08	8.69	0.09	0.09	1.55	1.14	3.12	0.00	7.74	7.30
1642003	5.00	5.40	16.30	19.30	0.70	0.90	2.40	2.80	4.40	5.20	10.40	8.30
1892003	9.70	8.30	20.30	24.30	<3.50	<3.5	3.70	4.40	9.70	6.90	13.70	12.2
1632003	1.90	1.80	3.50	3.70	0.00	0.00	0.00	0.00	1.70	1.60	2.50	1.50
1552003	5.97	6.50	14.9	13.3	1.08	1.10	3.00	2.74	6.16	5.70	9.60	9.98

Invalid data that was removed prior statistical analysis.
Outliers identified by statistical analysis.
[1] Fortified material.
[2] Naturally contaminated material.

Table 34. Individual results of patulin concentration in the fruit purees analysed with method A.

	PAT concentration [µg/kg] (target values)											
Lab ID	**10[1]**	**10[1]**	**25[1]**	**25[1]**	**bl**	**bl**	**5[2]**	**5[2]**	**10[2]**	**10[2]**	**15[2]**	**15[2]**
1752003	6.58	6.18	16.90	17.79	<4.80	<4.80	<4.8	<4.80	6.04	6.13	9.61	9.33
1862003	6.30	6.10	13.90	15.50	<0.30	<0.30	2.70	12.80	4.20	4.00	4.70	5.60
1712003	8.09	7.98	19.14	18.61	<0.77	<0.77	4.12	4.20	7.42	7.48	10.6	10.9
1622003	8.83	8.92	21.22	18.98	<2.40	<2.40	4.37	4.77	8.77	8.75	12.9	12.8
1872003	6.15	5.99	15.67	16.82	<1.00	<1.00	3.45	3.28	6.62	6.58	9.88	9.69
1572003	8.62	9.21	20.27	21.96	3.71	0.35	4.85	5.13	8.54	8.17	13.0	12.7
1832003	6.62	6.66	18.85	16.59	<0.01	<0.01	3.03	2.95	6.11	6.18	8.48	8.60
1822003	5.81	6.06	17.39	16.10	0.51	0.62	3.43	3.62	7.03	6.55	9.33	10.2
1792003	7.85	7.87	8.80	8.90	11.97	3.36	9.77	11.54	12.47	4.82	10.1	0.00
1772003	8.10	8.40	19.10	21.00	<1.00	<1.00	1.50	1.20	7.30	6.10	10.5	11.2
1742003	6.65	4.95	17.69	17.80	<4.80	<4.8	<4.8	<4.80	7.46	6.42	10.4	9.85
1702003	11.50	7.60	19.00	20.70	0.00	0.00	4.00	3.50	7.90	8.70	10.4	8.20
1662003	4.50	2.38	13.81	12.63	<0.10	<0.10	0.74	2.22	3.92	4.76	6.34	5.32
1642003	7.40	7.00	19.10	17.90	0.50	0.20	3.70	3.70	7.10	7.20	10.5	10.2
1892003	8.50	8.20	20.70	14.50	<3.50	<3.50	4.50	4.40	8.30	7.70	12.0	11.1
1632003	0.60	1.20	2.80	3.30	0.00	0.00	0.50	0.70	1.20	1.30	0.90	1.60
1552003	6.76	7.63	13.8	14.4	2.71	3.92	5.09	4.95	8.60	8.77	8.64	11.4

Invalid data that was removed prior statistical analysis.
Outliers identified by statistical analysis.
[1] Fortified material.
[2] Naturally contaminated material.

Table 35. Individual results of patulin concentration in the fruit juices analysed with method B.

	PAT concentration [µg/kg] (target values)											
Lab ID	**10[1]**	**10[1]**	**25[1]**	**25[1]**	**bl**	**bl**	**5[2]**	**5[2]**	**10[2]**	**10[2]**	**15[2]**	**15[2]**
1882003	7.57	7.40	18.53	19.72	<2.8	<2.8	3.51	2.08	5.94	5.96	10.41	9.35
1972003	9.00	6.58	17.45	19.38	1.81	3.48	6.76	2.28	7.74	3.90	8.52	12.16
1602003	11.32	9.65	23.11	24.23	<1.0	<1.0	4.03	4.14	7.96	8.52	13.57	13.50
1672003	13.20	11.80	29.00	26.80	0.00	5.00	7.40	5.20	11.40	8.00	20.40	19.60
1682003	8.90	8.20	18.00	16.00	<0.8	<0.8	3.40	3.00	7.00	6.50	11.80	13.20
1722003	10.10	8.10	20.70	18.70	0.00	0.00	3.00	3.40	7.40	8.00	12.60	12.50
1762003	6.70	5.40	13.80	10.40	0.00	12.00	8.80	32.60	5.10	23.60	6.10	10.10
1782003	7.30	7.40	20.50	20.60	2.90	2.10	2.30	4.60	8.90	8.40	13.50	14.40
1802003	17.40	13.20	23.30	24.00	0.00	3.60	5.30	10.30	2.70	2.80	15.70	26.90
1812003	9.00	9.80	22.70	22.50	2.40	2.50	3.80	3.80	7.50	8.20	13.70	14.00
1852003	7.61	6.79	20.06	20.44	0.00	0.00	1.76	1.61	5.15	4.18	10.68	15.23
1902003	11.40	11.40	24.71	25.00	0.00	0.00	5.10	5.30	8.40	8.20	13.90	14.80
1932003	8.39	8.72	21.25	22.58	<5.0	<5.0	3.71	3.97	8.51	9.12	15.24	14.59
1942003	8.00	9.40	20.00	20.10	-	-	-	-	-	-	12.20	13.30
1612003	11.40	12.70	25.80	24.10	1.25	2.79	9.98	9.64	10.90	19.30	17.10	15.10
1652003	3.41	6.07	17.01	0.00	<1.7	<1.7	0.00	<1.7	3.22	3.39	7.99	10.47
1842003	11.42	11.60	24.76	24.81	3.49	2.79	4.44	2.43	8.34	9.00	3.53	3.02

Invalid data that was removed prior statistical analysis.
Outliers identified by statistical analysis.
[1] Fortified material.
[2] Naturally contaminated material.

Table 36. Individual results of patulin concentration in the fruit purees analysed with method B.

	PAT concentration [µg/kg] (target values)											
Lab ID	**10[1]**	**10[1]**	**25[1]**	**25[1]**	**bl**	**bl**	**5[2]**	**5[2]**	**10[2]**	**10[2]**	**15[2]**	**15[2]**
1882003	7.73	7.32	19.79	21.17	<1.6	<1.6	3.23	3.60	5.20	6.60	10.79	10.50
1972003	19.83	17.58	43.15	42.04	1.37	1.35	5.72	2.67	7.53	9.00	12.66	11.78
1602003	8.61	8.05	21.68	21.64	<0.5	<0.5	4.26	3.78	7.96	8.06	12.95	12.90
1672003	27.60	23.60	39.40	39.40	22.40	22.00	16.60	16.20	23.20	26.80	20.20	31.60
1682003	7.10	6.40	18.10	18.40	<0.8	<0.8	3.10	3.30	7.60	7.30	11.80	11.80
1722003	7.90	7.60	19.80	20.50	0.00	0.00	3.50	3.80	7.80	7.70	10.80	11.60
1762003	7.20	6.50	11.90	21.20	7.30	8.20	3.70	5.50	8.00	9.10	12.30	6.20
1782003	3.80	3.90	9.20	8.70	0.00	0.00	0.00	0.00	2.50	0.00	3.50	3.90
1802003	0.00	0.00	52.50	40.00	22.10	32.20	0.00	25.00	31.40	26.90	0.00	31.90
1812003	3.10	9.00	3.10	23.90	4.80	4.60	4.40	4.20	9.20	9.50	14.00	12.70
1852003	6.72	7.76	15.22	0.00	0.00	0.00	2.69	2.44	6.20	6.04	10.78	10.64
1902003	21.40	20.50	38.50	34.70	24.30	15.70	15.20	16.60	21.60	19.60	22.80	18.00
1932003	8.93	9.91	22.29	21.13	1.02	0.70	5.08	5.10	8.65	9.09	14.01	13.42
1942003	16.10	13.40	39.00	34.10	-	-	-	-	-	-	11.60	10.60
1612003	13.80	15.50	27.70	21.50	3.98	4.08	7.96	8.70	8.12	13.30	17.60	16.90
1652003	6.73	6.27	16.11	15.43	6.65	<1.7	1.78	5.64	5.74	7.03	7.73	10.48
1842003	13.01	10.13	21.30	24.70	3.70	15.57	6.09	6.45	7.99	9.36	32.18	14.65

Invalid data that was removed prior statistical analysis.
Outliers identified by statistical analysis.
[1] Fortified material.
[2] Naturally contaminated material.

5.1.7.2. Statistical analysis of results

In some cases data were excluded from the statistical analysis. These cases were when either statistical evaluation was impossible because values were reported as zero or below the detection limit, or when data sets were identified as suspicious by the participants (sections 5.1.7.3 and 5.1.7.4). Other cases were when the accuracy of the data could not be resolved e.g. required calculations and chromatograms were not supplied for data sets. As a result, the number of invalid data sets was 4, giving valid data ranging from 13 to 15 laboratories for method A and 14 to 17 laboratories for method B.

Table 37. Results of an intercollaborative trial for the determination of patulin in apple juice using Method A.

Added [µg/kg][1]	No. of Labs$^{m(n)2}$	Average [µg/kg]	S_r [µg/kg]	S_R [µg/kg]	RSD_r [%]	RSD_R [%]	r [µg/kg]	R [µg/kg]	Horwitz value	Mean Recovery [%]
10	14 (0)	7.4	0.57	2.34	8.0	31.6	1.6	6.5	0.94	74
25	14 (0)	15.5	1.81	4.39	11.7	28.3	5.1	12.2	0.94	62
nc (0)	15 (0)	< 2	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]
nc (5)	12 (1)	3.0	0.36	1.17	12.2	39.5	1.0	3.3	1.03	n.a.[3]
nc (10)	11 (2)	6.0	0.89	1.18	14.3	19.8	2.4	3.3	0.57	n.a.[3]
nc (15)	15 (0)	10.7	1.29	2.60	12.1	24.3	3.6	7.3	0.77	n.a.[3]

[1]nc – naturally contaminated; [2]number of laboratories, where m = number of labs retained after outliers removed and (n) = number of outlier; S_R = Standard deviation for repeatability; S_r = Standard deviation for reproducibility.
R = reproducibility and r = repeatability are calculated according to the IUPAC Harmonized Protocol (*135*).
The parameters have been calculated using the Excel® template CLSTD.XLT (*136*).
n.a.[3] = not applicable.

Table 38. Results of an intercollaborative trial for the determination of patulin in fruit puree using Method A.

Added [µg/kg][1]	No. of Labs$^{m(n)2}$	Average [µg/kg]	S_r [µg/kg]	S_R [µg/kg]	RSD_r [%]	RSD_R [%]	r [µg/kg]	R [µg/kg]	Horwitz value	Mean Recovery [%]
10	13 (2)	7.4	0.26	1.09	3.5	14.7	0.7	3.0	0.44	74
25	13 (2)	17.9	1.04	2.25	5.8	12.5	2.9	6.3	0.43	72
nc (0)	15 (0)	< 2	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]
nc (5)	11 (2)	3.8	0.18	1.06	4.6	27.7	0.5	3.0	0.75	n.a.[3]
nc (10)	14 (1)	7.1	0.38	1.29	5.3	18.0	1.1	3.6	0.53	n.a.[3]
nc (15)	14 (0)	10.1	0.76	1.98	7.5	19.6	2.13	5.54	0.61	n.a.[3]

[1]nc – naturally contaminated; [2]number of laboratories, where m = number of labs retained after outliers removed and (n) = number of outlier; S_r = Standard deviation for repeatability; S_R = Standard deviation for reproducibility.
R = reproducibility and r = repeatability are calculated according to the IUPAC Harmonized Protocol (*135*).
The parameters have been calculated using the Excel® template CLSTD.XLT (*136*).
n.a.[3] = not applicable.

Precision estimates were obtained using a one-way analysis of variance approach according to the IUPAC Harmonized Protocol (*135*). Details of the average analyte concentration, the standard deviations for repeatability (RSD_r) and reproducibility (RSD_R), the number of statistical outlier laboratories, the Horwitz values and the percentage recovery are presented in Tab. 37 - 40. The collaborative trial results had previously been examined for evidence of individual systematic error ($p<0.025$) using Cochran's and Grubbs tests progressively (*135*).

Pairs of results that were identified as outliers are indicated with shaded background in Tab. 33 - 36. The maximum number of outliers identified was three laboratories (excluding the data for blank materials), giving acceptable data ranging from 11 to 15 laboratories for method A and 11 to 17 laboratories for method B.

Table 39. Results of an intercollaborative trial for the determination of patulin in apple juice using Method B.

Added [µg/kg][1]	No. of Labs$^{m(n)2}$	Average [µg/kg]	S_r [µg/kg]	S_R [µg/kg]	RSD_r [%]	RSD_R [%]	r [µg/kg]	R [µg/kg]	Horwitz value	Mean Recovery [%]
10	17 (0)	9.3	1.17	2.76	12.6	29.7	3.3	7.7	0.92	94
25	16 (0)	21.3	1.06	3.88	5.0	18.2	3.0	10.9	0.64	85
nc (0)	17 (0)	< 2	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]
nc (5)	13 (2)	4.1	1.54	1.92	37.6	46.9	4.3	5.4	1.28	n.a.[3]
nc (10)	14 (2)	6.9	1.03	2.27	14.9	32.7	2.9	6.4	0.97	n.a.[3]
nc (15)	16 (1)	12.4	1.45	3.88	11.7	31.3	4.1	10.9	1.01	n.a.[3]

[1]nc – naturally contaminated; [2]number of laboratories, where m = number of labs retained after outliers removed and (n) = number of outlier; S_r = Standard deviation for repeatability; S_R = Standard deviation for reproducibility.
R = reproducibility and r = repeatability are calculated according to the IUPAC Harmonized Protocol (*135*).
The parameters have been calculated using the Excel® template CLSTD.XLT (*136*).
n.a.[3] = not applicable.

Table 40. Results of an intercollaborative trial for the determination of patulin in fruit puree using Method B.

Added [µg/kg][1]	No. of Labs$^{m(n)2}$	Average [µg/kg]	S_r [µg/kg]	S_R [µg/kg]	RSD_r [%]	RSD_R [%]	r [µg/kg]	R [µg/kg]	Horwitz value	Mean Recovery [%]
10	16 (0)	11.0	1.57	6.30	14.2	57.1	4.4	17.6	1.81	110
25	14 (1)	24.7	2.54	10.21	10.3	41.3	7.1	28.6	1.48	99
nc (0)	17 (0)	< 2	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]	n.a.[3]
nc (5)	11 (3)	4.1	1.13	1.28	27.7	31.3	3.2	3.6	0.86	n.a.[3]
nc (10)	11 (3)	7.8	0.65	3.52	8.4	16.2	1.8	3.5	0.49	n.a.[3]
nc (15)	14 (2)	12.0	1.62	4.02	13.5	33.7	4.5	11.3	1.08	n.a.[3]

[1]nc – naturally contaminated; [2]number of laboratories, where m = number of labs retained after outliers removed and (n) = number of outlier; S_r = Standard deviation for repeatability; S_R = Standard deviation for reproducibility.
R = reproducibility and r = repeatability are calculated according to the IUPAC Harmonized Protocol (*135*).
The parameters have been calculated using the Excel® template CLSTD.XLT (*136*).
n.a.[3] = not applicable.

The parameters in Tab. 37 - 40 have been calculated using an Excel® template CLSTD.XLT (*136*). These values were compared with the performance requirements listed in Tab. 41 and summarised in Tab. 42 with remarks on the qualification of each contamination level tested. As a result, Method A fulfilled the requirements for recovery for both apple juice and apple puree at both 10 and 25 µg/kg levels. The requirements for precision (RSD_r and RSD_R) were fulfilled for apple juice down to 3.0 µg/kg patulin and for fruit puree down to 3.4 µg/kg patulin. In the case of Method B, it qualified for apple juice from a PAT contamination level of 6.9 µg/kg. For the determination of fruit puree the two fortification levels did not qualify (11.0 and 24.5 µg/kg) however indicating good overall recoveries for the respective levels. The reason for this could not be identified, as for juice and puree the same coded

fortification solutions were used and no comments were made that allowed the identification of any fortification problems.

Table 41. Acceptance criteria for analytical methods of patulin according to Commission Regulation (EC) N° 401/2006 (*69*).

Level [µg/kg]	PAT		
	RSD_r [%]	RSD_R [%]	Recovery [%]
< 20	≤ 30	≤ 40	50 - 120
20 – 50	≤ 20	≤ 30	70 - 105
> 50	≤ 15	≤ 25	75 - 105

Table 42. Method performance parameters obtained in the collaborative trial.

Method	Matrix	Target Value[1] [µg/kg]	Level[2] [µg/kg]	Obtained parameter			Qualified[3] YES/NO
				RSD_r [%]	RSD_R [%]	Recovery [%]	
Method A	Apple Juice	Nc (5)	3.0	12.2	39.5	-[4]	YES
		Nc (10)	6.0	14.3	19.8	-[4]	YES
		F (10)	7.4	8.0	31.6	74	YES
		Nc (15)	10.7	12.1	24.3	-[4]	YES
		F (25)	15.5	11.7	28.3	62	YES
	Fruit Puree	Nc (5)	3.8	4.6	27.7	-[4]	YES
		Nc (10)	7.1	5.3	18.0	-[4]	YES
		F (10)	7.4	3.5	14.7	74	YES
		Nc (15)	10.1	7.5	19.6	-[4]	YES
		F (25)	17.9	5.8	12.5	72	YES
Method B	Apple Juice	Nc (5)	4.1	37.6	46.9	-[4]	NO
		Nc (10)	6.9	14.9	32.7	-[4]	YES
		F (10)	9.3	12.6	29.7	94	YES
		Nc (15)	12.4	11.7	31.3	-[4]	YES
		F (25)	21.3	5.0	18.2	85	YES
	Fruit Puree	Nc (5)	4.1	27.7	31.3	-[4]	YES
		Nc (10)	7.8	8.4	16.2	-[4]	YES
		F (10)	11.0	14.2	57.1	110	NO
		Nc (15)	12.0	13.5	33.7	-[4]	YES
		F (25)	24.7	10.3	41.3	99	NO

[1]Nc - naturally contaminated, F - fortified; [2]Mean level as reported in the collaborative trial; [3]The qualification required that the performance parameters obtained in the collaborative trial fulfilled the requirements set in the Directive 2003/78/EC (*167*). Shaded fields indicate the parameters not fulfilling these requirements; [4]Materials for which no recovery data is given (marked with " – ") were naturally contaminated.

5.1.7.3. Comments from collaborative trial participants for method A

Comments were received via the on-line reporting sheets from 11 of the 17 collaborative trial participants. In all cases the participants regarded the method description as being adequate. For identification the participant numbers are reduced to the first 3 digits in the following descriptions.

Participant 166 reported that problems with the UV detector occurred and that quantification was problematic. Participant 163 reported consistently low values with an average factor of 4 below the mean value for each level but made no comments. As neither the requested chromatograms nor the calculation was supplied, it was decided to exclude this data set from statistical evaluation.

Some participants indicated that they had to adjust the mobile phase (participant 182) or even used another column type to achieve a better separation. As a result participant 189 used a "Synergy Luna RP18" HPLC column with a mobile phase gradient. No differences were observed for these two participants during statistical analysis. Participants 186, 157 and 174 reported interferences in the chromatograms, with respect to an unstable baseline. In the case of participant 157, who had this problem in the juice, PAT was baseline resolved after the collaborative trial by modifying the mobile phase to a lower organic solvent content and a higher content of acid. As a result, part of the reported values for juice was removed before statistical analyses.

5.1.7.4. Comments from collaborative trial participants for method B

Four out of the 17 participants made editorial comments. Six participants made comments on technical matters. Participant 197 used a diode array detector instead of a standard UV detector, but reported that due to the acidity and the low content of organic solvent no useful spectra could be obtained for verification. Participant 188 reported a shift in retention time for one sample (target level 15 µg/kg), but this value was not detected as outlier. Participant 178 reported the purification step to be not efficient for PAT separation and reported better values out of the scope of this trial with his in-house method. As a matter of fact two outliers were detected for this participant for the analysis of puree. Participants 181 and 180 reported interferences that may lead to misinterpretation of results. As a consequence results form participant 180 were excluded from the statistical analysis for puree. During statistical

analysis, one outlier was detected in juice (participant 180) and another in puree (participant 181), underpinning the observed statements by the participants.

5.1.7.5. Precision characteristics of the method

Due to differences in reporting limits for not detectable PAT the results for 'blank' materials were not analysed statistically. The results however indicated clearly that all participants could identify the blank pairs of samples as not containing detectable PAT or containing levels which were detectable but close to limits of determination.

The precision data for all samples are summarised in Tab. 42. Based on results for spiked samples (blind pairs at two levels), as well as naturally contaminated samples (blind pairs at three levels), the relative standard deviation for repeatability (RSD_r) in juice ranged from 8.0 - 14.3 % for method A and from 5.0 - 37.6 % for method B. For puree these values ranged from 3.5 - 7.5 % for method A and from 8.4 - 27.7 % for method B. The relative standard deviation for repeatability (RSD_R) in juice ranged from 19.8 - 39.5 % for method A and from 18.2 - 46.9 % for method B. In puree, these values ranged from 12.5 - 27.7 % for method A and from 16.2 - 57.1 % for method B.

The recovery values for PAT in apple juice derived from the spiked samples were found to range from 37 - 123 % with an average of 73 % for method A and from 47 - 124 % with an average of 88 % for method B. For puree the recovery values ranged from 60 - 87 % with an average of 70 % for method A and from 37 - 207 % with an average of 110 % for method B.

5.1.7.6. Interpretation of results

The acceptability of the precision characteristics of the method was assessed on the basis of Commission Regulation (EC) No 401/2006 (*69*) by comparing the obtained RSD_R values at the various levels with the legal limits given in Tab. 41.

Both methods gave satisfactory results for samples above 25 µg/kg while the advantage of the Method B was that recoveries were higher and close to 100 %. Nevertheless, the new developed method showed superior precision for less contaminated samples while the recovery was still at an acceptable, although lower range.

5.1.8. Conclusions

The results of this laboratory intercomparison showed precision characteristics (RSD_r, RSD_R and recovery) that fulfilled the criteria at very low contamination levels that have been stipulated by European legislation (Tab. 41). In conclusion, method A qualified for both matrices (juice and puree) at all levels, while method B qualified for juice but only partially for puree. A typical chromatogram of an apple juice sample spiked at the level of 10 µg/kg and analysed with method A is showed in Fig. 20. Good separation of the PAT peak in an area free of interferences was achieved.

CEN is currently publishing a standard method on the basis of method A.

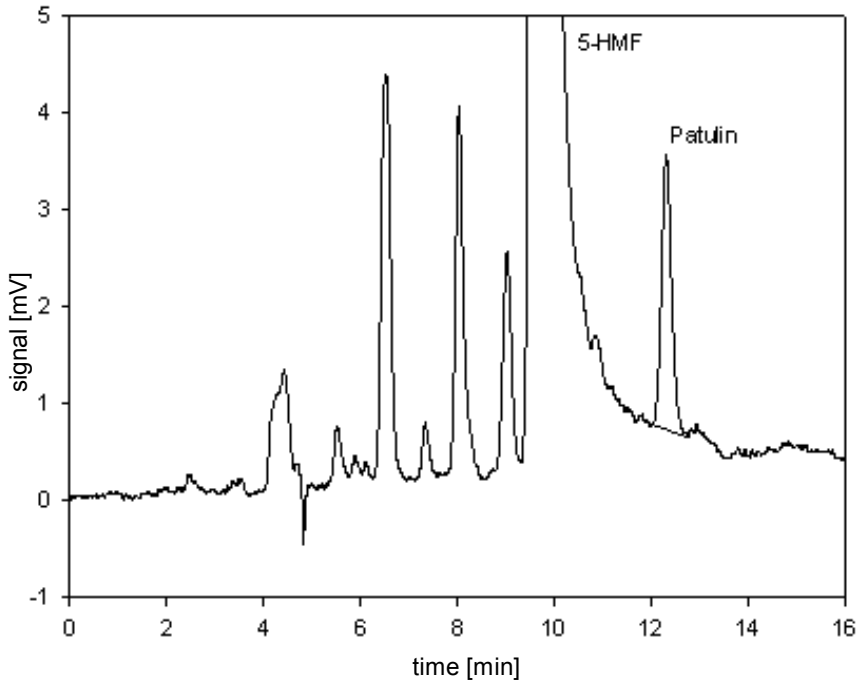


Figure 20. Typical chromatogram after sample preparation with the method described for apple juice sample spiked at 10 µg/kg.

5.2. Method development for a liquid chromatographic/mass spectrometry method

5.2.1. Introduction and scope of the work

One of the biggest improvements in the determination of patulin has been the introduction of commercial ^{13}C-labelled patulin as internal standard, which increases the possibility of exact quantification and has shown to improve the precision of the measurements. Some of the first approaches using it have been developed by Rychlik *et al*. (*104, 105*). The employment of GC/MS methods for the determination of patulin has been recently described in the literature (*49*) performing excellent quantification results (LOD of 0.4 µg/kg for apple juice). Of great importance are the LC/MS/MS multi-analyte methods, as they offer an idea of the variety of mycotoxins occurring in food products and this is an excellent tool as assessment of the hazards that these compounds pose to humans and animal health. Most of them are based on LC/MS/MS being the first method for *Penicillium* toxins published the one of Rundberget and Wilkins (*119*). A more recent simultaneous determination of 87 analytes in food and feed matrices is described by Sulyok *et al.* (*119*). This method is based on electrospray ionisation tandem mass spectrometry. The values obtained for the LOD are below the corresponding maximum concentrations of mycotoxins in foodstuff permitted in the EU with the exception of patulin, aflatoxin M_1, B_1 and ochratoxin A.

Besides the achievements in the development of such multitoxin LC/MS methods in the last years for all important mycotoxins groups (*119, 174*), there are some drawbacks still to be solved. For example, the ionisation efficiencies are not equal for all analytes and can be influenced by the instrument performance and the co-eluting matrix compounds, the complexity of food and feed matrixes and as well the wide range of physical and chemical properties of mycotoxins are some of them. For patulin, due to its small size and high polarity its quantification in the same run together with other mycotoxins may create some difficulties. Additionally, patulin has a different extraction procedure than other mycotoxins.

In this study an LC/MS/MS method has been developed to identify PAT in food samples offering a LOQ < 5 µg/kg with a retention time of 2.9 min. An advantage of this method is that interfering compounds like hydroxymethylfurfural, which are a big problem for HPLC-

UV methods, are minor for LC/MS/MS. Quantification of PAT is possible although on the other hand some other matrix effects or ion suppression difficulties are likely to happen and the final quantification result can be affected.

Sample preparation and clean up were performed according to the previously discussed method in this chapter and already published (*175*). Separation was performed with a 5 µm Hypercarb® Thermo HPLC column with mass spectrometric detection after negative electrospray ionisation.

After method development, the technique was applied in a survey of more than 200 samples (Fig. 21). The same extracts were quantified with both HPLC-UV and LC/MS/MS and both results were compared with special interest in some of the positive samples detected by HPLC- UV.

5.2.2. Test materials

Figure 21. Whole set of survey samples collected for the comparison between HPLC-UV and LC/MS methods.

The survey was carried out on a total of 227 samples purchased during the months of December 2006 and January 2007 in Austria, Belgium, Bulgaria, Czech Republic, Germany, Spain, Finland, France, Hungary, Ireland, Italy, Lithuania, Norway, Netherlands, Portugal, Rumania, Sweden, Slovakia and Turkey. The samples were classified into three groups, with 48 apple compote, 93 tomato concentrates, including tomato ketchup, from Spain, Germany and The Netherlands, and 86 baby food samples.

5.2.3. Method development

5.2.3.1. Instrumental parameters

Initially, the MS parameters were optimised by direct infusion of a PAT standard. A full scan acquisition from m/z 100 to m/z 200 was performed to determine the adduct formation pattern and whether the best ionisation was positive or negative mode.

Deprotonated PAT $[M\text{-}H]^-$ m/z 153 in negative ionisation mode generated the best signal/noise ratio. MS/MS experiments showed two strong daughter ions at m/z 109 and 81. Possible pathways to the m/z 109 are loss of carbon dioxide or acetaldehyde both having a mass of 44. An additional loss of carbon monoxide with a mass of 28 will then lead to the ion m/z 81 (*184*). Therefore, a selected reaction monitoring with the transitions of m/z 153 > to m/z 109 as the primary and of m/z 153 > to m/z 81 as the secondary was chosen (Fig. 22).

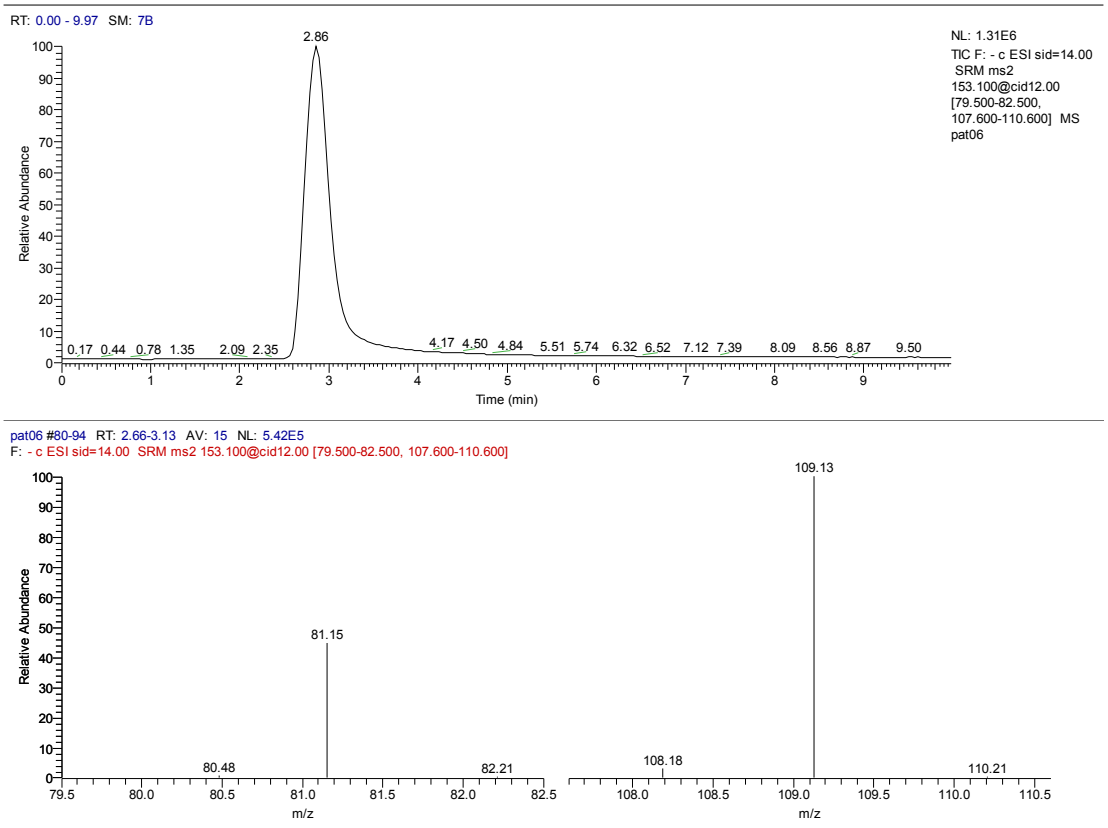


Figure 22. Total ion current and selected reaction monitoring mass spectrum of the two product ions of patulin following negative electrospray ionisation.

5.2.3.2. Performance characteristics

For the determination of the performance characteristics of the method, the spiking was carried out in the three different matrices - apple compote, tomato concentrates and baby food samples - at three levels 10, 20 and 50 µg/kg.

The accuracy and precision of the LC/MS/MS method was performed by comparing the retention times of the positive findings with the peak intensity ratios of the first and the second transitions (m/z 153 > m/z 109 and m/z 153 > m/z 81) with the expected peak intensity ratios from the standards.

For calibration, 6 standards were prepared according to the same HPLC method described in section 3.16 of Annex 7 resulting in concentrations of: 12.0, 24.0, 36.0, 48.0, 60.0 and 72.0 ng/mL. These standards were injected in order to determine the relation of response versus the injected amount of analyte. The linearity of the model was cheked by means of residual plot calculation (Annex 9 - Figure 1). This residual plot didn't show any indication of non-linearity but of heteroskedasticity (Annex 9 - Figure 2).

5.2.4. Experimental

5.2.4.1. Materials

- **Reagents**

As described in Annex 7 - Method A.

- **Apparatus**

Liquid chromatography with mass spectrometry from Thermo Fisher Scientific, Waltham (MA), USA:

a) Accela Pump
b) Accela Autosampler
c) Finnigan TSQ Quantum Ultra
d) Analytical column Hypercarb 5µm (2.1x50 mm) with guard column.

5.2.4.2. Methods

- **Sample preparation**

The sample extraction and clean-up was done according to the methodology described in Method A of Annex 7.

- **Preparation of standards solutions**

As described in the method A of Annex 7.

- **Mass spectrometry parameters**
  i. Spray voltage: -3000 V
  ii. Sheath gas pressure: 60 psi
  iii. Aux gas pressure: 10 psi
  iv. Capillary temperature: 300 °C
  v. PAT ions: m/z 153 > m/z 109 and m/z 153 > m/z 81.
- **HPLC conditions:**

The mobile phase consisted of 60% 2 mM ammonium acetate and 40 % acetonitrile at a flow rate of 200 µL/min. The total run time was 6 min and the retention time of PAT was around 3 min.

5.2.5. Results and discussion

The sample preparation was done according to the described method for HPLC with UV detection. The obtained extracts were divided in two parts, one was injected into the HPLC for UV detection and the other one was injected into the LC for MS/MS detection.

Previous experiments to clarify whether results from both techniques are comparable were performed by quantifying two naturally contaminated apple juice and apple puree samples with known levels of 10 and 50 µg/kg PAT.

All of the positive findings by both detections modes were directly compared and summarised in Tab. 43. For apple puree and baby food matrices comparable results were achieved, but in the case of tomato concentrate samples a peak was detected in two cases by HPLC-UV giving contamination levels of 100 µg/kg. These results led to a further investigation and therefore, they were evaluated with LC/MS/MS. With the results from the LC/MS/MS it became clear that these two positive tomato paste samples were not contaminated with PAT but some other interfering peak was detected instead.

As summarised, PAT could be quantified in six samples out of the 227 analysed, one apple puree (~25 µg/kg), four tomato concentrates (three at the level of ~8 µg/kg, and one at the level of ~50 µg/kg) and one baby food at the LOD, which is estimated to be around 3 µg/kg by signal/noise ratio.

Table 43. Patulin content determinated by HPLC-UV and LC/MS in the survey samples.

SAMPLES	Contamination level [µg/kg] HPLC-UV	Contamination level [µg/kg] LC/MS
Apple Puree 'Sample 46'	25	25
Tomato 'Sample 121'	10	8
Tomato 'Sample 128'	10	8
Tomato 'Sample 129'	10	8
Tomato 'Sample 152'	50	50
Baby food 'Sample 219'	n.d[1]	n.d[1]

[1]n.d Not detectable.

Compared to the HPLC-UV method, the LC/MS/MS method represents the advantage of speed and specificity. PAT eluted within three min, therefore it can be considered as a good routine laboratory technique especially in cases where large numbers of samples need to be analysed (Fig. 22).

Additionally, it offers an unambiguous confirmation of the identity of PAT, which is very useful in particular matrices like tomato concentrate since by HPLC-UV detection an interfering peak can lead to 'false positive' result.

5.2.6. Conclusions

Results were compared and confirmed, since after extraction and clean-up, the extract was divided in two portions and one injected in the HPLC and another one in the LC/MS system. Clear chromatograms with excellent signal-to-noise ratio were achieved with the developed method (Fig. 23).

Based on the results of the survey, no indication was found for an increased consumer risk from the products available in supermarkets for those countries mentioned in section 5.2.2 during 2006. Therefore since the growth rate and PAT production of *P. expansum* is influenced by several factors it can be concluded that 2006 was a year where environmental

conditions were not favourable for the growth of the mould. This statement is confirmed with the data reported in the 2006 RASFF-Annual Report (*176*), in which out of a total of 877 mycotoxins notifications, only 7 concerned PAT. The largest number of notifications concerned mainly aflatoxins (800 notifications), ochratoxin A (54 notifications) and fumonisin (15 notifications).

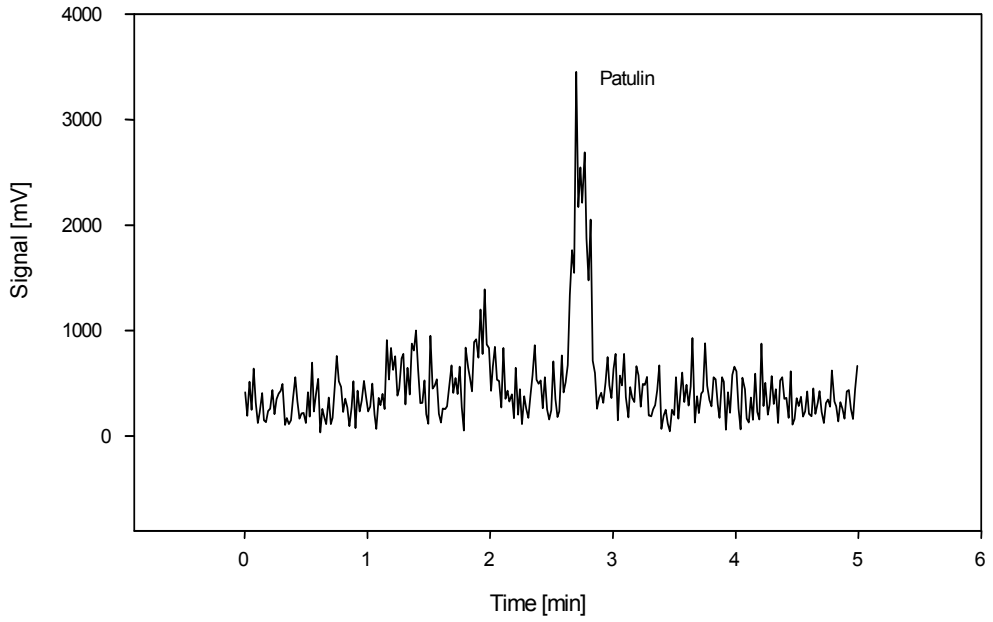


Figure 23. LC/MS chromatogram of a baby food sample naturally contaminated with PAT at the level of 3 µg/kg.

The method here described shows an excellent way to confirm the positive findings in food samples contaminated with patulin. Additionally, it is the first survey in such a big amount of samples giving an overview of the patulin risk in European food products. It offers several advantages to the use of more conventional techniques with improved sensitivity and reaching very low detention limits. Nevertheless, further research aiming simultaneous determination with other mycotoxins in combination with fully validated assays in agreement with EU regulations would be desiderable.

6. General conclusions

Several analytical methods have been developed and validated either in-house or by collaborative trial, following international procedures. The methods have been published or are accepted for publishing in peer reviewed journals. Several of them are currently under consideration by CEN to become international standards.

6.1. Determination of aflatoxin B_1 in medicinal herbs

A method for the determination of AfB_1 in the medicinal herbs devil's claw, senna pods and ginger root was developed in collaboration with the Institute for Public Health and the Environment, RIVM (*157*), The Netherlands, Instituto de Salud Carlos III del Ministerio de Sanidad y Consumo (*177*), Spain and PhytoLab (*132*), Germany. Several method variants were subject to validation by interlaboratory trial, after which it could be concluded that the existing methods for chemical, photochemical and electrochemical derivatisation have little influence on the analytical result, thus allowing laboratories to apply the derivatisation method of their preference. The method's main performance characteristics were established from the results of the study and resulted in Horwitz values ranging from 0.12 to 0.75 with mean recoveries from 78 to 91 % for the extraction with methanol-water and Horwitz values ranging from 0.10 to 1.03 with mean recoveries from 98 to 103 % for the extraction with acetone-water.

This work was carried out on request of the European Pharmacopoeia and the findings were published in a peer reviewed journal (*178*).

6.2. Development and in-house validation of aflatoxin B_1 in tiger nuts

A method for the determination of AfB_1 in tiger nuts was developed and in-house validated. After in-house development as small survey was carried out with approximately 20 different tiger nut products popular with children. Except in one sample in which traces of AfB_1 could be found, all test samples were free of aflatoxin B_1. The mean recovery of the method was 88% (n = 6). The method has been published in a peer reviewed journal (*179*).

6.3. Development of a method for the determination of zearalenone in infant food and animal feed

For the first time, a common method for animal feed and infant food matrices was elaborated. The main challenge was to allow the determination of ZON at rather low concentrations in infant food, while being also able to deal with other complex matrices and more challenging with respect to interferences such as compound animal feed. Previous works on this subject by other authors focused on the development of methods for cereal grains and at higher levels therein. The method was validated in an international interlaboratory trial according to the IUPAC Harmonized Protocol (*135*) and showed excellent performance characteristics.

Results were calculated for spiked samples based on blind duplicates at two levels and for naturally contaminated samples based on blind duplicates at three levels, the relative standard deviation for repeatability (RSD_r) in baby food ranged between 2.8 - 9.0 % and in animal feed between 5.7 - 9.5 %. The relative standard deviation for reproducibility (RSD_R) in baby food ranged between 8.2 - 13.3 % and between 15.5 - 21.4 % for animal feed.

The recovery values for ZON in baby food derived from the spiked samples were found to range between 65 - 123 % with an average of 92 % and between 39 - 138 % with an average of 74 % for animal feed.

The newly developed method allows the enforcement of EU legislative limits for ZON in foods for infants [Regulation 1881/2006] as well as current guidance levels in animal feed [Recommendation 2006/576/EC]. The method has been published in a peer reviewed journal (*180*). It has also been accepted by CEN TC275/WG5 (food for infants) and CEN TC327/WG1 (animal feed) as a good basis for a standard, and has been taken over in both CEN working groups as working document, thus indicating an international recognition by the scientific community of this work, and a long term impact.

6.4. Development and validation of a new analytical method to determine patulin in juices and purees for infants

The method developed is simpler to use than methods already available in the literature because it makes use of an improved single extraction procedure that saves time, money and effort, and also allows the direct extraction of purees without previous enzymatic treatment. Furthermore it displays good performance characteristics, at levels down to 10 µg/kg for infant foods, whereas previous methods were validated only at the level of 25 µg/kg or higher as experience at that time indicated that satisfying precision parameters at levels lower than 25 µg/kg would be difficult or not possible to achieve.

The method was validated in an international interlaboratory trial according to the IUPAC Harmonized Protocol *(135)* and has been published in a peer reviewed journal (*175*). The improvements were made possible by applying an optimised single liquid-liquid extraction in combination with a simplified solid phase extraction clean-up where PAT is purified by trapping the interferences on an unconditioned solid-phase extraction column. The separation and determination of PAT was performed by RP-HPLC and detected by UV absorption at 278 nm.

With the newly developed method, enforcement of the maximum residue limit for PAT, established by Regulation (EC) No 1881/2006 (*57*) is possible, unlike with previous methods. Additionally, CEN is currently publishing a standard method on the basis of the new developed method, thus indicating both international recognition by the scientific community of the work, and a long term impact. Harmonised methods across Europe help raise consumer confidence because scientists argue less about results, and they help reduce costs because analyses only need to be carried out once.

6.5. Development and validation of a method to determine patulin using LC/MS

A method based on liquid chromatography / mass spectrometry was developed. Compared with previous methods, reduced analysis time from 40 min per chromatographic run to 6 min was achieved by the implementation of new ultra high performance liquid chromatography (UHPLC). This new system operates at higher pressure and lower flow-rate compared to traditional HPLC, in combination with a triple quadruple mass spectrometric detector. It resulted in a better chromatographic and analyte selective separation within a shorter time. This is of interest especially for projects in which larger numbers of results are need to be generated. The method performs at a LOQ < 5 µg/kg with a retention time of less than 5 min.

With the above mentioned method, a survey with more than 200 samples of infant food, apple and tomato purees from the EU food market indicated that during the period of 2006 almost all products were free of PAT and all products were compliant with current EU legislation (*57*).

7. Future research

The main research points in mycotoxins analysis deal with the effort to reduce time and cost of analysis, to cut down the use or organic solvents, and to develop methods that are easy to use, more specific and that need less analysis time. Therefore LC/MS/MS is every day more in use due to its potential for screening large amount of samples in a short time and for the ability to quantify the presence of several mycotoxins simultaneously. Optimisation work in terms of reduced time, costs and use of organic solvents, while maintaining or improving the performance characteristics of analytical methods remain a research priority. UHPLC offers interesting possibilities in this regard. For simultaneous multiple analyte determination, multi-mycotoxin immunoaffinity clean up columns are a very convenient alternative providing that antibodies are available for the desired mycotoxins. Future work should ensure that suitable columns are available for all key mycotoxins, including PAT, which is not available at this moment.

Although significant advantages are offered by LC/MS, there still appear to exist some quantification difficulties. These are called matrix effects and are mainly caused by some of the other components of the sample except the specific compound to be quantified. They are common in food samples, for which optimisation in this field should be pursued.

A recent approach is envisaged by microarray experiments (*68*), where the detection of the expression pattern of the genes responsible for mycotoxin biosynthesis can be identified. This approach, which can be regarded as a supplement to the conventional official analytical methods for the detection of mycotoxins, can be viewed as a preventive measurement to avoid mycotoxin contamination.

Molecular imprinting is a new alternative for preparing artificial antibodies (*181, 182*), which are synthetic polymers offering more stability and resistance to chemicals than the natural antibodies. They can also be reused without losing their affinity and selectivity. They have been developed for several mycotoxins.

Finally, an aspect to consider is the availability of the analytical technologies to quantify mycotoxins in food and feed in countries with limited economical resources. This is a goal

that needs to be achieved in the future in order to protect the consumer from every corner of the world form the harmful effect of these compounds.

SUMMARY

This thesis concerns with the development and the validation of analytical methods for the determination of the mycotoxins aflatoxin B_1, zearalenone and patulin, which occur frequently in food and feed. The toxic syndromes produced by them when ingested are known as mycotoxicoses. One of the first reports in history of mycotoxicoses is ergotism, caused by the fungus *Claviceps purpurea*. Nowadays ergotism is of minor importance; however the problem of mycotoxicoses and long term sub-acute exposure has not faded. Therefore, regulations have been established in many countries, and reliable testing methodology is needed to implement and enforce the regulatory limits.

So far, several hundred different mycotoxins have been discovered, exhibiting different structural diversity, with various chemical and physicochemical properties, but only a few present significant food safety challenges. Among these are aflatoxins and ochratoxin A (produced by *Aspergillus* sp.), fumonisins, trichothecenes such as T-2, HT-2 toxins, deoxynivalenol and zearalenone (produced by *Fusarium* sp.), patulin (produced by *Penicillium* sp.) and ergot alkaloids (produced by *Claviceps* sp.) the most frequent occurring mycotoxins with the highest potential to adverse effects in humans and animals.

The work of this thesis can be clustered into three parts as follows:

(I) Method comparison and collaborative trial for the determination of aflatoxin B_1 in medicinal herbs. This study was initiated upon the request of the European Pharmacopoeia since the regulatory limits for aflatoxin B_1 in medicinal herbs were discussed in that moment.

The methodology used has been adopted from existing methods for the determination of aflatoxin B_1 in food. The food is extracted with an organic solvent followed by immunoaffinity clean-up and reversed-phase high performance liquid chromatography with fluorescence detection. The aim was to select the most suitable method parameters in order to obtain a method that allows the precise determination of aflatoxin B_1 in a variety of medicinal herbs. Therefore acetone-water and methanol-water were tested as extraction solvents. Further, the influence of different post-column derivatisation options with electrochemically generated bromine, photochemical reaction and chemical bromination was compared. In addition, two different calculation modes peak height versus peak area have

been investigated concerning the precision on the evaluation of the rather small peaks that are obtained for aflatoxin B_1 at low contamination levels. The different method parameters were applied in the collaborative study to three matrices: senna pods, ginger root and devil's claw root.

As a result, the method with all tested variations was found to be fit-for-purpose for the determination of aflatoxin B_1 in medicinal herbs at levels of 1 µg/kg and above with mean recoveries from 78% - 92% for the extraction with methanol-water and from 98% - 103% for the extraction with acetone-water. It could be concluded that the tested derivatisation methods had no influence on the analytical result in a range of 1 - 3 µg/kg for aflatoxin B_1 in medicinal herbs. This is an interesting conclusion as control laboratories often have a preference for one or the other derivatisation method depending on their experience with one or the other system and its availability.

A second method was adopted by single-laboratory validation for the determination of aflatoxin B_1 in tiger nuts. The interest on tiger nuts rose on the fact of recent entries in the Rapid Alert System for Food and Feed regarding contamination with aflatoxin B_1 in tiger nuts. This system allows the European Commission, EU member states and other associated countries to share information and take immediate action when potentially dangerous food or feed is detected on the market or at the border. Additionally a small survey of aflatoxin B_1 content in chufa, which is a tiger nuts based soft drink in Spain, was conducted with the adopted method. The detection limit and the quantification limit were 0.02 µg/kg and 0.06 µg/kg respectively. The mean recovery at a level of 2 µg/kg was 88 % (n = 6) and the coefficient of variation 9 %.

(II) Development and validation of an analytical method for the determination of zearalenone in infant food as well as in animal feed. For the first time the elaboration of a common method for both infant food and animal feed to determine zearalenone was achieved. The main challenge was to allow the determination of zearalenone at rather low concentrations in infant food, while being also able to deal with complex and more challenging matrices such as compound animal feed, due to their abundant interferences compounds. Previous work performed the determination of zearalenone in cereal grains and at higher levels.

The method was validated in an international interlaboratory trial in which laboratories from EU member states, China, Turkey and Uruguay participated.

Method performance parameters for both baby food and animal feed were calculated based on results for spiked samples blind duplicates at two levels and based on results for naturally contaminated samples blind duplicates at three levels.

Test portions of the samples were spiked at levels of 20 µg/kg and 30 µg/kg zearalenone in baby food and at levels of 100 µg/kg and 150 µg/kg zearalenone in animal feed. Mean recoveries from each participant ranged between 91 - 92 % for baby food and between 72 - 75 % for animal feed. The relative standard deviation for repeatability for baby food ranged between 2.8 - 9.0 % and for animal feed between 5.7 - 9.5 %. The relative standard deviation for repeatability for baby food ranged between 8.2 - 13.3 % and for animal feed between 15.5 - 21.4 %. As a result the method showed acceptable within-laboratory and between-laboratory precision for each matrix, as required by European legislation. Therefore, the newly developed method allows the enforcement of EU legislative limits for zearalenone in foods for infants at 20 µg/kg.

(III) Development and validation of an analytical method for the determination of patulin in juices and purees for infants. The main challenge was to stress the method to determin patulin reliably at levels of 10 µg/kg in products intended for infants and young children. Previously developed methods for patulin were either collaboratively tested at higher levels, indicating that the lower limit for reliable quantification of patulin in such products was around 25 µg/kg or higher, or no validation data except single-laboratory validation were available.

Method development focussed on improved and simplified extraction and clean-up procedures. A single liquid-liquid extraction in the presence of sodium sulfate as water binding agent showed sufficient extraction recovery rates for patulin in combination with a solid-phase extraction method, which trapped interfering substances and allowed the purification of patulin extracts without any pre-conditioning of the SPE cartridge. As a result, purees can be extracted without previous enzymatic treatment, as it is required by other methods that use multiple liquid-liquid extractions. Patulin was well separated from the main interfering compound 5-hydroxymethylfurfural during chromatography when using RP-HPLC columns that allow the separation of rather polar substances with mobile phases of more 99% of water.

Additionally to this method A and due to the large number of laboratories that intended to participate in the validation process, the participants were split into two groups and a second

method B was validated. This method B is a slightly modified version with the same principle as the one previously published by MacDonald *et al.* in 2000. The main modifications related to the aliquotation. Patulin is extracted three times from the juice or the de-pectinated puree with neat ethyl acetate. The combined ethyl acetate phases were re-extracted with sodium carbonate solution and evaporated. The residue was then re-dissolved in 0.1 % acetic acid solution and separated by HPLC as in method A. Both methods were tested for the determination of patulin in apple juice and fruit puree at the proposed European regulatory limit of 10 µg/kg.

The methods were validated in an international interlaboratory trial in which laboratories from EU Member States, Japan and Brazil participated. Method performance parameters for both apple juice and fruit puree were calculated based on results for spiked samples blind duplicates at two levels and based on results for naturally contaminated samples blind duplicates at three levels for both methods. Test portions of the samples were spiked at levels of 10 µg/kg and 25 µg/kg patulin for both apple juice and fruit puree.

Apple juice mean recoveries from each participant ranged between 37 - 123 % with an average of 73% for method A and between 47 - 124 % with an average of 88 % for method B. Fruit puree recovery values ranged between 60 - 87 % with an average of 70 % for method A and between 37 - 207 % with an average of 110 % for method B. The relative standard deviation for repeatability in apple juice ranged between 8.0 - 14.3 % for method A and between 5.0 - 37.6 % for method B. For fruit puree these values ranged between 3.5 - 7.5 % for method A and between 8.4 - 27.7 % for method B. The relative standard deviation for repeatability in juice ranged between 19.8 - 39.5 % for method A and between 18.2 - 46.9 % for method B. In puree, these values ranged between 12.5 - 27.7 % for method A and between 16.2 - 57.1 % for method B.

In conclusion, the new developed method A showed acceptable within-laboratory and between-laboratory precision for both juice and puree at all levels, while method B only fulfilled partially the performance parameters as required by current EU legislation. Therefore, the newly developed method allows the enforcement of EU legislative limits for patulin in foods for infants at 10 µg/kg.

Finally the development and in-house validation of a method for determination of patulin using ultra high pressure liquid chromatography in combination with a mass selective detector, resulting in a better chromatographic and analyte selective separation within a shorter time is described. This is of interest especially for projects in which larger amounts of results need to be generated. A survey with more than 200 samples of baby foods, apple purees and tomato purees from the EU food market was performed with this method. It indicated that during the period of 2006 almost all products were free of patulin and all products were compliant with current EU legislation.

ANNEXES

Annex 1. Draft standard operating protocol for the determination of aflatoxin B_1 in medicinal herbs

1. Scope

This method can be applied to the determination of aflatoxin B_1 (AfB_1) in medicinal herbs. The limit of quantification of the method has been demonstrated to be 2 µg/kg or better, depending on the equipment used.

2. Principle

A test portion is extracted with methanol or acetone in combination with water. The sample extract is filtered, diluted with water to a specified solvent concentration, and applied to an immunoaffinity column (IAC) containing antibodies specific to aflatoxins. AfB_1 is eluted from the IAC with methanol. AfB_1 is quantified by reverse-phase high performance liquid chromatography (RP-HPLC) with post-column derivatisation (PCD). The PCD is achieved with photochemical reaction (PHRED) or with bromination reaction, followed by fluorescence detection. The bromination reaction can be achieved with electrochemically generated bromine (KOBRA) or with pyridinium hydrobromide perbromide (PBPB).

3. Reagents

Use only reagents of recognised analytical grade and water complying with grade 3 of ISO 3696 9 (*183*) unless otherwise specified.

Note: Decontamination procedures for laboratory wastes were developed and validated by the International Agency for Research on Cancer (WHO).

3.1. Pyridinium hydrobromide perbromide (PBPB). Sigma Aldrich.
CAS: 39416-48-3.

3.2. Potassium bromide

3.3. Acetonitrile, HPLC grade

3.4. Methanol, HPLC grade

3.5. Methanol, technical grade

3.6. Toluene, HPLC grade

3.7. Extraction solvent

3.7.1. Mandatory extraction procedure: methanol-water (7+3, v/v)

3.7.2. Optional extraction procedure: acetone-water (85+15, v/v)

3.8. Nitric acid, *c* (HNO_3) = 4 mol/L

3.9. **Immunoaffinity column** (IAC)

The IAC must contain antibodies raised against AfB_1. The column must have a maximum capacity of not less than 100 ng of AfB_1 and must give a recovery of not less than 80% when applied as a standard solution in methanol-water (87.5+12.5, v/v) containing 5 ng. For this experiments IAC from R-Biopharm (*133*) were mandatory.

3.10. HPLC mobile phase A

For use with photochemical reactor or with PBPB (3.1): water - acetonitrile (3.3) - methanol (3.4) (6+2+3, v/v/v). Degas the solution before use.

3.11. HPLC mobile phase B

For use with electrochemically generated bromine: water - acetonitrile (3.3) - methanol (3.4) solution (6+2+3, v/v/v). Add 120 mg of potassium bromide (3.2) and 350 µL of nitric acid (3.8) per litre of mobile phase. Degas the solution before use.

3.12. Post-column reagent

Dissolve 50 mg of PBPB (3.1) in 1000 mL of water.

Note: The solution can be used for up to four days if stored in a dark place at room temperature.

3.13. Toluene-acetonitrile mixture

Toluene - acetonitrile (98+2, v/v). Mix 98 parts per volume of toluene (3.6) with 2 parts per volume of acetonitrile (3.3).

3.14. Preparation of AfB_1 standard solutions for HPLC.

The preparation of standards was the same for all method variations tested. The AfB_1 standard solution was supplied by RIVM (*157*). The mass concentration was 10.0 µg/mL in chloroform. From this, a stock solution containing 100 ng/mL was prepared by diluting with toluene-acetonitrile (3.13).

Use this stock solution (100 ng/mL, see instructions for mini collaborative study, section 4.) for pipetting the volumes as given in Tab. 1 into a set of 250 mL calibrated volumetric flasks. Evaporate the chloroform solution just to dryness under a stream of nitrogen at room temperature. To each flask, add 75 mL of methanol (3.4), let AfB_1 dissolve and fill up to the mark with water. Finally shake well.

Table 1. Preparation of AfB_1 standard solutions.

Standard solution	Taken from stock solution [µl]	Final concentration of standard solution [ng/mL]
1	25.0	0.010
2	50.0	0.020
3	125.0	0.050
4	250.0	0.10
5	500.0	0.20
6	750.0	0.30
7	1000.0	0.40

Calculate the plot for linearity.

The stock solution (100 ng/mL) should be stored in a cool place (approximately 4 °C) in the dark, well sealed and wrapped in aluminium foil. This solution is stable for at least four months.

Preparation of fortified material.

- **Mandatory extraction procedure:** to 5.0 g of blank material, add 100.0 µL of the 100 ng/mL AfB_1 stock solution.

- **Optional extraction procedure:** to 10.0 g of blank material, add 200.0 µL of the 100 ng/mL AfB_1 stock solution.

4. Apparatus

General: All glassware coming into contact with aqueous solutions of aflatoxins shall be washed with acid solution before use. Many laboratory washing machines do this as part of the washing program. Otherwise soak laboratory glassware coming into contact with aqueous solutions of aflatoxins in sulfuric acid (2 mol/L) for several hours, then rinse well (e.g. three times) with water to remove all traces of acid. Check the absence of acid with pH paper.

Note: This treatment is recommended, because the use of non-acid washed glassware may cause losses of aflatoxins. In practice, the treatment is necessary for round bottomed flasks, volumetric flasks, measuring cylinders, vials or tubes used for calibration solutions and final extracts (particularly autosampler vials), and Pasteur pipettes, if these are used to transfer calibration solutions or extracts.

4.1. Usual laboratory apparatus and, in particular, the following

4.2. Ultrasonic bath, e.g. frequency: 35 kHz.

4.3. Filter paper, Schleicher & Schuell 5951/2 or equivalent.

4.4. Conical flask, with screw top or glass stopper.

4.5. Glass microfiber filter paper, Whatman GF/A 1820 090 or equivalent.

4.6. Reservoir, 50 mL with luer tip connector for immunoaffinity column.

4.7. Vacuum manifold.

4.8. Volumetric glassware, 5 and 50 mL flasks, with an accuracy of at least 0.5 %.

4.9. HPLC apparatus, consisting of:

- HPLC pump, suitable for flow rate at 1.0 mL/min.

- Injection system (manual or automatic), capable of injecting 500 µL by partial or total loop filling (see instructions of manufacturer). Smaller volumes are acceptable, provided that enough sensitivity is obtained.

- RP-HPLC column, e.g. LC-18 or ODS-2, which ensures a baseline resolved resolution of the aflatoxin B_1 peak from all other peaks. The maximum overlapping of peaks must be less than 10% (it might be necessary to adjust the mobile phase for a sufficient baseline resolution). A suitable pre-column should be used.

4.10. Post-column derivatisation system. Three derivatisation systems have been found appropriate:

4.10.1. System for derivatisation with pyridinium hydrobromide perbromide.

The derivatisation solution made of 50.0 mg of PBPB (3.1) in 1000 mL of water is pumped with a second pulseless HPLC pump via a zero-dead volume T-piece to the mobile phase after separation, at a flow rate of 0.3 mL/min. The reaction takes place in a PTFE reaction tubing of 45 cm x 0.5 mm i.d. to allow a reaction time of 4 s. Only to be used with mobile phase A (3.10).

4.10.2. System for derivatisation with photochemical reactor.

UV-reactor unit with a 254 nm low pressure mercury ultraviolet bulb, a knitted UV-transparent reactor coil, length 25 m, ID 0.25 mm, nominal void volume 1.25 mL. The device is mounted between HPLC column and detector to be used with mobile phase A (3.10).

4.10.3. System for derivatisation with electrochemically generated bromine.

KOBRA-cell (R-Biopharm Rhone Ltd., Glasgow, Scotland), DC-supply in series with the KOBRA-cell, capable of providing a constant current of ca. 100 µA, reaction tube Teflon. Dimensions of 120 mm x 0.25 mm ∅ have found to be appropriate. Only to be used with mobile phase B (3.11).

4.11. Fluorescence detector, with a wavelength of λ = 360 nm excitation filter and a wavelength of λ > 420 nm cut-off emission filter, or equivalent. Recommended settings for adjustable detectors are 365 nm (excitation wavelength), 435 nm (emission wavelength).

4.12. Disposable filter unit (0.45 μm). Prior to usage, verify that no aflatoxin losses occur during filtration (recovery testing).

Note: There is a possibility that various filter materials can retain aflatoxins.

4.13. Pipettes, 10 mL capacity

4.14. Analytical balance, capable of weighing to 0.1 mg

4.15. Laboratory balance, capable of weighing to 0.01 g

4.16. Calibrated microliter syringes or microliter pipettes, 25 μL to 200 μL.

5. Procedures

5.1. Conditioning of immunoaffinity columns

Allow the immunoaffinity columns (3.9) to reach room temperature.

5.2. Extraction

Mandatory extraction procedure (Annex 2, Figure 1): Weigh, to the nearest 0.1 g, approximately 5.0 g of the test portion into a 150 mL conical flask (4.4). Add 100.0 mL of extraction solvent (3.7.1). Extract by sonication (4.2) for 30 min. Filter the extract using a prefolded filter paper (4.3).

Optional extraction procedure (Annex 2, Figure 2): Weigh, to the nearest 0.1 g, approximately 10.0 g of the test portion into a 150 mL conical flask (4.4). Add 100.0 mL of extraction solvent (3.7.2). Extract by sonication (4.2) for 30 min. Filter the extract using a prefolded filter paper (4.3).

5.3. Immunoaffinity clean up

Mandatory extraction procedure: Pipette 10.0 mL of the clear filtrate into a 150 mL conical flask (4.4). Dilute with 70.0 mL water. In case of turbidity, filter extract with a glass microfiber filter paper (4.5). Connect the IAC (3.9) to the vacuum manifold (4.7) and attach the reservoir (4.6) to the IAC. Add 40.0 mL diluted sample extract to the reservoir and pass through the IAC at a flow rate of approximately 3 mL/min (approx. 1 drop/s, gravity). Do not exceed a flow rate of 5 mL/min. Wash the column twice with 10 mL of water at a flow rate of max. 5 mL/min and dry by applying little vacuum for 5 s or passing air through the IAC by means of a syringe for 10 s.

Optional extraction procedure: Pipette 5.0 mL of the clear filtrate into a 150 mL conical flask (4.4). Dilute with 70.0 mL water. In case of turbidity, filter extract with a glass microfiber filter paper (4.5). Connect the IAC (3.9) to the vacuum manifold (4.7) and attach the reservoir (4.6) to the IAC. Add 40.0 mL diluted sample extract to the reservoir and pass through the IAC at a flow rate of approximately 3 mL/min (approx. 1 drop/s, gravity). Do not exceed a flow rate of 5 mL/min. Wash the column twice with 10 mL of water at a flow

rate of max. 5 mL/min and dry by applying little vacuum for 5 s or passing air through the IAC by means of a syringe for 10 s.

5.4. Elution of the aflatoxins in a three step procedure.

Apply 0.5 mL methanol (3.5) on the column and let it pass through by gravity. Collect the eluate in a calibrated volumetric flask of 5 mL (4.8). Wait for 1 min and apply a second portion of 0.5 mL methanol (3.5). Wait for 1 min and apply a third portion of 0.5 mL methanol (3.5). Collect most of the applied elution solvent by pressing air or vacuum through.

Fill the flask to the mark with water and shake well. If the solution is clear it can be used directly for HPLC analysis. If the solution is not clear, pass it through a disposable filter unit (4.14) prior to HPLC injection.

5.5. High performance liquid chromatography (HPLC)

The aflatoxins are separated by isocratic reversed-phase HPLC (RP-HPLC) at ambient temperature with a reversed-phase column (4.9) and an appropriate mobile phase respectively. The recommended HPLC operating conditions are:

- Flow rate mobile phase: 1.0 mL/min (for 4.6 mm inner diameter).
- Injection volume: 500 µL. Smaller volumes are acceptable, provided that enough sensitivity is obtained.
- Fluorescence detector settings: see 4.13.

The aflatoxins elute in the order G_2, G_1, B_2 and B_1 respectively, and should be baseline resolved. The mobile phase may be adjusted by addition of water, methanol or acetonitrile for maximum peak resolution and chromatographic performance.

5.6. Post-column derivatisation

- Pyridinium hydrobromide perbromide. When using PBPB, mount the mixing T-piece and reaction tubing mentioned under (4.10.1.), and then operate using the flow rate of 0.30 mL/min for the post-column reagent.
- Photochemical reactor. When using a photochemical reactor, mentioned under (4.10.2.), follow the instructions for the installation of the photochemical reactor as supplied by the manufacturer.
- Electrochemically generated bromine. When using electrochemically generated bromine, mentioned under (4.10.3.), follow the instructions for the installation.

5.7. Calibration curve

Prepare the calibration curve using the AfB_1 standard solutions described (3.14). These solutions cover the range of 0.2 ng/mL to 8 ng/mL for AfB_1. After injection of the standard solutions calculate the linearity. In case that the content of aflatoxin B_1 in the test portion will be outside of the calibration range, the test solution for HPLC analysis can be diluted to an aflatoxin content appropriate for the established calibration curve.

6. Calculation

Plot the data - concentration of AfB_1 [ng/mL] (x-axis) against the signal [units] (y-axis) - from the calibrant solution experiments into a table and calculate the calibration curve using linear regression. Use the resulting function:

(y = ax + b)

to calculate the concentration of aflatoxin in the measured solution.

The calibration curve (function) obtained by linear regression for calculation of the concentration of the measured solution is as follows:

C_{smp} [ng/mL] = *(*$Signal_{smp}$ [units] – *b) / a*

$Signal_{smp}$: signal of aflatoxin peak obtained from the measured solution [units]

For the calculation of the contamination level [ng/g] use the obtained concentration *(*C_{smp}*)* in the following equation:

$$C_{afla} = \frac{C_{smp} \times V_e \times V_{final}}{m_s \times V_{IAC}} \left[\frac{\text{ng} \times \text{mL} \times \text{mL}}{\text{mL} \times \text{g} \times \text{mL}} \right]$$

m_s : test portion taken for analysis [g] - (5.0 g);

V_e : solvent taken for extraction [mL] - (100.0 mL);

V_{IAC} : aliquot taken for immunoaffinity clean-up [mL] - (5.0 mL)

V_{final} : final volume achieved after elution from IAC [mL] - (5.0 mL)

C_{smp} : concentration of aflatoxin calculated from linear regression [ng/mL]

7. Confirmation of identity of AfB_1

HPLC without post-column derivatisation decreases with a factor of 10 or more the fluorescence response of AfB_1 (also for AfG_1). This can be used to confirm the identity of AfB_1 in the test solutions obtained in 5.3. These test solutions can be stored in the dark at room temperature for at least a week, if desired. In order to confirm the identity of aflatoxin B_1 in a test solution, proceed as follows:

7.1. Using a photochemical reactor (PHRED). Switch-off the electrical current. Reinject the relevant test solution.

7.2. Using electrochemically generated bromine (KOBRA cell). Disconnect the HPLC column from the bromination device and connect it directly to the fluorescence detector. Reinject the relevant test solution. Switching-off the electrical current with the bromination device still in line is not recommended due to the possibility of remaining bromine in the cell membrane of the device.

Annex 2. Schematic of methodology of extraction procedure for aflatoxin B_1 in medicinal herbs

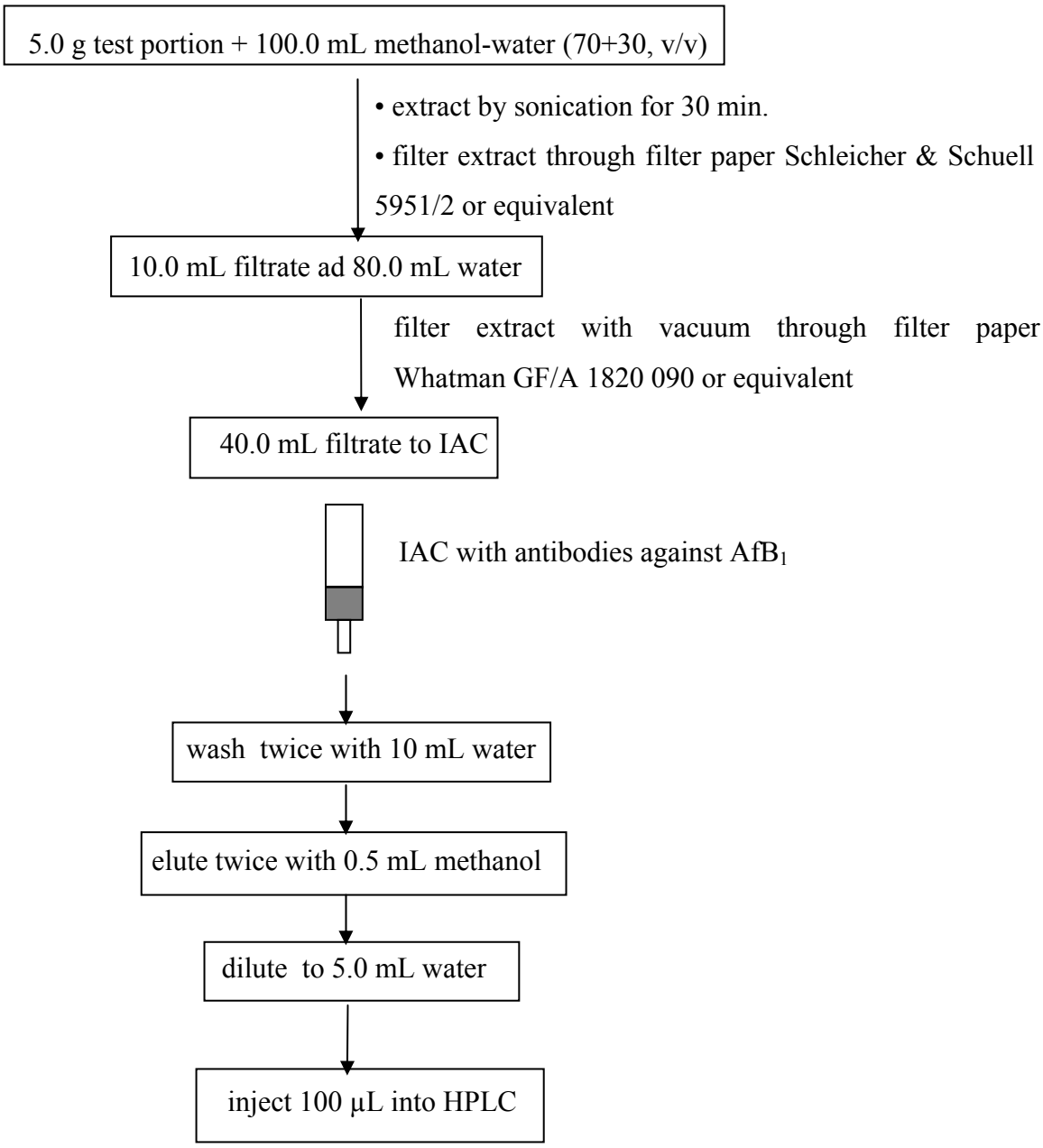


Figure 1. Schematic methodology of mandatory extraction procedure.

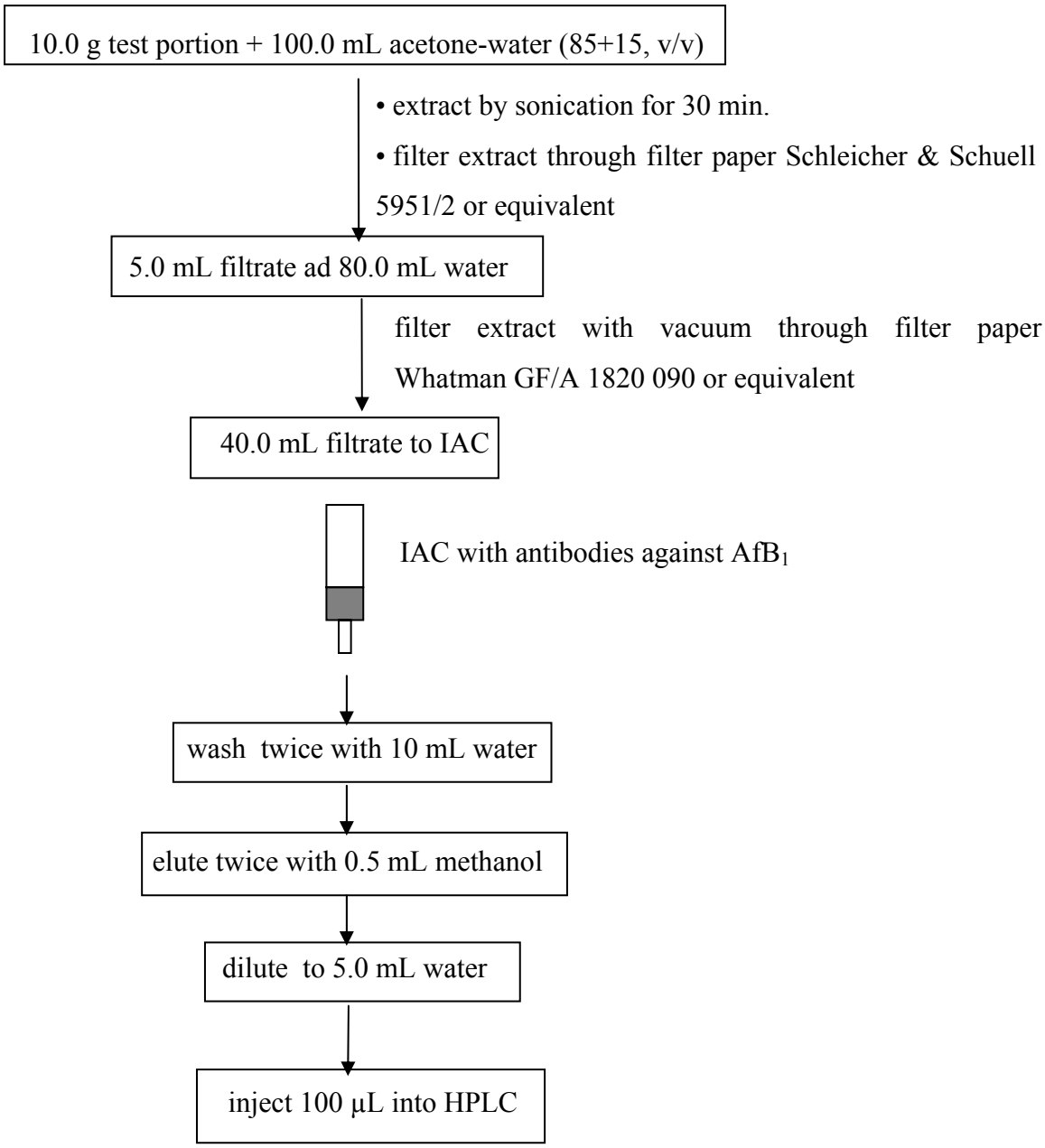


Figure 2. Schematic methodology of optional extraction procedure.

Annex 3. Collaborative trial results on determination of aflatoxin B_1 in medicinal herbs

Table 1. Results from duplicate analysis (A and B) obtained with mandatory extraction procedure, post-column derivatisation by KOBRA, and integration by area.

	Aflatoxin B_1 [µg/kg]							
	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4	
Material	A	B	A	B	A	B	A	B
Senna pods 1 (NC)[a]	1.78	2.03	1.85	1.88	2.83	2.82	1.47	1.66
Senna pods 2 (NC)	13.03	12.81	15.73	15.41	18.61	18.14	11.76	13.82
Ginger root 1 (NC)	<0.4	<0.4	<0.07	<0.07	<LOD	<LOD	<0.06	<0.06
Ginger root 2 (NC)	1.39	1.46	2.37	2.22	2.85	2.96	1.62	2.19
Devil's claw root 1 (NC)	<0.4	<0.4	<0.07	<0.07	0.17	0.16	<0.06	<0.06
Devil's claw root (F)	0.91	1.06	0.89	0.86	0.93	0.86	0.95	0.88
Senna pods recovery [%][b]	72	63	107	105	26	73	92	85
Ginger root recovery [%]	82	87	88	97	93	90	95	95
Devil's claw root recovery [%]	81	64	90	90	83	87	86	81

[a] NC: Naturally contaminated material; F: fortified (1 µg/kg).
[b] The fortification level for the recovery experiments was 2 µg/kg for all matrices.

Table 2. Results from duplicate analysis (A and B) obtained with mandatory extraction procedure, post-column derivatisation by KOBRA, and calculation by height.

	Aflatoxin B_1 [µg/kg]							
	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4	
Material	A	B	A	B	A	B	A	B
Senna pods 1 (NC)[a]	1.89	2.14	-	-	-	-	1.57	1.80
Senna pods 2 (NC)	13.84	13.10	-	-	-	-	12.80	14.57
Ginger root 1 (NC)	<0.4	<0.4	-	-	-	-	-	-
Ginger root 2 (NC)	1.38	1.47	-	-	-	-	1.72	2.27
Devil's claw root 1 (NC)	<0.4	<0.4	-	-	-	-	-	-
Devil's claw root (F)	0.86	0.95	-	-	-	-	1.00	0.95
Senna pods recovery [%][b]	79	61	-	-	-	-	97	93
Ginger root recovery [%]	78	81	-	-	-	-	101	98
Devil's claw root recovery [%]	62	61	-	-	-	-	93	86

[a] NC: Naturally contaminated material; F: fortified (1 µg/kg).
[b] The fortification level for the recovery experiments was 2 µg/kg for all matrices.
- Not determined (not mandatory requirement).

Table 3. Results from duplicate analysis (A and B) obtained with optional extraction procedure, post-column derivatisation by KOBRA, and integration by area.

	Aflatoxin B_1 [µg/kg]							
	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4	
Material	A	B	A	B	A	B	A	B
Senna pods 1 (NC) [a]	2.30	1.43	1.75	1.83	2.80	3.94	1.77	1.45
Senna pods 2 (NC)	14.42	13.32	18.44	19.52	22.53	22.65	15.83	16.34
Ginger root 1 (NC)	<0.4	<0.4	<0.07	<0.07	< LOD	< LOD	<0.06	<0.06
Ginger root 2 (NC)	2.35	3.58	2.56	2.94	3.08	3.24	2.31	2.80
Devil's claw root 1 (NC)	<0.4	<0.4	<0.07	<0.07	0.18	0.18	<0.06	<0.06
Devil's claw root (F)	0.94	1.06	1.08	1.02	1.09	1.21	1.08	1.02
Senna pods recovery [%][b]	87	102	86	114	102	96	110	83
Ginger root recovery [%]	96	93	100	104	112	114	104	104
Devil's claw root recovery [%]	96	104	102	100	92	96	104	102

[a] NC: Naturally contaminated material; F: fortified (1 µg/kg).
[b] The fortification level for the recovery experiments was 2 µg/kg for all matrices.

Table 4. Results from duplicate analysis (A and B) obtained with optional extraction procedure, post-column derivatisation by KOBRA, and calculation by height.

	Aflatoxin B_1 [µg/kg]							
	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4	
Material	A	B	A	B	A	B	A	B
Senna pods 1 (NC) [a]	2.83	1.46	1.72	1.84	-	-	1.87	1.56
Senna pods 2 (NC)	14.87	12.96	18.33	19.43	-	-	17.09	17.42
Ginger root 1 (NC)	-	-	-	-	-	-	-	-
Ginger root 2 (NC)	2.31	3.57	2.99	2.59	-	-	2.43	2.94
Devil's claw root 1 (NC)	-	-	-	-	-	-	-	-
Devil's claw root (F)	0.93	0.95	1.09	1.02	-	-	1.14	1.12
Senna pods recovery [%][b]	87	102	87	111	-	-	119	91
Ginger root recovery [%]	95	92	102	105	-	-	112	109
Devil's claw root recovery [%]	93	99	102	100	-	-	115	112

[a] NC: Naturally contaminated material; F: fortified (1 µg/kg).
[b] The fortification level for the recovery experiments was 2 µg/kg for all matrices.
- Not determined (not mandatory requirement).

Table 5. Results from duplicate analysis (A and B) obtained with mandatory extraction procedure, post-column derivatisation by photochemical derivatisation, and integration by area.

	Aflatoxin B_1 [µg/kg]							
	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4	
Material	A	B	A	B	A	B	A	B
Senna pods 1 (NC)[a]	1.50	2.29	-	-	-	-	1.59	1.83
Senna pods 2 (NC)	13.66	12.97	-	-	-	-	12.86	13.07
Ginger root 1 (NC)	<0.4	<0.4	-	-	-	-	<LOD	<LOD
Ginger root 2 (NC)	1.32	1.40	-	-	-	-	1.66	2.19
Devil's claw root 1 (NC)	<0.4	<0.4	-	-	-	-	<LOD	<LOD
Devil's claw root (F)	0.89	0.66	-	-	-	-	0.99	0.93
Senna pods recovery [%][b]	70	71	-	-	-	-	91	88
Ginger root recovery [%]	82	78	-	-	-	-	96	98
Devil's claw root recovery [%]	37	65	-	-	-	-	99	93

[a] NC: Naturally contaminated material; F: fortified (1 µg/kg).
[b] The fortification level for the recovery experiments was 2 µg/kg for all matrices.
- The mycotoxin was not detected (below limit of detection).

Table 6. Results from duplicate analysis (A and B) obtained with optional extraction procedure, post-column derivatisation by photochemical derivatisation / pyridinium hydrobromide perbromide and calculation by area [a].

	Aflatoxin B_1 [µg/kg]							
	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4	
Material	A	B	A	B	A	B	A	B
Senna pods 1 (NC)[b]	1.66	2.35	1.59	1.62	-	-	1.75	1.59
Senna pods 2 (NC)	15.16	13.34	17.96	18.84	-	-	17.10	15.84
Ginger root 1 (NC)	<LOD	<LOD	<LOD	<LOD	-	-	<LOD	<LOD
Ginger root 2 (NC)	2.27	3.41	3.14	2.43	-	-	2.29	2.76
Devil's claw root 1 (NC)	<LOD	<LOD	<LOD	<LOD	-	-	<LOD	<LOD
Devil's claw root (F)	1.02	1.05	1.11	0.88	-	-	1.08	1.02
Senna pods recovery [%][c]	73	97	89	108	-	-	122	92
Ginger root recovery [%]	94	96	103	101	-	-	107	107
Devil's claw root recovery [%]	102	105	110	103	-	-	105	107

[a] photochemical derivatisation was used by Laboratories 1 and 4, and pyridinium hydrobromide perbromide by laboratory 2.
[b] NC: Naturally contaminated material; F: fortified (1 µg/kg).
[c] The fortification level for the recovery experiments was 2 µg/kg for all matrices.
- Not determined (not mandatory requirement).

Annex 4. Experimental methodology for the determination of aflatoxin B_1 in tiger nuts

1. Scope

This method can be applied to the determination of aflatoxin B_1 (AfB_1) in tiger nuts. The limit of quantification of the method has been demonstrated to be 2 µg/kg or better, depending on the equipment used.

2. Principle

A test portion is mixed with phosphate buffered saline and filtered. This filtrate is applied to an immunoaffinity column (IAC) containing antibodies specific to aflatoxins. AfB_1 is eluted from the IAC with methanol. AfB_1 is quantified by reversed-phase high performance liquid chromatography (RP-HPLC) with electrochemically generated bromine (KOBRA) post-column derivatisation (PCD).

3. Reagents

3.1. Potassium bromide

3.2. Acetonitrile, HPLC grade

3.3. Methanol, HPLC grade

3.4. Methanol, technical grade

3.5. Nitric acid, $c(HNO_3) = 4$ mol/L

3.6. Phosphate buffered saline. Dissolve 0.20 g of potassium chloride (KCl), 0.20 g of potassium dihydrogen phosphate (KH_2PO_4), 1.16 g of disodium hydrogen phosphate water free (or 2.92 g of disodium phosphate dodecahydrate, $Na_2HPO_4 \times 12H_2O$) and 8 g of sodium chloride (NaCl) in 0.90 L of water. Adjust the pH to 7.4 with HCl (0.1 mol/L) or NaOH (0.1 mol/L) as appropriate. Dilute to 1.0 L with water. Commercially available phosphate buffered saline tablets with equivalent properties may be used.

3.7. Immunoaffinity column. It must contain antibodies raised against AfB_1. The column must have a maximum capacity of not less than 100 ng of AfB_1 and must give a recovery of not less than 80 % when applied as a standard solution in methanol-water (87.5+12.5, v/v) containing 5 ng. Immnunoaffinity columns from R-Biopharm (*133*) were mandatory for this study.

3.8. HPLC mobile phase: water - acetonitrile (3.2) - methanol (3.3) solution (6+2+3, v/v/v). Add 120 mg of potassium bromide (3.1) and 350 µL of nitric acid (3.5) per litre of mobile phase. Degas the solution before use.

The AfB_1 standard solution was supplied by RIVM (*157*). The mass concentration was 10.0 µg/mL in chloroform.

4. Apparatus

General: All glassware coming into contact with aqueous solutions of aflatoxins shall be washed with acid solution before use. Many laboratory washing machines do this as part of the washing program. Otherwise soak laboratory glassware coming into contact with aqueous solutions of aflatoxins in sulfuric acid (2 mol/L) for several hours, then rinse well (e.g. three times) with water to remove all traces of acid. Check the absence of acid with pH paper.

Note: This treatment is recommended, because the use of non-acid washed glassware may cause losses of aflatoxins. In practice, the treatment is necessary for round bottomed flasks, volumetric flasks, measuring cylinders, vials or tubes used for calibration solutions and final extracts (particularly autosampler vials), and Pasteur pipettes, if these are used to transfer calibration solutions or extracts.

Usual laboratory apparatus and**,** in particular, the following:

4.1. Conical flask, with screw top or glass stopper

4.2. Glass microfiber filter paper, Whatman GF/A 1820 090 or equivalent.

4.3. Reservoir, 50 mL with luer tip connector for immunoaffinity column

4.4. Vacuum manifold

4.5. Volumetric glassware, 5 and 50 mL flasks, with an accuracy of at least 0.5 %

4.6. HPLC apparatus, consisting of:

- Gilson HPLC pump, type 306 and 307.
- Gilson ASPEC, Automated Sample Preparation, Extraction and Collection device, model 'ASPEC XL'
- Waters fluorescence detector model 474; 365 nm excitation wavelength, 435 nm (emission wavelength. A s/n-ratio of more than 6 should be obtained for the calibration solution of 0.02 ng/mL
- Gilson Software Unipoint version 5.0
- RP-HPLC column, Supelcosil® 5 µm LC-18 120 Å, 12.0 % carbon loading, 250 x 4.6 mm, or similar.
- Post-column derivatisation system with electrochemically generated bromine KOBRA-cell (R-Biopharm Rhone Ltd, Glasgow, Scotland) with direct current power supply providing a constant current of ca. 100 µA. The device was mounted according to the manufacturer's specifications.

4.7. Pipettes, 10 mL capacity

4.8. Analytical balance, capable of weighting to 0.1 mg

4.9. Laboratory balance, capable of weighting to 0.01 g

4.10. Calibrated microliter syringes or microliter pipettes, 25 µL to 200 µL.

5. Method

5.1. Sample extraction

25 mL of phosphate buffered saline (3.6) and 25.0 mL of the horchata were weighed into a 150 mL conical flask (4.1) mixed and filtered over a glass micro fibre filter (4.2).

5.2. Immunoaffinity clean-up

25.0 mL of this filtrate were passed through an IAC (3.7) at a flow rate of approximately 3 mL/min. The IAC was washed twice with 10 mL of water at the same speed. For eluting the aflatoxin from the IAC a three step procedure was necessary:

First 0.5 mL methanol (3.3) was applied on the IAC (3.7) and passed through by gravity. The eluate was collected in a calibrated volumetric flask of 5 mL (4.5). After waiting for 1 min a second portion of 0.5 mL methanol (3.3) was applied and collected. In the same way a third portion of 0.5 mL methanol (3.3) was applied. Most of the applied elution solvent was collected by pressing air or vacuum through the column. The flask was filled to the mark with water and shaken. If the solution was clear it could be used directly for HPLC analysis. If the solution was not clear, it was passed through a disposable filter unit prior to HPLC injection (4.6).

5.3. Preparation of standard solutions

From the AfB_1 standard solution with a concentration of 10.0 µg/mL dissolved in chloroform, a dilution with a final concentration of 0.10 µg/mL solution was used for calibration standard purposes. Portions of 125.0, 250.0, 500.0, 750.0 and 1000.0 µL from this standard were evaporated in 250 mL volumetric flasks (4.5), afterwards re-dissolved with 75 mL of methanol (3.3) and filled up with water. The following concentrations were achieved: 0.050, 0.10, 0.20, 0.30 and 0.40 ng/mL.

For recovery experiments, 5.0 mL of a 0.40 µg/mL AfB_1 standard solution was used to fortify 1.0 L of blank matrix the day prior to analysis, resulting in a fortification level of 2.0 µg/L. After injection of the standard solutions calculate the linearity.

The blank matrix consisted of an AfB_1-free sample of horchata.

The stock solution (100.0 ng/mL) should be stored in a dark cool place (approximately 4 °C), well sealed and wrapped in aluminium foil. This solution is stable for at least 4 months.

Annex 5. Standard operating protocol for the determination of zearalenone in baby food and animal feed

ABSTRACT

This method can be applied to the determination of zearalenone (ZON) in baby food and animal feed. The limit of quantification has been demonstrated to be >10 µg/kg. The method involves extraction of a test portion with a mixture of methanol-water, followed by purification using an immunoaffinity column. The purified sample extract is eluted and ZON is determined by high performance liquid chromatography (HPLC) with fluorescence detection.

1. Scope

This draft specifies a method for the determination of ZON in baby food and animal feed at concentrations above 10 µg/kg.

2. Principle

A known quantity of test material is extracted with a mixture of methanol-water extraction solvent. The extract is filtered, diluted with PBS, and then purified with immunoaffinity column (IAC) chromatography. The purified ZON is eluted with neat methanol, brought to a defined volume with water and quantitatively determined by HPLC with fluorescence detection.

Caution: Wear protective clothing, gloves, and eye protection. WARNING: ZON is an endocrine disruptor. Handle with due regard to its biological activity. Dispose of waste solvents according to applicable environmental rules and regulations.

3. Reagents

3.1. **Methanol,** technical grade.

3.2. **Methanol**, HPLC grade.

3.3. **Water**, HPLC grade or equivalent, and distilled or de-ionised.

3.4. **Sodium chloride**, minimum 99 % purity.

3.5. **Disodium hydrogen phosphate** water free, minimum 99 % purity.

3.6. **Potassium dihydrogen phosphate**, minimum 99 % purity.

3.7. **Potassium chloride**, minimum 99 % purity.

3.8. **Sodium hydroxide**, minimum 99 % purity.

3.9. **Sodium hydroxide**, 0.2 M. Dissolve 8.0 g NaOH ad 1.0 L water.

3.10. **Acetonitrile,** HPLC grade.

3.11. Phosphate buffered saline (PBS).

Dissolve 8.0 g NaCl (3.4), 1.16 g Na_2HPO_4 (3.5), 0.2 g KH_2PO_4 (3.6), and 0.2 g KCl (3.7) in 1.0 L water. Adjust pH to 7.4 with 0.2 M NaOH (3.9). Alternatively, PBS tablets can be purchased.

3.12. Extraction solvent, methanol - water (75+25, v/v).

Mix 750 mL of methanol (3.1) ad 1.0 L of water (3.3). Mix well

3.13. Washing solvent, 15 % methanol in PBS.

Mix 150 mL of Methanol (3.2) with 850 mL of PBS (3.11). Mix well

3.14. Dilution solvent, methanol - water (50+50, v/v).

Mix 500.0 mL of methanol (3.2) with 500.0 mL of water (3.3). Mix well

3.15. HPLC mobile phase, methanol - water (75+25, v/v).

Mix 750.0 mL of methanol (3.2) ad 1.0 L of water (3.3). Mix well and degass.

3.16. ZON stock solution.

For baby food: 400 ng/mL in acetonitrile.
For animal feed: 2000 ng/mL in acetonitrile.
The corresponding solution will be provided for this collaborative trial.

3.17. Working standard solutions for calibration:

For baby food samples: Pipette amounts of 50.0, 200.0, 350.0, 500.0, 650.0 µL of the provided stock solution (3.16) into different 10 mL volumetric flasks using either micropipette or Hamilton syringe (4.10). Fill the flask up to the mark with dilution solvent (3.14) and shake. This will result in ZON solution with concentrations of 2.0 8.0, 14.0, 20.0, 26.0 ng/mL. These concentrations reflect contamination levels of approx. 5.0, 18.0, 32.0, 45.0, 59.0 µg/kg ZON in the sample and can be used directly for injection into the HPLC system.

For animal feed samples: Pipette amounts of 50.0, 250.0, 450.0, 650.0, 850.0 µL of the provided stock solution (3.16) into different 10 mL volumetric flasks using either micropipette or Hamilton syringe (4.10). Fill the flask up to the mark with dilution solvent (3.14) and shake. This will result in ZON solution with concentrations of 10.0, 50.0, 90.0, 130.0, 170.0 ng/mL. These concentrations reflect contamination levels of approx. 23.0, 115.0, 207.0, 299.0, 390.0 µg/kg ZON in the sample and can be used directly for injection into the HPLC system.

3.18. Spiking solutions - you will be provided with 4 vials containing spike solutions of unknown ZON concentration in acetonitrile (3.10) (please note the attached spiking protocol for the collaborative trial).

3.19. Immunoaffinity columns - the columns must contain antibodies specific to ZON, with a minimum capacity of retaining 1500 ng of ZON, resulting in a recovery of at least 70% at the maximum capacity, when applied in a 10 mL solution of 15% methanol in PBS (3.11). For this trial IAC from R-Biopharm (*133*) were provided.

4. Apparatus

Usual laboratory apparatus and, in particular, the following:

4.1. Common laboratory glassware, such as graduated cylinders, volumetric flasks, volumetric pipettes.

4.2. Horizontal or vertical shaker.

4.3. Automated SPE Vacuum System, Supelco Visiprep® Extraction Manifold, or equivalent.

4.4. Volumetric flasks, 3 mL and 10 mL.

4.5. Filter paper folded, Whatman No. 113 V, 18,5 cm or equivalent.

4.6. Screw-cap flasks (100 and 250 - 500 mL).

4.7. Glass funnels, 9 cm ID.

4.8. Reservoirs, polypropylene, suitable for attachment to top of immunoaffinity column, 50 to 75 mL size.

4.9. Plastic syringes, 5 mL.

4.10. Displacement micropipette or Hamilton syringes, gas tight with a volume of 100, 500 and 1000 µL.

4.11. Solvent vacuum filtration system - suitable for 47 mm filter**.**

4.12. Glass microfibre filter paper, Whatman GF/A (47 mm), or equivalent.

4.13. HPLC syringe filter cartridges, Nylon with 0.45 µm pore size.

4.14. Ultrasonic bath.

4.15. HPLC apparatus, consisting of:

- Pump, pulse free, flow capacity 0.5 mL/min to 1.5 mL/min.
- Injector system, manual or autosampler, with loop suitable for 100-300 µl injections.
- Fluorescence detector, suitable for measurements with excitation wavelength 274 nm, and emission at 446 nm.
- Integrator or PC workstation.
- HPLC column. A suitable column is e.g.: Supelcosil® 5 µm LC-18 120 Å, 12.0 % carbon loading, 250 x 4.6 mm, or similar.
- Pre-column, with preferably the same stationary phase material as the analytical column, stationary phase with particles of size 5 µm and internal diameter of 4.0 mm.

5. Procedure

5.1. Preparation of the test materials

The test materials must be sufficiently homogenised to be representative for the lot under investigation. The material must be ground to a particle size of less than 0.5 mm. For this collaborative trial NO preparation is required.

5.2. Extraction

Weigh 20.0 g test portion into a screw-cap flask of 250 - 500 ml (4.6). Add 150 ml extraction solvent (3.12). Mix shortly by hand for a few sec to obtain a homogeneous suspension, then either 1 h in a shaker (4.2) or sonicate for 15 min in an ultrasonic bath (4.14) and shake on a shaker (4.2) for another 15 min.

Filtrate extract through folded filter paper (4.5) and collect the extract in a flask of 100 mL (4.6). Proceed immediately with the IAC cleanup procedure (5.3).

5.3. Immunoaffinity Column Cleanup

Transfer 30 ml of the filtrate extract into a 250 mL volumetric cylinder with stopper. Dilute the extract with PBS (3.11) to achieve a final volume of 150.0 mL. Shake and filter approx. 20 mL of this diluted extract through glass membrane filter paper (4.12) by applying a slight vacuum (4.11) into a glass beaker. Discard these 20 mL and filter again approx. 70 mL for further analysis.

Note: Do not apply a strong vacuum in the beginning of the filtration process, as this can lead to turbid filtered extracts after filtration.

Attach an IAC to the port of the SPE vacuum system (4.3). Attach a reservoir (4.8) on the top of the immunoaffinity column.

Pipette 50.0 ml of the filtrate into the reservoir (4.8). Draw extract through the column by gravity at a steady flow rate until all extract has passed the column and the last solvent portion reaches the frit of the column; the flow rate should result in a dropping speed of 1-2 drops/s.

After the extract has passed through the column, wash it with 5 ml of washing solvent (3.13). Then wash with 15 mL of water (3.3) at a rate of 1-2 drops/s.

Dry the column by passing nitrogen or air through it for about 1 - 2 s. Then discard all the eluent from this stage of the clean-up procedure. Finally, place a 3.0 mL volumetric flask (4.4) under the column and pass 0.75 mL of methanol (3.2) through the column, collecting the eluate. After the last drops of methanol have passed through the column allow the methanol to remain on the column for approximately 1 min. Then add a further 0.75 mL of methanol (3.2) and continue to collect the eluate. Carefully pass air through the column in order to collect any final drops.

Fill the volumetric flask up to the mark with water and shake. In case of turbid samples, filter test solution through a HPLC syringe filter (4.13) with a plastic syringe (4.9).

Note: Methanol and water undergo volume contraction when mixed. Adjust volume if necessary after shaking.

Note: Alternatively to a manual procedure the IAC clean-up and elution can be performed with an automatic sample preparation unit, provided that volumes and aliquots remain unchanged.

5.4. Spike recovery

Using a displacement micropipette or preferably a Hamilton syringe (4.10) add 1000.0 µL of a spike recovery solution (3.16) to 20.0 g of blank matrix, allow to stand for at least 30 min and extract as described in section (5.2). A spike recovery should be carried out with each analytical batch. You will be supplied with 4 different spiking solutions for spiking of the "blank" materials.

5.5. HPLC operating conditions

- Flow rate: 0.70 - 1.0 mL/min.
- Injection volume: 100 - 300 µL.
- Detector wavelength: excitation 274 nm, emission 446 nm.

5.6. Preparation of the calibration graph

Plot the data - concentration of ZON [ng/mL] (x-axis) against the peak signal as area or height (y-axis) from the calibration experiments into a table and calculate the linearity.

Use the resulting function (y = ax + b) to calculate the concentration of ZON in the measured solution (where a is the value of the slope of the linear function and b is the value where the calibration function intercepts the y-axis of the co-ordinate system).

Calculation of the calibration curve (function) obtained by linear regression:

C_{smp} [ng/mL] = a x $Signal_{smp}$ [units] + b

$Signal_{smp}$: signal of ZON peak obtained from the measured solution

5.7. Determination of ZON in test material

Inject aliquots of the working standards (3.17) into the chromatograph using the same conditions used for the preparation of the calibration graph. Identify the ZON peak of the test solution by comparing the retention time of the sample with that of the calibrants.

5.8. Calculation of results

Quantitative determination is carried out by the calculation of the peak signal (area or height). Determine the content of ZON in the test material, in ng/mL, directly from the calibration graph (5.6).

To calculate the contamination level of ZON in the test material according to:

$$ZON\ [\mathrm{ng/g}] = \frac{C_{smp} \times Solvent \times Elution}{W \times Aliquot} \left[\frac{\mathrm{ng} \times \mathrm{mL} \times \mathrm{mL}}{\mathrm{mL} \times \mathrm{g} \times \mathrm{mL}} \right]$$

$$ZON\ [\text{ng/g}] = C_{smp} \times 2.25$$

W [g] : sample material taken for analysis (20.0 g)

Solvent [mL] : solvent taken for extraction (150.0 mL)

Aliquot [mL] : extract aliquot used for immunoaffinity clean-up (10.0 mL)

(30 mL x 50 mL / 150 mL = 10 mL)

Elution [mL] : final volume achieved after elution from IAC (3.0 mL)

C_{smp} [ng/mL] : concentration of ZON in the injected solution calculated from linear regression

Annex 6. Youden Plots from the zearalenone trial

BABY FOOD	
Original Lab. ID (Tab. 21)	New Lab ID
1	A
2	B
3	C
4	D
5	E
6	F
7	G
8	H
9	I
10	J
11	K
12	L
13	M
14	N
15	O
16	P
18	Q
19	R
20	S
21	T

ANIMAL FEED	
Original Lab ID (Tab. 22)	New Lab ID
1	a
2	b
3	c
4	d
5	e
6	f
7	g
8	h
9	i
10	j
11	k
12	l
13	m
14	n
15	o
16	p
18	q

Labelling codes for the identification of the Youden data from the laboratories participating in the zearalenone collaborative trial.

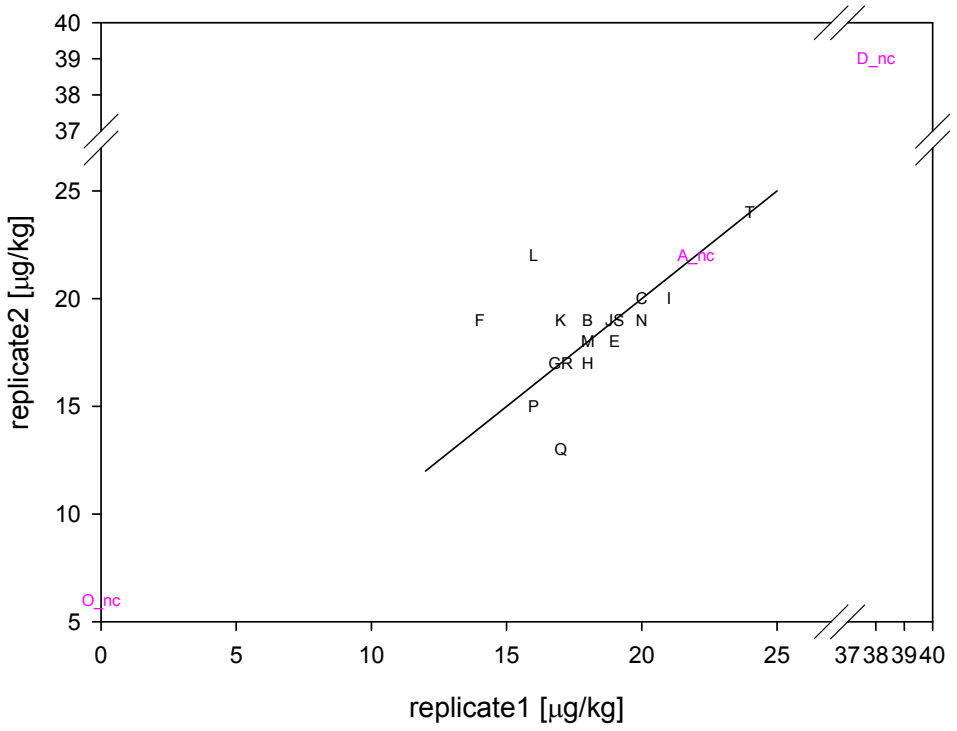


Figure 1. Baby food spike low level (data in Tab. 21).

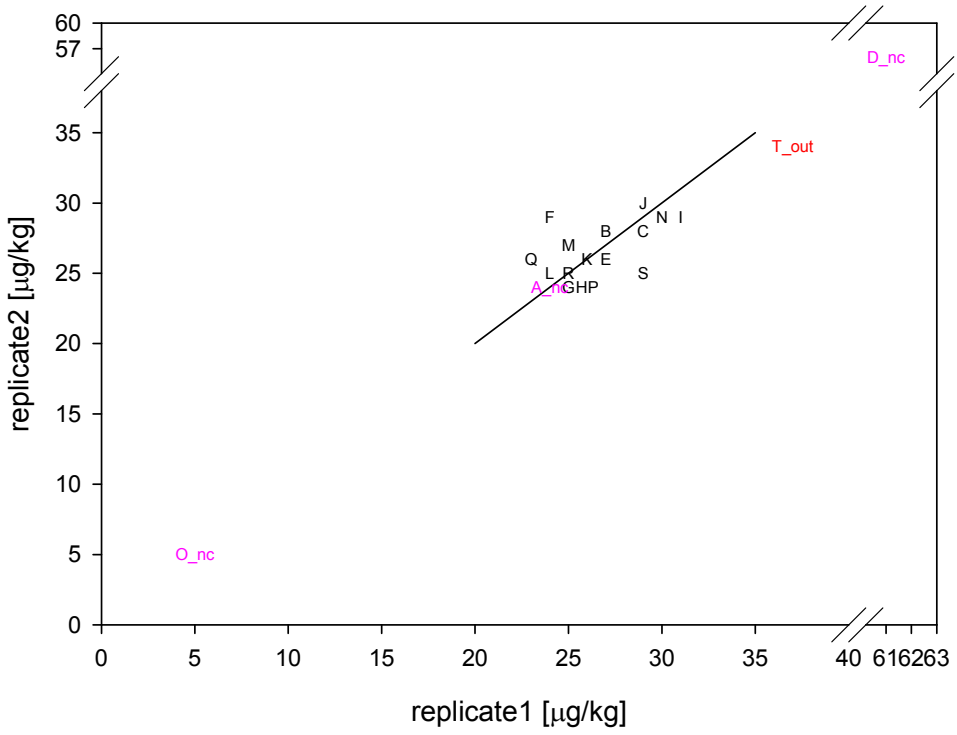


Figure 2. Baby food spike high level (data in Tab. 21).

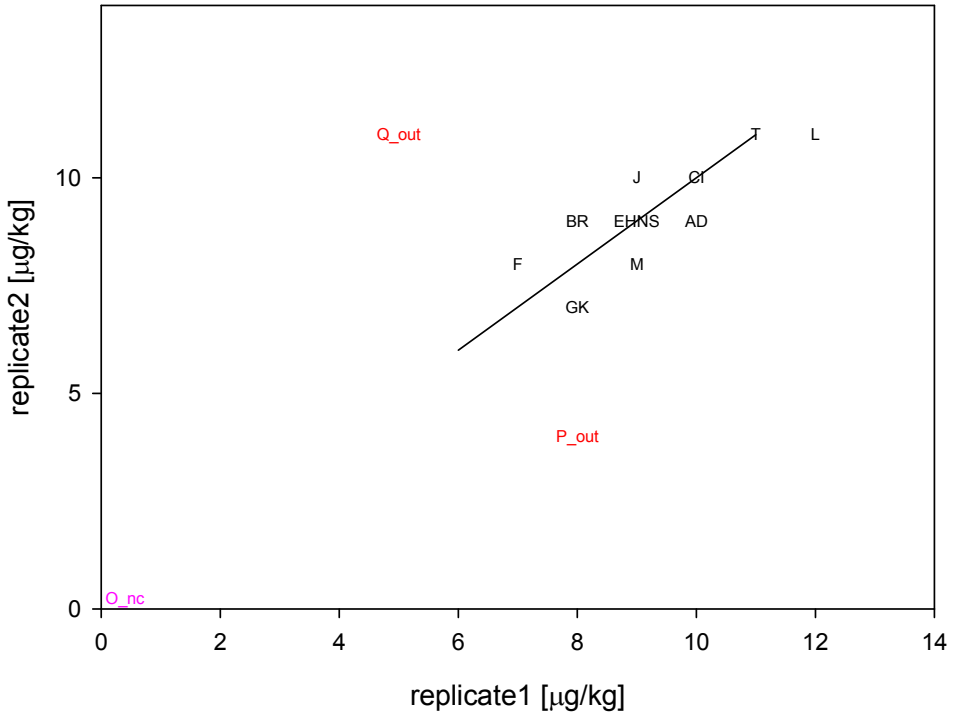


Figure 3. Baby food naturally contaminated low level (data in Tab. 21).

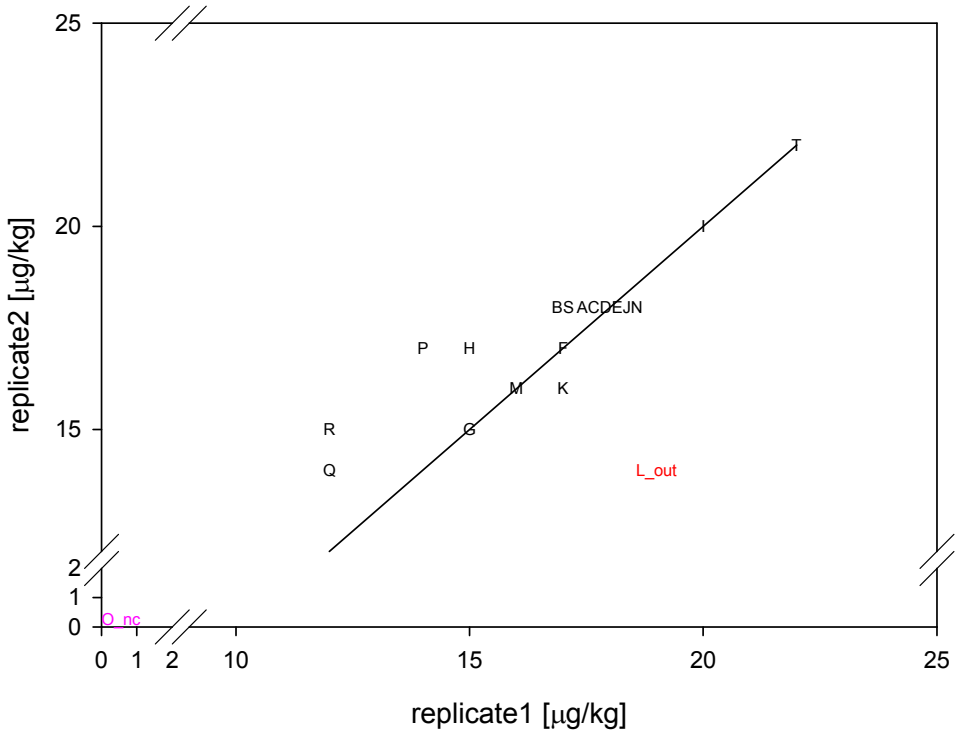


Figure 4. Baby food naturally contaminated medium level (data in Tab. 21).

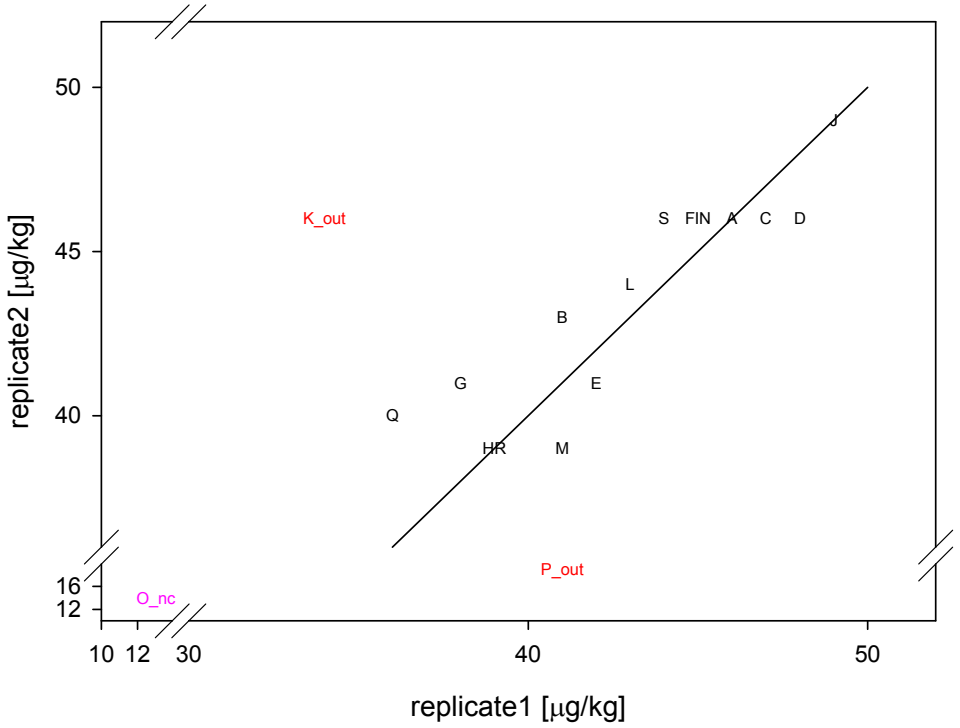


Figure 5. Baby food naturally contaminated high level (data in Tab. 21).

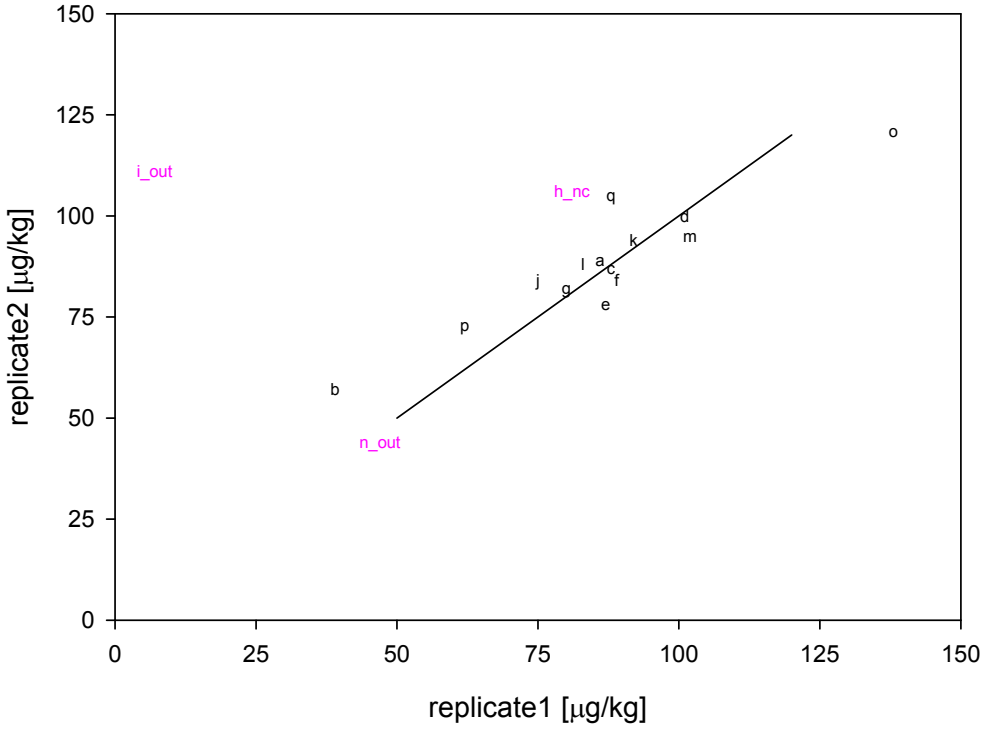


Figure 6. Animal feed spike low level (data in Tab. 22).

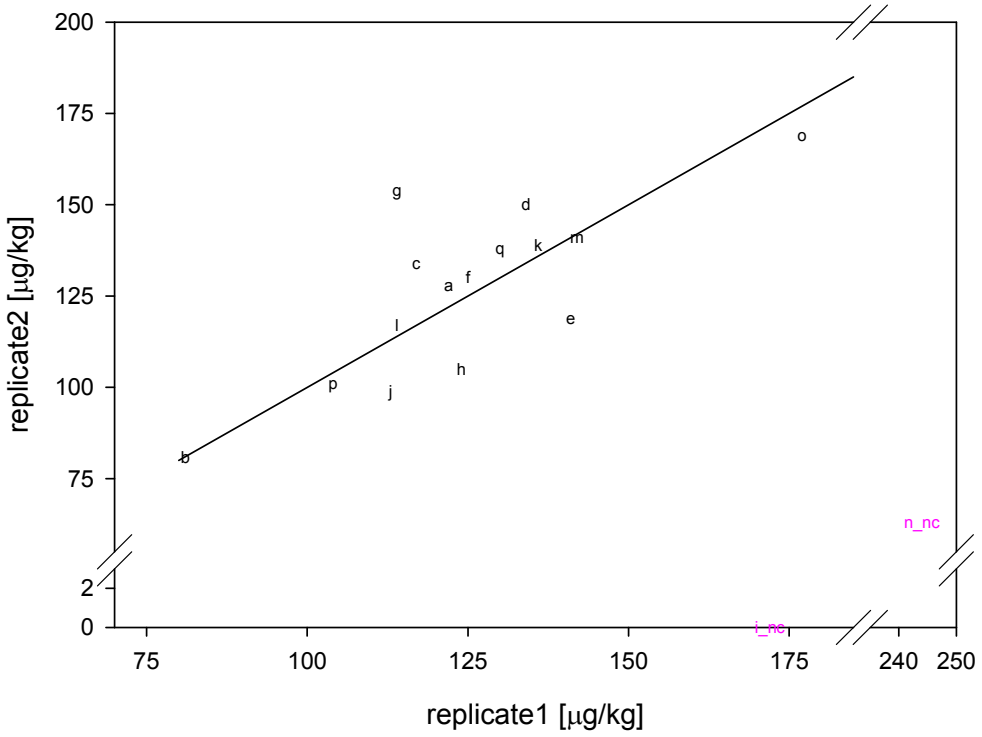


Figure 7. Animal feed spike high level (data in Tab. 22).

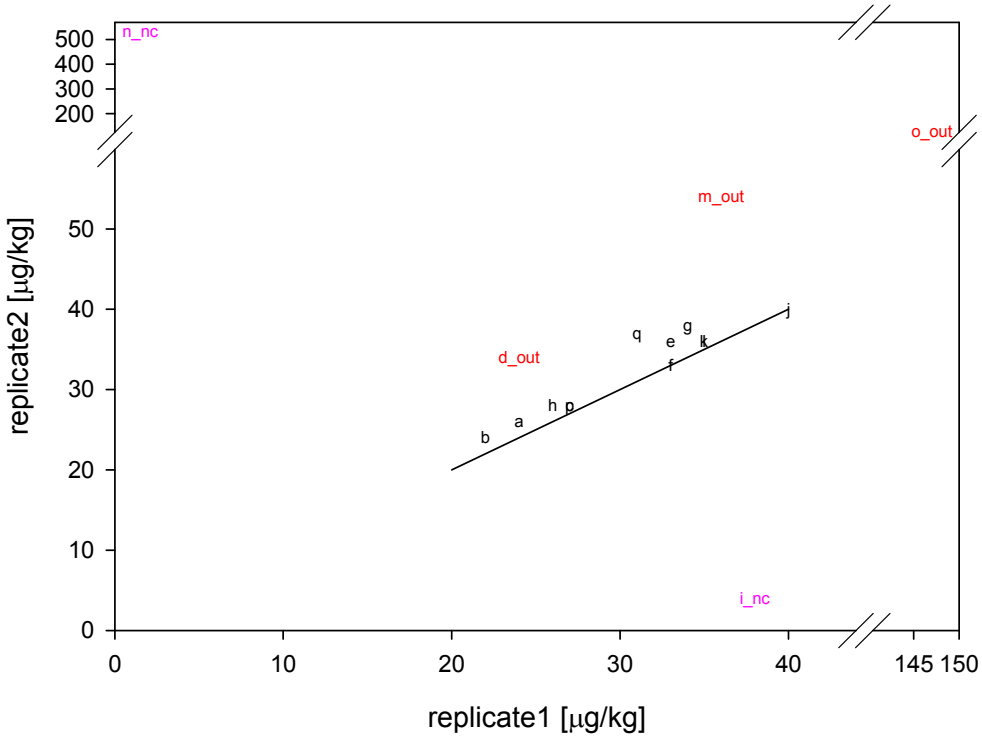


Figure 8. Animal feed naturally contaminated low level (data in Tab. 22).

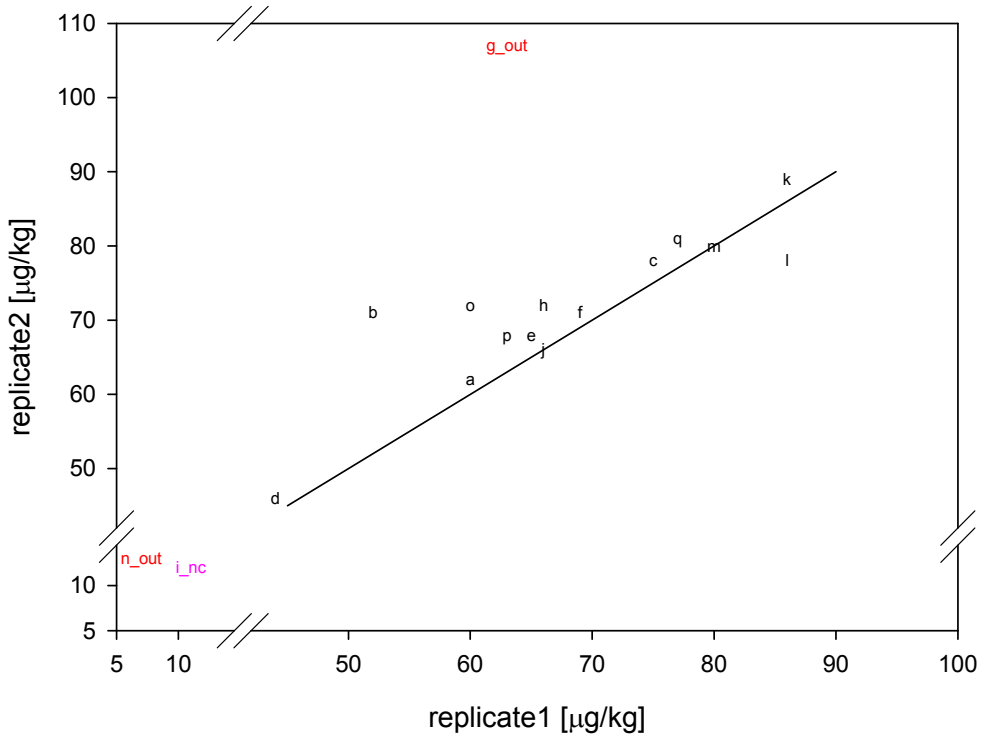


Figure 9. Animal feed naturally contaminated medium level (data in Tab. 22).

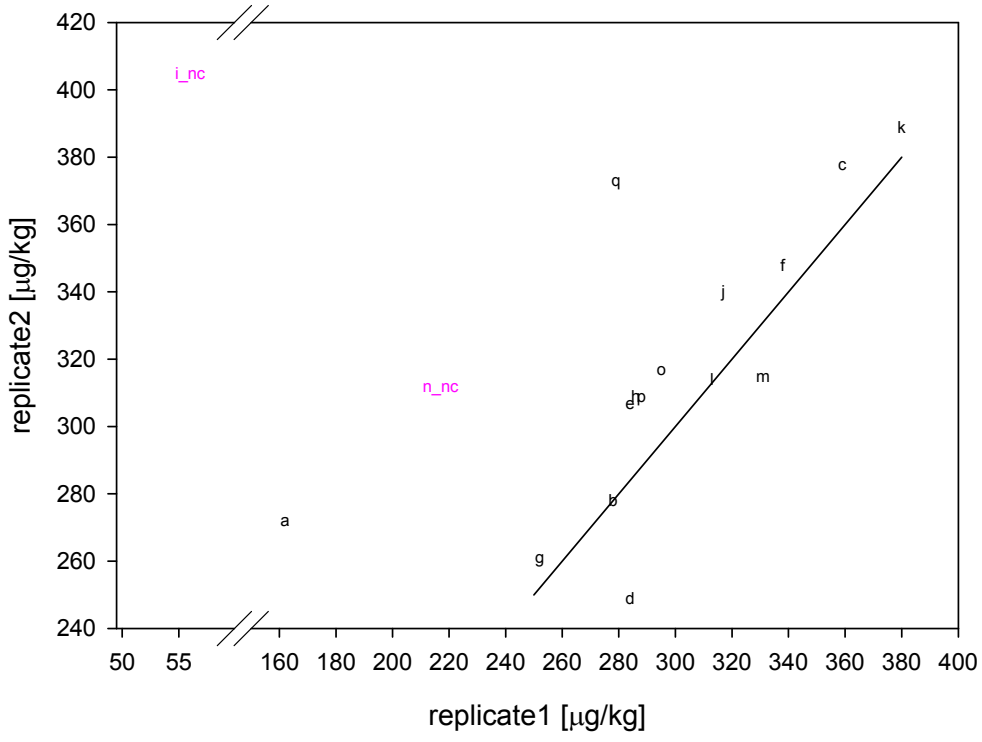


Figure 10. Animal feed naturally contaminated high level (data in Tab. 22).

Annex 7. Standard operating protocol for the determination of patulin in apple juices and fruit purees.

COLLABORATIVE STUDY MATERIALS RECEIPT FORM METHOD A

Name of Participant and Laboratory Code:	

Please ensure that the items listed below have been received undamaged, and then describe the relevant statement:

Date of the receipt	
All items have been received undamaged	yes/no
Items are missing/damaged. I require an additional series of samples	yes/no

Contents of parcel

a) Apple Juice and Fruit/Cereal Puree test materials (24 containers)
b) One amber vial identified as "PAT Standard" (for calibration curve)
c) Eight coded vials identified as "Spiking solution"
d) 24 Silicagel SPE columns
e) Receipt form
f) One copy of the collaborative study method
g) Spiking Protocol
h) Annex (Technical notes and chromatograms)
i) Please fax or email the completed form to:

Isabel Arranz
European Commission
DG Joint Research Center
Institute for Reference Materials and Measurements
B-2440 Geel

Liquid Liquid Extraction Method A

Draft of a method for the determination of patulin in apple juices and fruit purees in a suitable format for intercomparison purposes.

ABSTRACT

This method can be applied to the determination of patulin (PAT) in clear and cloudy apple juices and in fruit purees. The limit of quantification of the method for PAT has been demonstrated to be >10 µg/kg. The method involves extraction of a test portion of juices with an ethyl acetate extraction solvent followed by a solid-phase extraction (SPE) clean-up. The sample extract is dried, concentrated and PAT is determined by high performance liquid chromatography (HPLC) with ultra-violet (UV) detection.

1. Scope

This draft specifies a method for the determination of PAT in apple juices and apple puree using HPLC.

2. Principle

A known quantity of apple juice / puree is extracted with an ethyl acetate extraction solvent in the presence of sodium sulfate and sodium-hydrogen-carbonate and is then cleaned up by SPE. The purified extract is evaporated to dryness, re-dissolved in aqueous acetic acid solution and quantitatively determined by HPLC with UV detection.

Caution: Wear protective clothing, gloves, and eye protection. PAT has antibiotic activity. Various in vitro and in vivo laboratory tests have shown mutagenic, immunotoxic, neurotoxic and adverse gastrointestinal effects. Data on the carcinogenic potential of PAT are incomplete. Dispose of waste solvents according to applicable environmental rules and regulations.

3. Reagents

3.1. General - Use only reagents of a recognised analytical grade.

3.2. Acetic acid glacial

3.3. Acetonitrile (HPLC grade)

3.4. Ethyl acetate (glass distilled grade or better) - PAT free

3.5. *n*-Hexane (glass distilled grade or better) - PAT free

3.6. Extraction solution – ethyl acetate (3.4)-*n*-hexane (3.5) (60+40, v/v)

3.7. Perchloric acid 60 %

3.8. Sand (washed seasand)

3.9. Sodium hydrogencarbonate - anhydrous

3.10. Sodium sulfate – anhydrous

3.11. Deionised water

3.12. Acetic acid in ethyl acetate - add 3.0 mL acetic acid (3.2) ad 100.0 mL of ethyl acetate (3.4).

3.13. Water pH 4. Adjust deionised water (3.11) with acetic acid (3.2) to pH 4.

3.14. 5-Hydroxymethylfurfural (HMF) solution. Disolve 5 mg HMF in 1.0 mL ethyl acetate (3.4) and dilute to 25.0 mL water pH 4 (3.13).

3.15. Mobile phase for HPLC. Water – acetonitrile (3.3) – perchloric acid 60 % (3.7) (990+10+1, v/v/v). The exact amount of acetonitrile used will depend upon the sample extract and the HPLC column chosen for analysis. Degas this solution before use.

3.16. PAT – stock solution of 10 µg/mL in water pH 4 (3.13). This solution will be provided for this collaborative study.

3.17. Working standard solutions for calibration: Pipette amounts of 60.0, 120.0, 180.0, 240.0, 300.0, 360.0 and 420.0 µL of the stock solution (3.16) into different 50 mL volumetric flasks. Fill the flasks up to the mark with water pH 4 (3.13) and shake. This will result in PAT solutions with concentrations of: 12.0, 24.0, 36.0, 48.0, 60.0, 72.0 and 84.0 ng/mL. These concentrations reflect contamination levels of 4.8, 9.6, 14.4, 19.2, 24.0, 28.8 and 33.6 µg/kg PAT in the sample and can be used directly for injection into the HPLC system.

3.18. Spike recovery solutions - you will be provided with 4 vials containing spike solutions of unknown PAT concentration in water pH 4 (3.13).

3.19. Silicagel SPE columns: these will be provided.

4. Apparatus

Usual laboratory apparatus and in particular the following:

4.1. HPLC apparatus comprising the following; an eluent reservoir,

- HPLC pump(s), an injection system of 200 µL that allows a full loop injection at a fixed volume, an UV detector at λ = 278 nm.

- Analytical reversed phase HPLC column**.** The column must allow a sufficient separation of PAT from other interfering components. Suitable columns are e.g.: Polar endcapped or polar embedded alkyl phases that are designed to run with mobile phases of 100 % water (e.g. Synergi® 4 µm HydroRP 80Å, 250 x 4.6 mm) provided that the available column type allows a sufficient separation.

- Pre-column, similar nature as the analytical column, with preferably an internal diameter of 4.0 mm and stationary phase with particles of size 5 µm

4.2. Centrifuge tubes of 50 mL capacity with screw cap

4.3. Micro-pipettes of 10 - 100 µL and 100 - 1000 µL capacity.

4.4. Hamilton syringes with a total capacity of 3 to 5 mL (or equivalent).

4.5. Evaporatation block, for 40 °C with nitrogen supply

4.6. Volumetric flasks of 5, 20 and 50 mL.

5. Procedure

5.1. Preparation of the test materials. For all types of sample NO preparation is required.

5.2. Test procedure

5.2.1. Extraction of PAT from the sample

To a clean centrifuge tube (4.2) add 2 g of sand (3.8), 15.0 g of Na_2SO_4 (3.10), 2.0 g of $NaHCO_3$ (3.9) and shake. Add 10 mL of the extraction solvent (3.6) to the prepared tube and close tightly. The centrifuge tube is now ready for analysis.

Transfer 10.0 g of sample into the prepared centrifuge tube and shake vigorously by hand for a few seconds, and then for exactly 5 min on a mechanical shaker. Subsequently centrifuge the extraction mixture at low speed for 30 s to force layer separation.

Note: PAT is not stable in alkaline solutions (e.g. $NaHCO_3$), therefore this stage of the method must be carried out as quickly as possible to avoid any losses.

5.2.2. SPE clean-up

Add 50.0 µL of the acetic acid solution in ethyl acetate (3.12) to a 6 mL glass vial with screw cap and place it under an unconditioned Silicagel SPE column. Immediately transfer exactly 2.50 mL of the centrifuged extract onto the unconditioned SPE column. Collect the eluate in the glass vial at a speed of 1 drop per s (e.g. by gravity or slight air pressure). Immediately wash the SPE column with 3 mL of the ethyl acetate-hexane mixture (3.6) to elute the PAT-containing extractant quantitatively from the column. When most of the washing solution has passed through, push with an air filled syringe the remaining solvent from the column into the vial.

Evaporate the collected solvents at max. 40 °C under a gentle stream of nitrogen just to dryness. This should not take longer than 10 min. Don't allow the samples to evaporate any further beyond dryness, in order to avoid any PAT losses. Add 1.0 mL of water pH 4 (3.13) to the vial and close it with the screw cap.

Shake (or vortex) the vial for at least 3 min to ensure that the PAT is fully re-dissolved. Transfer the solution into a suitable injector vial (e.g. 2 mL or smaller).

5.3. Spike recovery

You will be supplied with 4 different spiking solutions for each matrix (see Spiking Protocol).

5.4. HPLC operating conditions

- Flow rate: 1.0 mL/min.
- Injection volume: 200 µL.
- Detection: UV detector set at 278 nm.

Note: in most cases it is necessary to wash the HPLC system with 65-100 % acetonitrile after each sample injection to wash "late eluting peaks" from the column. After such a wash re-equilibrate the system with mobile phase prior to the next injection. In case of doubt of peak identification, confirm the patulin peak by tracking with the patulin / 5-HMF ratio.

5.5. Preparation of the calibration graph

Prepare the calibration graph by injecting 100-200 µL of at least five of the seven standard solutions proposed in 3.17 into the chromatograph. Plot the peak area or peak height values of the PAT calibration solutions (3.17) against the concentration in µg/kg of PAT in the sample.

5.6. Determination of PAT in test solutions

Inject aliquots of the test solutions into the chromatograph using the same conditions used for the preparation of the calibration graph. Identify the PAT peak of the test solution by comparing the retention time of the sample with that of the calibrants.

5.7. Calculation

Quantitative determination is carried out by the calculation of the peak height or peak area. Determine the content of PAT in the test material, in µg/kg, directly from the calibration curve or via the given formula:

$$PAT\,[\mu g/kg] = c\,[ng/mL] \times \frac{v3\,[mL] \times v1\,[mL]}{v2\,[mL] \times m1\,[g]}$$

$$PAT\,[\mu g/kg] = c\,[ng/mL] \times \frac{1.0 \times 10.0}{2.5 \times 10}$$

$$PAT\,[\mu g/kg] = c\,[ng/mL] \times 0.4$$

Where:

c : Concentration of the injection solution calculated form linear regression

m1 : Mass of sample taken for analysis (10.0 g)

v1 : Amount of extraction solvent (10.0 mL)

v2 : Aliquot taken for SPE clean-up (2.5 mL)

v3 : Volume of acetic acid solution (3.12) used to re-dissolve (1.0 mL)

Spiking Protocol:

In order to calculate the recovery of the method you are supplied with 8 different vials (4 vials for apple juice and 4 vials for fruit puree). These vials contain 1.2 mL of PAT standard in frozen in water pH 4 (3.13). Prior to analysis defrost the vials and shake them vigorously (e.g. with a vortex shaker).

Four vials are marked with A, B, C and D and have to be used for apple juice spiking.

Four vials are marked with 1, 2, 3 and 4 and have to be used for fruit puree spiking.

For spiking you will need, as well as to the spiking solutions the sample containers identified as 'blank'. These containers are exclusively reserved for the spiking experiments and MUST NOT be used for direct analysis.

For JUICE (vials A, B, C and D):

After defrosting, shake the container and weigh 19.0 g of the juice into a 50 mL beaker and add 1.0 mL of the spiking solution. Stir the solution until completely mixed. From this 20.0 g fortified solution (19+1) use 10.0 g for analysis according to the method description.

For PUREE (vials 1, 2, 3, and 4):

After defrosting, stir the contents of the container with a spatula until homogeneous and weigh 19.0 g of the puree into a 50 mL beaker. Add 1.0 mL of the spiking solution to the puree and mix thoroughly with a spatula for at least 3 min. Make sure that the contents are mixed thoroughly. If necessary, use the spatula to scratch puree traces from the beaker wall and include these in the mixing process. Alternatively, after pre-stirring with a spatula, you can also use a syringe and mix the puree by repeated aspiration and rapid dispensing of the spiked puree. From the resulting 20.0 (19+1) use 10.0 g for analysis according to the method description.

COLLABORATIVE STUDY MATERIALS RECEIPT FORM METHOD B

Name of Participant and Laboratory Code:	

Please ensure that the items listed below have been received undamaged, and then describe the relevant statement:

Date of the receipt	
All items have been received undamaged	yes/no
Items are missing/damaged. I require an additional series of samples	yes/no

Contents of parcel

a) Apple Juice and Fruit/Cereal Puree test materials (24 containers)
b) One amber vial identified as "PAT Standard" (for calibration curve)
c) Eight coded vials identified as "Spiking solution"
d) A container identified as "Pectinase enzyme solution"
e) One copy of the collaborative study method
f) Annex (chromatogram)
g) Report form
h) A CD with training videos (**for understanding the method principle ONLY, the method on the CD is not the same as the method used here**)
i) Please fax or email the completed form to:

Isabel Arranz
European Commission
DG Joint Research Center
Institute for Reference Materials and Measurements
B-2440 Geel

Liquid Liquid Extraction Method B

Draft of a method for the determination of PAT in apple juices and fruit purees in a suitable format for intercomparison purposes.

ABSTRACT

This method can be applied to the determination of PAT in clear and cloudy apple juices and in fruit purees. The limit of quantification of the method for PAT has been demonstrated to be >10 µg/kg. The method involves extraction of a test portion of juice with ethyl acetate followed by extraction with sodium carbonate solution. Cloudy juices and fruit purees are treated with pectinase prior to extraction. The sample extract is dried, concentrated and PAT is determined by high performance liquid chromatography (HPLC) with ultra violet (UV) detection.

1. Scope

This draft specifies a method for the determination of PAT in apple juices and apple puree using HPLC.

2. Principle

A known quantity of apple juice or puree is extracted with ethyl acetate and then cleaned up by extraction with sodium carbonate solution. The ethyl acetate extract is dried with anhydrous sodium sulfate. After evaporation of ethyl acetate, PAT is quantitatively determined by HPLC with UV detection**.**

Caution: Wear protective clothing, gloves and eye protection. PAT has antibiotic activity. Various in vitro and in vivo laboratory tests have shown mutagenic, immunotoxic, neurotoxic and adverse gastrointestinal effects. Data on the carcinogenic potential of PAT is incomplete. Dispose of waste solvents according to applicable environmental rules and regulations.

3. Reagents

3.1. General - Use only reagents of a recognised analytical grade.

3.2. Ethanol (HPLC grade)

3.3. Acetic acid glacial

3.4. Acetonitrile (HPLC grade)

3.5. Ethyl acetate (glass distilled grade or better) - PAT free

3.6. Pectinase enzyme solution - (endo-galacturonase), typical activity 1400 U/g. Unit definition - the amount of enzyme which catalyses the decrease in viscosity of a 1 % pectin solution by 20 % in 5 min at pH 3.4 and 25 ºC.

3.7. Perchloric acid 60 %

3.8. Sodium carbonate (anhydrous)

3.9. Sodium sulfate (anhydrous)

3.10. Deionised water

3.11. Sodium carbonate solution 1.5 % - dissolve 1.5 g sodium carbonate (3.8) in 100 mL deionised water (3.10).

3.12. Water pH 4 - adjust deionised water (3.10) with acetic acid (3.3) to pH 4.

3.13. Mobile phase for HPLC - Water (3.10)-acetonitrile (3.4)-perchloric acid 60 % (3.7) (980+20+1, v/v/v). The exact amount of acetonitrile used will depend upon the sample extract and the HPLC column chosen for analysis. Degas this solution before use.

3.14. PAT - stock solution of 10 µg/mL in water pH 4 (3.12). This solution will be provided.

3.15. Working standard solutions for calibration: Pipette amounts of 50.0, 100.0, 150.0, 200.0, 250.0, 300.0 and 350.0 µL of the provided calibrant solution (3.14) into different 20 mL volumetric flasks. Fill the flasks up to the mark with water pH 4 (3.12) and shake. This will results in PAT solutions with concentrations of 25.0, 50.0, 75.0, 100.0, 125.0, 150.0 and 175.0 ng/mL. These concentrations reflect contamination levels of 5.0, 10.0, 15.0, 20.0, 25.0, 30.0 and 35.0 µg/kg PAT in the sample (provided that the method protocol is followed) and can be used directly for injection into the HPLC system.

3.16. Spike recovery solution - you will be provided with 4 vials containing spike solutions of unknown PAT concentration in water pH 4 (3.12).

3.17. 5-Hydroxymethylfurfural (HMF) solution. Disolve 5 mg HMF in 1.0 mL ethyl acetate (3.5) and dilute to 25.0 mL water pH 4 (3.12).

4. Apparatus

Usual laboratory apparatus and, in particular, the following:

4.1. HPLC apparatus comprising the following:

- HPLC pump(s).
- Injection system of 100 µL, 200 µL or volumes between that allows a full loop injection at a fixed volume.
- UV detector at $\lambda = 278$ nm.
- Analytical reversed phase HPLC column. The column must allow a sufficient separation of PAT from other interfering components. Suitable columns are e.g.: Octadecylsilane endcapped stationary phase particles of size 5 µm, 250 Å pore size. Or polar endcapped or polar embedded alkyl phases that are designed to run with mobile phases of 100 % water (e.g. Synergi® 4 µm Hydro RP 80 Å, 250 x 4.6 mm) provided that the available column type allows a sufficient separation.
- Pre-column, ODS, with preferably particles of size 5 µm

4.2. Spectrophotometer - suitable for measurement from $\lambda = 250$ nm to $\lambda = 350$ nm.

4.3. **Quartz cells**, of optical path length 1.0 cm.

4.4. **Centrifuge**

4.5. **Centrifuge tubes** of 50 mL capacity with screw cap.

4.6. **Rotary evaporator**, or similar.

4.7. **Round bottomed flasks** of 500 mL (suitable for vacuum evaporation).

4.8. **Fluted filter funnel**, (e.g. so called Urbanti funnel)

4.9. **Watchglass**, suitable to cover funnel (4.10)

4.10. **Micro-pipettes** of 10 - 100 µL and 100 - 1000 µL capacity.

4.11. **Volumetric flasks** of 5 and 20 mL.

4.12. **Separation funnel** of 250 mL capacity.

4.13. **Filter paper** 11 cm diameter (e.g. Schleicher & Schuell "Whiteband" or Whatman #40)

5. Procedures

5.1. Preparation of the test materials

For clear apple juice no preparation is required. For cloudy juices measure 20.0 g of sample into a centrifuge tube (4.4) and add 10 drops of enzyme solution (3.6). Leave overnight at room temperature, or for 2 h at 40 ^{o}C, after which centrifuge the sample at 4500 g for 5 min. For fruit puree weigh 20.0 g of sample into a centrifuge tube (4.4), add 20 drops of enzyme solution (3.6) followed by 20.0 mL of H_2O (3.10) and thoroughly mix together. Leave the sample at room temperature overnight, or for 2 h at 40 ^{o}C in a closed container and centrifuge at 4500 g for 5 min.

5.2. Test procedure

5.2.1. Extraction of PAT from the sample

Transfer 20.0 g of sample of clear juice (or cloudy juice or puree as prepared in 5.1) into a 100 mL separation funnel. Add 30 mL of ethyl acetate (3.5) and shake for 1 min. Allow the layers to separate then drain them into two separate conical flasks.

Transfer the aqueous layer back into the same separation funnel and re-extract with a second 30 mL portion of ethyl acetate (3.5). Allow the layers to separate and drain the lower aqueous layer into an empty conical flask and the top layer into the conical flask containing the ethyl acetate layer from the first extraction. Repeat this extraction procedure for a third time, but after allowing the layers to separate pour the lower aqueous to waste. Combine the three ethyl acetate phases into the separation funnel.

Rinse the conical flask used to collect the ethyl acetate phases with a further 5 mL ethyl acetate (3.5) and add this to the ethyl acetate extract in the separation funnel.

5.2.2. Removal of interfering acidic compounds

Prepare a funnel (4.7) and filter paper (4.12) containing 25 g anhydrous sodium sulfate (3.9). Ensure that the sodium sulfate has a concave shape within the filter paper. Use only fluted funnels (4.7) with a rim at least 0.5 cm higher than the filter paper (4.12).

Add 8.0 mL sodium carbonate solution (3.11) to the separation funnel and shake for 0.5 min. Allow the layers to separate, and then pour off the lower aqueous layer into a conical flask. Pour the top layer into a round bottomed flask (4.9) through the prepared funnel containing the 25 g anhydrous sodium sulfate (3.9). After all the ethyl acetate has been poured onto the filter, cover it with the watchglass (4.8).

Transfer the aqueous layer back into the separation funnel, rinse the conical flask with 10.0 mL ethyl acetate (3.5), and add this to the separation funnel and shake for 0.5 min. Allow the layers to separate, pour off the lower layer to waste, remove the watchglass from the funnel and pour the top layer through the sodium sulfate (3.9) into the round bottomed flask (4.6). Wash the sodium sulfate (3.9) with 2 x 25 mL of ethyl acetate (3.5) and collect in the round bottomed flask (4.6). During the washing step make sure that the upper rim of the filter paper and the sodium sulfate is sufficiently washed. In cases where a sodium sulfate clump has formed, remove it from the filter and squeeze the filter slightly with the remaining sodium sulfate against the funnel wall in order to recover most of the washing solution.

Note: PAT is not stable in alkaline solutions, therefore this stage of the method must be carried out as quickly as possible to avoid any losses.

5.2.3. Preparation of test sample for HPLC analysis

Evaporate the combined sample and washing extracts to dryness on a rotary evaporator.

Make sure that no further evaporation occurs after dryness. Immediately cool the round bottom flask to ambient temperature when evaporation has been carried out at 40 °C and re-dissolve in a final volume of 4.0 mL (2.0 mL for puree samples), water pH 4 (3.12).

Make sure that the residue is re-dissolved. Transfer to an HPLC vial. If necessary the sample may be filtered before analysis by HPLC. A check should be made with a standard solution to assess any loss of PAT before the test extracts are filtered.

5.3. Spike recovery

For the collaborative trial, please perform spike experiments according to the instructions in the sheet named **SPIKING PROTOCOL**.

5.4. HPLC operating conditions

- Flow rate: 1.0 mL/min.
- Injection volume: 100 - 200 µL.
- Detection: UV detector set at 278 nm.

Note: it may necessary to wash the HPLC system thoroughly with 100 % acetonitrile (3.4) after each sample injection to ensure that there are no materials retained on the column. After

such a wash re-equilibrate the system with mobile phase (3.13) prior to the next injection. In case of doubt of peak identification, confirm the patulin peak by tracking with the patulin/5-HMF ratio.

5.5. Preparation of the calibration graph

Prepare the calibration graph by injecting 100-200 µL of at least five standard solutions of different suitable concentrations (3.15) into the chromatograph. Independent from the injection volume chosen, you must make sure that for all injections (standard solutions and samples extracts) the same injection volume is used. Plot the peak height (or area) values of the PAT calibration solutions (3.15) against the concentration in ng/mL (injected solution) or directly in µg/kg of PAT in the sample.

5.6. Determination of PAT in test solutions

Inject aliquots of the test solutions into the chromatograph using the same conditions used for the preparation of the calibration graph. Identify the PAT peak of the test solution by comparing the retention time of the sample with that of the calibrants.

5.7. Calculation

Quantitative determination is carried out by the calculation of the peak height or peak area. Determine the content of PAT in the test material, in µg/kg, directly from the working standard solution for calibration (3.15) or from the formula given below:

$$PAT\,[\mu g/kg] = c\,[ng/mL] \times \frac{v1\,[mL]}{m1\,[g]}$$

$$PAT\,[\mu g/kg] = c\,[ng/mL] \times 0.2$$

$$PAT\,[\mu g/kg] = c\,[ng/mL] \times \frac{4}{20}$$

c : Concentration of the injection solution calculated from linear regression.

$m1$: Mass of sample taken for analysis 20.0 g for juices and 10.0 g for purees.

$v1$: Volume of water pH 4 (3.12) used to re-dissolve 4.0 mL for juices and 2.0 mL for purees.

Spiking Protocol:

In order to calculate the recovery of the method you are supplied with 8 different vials (4 vials for apple juice and 4 vials for fruit puree). These vials contain 1.2 mL of PAT standard in frozen water pH 4 (3.12). Prior to analysis defrost the vials and shake them vigorously (e.g. with a vortex shaker).

Four vials are marked with A, B, C and D and have to be used for apple juice spiking.

Four vials are marked with 1, 2, 3 and 4 and have to be used for fruit puree spiking.

For spiking you will need, as well as to the spiking solutions the sample containers identified as '**blank**'. These containers are exclusively reserved for the spiking experiments and **MUST NOT** be used for direct analysis.

For JUICE (vials A, B, C and D):

After defrosting, shake the container and weigh 19.0 g of the juice into the separation funnel. Then add 1.0 mL of the spiking solution. Stir the solution until completely mixed and proceed according to the method description.

For PUREE (vials 1, 2, 3, and 4):

After defrosting, stir the contents of the container with a spatula until homogeneous and weigh 19.0 g of the puree (instead of 20.0 g) into the centrifuge tube (see method description section 5.1). Add 1.0 mL of the spiking solution to the puree and then add 20 drops of enzyme solution (3.6) according to the method description.

Annex 8. Youden plots from the patulin trial

METHOD A	
Original Lab ID (Tab. 33-34)	New Lab ID
1752003	A
1862003	B
1712003	C
1622003	D
1872003	E
1572003	F
1832003	G
1822003	H
1792003	I
1772003	J
1742003	K
1702003	L
1662003	M
1642003	N
1892003	O
1552003	P

METHOD B	
Original Lab ID (Tab. 35-36)	New Lab ID
1882003	a
1972003	b
1602003	c
1672003	d
1682003	e
1722003	f
1762003	g
1782003	h
1802003	i
1812003	j
1852003	k
1902003	l
1932003	m
1942003	n
1612003	o
1652003	p
1842003	q

Labelling codes for the identification of the Youden data from the laboratories participating in the patulin collaborative trial.

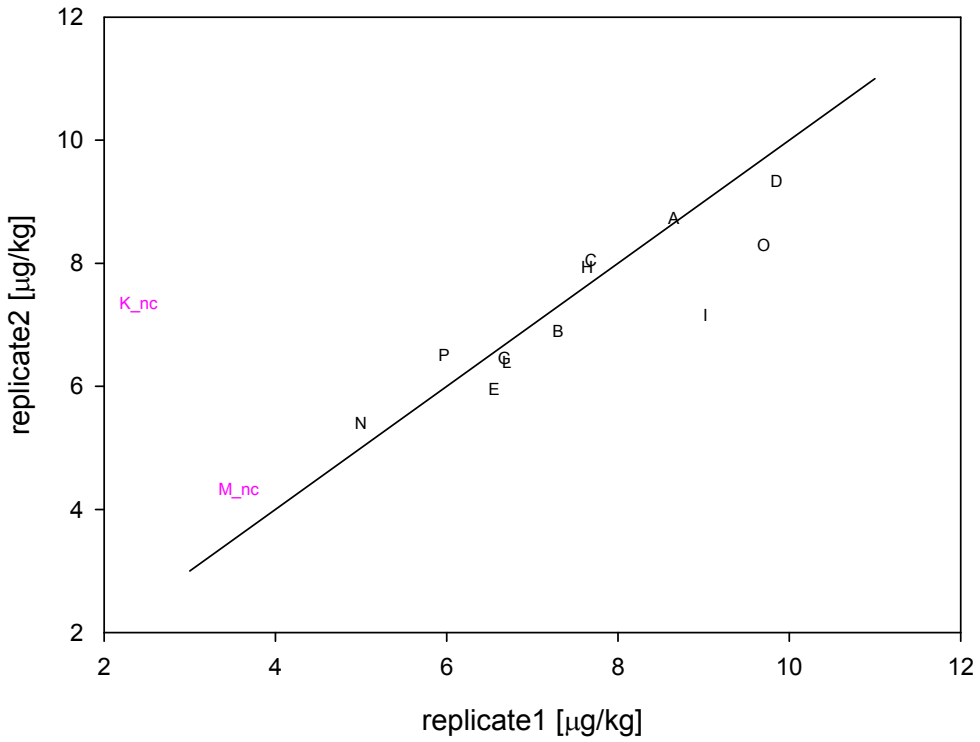


Figure 1. Method A juice spike low level (data in Tab. 33).

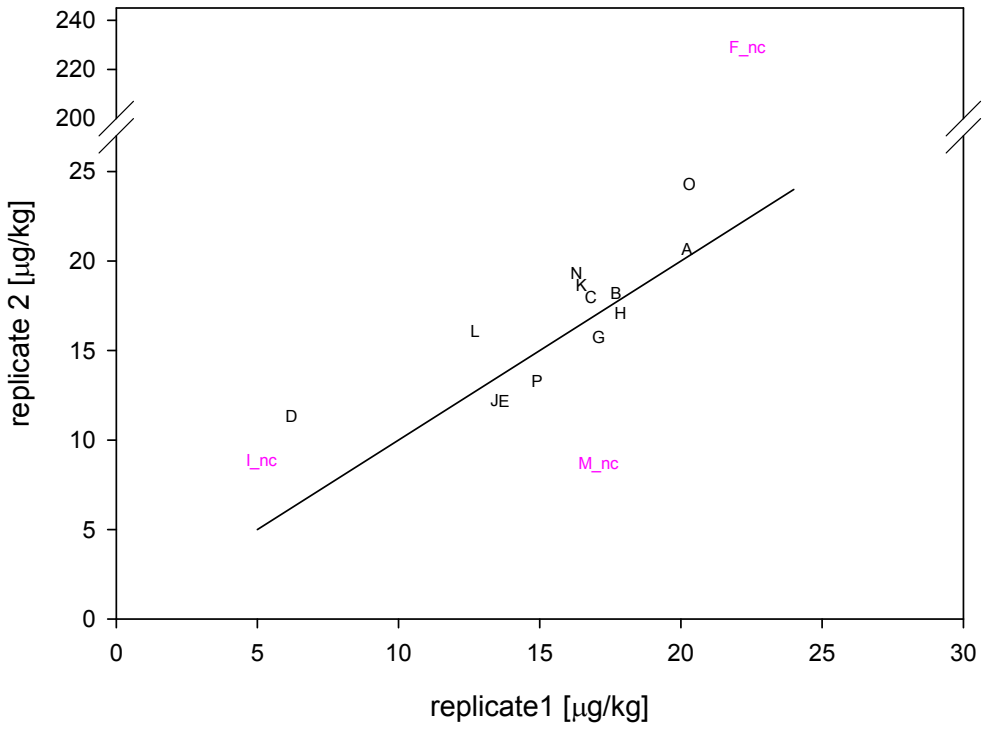


Figure 2. Method A juice spike high level (data in Tab. 33).

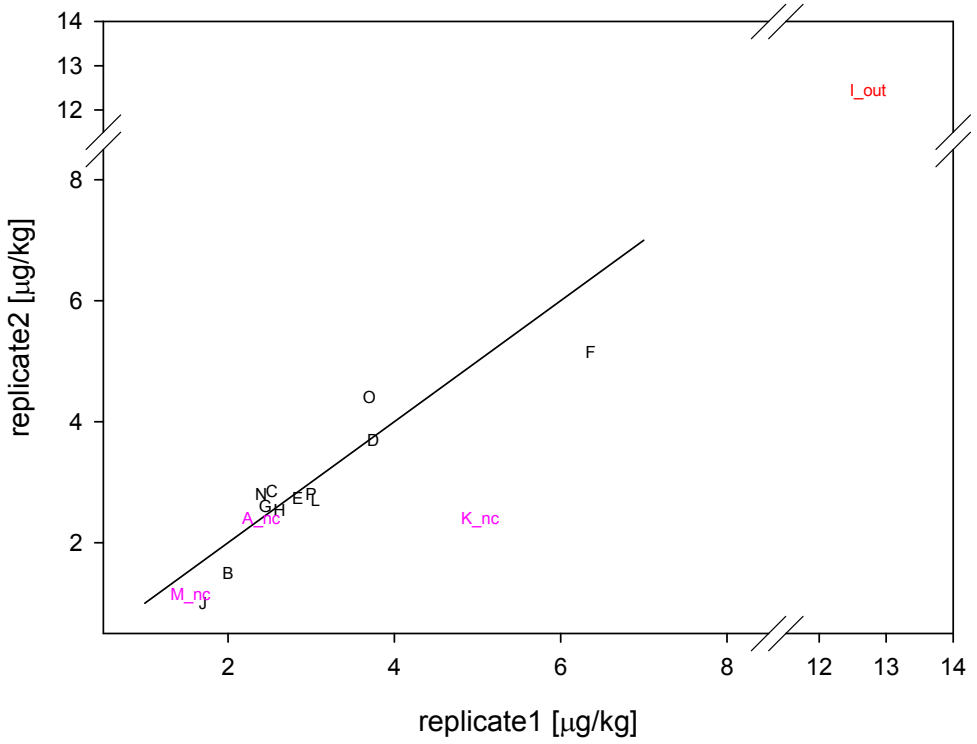


Figure 3. Method A juice naturally contaminated low level (data in Tab. 33).

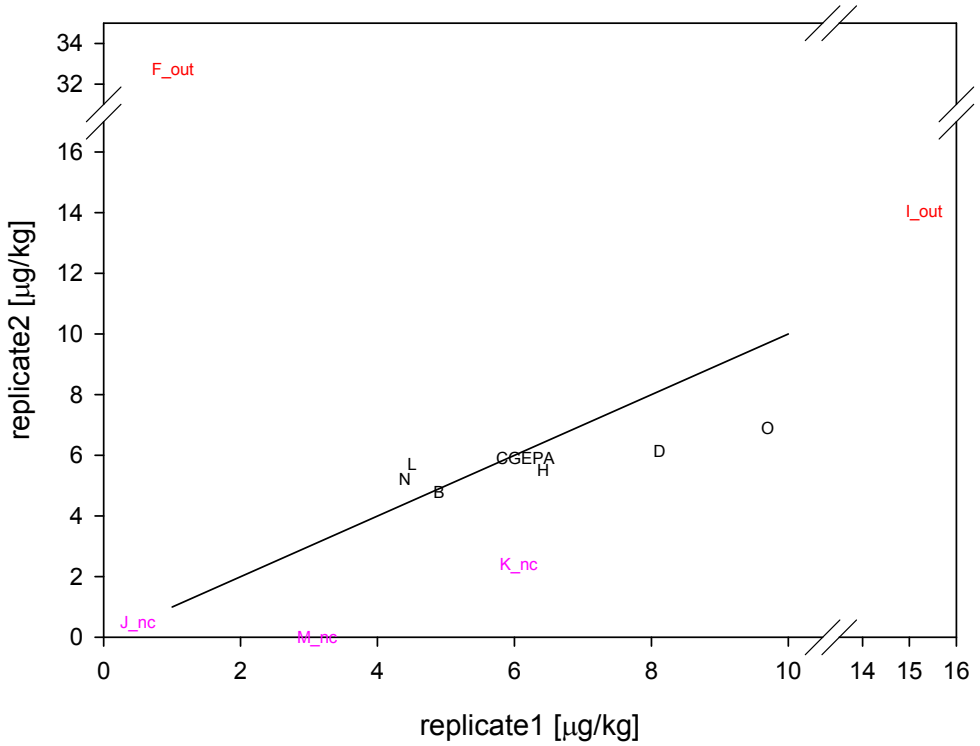


Figure 4. Method A juice naturally contaminated medium level (data in Tab. 33).

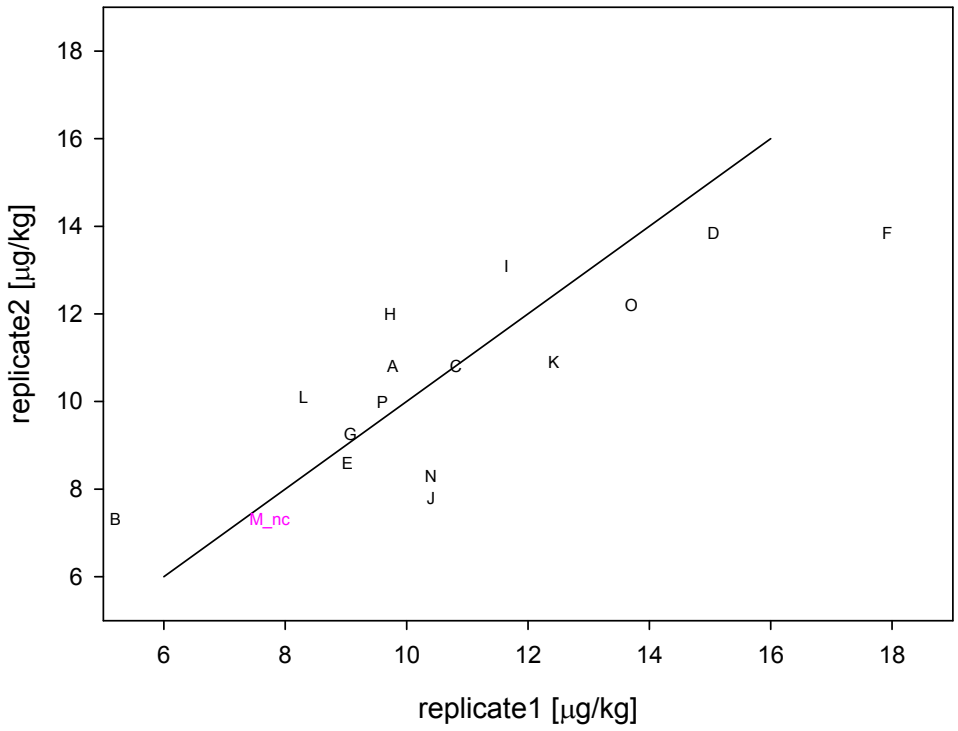


Figure 5. Method A juice naturally contaminated high level (data in Tab. 33).

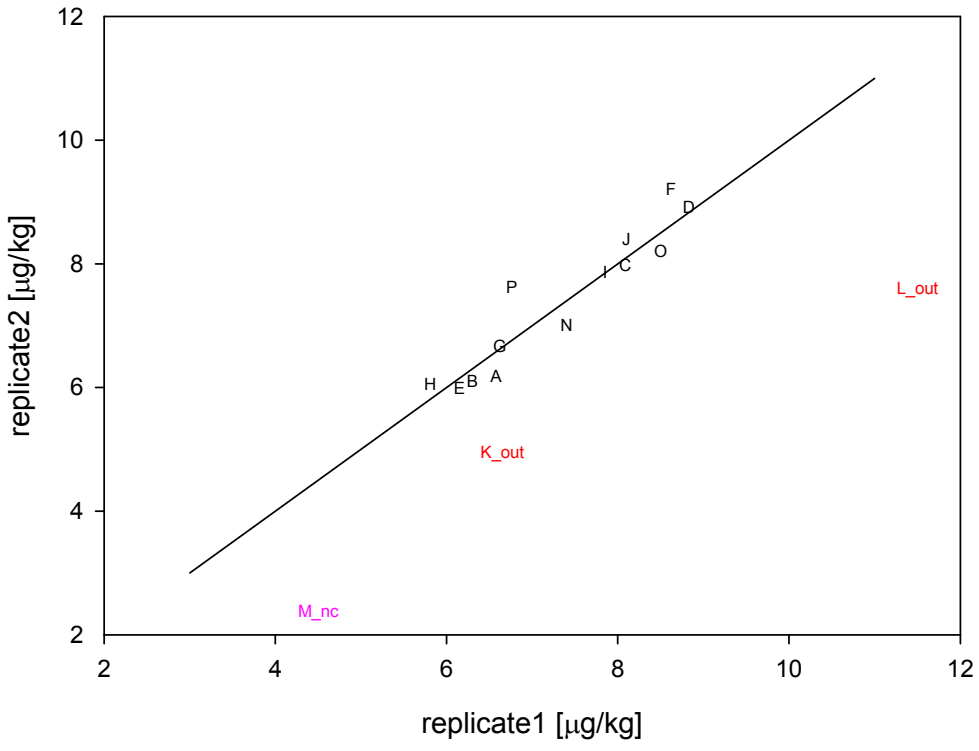


Figure 6. Method A puree spike low level (data in Tab. 34).

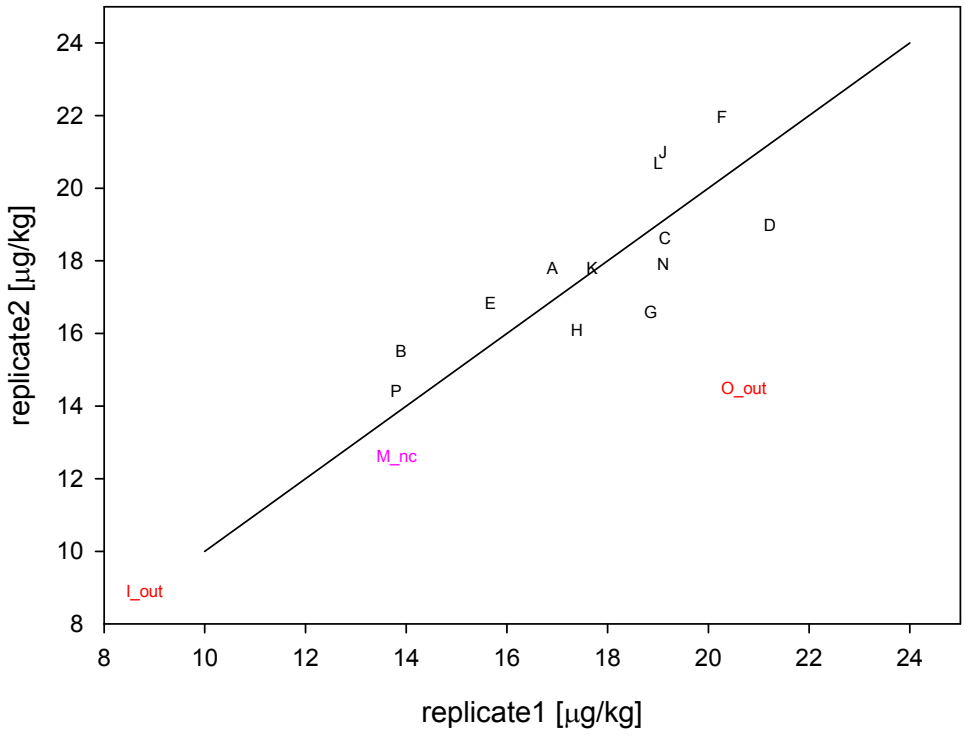


Figure 7. Method A puree spike high level (data in Tab. 34).

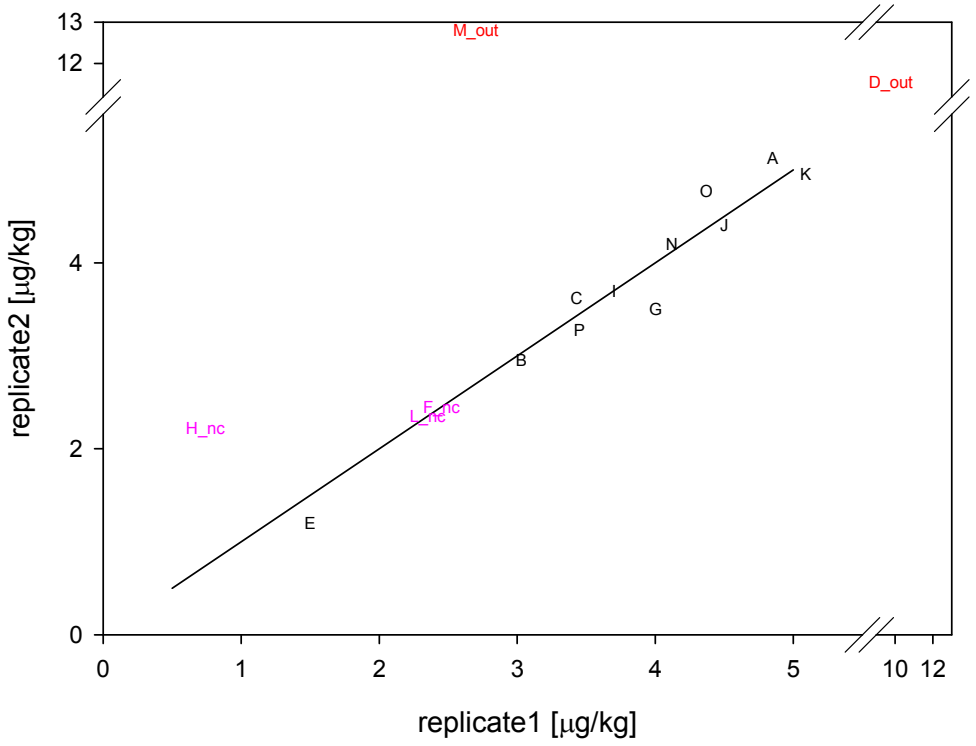


Figure 8. Method A puree naturally contaminated low level (data in Tab. 34).

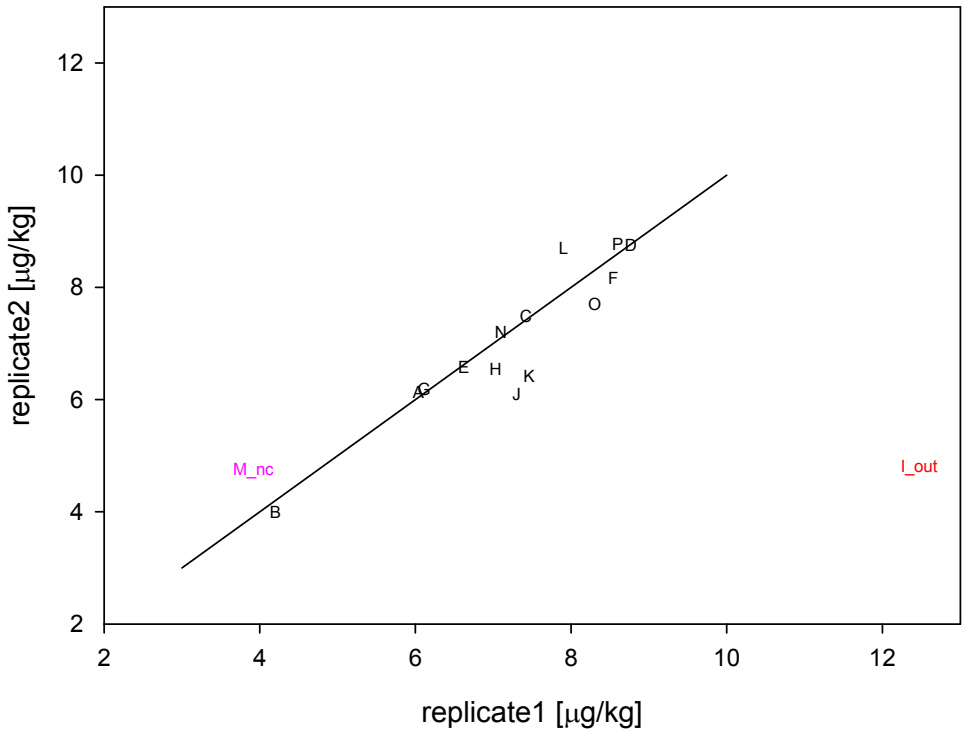


Figure 9. Method A puree naturally contaminated medium level (data in Tab. 34).

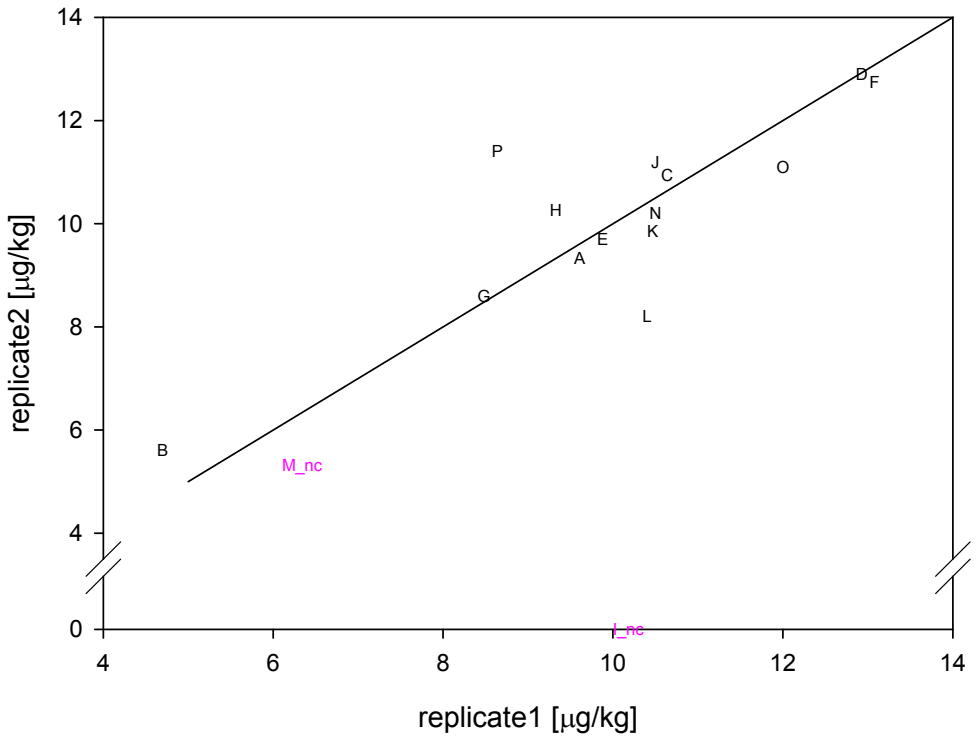


Figure 10. Method A puree naturally contaminated high level (data in Tab. 34).

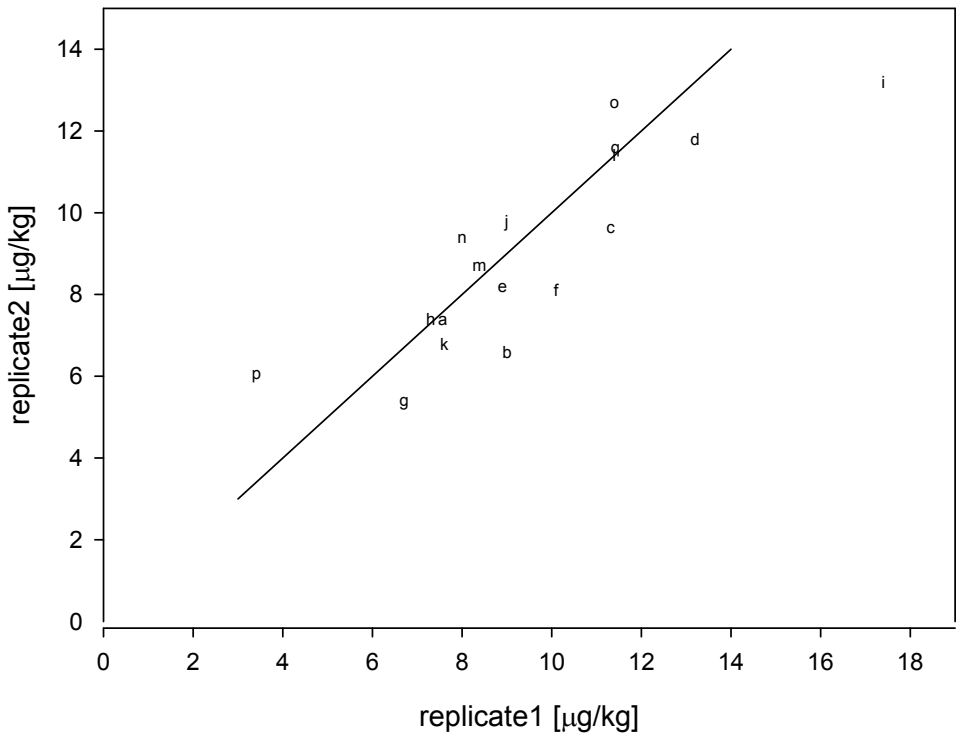


Figure 11. Method B juice spike low level (data in Tab. 35).

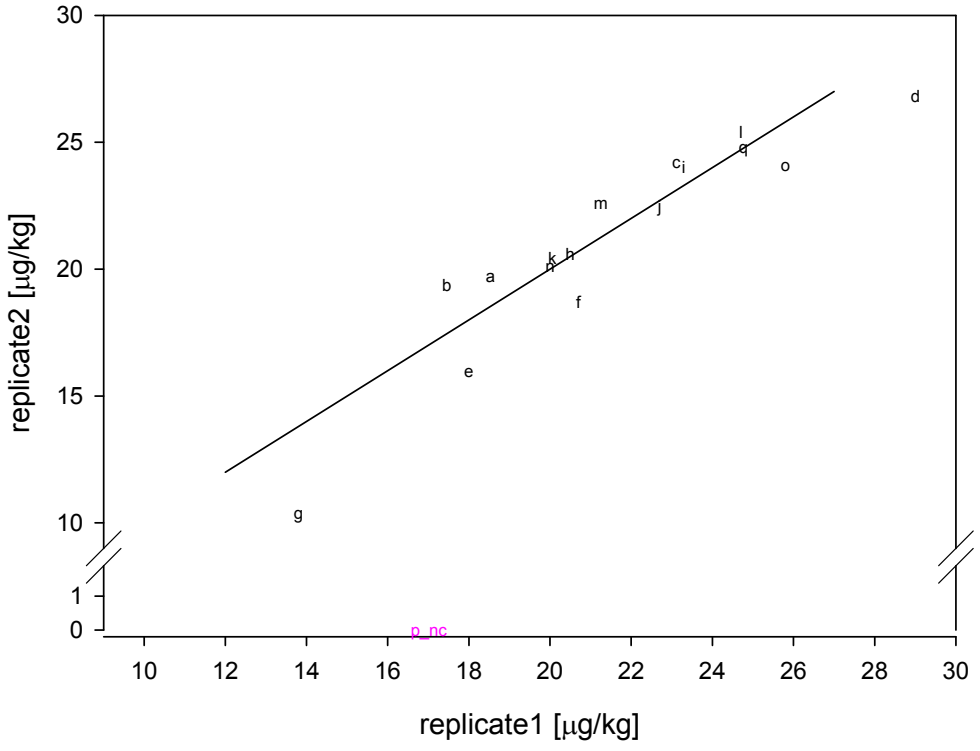


Figure 12. Method B juice spike high level (data in Tab. 35).

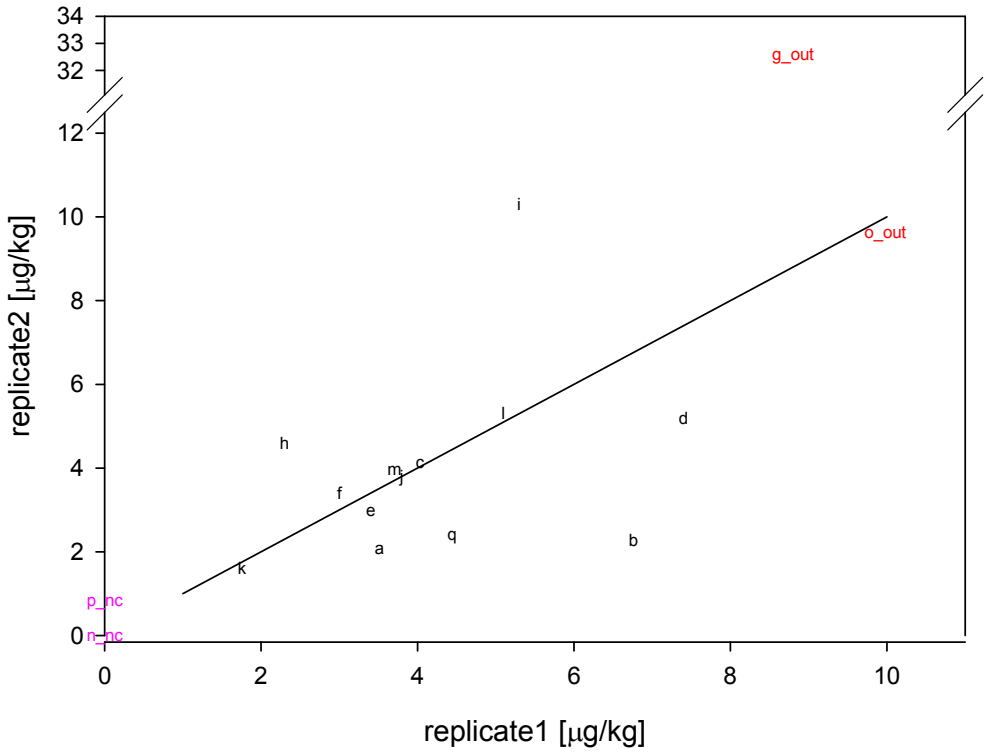


Figure 13. Method B juice naturally contaminated low level (data in Tab. 35).

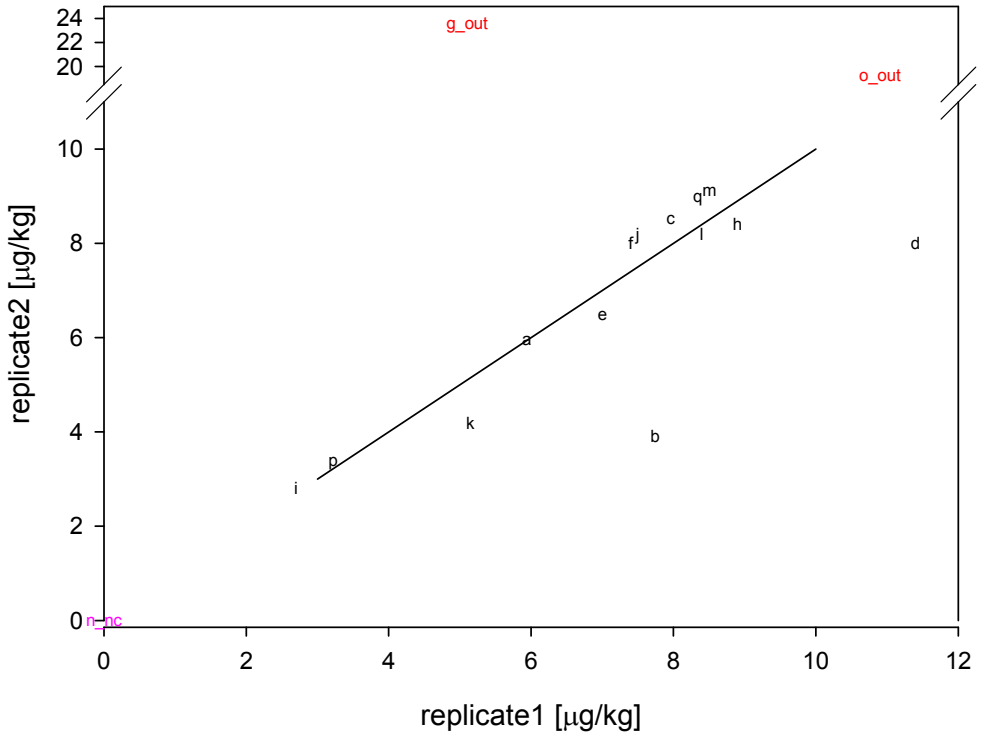


Figure 14. Method B juice naturally contaminated medium level (data in Tab. 35).

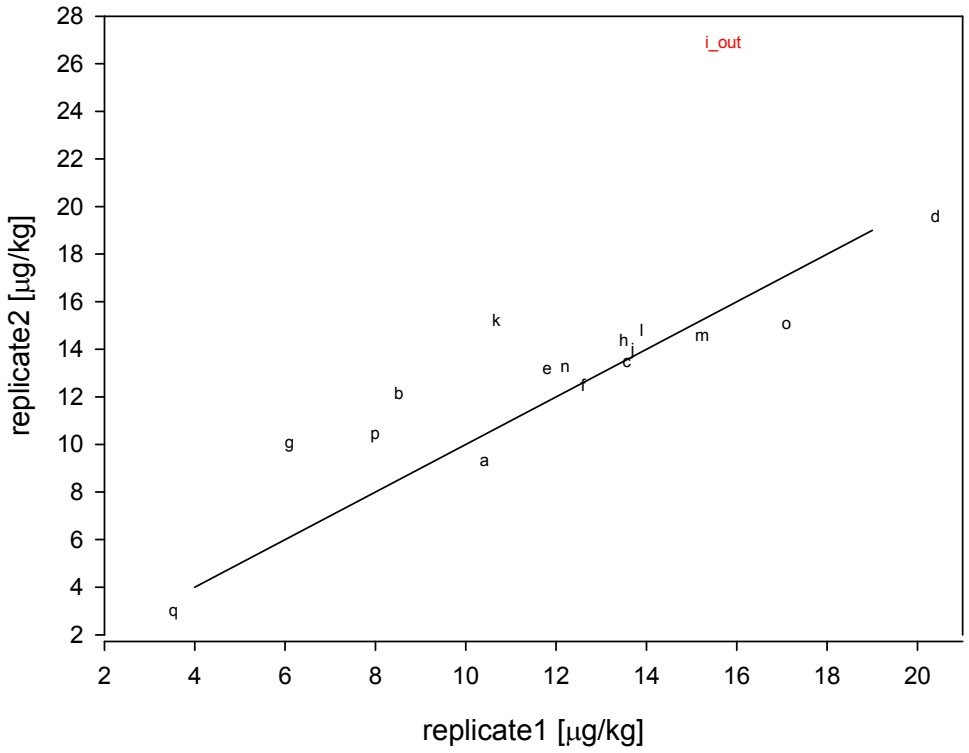


Figure 15. Method B juice naturally contaminated high level (data in Tab. 35).

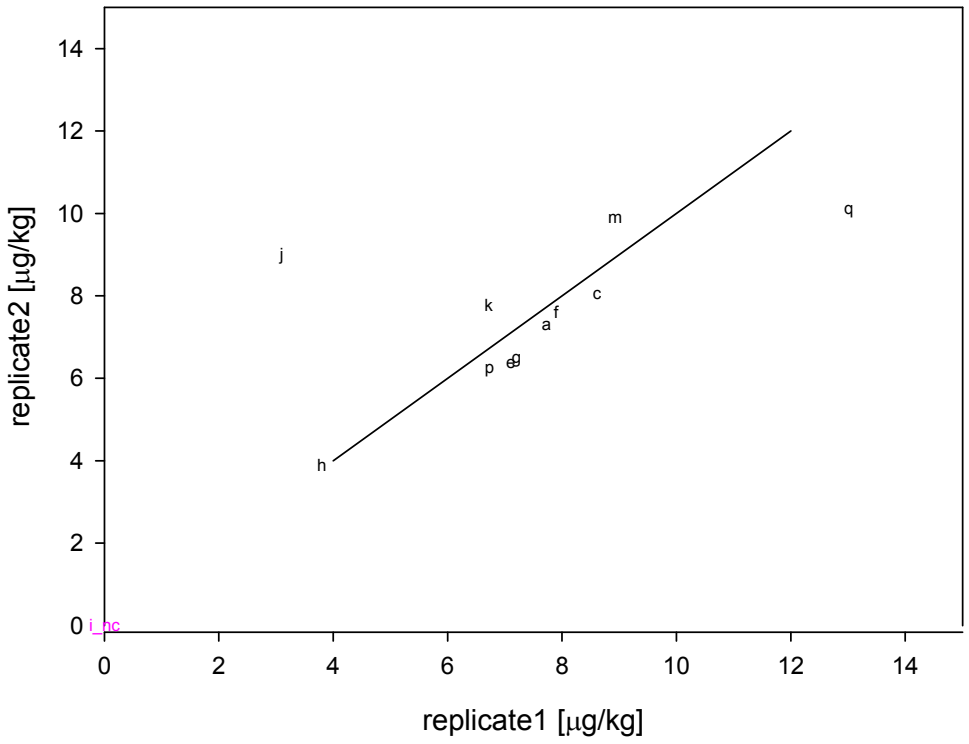


Figure 16. Method B puree spike low level (data in Tab. 36).

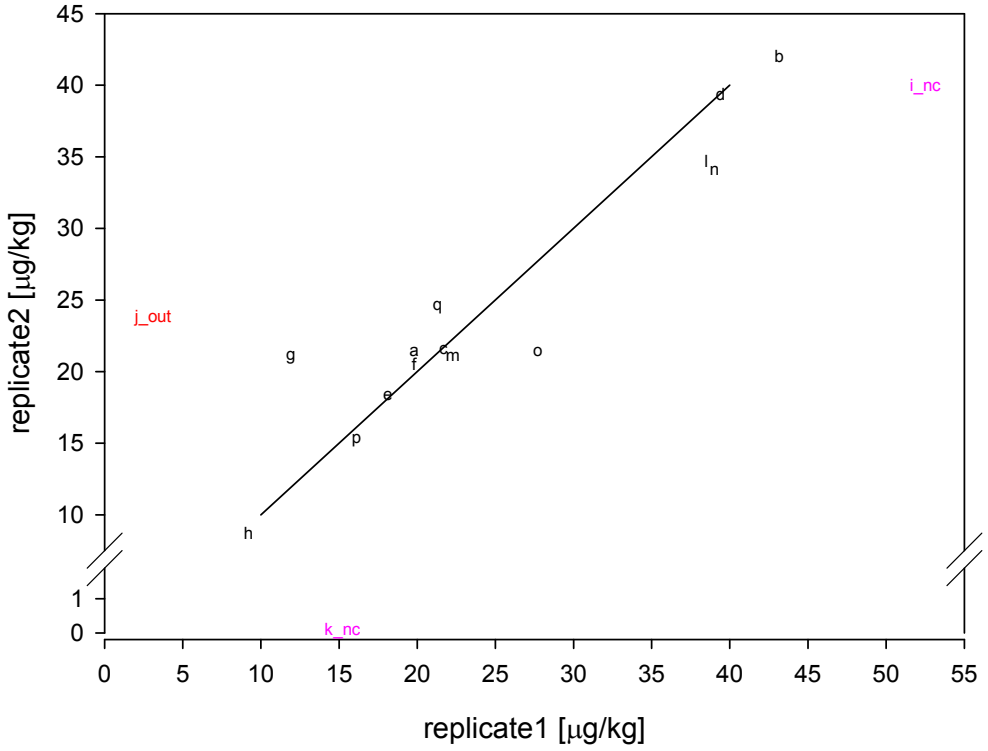


Figure 17. Method B puree spike high level (data in Tab. 36).

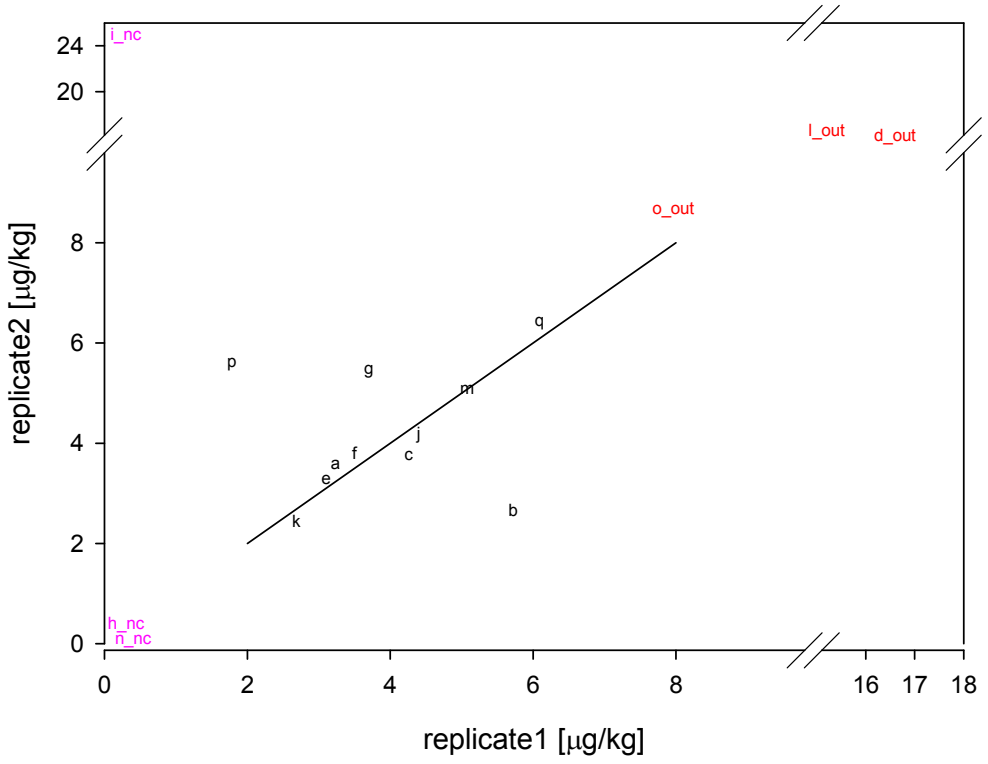


Figure 18. Method B puree naturally contaminated low level (data in Tab. 36).

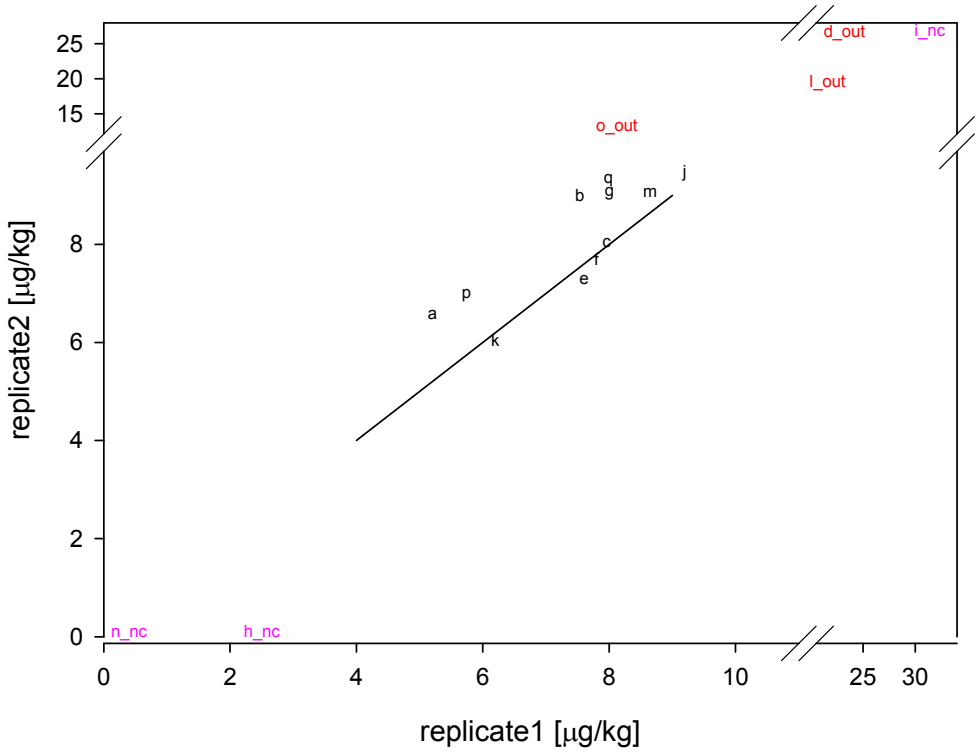


Figure 19. Method B puree naturally contaminated medium level (data in Tab. 36).

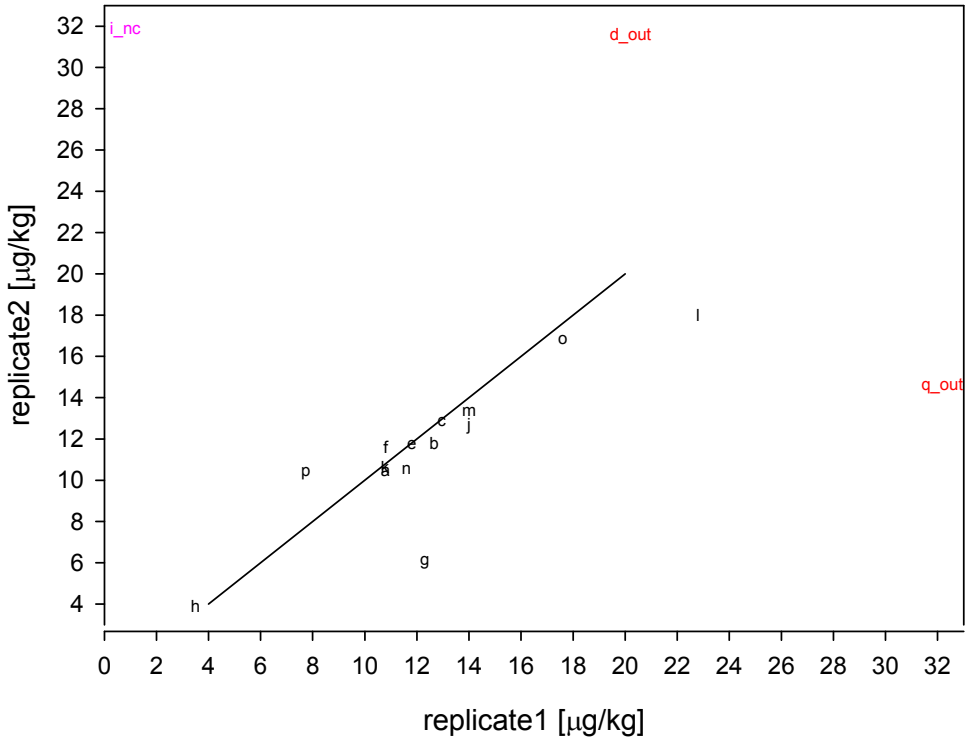


Figure 20. Method B puree naturally contaminated high level (data in Tab. 36).

Annex 9. Linearity calculation for patulin standards by LC/MS

STD [ng/mL]	Area	Height
12.0	42214	3821
24.0	83741	6284
36.0	133167	9798
48.0	166890	12582
60.0	216771	15575
72.0	270046	18744

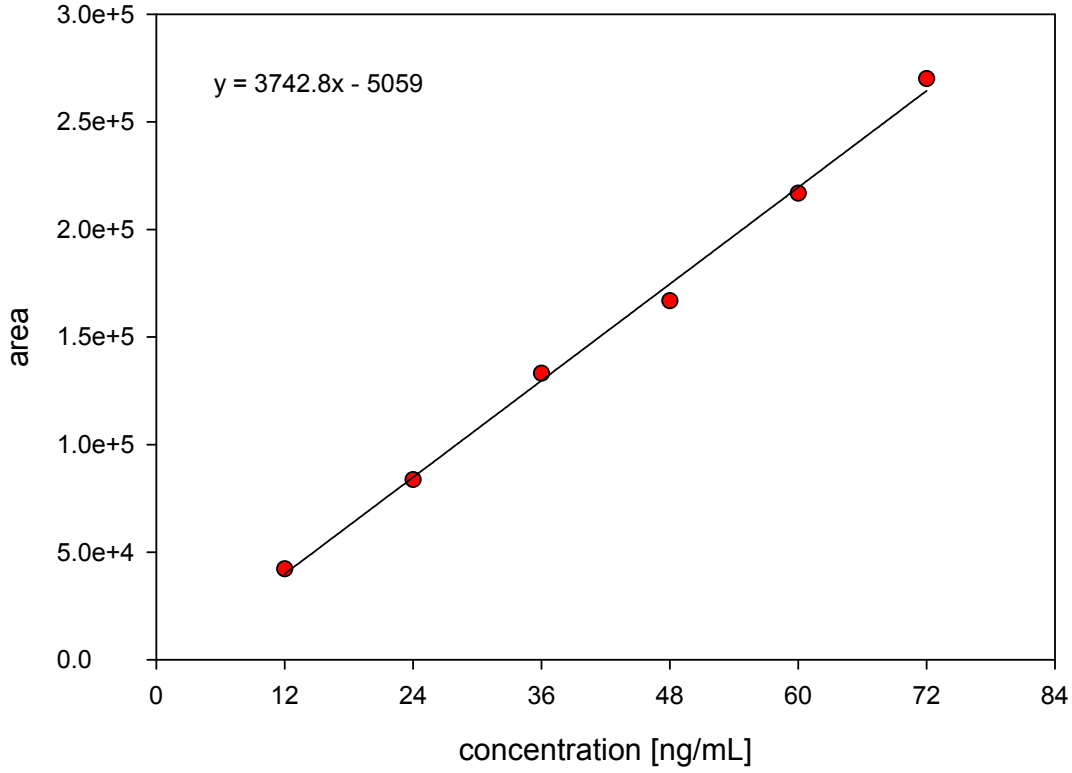


Figure 1. Calibration curve of the concentration of patulin standards against the area obtained by LC/MS.

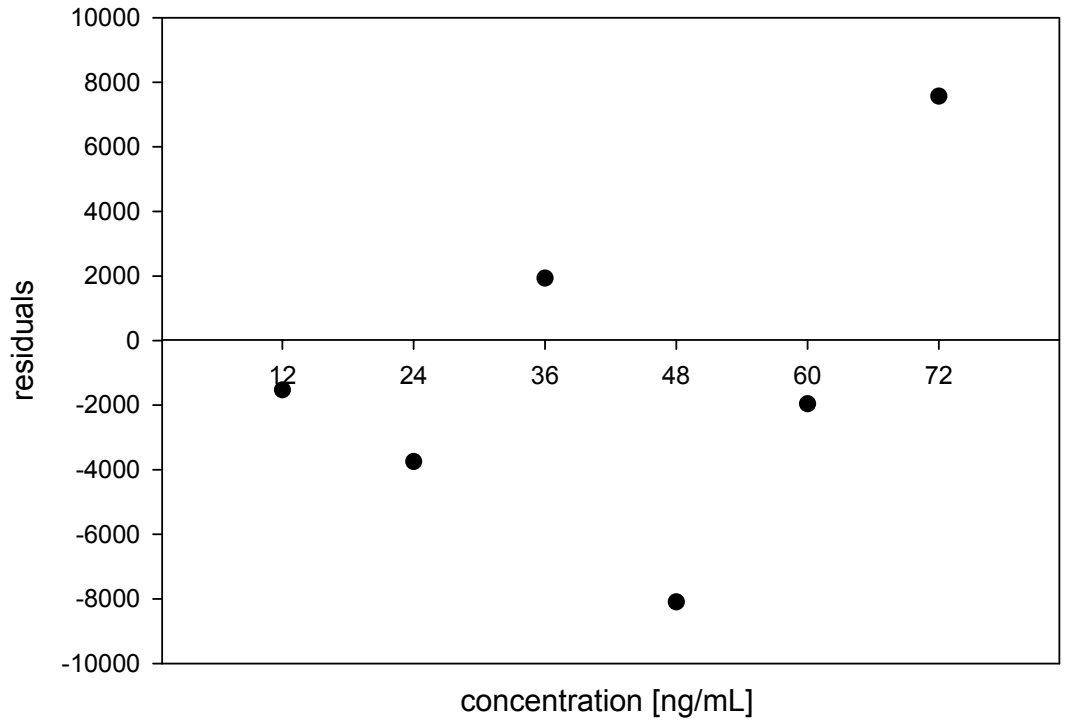


Figure 2. Residual plot for the above patulin standard concentrations.

Curriculum Vitae

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EDUCATION

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Dissertation thesis topic: Development and validation of analytical methods for mycotoxins in food, medicinal herbs and feed.
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2000 - 2002 **Postgraduate Master degree in Pharmaceutical Chemistry**
University Complutense – Facultad de Farmacia. Madrid (Spain)

1993 - 1999 **Degree in Pharmacy.**
Faculty of Pharmacy, University San Pablo CEU, Madrid (Spain).

EMPLOYMENT HISTORY

2002 - 2004 **Auxiliary agent in the Food Safety and Quality Unit.**

Joint Research Centre - Institute Of Reference Materials And Measurements

2001 - 2002 **Faculteit Farmaceutische Wetenschappen.**
Farmaceutische Analyse. Laboratory of drug quality control. University of Gent (Belgium)

2001 **LANDTOOLS – DOMINION. Sistemas de Información, S.L. Madrid (Spain).**
Development and distribution for Pharmacy Information and Management Software.

1999 - 2000 **GLAXO SMITHKLINE. (Fellowship)**
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PUBLICATIONS

- Arranz, A., Derbyshire, M., Kroeger, K., Mischke, C., Stroka, J., and Anklam, E. (2005) Liquid chromatographic method for quantitation of patulin at 10 ng/mL in apple-based products intended for infants: interlaboratory study. *Journal of AOAC International 88*, 518-525.

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