

GENERAL REFEREE REPORTS

Committee on Natural Toxins and Food Allergens

Mycotoxins

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Summary

As the newly appointed AOAC General Referee for Mycotoxins, I would like to thank the previous General Referee, Mary Trucksess, for her long and diligent efforts in serving AOAC and its members. In addition, Winston Hagler has resigned and Benedicte Hald retired as Topic Advisors for zearalenone (ZON) and ochratoxins, respectively. I would also like to thank them both for their input to AOAC over many years. Following this, I would like to welcome Mary Trucksess back as Topic Advisor for ochratoxins and Rudolf Krska as our new Topic Advisor for ZON. Krska has published widely in the field of emerging mycotoxin methods. The Topic Advisors below have all reported on recent advances in analytical methods. An overall impression is the advancement of the science in developed economies, with multitoxin methods using liquid chromatography/mass spectrometry (LC/MS) at the forefront, whereas surveys in developing countries still rely on older methods. The development of screening methods is to be welcomed, but care needs to be exercised on proper validation of methods in individual laboratories. The use of such methods in matrixes for which no validation has been performed can lead to the generation of false-positive results.

A number of international meetings have been held over the past year, including those held under the European Union (EU) Myco-Globe Project, namely in Accra, Ghana (September, 2005); Sydney, Australia (February, 2006); Cordoba, Argentina (March, 2006); and Bari, Italy (September, 2006). The World Mycotoxin Forum held meetings in Noordwijk-aan-Zee, The Netherlands, in November, 2005 and in Cincinnati, OH, in November, 2006, while special sessions of the European Association for Food Safety (SAFE) Consortium First International Congress on Food Safety (Budapest, Hungary, June, 2006) and of the American Chemical Society (San Francisco, CA, September, 2006) were devoted to mycotoxins. The 28th Mycotoxin Workshop was held in Bydgoszcz, Poland, by the Society for

Mycotoxin Research, and the Fifth Latin American Congress of Mycotoxicology was held in Florianopolis, Brazil (June 2006).

Books on mycotoxins continue to be published. Hamed K. Abbas has edited a book on *Aflatoxin and Food Safety*, with 27 chapters covering a wide range of subjects including economic costs, natural occurrence, and recent developments in analytical methods and sampling (1). A book on *Fusarium* toxins with information on chemistry and the producing fungi has also appeared (2).

A new society, the International Society for Mycotoxicology, has been established with the aims of increasing scientific knowledge concerning biology, chemistry, and any sciences or disciplines related to mycotoxins and toxigenic fungi. Further information and membership application can be found on the Website <http://www.mycotox-society.org>.

In 2005, the following method was adopted as Official First Action:

2005.08 Liquid Chromatographic Analysis of Aflatoxin Using Post-Column Derivatization (Modification of **991.31**, **999.07**, and **970.45**).

The following method was approved as Official Final Action:

2003.02 Determination of Aflatoxin in Animal Feed by Immunoaffinity Column Cleanup and LC with Post-Column Derivatization.

Codex Activities

The 38th Session of the Codex Committee on Food Additives and Contaminants (CCFAC) was held in The Hague, The Netherlands, April 24–28, 2006. The documents listed below were forwarded by CCFAC to the Codex Alimentarius Commission (CAC) for adoption by the 29th Session of the CAC, July 3–7, 2006, in Geneva, Switzerland.

(1) Appendix to the Codex “Code of Practice for the Prevention and Reduction of Aflatoxin Contamination in Tree Nuts” (CAC/RCP 55-2004)—Additional Measures for the Prevention and Reduction of Aflatoxin Contamination in Brazil Nuts—adopted by the CAC at Step 5/8, with the omission of Steps 6 and 7.

(2) Draft Maximum Level of 8 µg/kg for Total Aflatoxins in “ready-to-eat” tree nuts (almonds, hazelnuts, and pistachios)—adopted by the CAC at Step 5, advanced to Step 6.

(3) Project Document—Proposal for “New Work” on a Code of Practice for the Prevention and Reduction of Ochratoxin A (OTA) in Wine—approved by the CAC as “New Work” for the CCFAC.

Study Director Topics

Sampling and Subsampling

Topic Advisor Thomas B. Whitaker, U.S. Department of Agriculture, Agricultural Research Service, North Carolina State University, PO Box 7625, Raleigh, NC 27695-7625, Tel: 919-515-6731, Fax: 919-515-7760, E-mail: tom_whitaker@ncsu.edu. Whitaker reported that improving sampling methods to detect mycotoxins and other quality attributes in food and feed products continues to be a high priority among regulatory agencies, international organizations, and commodity industries worldwide. Examples of several worldwide efforts are discussed below.

In April 2006, the 38th CCFAC continued discussing the harmonization of aflatoxin limits and sampling plans for the following 4 tree nuts: almonds, hazelnuts, pistachios, and Brazil nuts. A contaminants working group has recommended that different limits and sampling plans be developed for raw tree nuts destined for further processing and for ready-to-eat tree nuts. Aflatoxin limits of 15 ng/g total aflatoxins are being discussed for tree nuts destined for further processing while lower limits of 8–10 ng/g total aflatoxins are being discussed for ready-to-eat tree nuts. CCFAC will have to establish limits before final sampling plan designs can be developed.

While limits are being discussed, sampling studies have been completed on almonds and hazelnuts, and sampling studies are in the planning stages for Brazil nuts and pistachios. The sampling working group led by the United States with assistance from Argentina, Brazil, Iran, European Community, and the International Nut Council have prepared sampling plans to detect aflatoxin in almonds and hazelnuts for circulation, consideration, and comments by CCFAC member nations. The working group proposed a sampling plan that uses a single 20 kg sample and an accept/reject limit of 15 ng/g total aflatoxins for almonds and hazelnuts destined for further processing.

Sampling studies were completed by The Scientific and Technical Research Council of Turkey to determine the uncertainty and distribution among sample test results used to quantify aflatoxin in hazelnuts and develop a computer model to predict the performance of aflatoxin sampling plan designs for hazelnuts. The computer model can be used by the hazelnut industry to predict the buyers' risk (bad lots accepted) and the sellers' risk (good lots rejected) associated with specific aflatoxin sampling plan designs. Aflatoxin sampling plans can be designed to minimize misclassification risks when detecting aflatoxin in domestic and export hazelnut shipments. The performance curves will be used by CCFAC to design sampling plans for aflatoxin in tree nuts.

A manuscript on predicting aflatoxin and fumonisin in shelled corn lots using poor-quality grade components has been published (3). Two manuscripts, one describing the uncertainty associated with sampling almonds and one associated with sampling hazelnuts for aflatoxin, have been submitted to AOAC for publication.

Aflatoxin M₁

Topic Advisor Hans P. van Egmond, National Institute for Public Health and the Environment, Laboratory for Food and Residue Analysis (ARO), PO Box 1, 3720 BA Bilthoven, The Netherlands, Tel: 31-30-2742440, Fax: 31-30-2744403, E-mail: hp.van.egmond@rivm.nl. van Egmond reported that during 2004, the EU installed new limits for aflatoxin M₁ in infant formulas and follow-on formulas, including infant milk and follow-on milk and in dietary foods for special medical purposes intended specifically for infants (4). The limits have been set at 0.025 µg/kg for both categories of food, and apply to all 25 EU member states since November, 2004. It is not known whether these limits are easily enforceable in the EU, as no publications on the occurrence of aflatoxin M₁ in these products have appeared yet.

Papers about analytical methodology for aflatoxin M₁ showed that LC in combination with tandem mass spectrometry (MS/MS) is increasingly used now (5–8), but it remains unclear at this moment whether this sophisticated technique has real advantages above the more conventional approaches, such as LC with fluorescence detection. Interlaboratory validation studies of LC/MS/MS methods have not yet been carried out and do not seem to be planned. In one of the papers on LC/MS/MS (5), the authors also studied the effectiveness and cleanup efficacy of immunoaffinity columns as compared to that of Mycosep multifunctional cleanup columns. Average recovery and detection limits of whole milk and low-fat milk cleaned up by immunoaffinity columns were found to be significantly superior to those obtained with the multifunctional cleanup columns.

A newer approach to determine aflatoxin M₁ in milk uses an electrochemical immunosensor with screen-printed electrodes (9). This technique, which is currently also being explored for the determination of trichothecenes in cereals within the European BioCop project (*see* <http://www.BioCop.org>) combines the high selectivity of immunoanalysis with the ease and low price of electrochemical probes. In the development of the technique, antibodies were immobilized directly on the surface of the screen-printed electrodes, allowing competition to occur between free aflatoxin M₁ and that conjugated with horseradish peroxidase. The electrochemical technique chosen was chronoamperometry. The authors claim a detection limit of 0.025 µg/kg and a working range of 0.030–0.160 µg/kg. This would make the technique suitable to detect aflatoxin M₁ in the area of current legal limits.

In 2005–2006, some articles were also published about the transfer of aflatoxin B₁ from feed to aflatoxin M₁ in milk and on the effects of processing of naturally contaminated milk (10, 11). The results found do not differ much from earlier published observations, i.e., up to a few % of aflatoxin B₁ in the feed appears as aflatoxin M₁ in the milk, and further processing and storage does not significantly reduce the content of aflatoxin M₁ in dairy products.

An interesting study on human exposure to some principal food mycotoxins, including aflatoxin M₁, was done in

France (12). This total diet study showed that the estimated average intake of aflatoxin M₁ in the French population is 0.09 ng/kg body weight/day for adults and 0.22 ng/kg body weight/day for children, with 95th percentile values of 0.21 ng/kg body weight/day for adults and 0.55 ng/kg body weight/day for children. These results were of the same order of magnitude as those estimated during a previous French evaluation in 1999, when the average aflatoxin M₁ contamination levels were close to the detection limits, which was also the case during the present study.

Recently published surveys on the occurrence of aflatoxin M₁ in milk and milk products involved countries such as Brazil (13), Italy (14), Iran (15, 16), and Turkey (17–20). The levels found were usually low, often fulfilled national legal requirements, and were not of health concern.

Aflatoxin Methods

Topic Advisor Gordon S. Shephard, Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, PO Box 19070, Tygerberg 7505, South Africa, Tel: 27-21-938-0279, Fax: 27-21-938-0260, E-mail: gordon.shephard@mrc.ac.za. Shephard reported that the results of studies undertaken during the tragic outbreak of human aflatoxicosis in Kenya in 2004 have been published. A survey of maize markets and vendors in the affected areas showed 55% of maize products to be contaminated with total aflatoxin above 20 µg/kg, and 7% had levels above 1000 µg/kg, with a maximum level of 58 000 µg/kg (21, 22). A case control study in the affected area showed that high aflatoxin levels in home-grown maize and high serum aflatoxin B₁-lysine adduct concentrations were both associated with case status (23). Case patients stored wet maize inside their homes more often than controls.

Studies on human exposure to aflatoxins have continued in West Africa. Investigations into the immune status of Ghanaians have shown that immunological parameters in persons with high aflatoxin-albumin adduct levels could result in impairment of cellular immunity with decreased resistance to infections (24). In Benin and Togo, aflatoxin-albumin adduct levels were associated with high *Aspergillus flavus* infection and aflatoxin contamination of maize, which is a more important dietary source of aflatoxin than groundnuts in the study area (25). An intervention study undertaken in Guinea, West Africa, has shown the potential of simple postharvest measures to reduce aflatoxin exposure in subsistence farming communities consuming home-grown groundnuts (26). A new method for the determination of aflatoxin B₁-lysine adduct in serum using isotope-dilution LC/MS/MS has been developed (27) and compared with the conventional enzyme-linked immunosorbent assay (ELISA) method (28). Although the methods correlated well, the ELISA method gave results 2.6-fold higher than the MS method, suggesting that the ELISA antibody was capable of reacting to other aflatoxin adducts.

Interest in aflatoxin methods has continued unabated. MS is increasingly being used as a detection and confirmation method for mycotoxin determination (29). Many of these

methods are capable of multitoxin determination, provided adequate extraction can be achieved. Examples of this are the determination of aflatoxins, trichothecenes, and ZON in foodstuffs (30) and determination of a mixture of 16 toxins possibly related to sick building syndrome (31). Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS has also been suggested as a rapid method with detection limits as low as 50 fmol for aflatoxins (32). Researchers are investigating aflatoxin contamination of traditional medicines and herbs. Methods have been developed for analysis of the herb *Maytenus ilicifolia* in Brazil (33), medicinal herbs in Spain (34), and for traditional medicines in Malaysia and Indonesia (35). All these publications report immunoaffinity column purification of extracts with high-performance liquid chromatography (HPLC) separation and fluorescence quantification. Results from samples collected in the latter countries showed a high incidence of aflatoxin B₁ contamination (70%), although mean levels were low (0.26 µg/kg; 35). An analytical method for the determination of aflatoxin B₁ in horchata, a tiger nut-based drink, has also been developed with immunoaffinity column purification and HPLC-fluorescence quantification (36). A small survey conducted on samples from Spain and Belgium failed to detect significant levels of contamination. A sensitive LC/MS/MS method (limit of column detection 3.75 pg) was developed to determine the possible presence of aflatoxin B₁ in sidestream cigarette smoke (37). However, researchers failed to detect any aflatoxin during laboratory experiments with unspiked cigarette samples. A modification of AOAC Official Method 991.31 (Aflatoxins in corn, raw peanuts, and peanut butter), which uses immunoaffinity columns for cleanup, has shown significant savings in organic solvent consumption and analysis time by extracting a 5 g rather than a 25 g aliquot of creamy or crunchy peanut butter and using 2 sequential steps with mixing on a Vortex mixer of the organic extractant as opposed to a single blending step (38).

Other publications have reported the further development of rapid methods based on immunoassay principles. An array biosensor has been described which has the ability to measure multiple analytes, including bacteria and toxins, simultaneously using different assay formats on a single substrate (39). For aflatoxin B₁, an array biosensor based on indirect competitive immunoassay was developed and tested on spiked foods (40). The limit of detection (LOD) depended on sample matrix, being between 1.5 and 5.1 and 0.6 and 1.4 µg/kg for various corn and nut products, respectively. The rapid detection of aflatoxin B₁ in pig feed can be achieved within 10 min with an immunoassay-based lateral flow dipstick, which has a visual detection limit of 5 µg/kg (41). The determination of aflatoxin B₁ in pig liver was described with an improved method using aqueous methanol extraction, immunoaffinity column cleanup, and direct fluorometric quantification with a detection limit of 1 µg/kg (42). A survey of 50 pig livers collected on farms in northern Italy failed to show any sample above this limit. A lateral flow test strip using colloidal gold-labeled polyclonal antibodies was

developed with a detection limit in food of about 2 µg/kg and a test time of less than 10 min (43). In a comparison with 67 naturally contaminated food samples, the strip method showed a good correlation with results obtained from a commercial ELISA. An immunoassay method which uses in situ sample cleanup has been described (44). The antibody is immobilized on a membrane over which the sample extract, cleaning solution, and enzyme-labeled aflatoxin B₁ conjugate are sequentially passed. For corn, wheat, and groundnuts, a detection limit of 5 µg/kg was achieved.

Results from The First French Total Diet Study have been published (12). Composite cooked "as consumed" food samples were prepared from individual raw ingredients and analyzed for a range of mycotoxins, including aflatoxin. None of the composite food samples contained detectable aflatoxin [limit of quantification (LOQ) 1 µg/kg fresh weight]. The natural occurrence of aflatoxin remains an area of intense publishing activity. Some of the recent publications include determination of aflatoxins in dried figs in Turkey (45, 46), beer in Canada (47), rice in the Philippines (48), baby food, peanut, and corn products in Indonesia (49), polished rice in Korea (50), spices in Hungary (51), corn in Arkansas (52), seed-, pulses-, and cereal-flours in Turkey (53), sorghum-based malt and beer in Botswana (54), ginseng roots in the United States (55), melon seeds in Nigeria (56), cereals and nuts in Pakistan (57), parboiled rice in India (58), peanuts in Niger (59), cereals and spices in Morocco (60), corn silage in France (61), pearl millet in Georgia (62), poultry feed in Kuwait (63), and food and feed in Nepal (64).

The fate of aflatoxin during various food cooking processes has been investigated. Some reductions in aflatoxin B₁ levels have been reported in wheat by cooking (65), in rice by pressure-cooking (66) or steaming (67), and in corn by nixtamalization (68), or by lactic acid fermentation (69). Other areas of aflatoxin research that were reported during the year were the use of adsorbents such as montmorillonite in poultry feed (70). Adsorption of aflatoxins was investigated for organozeolites at various pH values (71) and for a range of adsorbents in bovine ruminal fluid (72). Studies on the inhibitory effect of thyme oils demonstrated their potential to protect food from fungal infection (73). Lignans from the creosote bush showed their potential to control aflatoxigenic fungi (74), while propionic acid was effective under certain conditions as a grain preservative in protecting damp barley from aflatoxin contamination (75).

Alternaria Toxins

Topic Advisor Michele Solfrizzo, Institute of Sciences of Food Production, National Research Council, Via Amendola 122/o, Bari 70126, Italy, Tel: 39-080-5929367, Fax: 39-080-5929374, E-mail: michele.solfrizzo@ispa.cnr.it. Solfrizzo reported that no improvements of analytical methods for *Alternaria* mycotoxins have been reported in the last year. Some data have been published on the occurrence of these mycotoxins or the toxigenic fungus *Alternaria alternata* in sunflower, malted barley, vine fruits, and wheat. HPLC analysis of 50 grain samples of sunflower produced in Brazil

showed that 18% of the samples were contaminated with alternariol (AOH; 24.9–170.9 mg/kg) and 10% with AOH monomethyl ether (AME; 14.1–108.6 µg/kg). Mean incidence of *A. alternata* ranged from 3 to 42% in the examined samples collected during different stages of plant development (76). AOH was found in 20/27 red wines and 2/23 white wines produced in Canada, Italy, the United States, Chile, and Argentina at concentrations of 0.03–19.4 µg/L, usually accompanied by lower concentrations of AME. As previously observed for OTA, red wines contained a much higher incidence of AOH than white wines. The samples were cleaned up on aminopropyl solid-phase extraction (SPE) columns and analyzed by HPLC-UV. Positive samples were confirmed by HPLC-tandem negative ion electrospray mass spectrometry (MS/MS) in multiple reaction mode (77). *A. alternata* was isolated in moderate percentage (5.4%) from 11 dried vine fruits samples in Argentina. The production of AOH, AME, and tenuazonic acid (TeA) was demonstrated for most of the tested isolates of *A. alternata* but the vine fruit samples were not analyzed for the presence of these mycotoxins (78). The dominant fungal species in the 187 Spanish malting barley samples were members of the genus *Alternaria*. Within the 90 isolates of *Alternaria* spp., 24 isolates were toxigenic and identified as *A. alternata* (79). Occurrence of altenuene and AOH, at low concentrations (6.3–41.0 µg/kg), but neither AME nor TeA has been reported in winter wheat produced in the Czech Republic and analyzed by high-performance thin-layer chromatography (HPTLC; 80). New data on the toxicity of AOH have been published. The mycotoxin exhibited estrogenic potential in vitro comparable to that of the phytoestrogen daidzein or the environmental estrogen bisphenol A. Furthermore, AOH inhibited cell proliferation of Ishikawa and Chinese hamster V79 cells by interfering with the cell cycle (81).

Citrinin

Topic Advisor David Abramson, Agriculture and Agri-Food Canada, Cereal Research Center, 195 Dafoe Rd, Winnipeg MB R3T 2M9, Canada, Tel: 204-984-5536, Fax: 204-983-4604, E-mail: dabramson@agr.gc.ca. Abramson reported ongoing progress on the problem of citrinin production by *Monascus* species, which are used as natural sources of red food colorants. One study on citrinin in commercial *Monascus* products from Taiwan indicated 0.28–1.32 µg/g in tablets and capsules, and 2.83–6.29 µg/g in rice culture material (82). A survey of 23 isolates of 8 *Monascus* species from various culture collections found citrinin production by all species and isolates in yeast extract sucrose broth at levels of 65–480 mg/L (83). Two research groups achieved high production of the pigment monacolin K with low production of citrinin using mutant strains of *Monascus* on rice cultures (84, 85).

Other studies indicated that citrinin could also be easily produced in grains, grain products, and meats. A survey of breakfast cereals in France found 20% of 45 samples containing citrinin at 1.5–42 ng/g (86). A granary storage study of durum wheat at 20% moisture content found citrinin

levels at 11.6 µg/g after 20 weeks, and good correlation with chemosensor data from an “electronic nose” analyzer (87). Using dry cured ham substrate inoculated with *Penicillium citrinum*, Bailly et al. (88) found 87 µg/g after 16 days at 20°C.

Cyclopiazonic Acid

Topic Advisor Joe Dorner, U.S. Department of Agriculture, Agricultural Research Service, National Peanut Research Laboratory, PO Box 509, 1011 Forrester Dr SE, Dawson, GA 31742, Tel: 912-995-7408, Fax: 912-995-7416, E-mail: jdorner@nprl.usda.gov. Dorner reported that Bailly et al. (88) published that cyclopiazonic acid (CPA) was produced by *P. viridicatum* when cultured on dry meat at 20°C. Contamination reached 50 mg/kg after 16 days. It was further shown that CPA was much more stable on dry meat than OTA, citrinin, and patulin (PAT). After 192 h of incubation at 20°C, more than 80% of the initial CPA content was still present in the meat compared with less than 50% of the ochratoxin A and citrinin and none of the PAT. Given that several studies have shown that species of *Penicillium* can be isolated from cured meat products, it is important to ensure that growth of CPA-producing species on dry meat products is not allowed.

Oliveira et al. (89) reported natural occurrence of CPA in commercial milk samples in Brazil. Milk was extracted with methanol + 2% sodium bicarbonate in water (7 + 3, v/v), cleaned up by solvent partition and column chromatography, and quantified by LC. Two of 46 milk samples contained CPA at concentrations of 6.4 and 9.7 µg/L, respectively. These plus other samples also contained aflatoxin M₁, but at much lower concentrations (0.098 and 0.053 µg/L, respectively). Because there are many strains of *Aspergillus flavus* contaminating animal feed ingredients that produce CPA but not aflatoxins (as well as both), further evaluations of the natural occurrence of CPA in milk and its potential effect on infants is warranted.

Ergot Alkaloids

Topic Advisor George M. Ware, U.S. Food and Drug Administration (FDA), 60 8th St, Atlanta, GA 30309, Tel: 404-347-2131 ext. 5215, Fax: 404-347-4225, E-mail: gware@fda.hhs.gov. Ware reported that a confirmatory method for detecting 5 ergot alkaloid, (ergocristine, ergotamine, ergonovine, ergocornine, and alpha-ergokryptine) in rye flour has been described using HPLC coupled to MS/MS detection by monitoring 2 transition reactions per analyte (90). The procedure entailed a liquid-liquid extraction followed by a cleanup step using a C18 SPE cartridge. An analogue compound, methysergide hydrogen maleinate, was used to assess both repeatability of sample preparation and potential MS response fluctuations. Detection and quantification limits of all analytes were calculated ranging from 7 to 11 µg/kg and from 23 to 37 µg/kg, respectively. Fifteen rye flour samples were investigated with the newly developed method, and none of them were above the current Swiss limits of 200 mg/kg for total ergot alkaloids.

Another paper demonstrates the facility of using electrospray(+) MS with multiple reaction monitoring (MRM) detection during chromatographic examination of ergot alkaloid standards of lysergic acid, lysergol, ergonovine, ergovaline, ergotamine, ergocornine, ergocryptine, and ergocristine by HPLC (91). Ergoline-8 position epimers could be separated on the gradient HPLC system for ergocornine, ergocristine, and ergonovine and appeared as shoulders for ergotamine and ergovaline; epimers generally showed different patterns of relative intensity for specific MRM transitions. It is anticipated that findings such as these will provide impetus to future development of analytical methodology for these heretofore relatively rare ergot alkaloid species.

Fumonisin

Topic Advisor Chris Maragos, USDA, ARS National Center for Agricultural Utilization Research, 1815 N. University St, Peoria, IL 61604, Tel: 309-681-6266, Fax: 309-681-6689, E-mail: maragocm@ncaur.usda.gov. Maragos reported that the occurrence of fumonisins in foods, risk assessment, and the development of analytical methods continue to be active areas of research. Studies over a number of years have demonstrated the worldwide occurrence of fumonisins. The widespread distribution of these toxins was reinforced by publications within the past year on the occurrence of fumonisins in Belgium, Botswana, the People's Republic of China, Croatia, France, Italy, Korea, Kuwait, Morocco, Portugal, Slovakia, South Africa, Taiwan, and the United States. Most of the reports describe the occurrence of fumonisins in maize or maize-based products. In Belgium, 205 samples of cornflakes from retail stores collected in 2003–2004 were tested by HPLC-MS. Mean concentrations of fumonisins found were 104 (FB₁), 12 (FB₂), and 21 (FB₃) µg/kg (92). In Croatia, 49 maize samples from 2002 were tested, all of which contained FB₁ and 3 of which contained FB₂. The mean FB₁ level was 459 µg/kg, while FB₂ ranged from 68 to 3084 µg/kg (93). In France, exposure to mycotoxins, including fumonisins, was estimated as part of the First French Total Diet Study (12). In that study, 456 composite samples were prepared from 2280 individual samples and analyzed for between one and 14 mycotoxins, depending upon the type of food. Twelve of 34 composite samples contained detectable FB₁, and one contained detectable FB₂. In Morocco, 20 samples of maize were tested for FB₁ and found to contain an average of 1930 µg/kg (60). In Portugal, 31 samples of maize and maize-based products were tested, 14 of which were positive for fumonisins with a range of 113–2026 µg/kg. The daily intake of fumonisins was estimated as 0.14 µg/kg body weight/day (94). In Taiwan, 76 maize and maize products including corn snacks, corn flakes, corn starch, and canned corn were tested, and 11 contained fumonisins. Seven of the corn samples contained between 0.05 and 0.13 ppm, while the highest level of contamination, in a corn snack, was only 0.16 ppm (95). In the United States (Arkansas), 65 maize hybrids naturally infected with *Fusarium* and *Aspergillus* spp. were evaluated. In 2001,

the fumonisin levels ranged from 8 to 83.6 ppm, and a positive correlation was seen between fumonisin and aflatoxin levels (52). Home-brewed maize beer, produced by a traditional process in the Centane and Bizana districts of South Africa, also was found to contain fumonisins. Total fumonisins (FB₁, FB₂, FB₃) found averaged 369 ng/mL, with a range of 43–1329 ng/mL. Although data on consumption of the home-brewed beer were not available, the levels suggest that the fumonisin consumption in these districts may be above the provisional maximum tolerable daily intake of 2 µg/kg body weight/day (96). A study reported on the agronomic and environmental effects upon fumonisin levels in Ontario maize over the years 1993–2000 (97). From 1997 to 2000, between 17 and 56% of the samples were contaminated with ≥1 µg/g FB₁. Effects of hybrid variety, crop year, and rotation with wheat were reported. Interestingly, a higher incidence of fumonisin and deoxynivalenol (DON) was detected for maize grown after wheat than maize grown after maize. In Botswana, sorghum-based traditional malt, wort, and beer were collected from 3 villages around Gaborone. FB₁ was found in 3 of 46 malt samples at concentrations ranging from 47 to 1316 µg/kg (54).

Other commodities examined were rice, poultry feed, and asparagus. Of 88 samples of Korean polished rice, 2 contained FB₁, at an average level of 54.4 µg/kg, which was attributed to the presence of *F. proliferatum* (50). Poultry feed was tested in Slovakia and Kuwait. In Slovakia, 50 samples were tested, 49 of which contained FB₁ over a range of 43–798 µg/kg and 42 of which contained FB₂ over a range of 26–362 µg/kg (98). In Kuwait, fumonisins were detected in commodities used to make poultry feed, and poultry feed, over the range of 1.4–3.2 ppm (63). Two reports indicated contamination of asparagus with fumonisins. The first of these found FB₁ in asparagus spears collected in Poland (2002–2003) with levels up to 5.6 µg/kg fresh weight (99). In the same report, fumonisins were not found in asparagus spears collected in Germany in 2002. The second report examined asparagus collected in the Shandong province of the People's Republic of China. Thirty samples from 2004 were tested, of which 24 contained FB₁ ranging from 24 to 670 µg/kg (average 123 µg/kg) and FB₂ ranging from 17 to 138 µg/kg (average 35 µg/kg). The total amount of fumonisins (FB₁, FB₂) averaged 158 µg/kg, based on dry weight (100).

Sampling is an integral part of mycotoxin analysis. A study by an FAO/IAEA laboratory to estimate the uncertainty of fumonisin analysis associated with subsampling in maize samples was reported (101). The distribution of aflatoxins and fumonisins in components of shelled maize was also reported (3). Test samples were divided into 3 components: damaged kernels, broken corn and foreign material (BCFM), and whole kernels. The fumonisin levels averaged 148.3, 51.3, and 1.8 ppm, respectively. The damaged kernels and BCFM components comprised only 5% of the sample mass, but accounted for 77.5% of the total mass of fumonisin. The authors suggest that this will allow the use of fumonisin in the latter 2 components to predict fumonisin in the bulk lot.

Fluorescence derivatization of the primary amine of the fumonisins continues to be a widely used technique for detecting these toxins, especially with the *o*-phthalaldehyde (OPA) derivatization reagent. The re-use of commercial “single-use” immunoaffinity columns (IAC) for analysis of spiked maize was reported. The IACs could be used 5 consecutive times without regeneration by adding successive diluted extracts after each toxin elution and then an additional 5 times after regeneration achieved by leaving phosphate-buffered saline (PBS) in the column for 1 day at 4°C (102). Regeneration involved holding the IAC in PBS for a day at 4°C. The use of IAC cleanup and direct measurement of fluorescence (that is, without a further chromatographic step) may be problematic. Shephard et al. (103) re-examined wheat that had been previously tested and found to contain fumonisin with an IAC-direct fluorescence method. The re-examination used a modification of an AOAC-approved HPLC method and found no detectable (<5 µg/kg) fumonisin. This suggests caution should be exercised when assays are used outside of the matrix for which they have been validated. The effects of mobile phase pH, chromatography column temperature, extraction method, and derivatization time on an HPLC–OPA derivatization fluorescence method for maize were examined (104). Column temperature was negatively correlated, and mobile phase pH was positively correlated with fluorescence response.

Mass spectrometric methods for mycotoxins continue to be heavily investigated, and fumonisins are no exception. A review of chromatographic-MS techniques for mycotoxin analysis was recently published (29). HPLC with electrospray ionization–MS/MS detection (HPLC-ESI-MS/MS) methods were reported for multiple mycotoxins, including fumonisins in corn meal (105) and bovine milk (106). The method for corn meal detected FB₁, FB₂, and FB₃ as well as 8 trichothecenes, ZON, and α -zearalenol (105). FB₁, FB₂, and FB₃ were also detected in cornflakes by HPLC-ESI-MS/MS following IAC cleanup (107). In the latter case, a C12 sphinganine analog was used as an internal standard. Three extraction solutions were examined: methanol–water (70 + 30; pH 4), acetonitrile–methanol–water (25 + 25 + 50), and acetonitrile–methanol–water (25 + 25 + 50; pH 4). Of these, the methanol–water (70 + 30) pH 4 gave the best extraction (107). HPLC-ESI-MS/MS was also applied to 18 mycotoxins, including FB₁ and FB₂ in bovine milk. Cleanup involved a liquid–liquid partitioning and SPE. Mean recoveries were in the range of 78–108% at levels between 0.2 and 10 µg/L. Application of the method to 42 bulk milk samples from dairies in Denmark did not yield any positive samples (106). Recent reviews of MS techniques include those of Sforza et al. (29), Lino et al. (108), and Sugita-Konishi and Tanaka (109). The latter 2 articles are in Portuguese and Japanese, respectively.

Screening methods for fungal contamination might assist in diverting mycotoxin-contaminated commodities from the food supply. A near-infrared (NIR) reflectance spectroscopy method was applied to 280 maize samples, some of which were artificially inoculated with *F. verticillioides*. The NIR

method could be used to predict the incidence of kernel infection, the quantity of ergosterol, and the FB₁ content (110). Volatile compounds in the headspace have also been reported as an indicator for the screening of fungal contamination of maize using an electronic nose (111).

Antibody-based methods, especially traditional ELISAs and the newer lateral flow assays, remain popular for screening for fumonisins in commodities. A flow-through enzyme immunoassay (EIA) was compared to an HPLC/MS/MS method for 90 samples of cornflakes (92). The assay was established with a visual detection limit of 275 µg/kg. Of the 90 samples, 26 exceeded this limit when tested by EIA. The HPLC/MS/MS method confirmed that in 14 of these 26 samples, the fumonisin concentrations were greater than the visual detection limit of the EIA. All of the cornflake samples having responses below the visual LOD of the EIA were also confirmed to be below this level using the HPLC/MS/MS method (92). Both flow-through assays and lateral flow membrane assays using colloidal gold were reported. The membrane-based colloidal gold immunoassays had a visual detection limit of 1 µg FB₁/L and a detection time of 10 min (112). A surface plasmon resonance biosensor for fumonisins was also reported (113). Lastly, an array biosensor capable of detecting multiple foodborne contaminants simultaneously was reported. The sensor is capable of detecting *Campylobacter jejuni*, OTA, fumonisin B, aflatoxin B₁, and DON (39).

Mycotoxins in Botanicals

Topic Advisor Bruce Malone, Trilogy Analytical Laboratory, 111 West Fourth St, Washington, MO 63090, Tel: 636-239-1521, Fax: 636-239-1531, E-mail: bruce@trilogylab.com. Malone reported that several papers regarding mycotoxins in botanicals have been published. A reversed-phase HPLC method with fluorescence detection was reported for the analysis of aflatoxins B₁, B₂, G₁, and G₂ in ginseng roots (55). The samples were extracted with methanol–water (8 + 2), diluted and passed through an IAC packed with antibodies specific for aflatoxins, and derivatized with trifluoroacetic acid (TFA) prior to HPLC analysis. Recoveries of total aflatoxins at 2, 4, 8, and 16 ng/g added to toxin-free ginseng were 92, 77, 91, and 83%, respectively. All cultured ginseng roots analyzed were found to contain <0.1 ng/g total aflatoxins. The wild simulated ginseng roots were more susceptible to contamination with 2 samples containing total aflatoxins at 15.1 and 15.2 ng/g.

Another paper described the determination of aflatoxins and OTA in ginseng and other botanical roots by IAC cleanup and LC with fluorescence detection (114). Two types of IAC cleanups were evaluated and 3 derivatization techniques to enhance the fluorescence of aflatoxins were compared. These techniques were TFA, postcolumn bromination, and postcolumn UV radiation. The authors concluded that an IAC containing anti-aflatoxin antibodies was suitable for aflatoxin determination in botanical roots. An IAC packed with a combination of antibodies specific for aflatoxins and ochratoxin gave recoveries of added aflatoxin lower than an

IAC for aflatoxin alone. Recoveries of added OTA from the multitoxin column were <60%.

An interlaboratory study was conducted for the determination of aflatoxin B₁ in medical herbs (115). The method, which was evaluated in a mini collaborative study by 4 laboratories, was based on an IAC cleanup followed by reversed-phase HPLC separation and fluorescence detection after postcolumn derivatization. The method was tested on 3 different matrixes, including senna pods, ginger root, and devil's claw. Several options in extraction solvents, postcolumn derivatization methods, and integration modes were evaluated. The method, with all tested variations, was found suitable for the determination of aflatoxin B₁ in medical herbs at levels of 1 µg/kg and above.

Ochratoxins

Topic Advisor Mary Trucksess, U.S. Food and Drug Administration, 5100 Paint Branch Pkwy, College Park, MD 20740, Tel: 301-436-1957, Fax: 301-436-2665, E-mail: mary.trucksess@fda.hhs.gov. Trucksess reported that LC with fluorescence detection after extraction and purification continue to be widely used for OTA analysis. A recent report of extracting Italian salami with ethyl acetate, purification by IAC, and quantitation by LC could determine OTA at the ppt level (116). Recovery at 0.5 and 1 ng/g averaged 77%. Of 30 salami samples analyzed, the most contaminated sample was found to contain an OTA level of 0.4 ng/g. Lead hydroxyacetate precipitation and liquid partition were used to isolate OTA in beer marketed in Spain prior to LC analysis (117). Recoveries were about 96% at spiked levels of 0.01–0.5 ng/mL. Of 88 samples analyzed, 82.9% were found to contain OTA ranging from 0.007 to 0.204 ng/mL. SPME with a 60 µm thick poly(dimethylsiloxane)/divinylbenzene fiber was used for pretreatment of OTA-spiked beer samples before isocratic elution and LC separation (118). The factors influencing fiber adsorption and desorption of OTA were investigated; the linear range investigated in beer was 0.03–2 ng/mL. The LOQ in spiked beer was 53 pg/mL. A pressurized liquid extraction procedure was applied for the extraction of OTA from rice (119). The operating conditions were methanol as extraction solvent, 1500 psi, 40°C, 5 min static time, 50% flush volume, 60 s of purge, 1 cycle, and 11 mL cell size. Twelve samples of rice were analyzed; only one sample was contaminated with OTA at 4.17 ng/g.

Instead of using fluorescence detection, a nano-reversed-phase column coupled online with mass spectrometer (nano-LC/ESI-MS) was used for the determination of OTA in must and grapes (120). Intact or slightly mashed grapes were extracted with chloroform, lyophilized, redissolved in acetonitrile, and injected onto a capillary LC column. The LOD and LOQ of this method were 1 and 2 pg/g, respectively. The identity of OTA was confirmed by MS/MS. A 2-dimensional TLC (2D-TLC) method was developed for OTA at 5 ng/g in green coffee (121). Coffee beans were extracted with methanol–aqueous sodium bicarbonate solution. After OTA was partitioned into toluene, it was separated by 2D-TLC using toluene–methanol–formic

acid (8 + 2 + 0.3) and petroleum ether–ethyl acetate–formic acid (8 + 10 + 1) as 1st and 2nd dimensional developer, respectively. A monoclonal antibody against OTA was developed and applied to an immunochromatographic assay for detection of OTA up to 500 ng/mL in <10 min (122).

Two studies were reported on the designing of sampling plans. One study was for the detection of OTA in green coffee (123). Twenty-five lots of green coffee were sampled using probing devices, and 16 test samples were taken from each lot (16 kg). Each test sample (1 kg) was ground and two 25 g subsamples were analyzed. A total of 32 analyses were performed for each lot and 800 analyses for the 25 lots. With this experimental protocol, the sampling, sample preparation, and analytical variances were 7.80, 2.84, and 0.11, respectively, and accounted for about 73, 26, and 1% of the total variability, respectively. The other study was for the assessment of OTA content before grape harvesting (124). Grapes of 2 vineyards with “Negroamaro” and “Sangiovese” grape varieties were sampled according to the same sampling design. Ten plants were collected from the X-shaped path of the whole vineyard and bunches from each plant were crushed separately. Another 10 plants were collected from the 2 central cross-perpendicular lines, and bunches were crushed plant by plant. Juices obtained were analyzed. High and random variability was observed in OTA levels both among bunches and among plants. The best results were obtained when sampling involved one bunch per plant in a predefined position, from at least 10 plants.

There are papers reporting the occurrence of OTA in dried figs (125), spice (126), organic and traditional grown rice and rice products (127), kava-kava and licorice roots (114). One paper investigated the occurrence of OTA in cocoa beans and the effect of shelling (128). Twenty-two cocoa samples were analyzed before and after the shelling process. It was found that OTA contamination in cocoa beans is concentrated in the shell. The shelling process could prevent OTA occurrence in cocoa products. Polished rice naturally contaminated with OTA was used to determine the effect of cooking on OTA (129). Ordinary cooking and pressure cooking reduced OTA concentrations by about 59–795%.

Patulin

Topic Advisor Myrna Sabino, Instituto Adolfo Lutz, Av. Dr Arnaldo 355–01246-902, São Paulo, Brazil, Tel: 55-11-3068-2921, Fax: 55-11-3085-3505, E-mail: mysabino@ial.sp.gov.br. Sabino reported that estimates on dietary exposure from the First French Total Diet Study have been published (12). The results show that 16 composite samples exhibited contamination levels below the detection limit. Two composite samples showed contamination levels between the detection limit and 50 µg/kg, namely, the composite concentrate-based apple juice sample and the composite cider sample. This finding might suggest that apple juice may be added to cider fermentation. Levels exceeding 50 µg/kg were found in the composite apple tart sample (60 µg/kg) and the composite apple doughnut sample (100 µg/kg).

A method was developed and validated in-house for the determination of PAT in apple juice using ethyl acetate–hexane extraction and LC equipped with a C18 column and diode array detector. The mobile phase used for the quantification was water–ethanol, at a flow rate of 0.5 mL/min (130). The method showed a mean recovery of 84.8%, the relative standard deviation (RSD) obtained in the precision study was <7.7%, LOQ and LOD values were 7 and 3 µg/L, respectively, and the linear range for PAT in apple juice was 2.6–650 µg/L. The ruggedness was evaluated by an intralaboratory experiment, in which 5 factors were studied, and only one was found to influence the observed results. The developed method was fast, practical, and simple; the solvents (except hexane) and reagents used were nontoxic. An interlaboratory study for validation of a Japanese official analytical method for determination of PAT in apple juice was performed in 11 laboratories using a noncontaminated sample, 2 naturally contaminated samples, and 2 spiked samples of apple juice (131). For naturally contaminated apple juices, the RSDs for repeatability and reproducibility were 3.2, 7.1, and 10.0, 21.7%, respectively. HorRat values were 0.4 and 0.9. The average recovery of PAT from the spiked samples was 83.7%. The LOQ was calculated as 10 µg/kg. In a review of recent advances in mycotoxin determination in food and feed by hyphenated chromatographic techniques/MS, the use of MS in connection with the analysis of PAT, aflatoxins, OTA, ZON, trichothecenes, and fumonisins was discussed (29). Although the selectivity of MS is unchallenged if compared to conventional gas chromatography (GC) and LC detection methods, accuracy, precision, and sensitivity may be extremely variable concerning the different mycotoxins, matrixes, and instruments. A stable isotope dilution assay (SIDA) was developed for PAT using a synthesized ¹³C isotopomer (132). After extraction, the isotopomers were derivatized and determined by GC coupled to low- or high-resolution MS.

A study reinvestigated the production of polyclonal antibodies against PAT and their purification since crude antiserum could react nonspecifically in immunoassays (133). PAT-hemiglutarate (Pat-HG) was synthesized and conjugated to bovine serum albumin (BSA) as the immunogen for the immunization of 5 New Zealand white rabbits. The polyclonal antibodies generated against the mycotoxin PAT showed high titers due to the recognition of the whole immunogen Pat-HG-BSA. Crude and purified antisera using the Sepharose-LS affinity gel gave unsatisfied displacement results by the indirect competitive ELISA assay. Only 30% of the antibodies were specific of free PAT, and this result was not sufficient to develop a useful immunoassay for food analysis. SDS-PAGE analysis showed the presence of a contaminant protein with a molecular weight of 67 kilodaltons (kD). The polyclonal antibodies are more specific to the whole Pat-HG-Pat conjugate than to the free mycotoxin PAT molecule. The polymer-assisted reaction of hydroxymethylfuranone with 21 carboxylic acids using polystyrene-carbodiimide yielded an ester library (134). Four of the resulting esters were tested as internal standards for the

quantification of PAT in apple juice by GC/MS in the selected-ion monitoring mode (GC/MS-SIM). The developed method combines an AOAC official extractive step and a GC/MS-SIM analysis. Using a chromatographic column containing trifluoropropylmethylpolysiloxane as the stationary phase, it was possible to perform an accurate and precise quantification of underivatized PAT in apple juice at concentrations down to 6 µg/L. A detection limit of 1 µg/L was established (134). The effects of *trans*-2-hexenal on blue mold disease, PAT content, and fruit quality in "Conference" pears was investigated (135). *Trans*-2-hexenal treatment was effective in the reduction of blue mold infection and PAT content in Conference pears when applied 24–48 h after pathogen inoculation. It could be a natural alternative to fungicides in the control of *P. expansum* infections. Further work is needed to study the methods and conditions to avoid the persistence of off-odors and off-flavors in pears after their exposure to *trans*-2-hexenal vapors. An investigation of mycological contamination of stored and newly harvested vegetables was carried out (136). In these investigations, samples of carrots, onions, and cabbage were taken from storehouses with different storage periods and conditions. *P. expansum*, *P. nalgiovense*, *Mucor silvaticus*, and *P. verrucosum* were more frequently detected on carrots, *P. expansum* on onions, and *Aspergillus niger*, *Botrytis cinerea*, *Mucor hiemalis*, *P. funiculosus*, and *P. expansum* on cabbages. The storage conditions of vegetables influenced the distribution of different fungal species. The ability of *Penicillium expansum* Sv-168-1 growing on different foodstuffs and especially potato to produce PAT was confirmed quantitatively (136). The possible production of PAT in dry cured meat products sold on the French market was studied (88). The result demonstrated no production of PAT by toxigenic strains. After direct contamination, the initial content of PAT decreased to become undetectable after only 6 h of incubation at 20°C.

The cytotoxicity of PAT was characterized by analysis of the yeast transcriptome upon challenge with PAT (137). PAT-induced yeast gene expression profiles were found to be similar to gene expression patterns obtained after treatment with the antifungal agricultural chemicals thiuram, maneb, and zineb. Moreover, PAT treatment was found to activate protein degradation, especially proteasome activities, sulfur amino acid metabolism, and the defense system for oxidative stress. The results suggest the possibility of applying the yeast transcriptome system for the evaluation of chemicals, especially for natural chemicals that are difficult to obtain by organic synthesis (137). PAT, at a dose of 0.1 mg/kg body weight/day, was administered orally to growing male rats 5–6 weeks for a period of 60 or 90 days (138). The dose of PAT used in the present study was based on estimated human exposure levels. At the end of these periods, the thymus glands of PAT-treated and control Wistar rats were removed, and ultrastructural changes in capillary cells of the thymus of PAT-treated Wistar rats were determined by electron microscopy. The results obtained from this study may provide a guide to research dealing with the toxic effects of PAT on

tissue and organ ultrastructure (138). In a study of cloning and molecular characterization of *P. expansum* genes, 5 genes were cloned (full-length *idh* gene, 470 base pair (bp) fragment of the *6-msas* gene, 715 bp fragment of a putative adenosine triphosphate (ATP)-binding cassette transporter gene *peab1*, together with a part of 2 putative cytochrome P450 monooxygenase genes *P-450 1* and *P-450 2*). Increased expression of all 5 genes was observed under PAT-permissive conditions, indicating not only their likely involvement in PAT biosynthesis, but indicating for the first time that regulation of PAT biosynthesis in *P. expansum* is mediated at the level of gene transcription (139).

Trichothecenes

Topic Advisor Gary A. Lombaert, Health Canada, 510 Lagimodiere Blvd, Winnipeg, MB, R2J 3Y1, Canada, Tel: 204-984-2088, Fax: 204-983-5547, E-mail: gary_lombaert@hc-sc.gc.ca. Lombaert reported that an excellent review of recent advances in mycotoxin methods touches on classical physicochemical methods, biosensors, and rapid kits based on immunoanalytical principles and molecularly imprinted polymers (140). Rapid method technologies for toxigenic fungi and mycotoxins were reviewed in 2 articles (141, 142). Hyphenated chromatographic-MS techniques for the analysis of mycotoxins were also recently reviewed (29). All of these reviews include discussion and/or reference to methods for trichothecenes.

Nuclear magnetic resonance, infrared and UV spectrometry, ion and HPLC were used to characterize purified B-trichothecene standards of DON, 3-acetyl-DON (3-ADON), 15-acetyl-DON (15-ADON), and nivalenol (NIV; 143).

With respect to methods of analysis for mycotoxins in foods, feeds, and the environment, LC with either UV or fluorescence detection (FLD) remain the methods of choice, while LC/MS methods continue to gain favor as this technology becomes routinely available to more laboratories. Array biosensors and similar rapid methods will surely continue to find application within the sphere of mycotoxin analysis, while interest in GC methods appears to wane.

A comparison of cleanup procedures (using commercial IAC and charcoal/alumina columns) and LC detection techniques for DON in extracts of cereal grains and cereal-based foods was reported (144). Analytical parameters were optimized and the authors concluded that IAC cleanup, postcolumn derivatization to the DON derivative dihydrolutidine, and detection by LC-FLD provided the best results. Two LC methods for the analysis of DON were subjected to interlaboratory studies. The first, a method for DON in cereals and cereal products, was based upon IAC cleanup and UV detection (145). At spiking levels of 200–2000 ng/g, recoveries ranged from 78 to 87%. The RSDs for repeatability were from 3 to 14%, while those for reproducibility were 11 to 26%. The second method used a multifunctional cleanup column for the analysis of wheat intended for food or feed (146). This publication reported

recoveries of 100%, RSDs for repeatability and reproducibility of 11 and 10%, respectively, and an LOQ of 100 ng/g. An LC method for the analysis of T-2 and HT-2 toxins at ng/g levels in wheat, maize, and barley using commercial IACs was recently developed (147). Samples were extracted with methanol–water and cleaned up on IACs containing monoclonal anti-T-2 antibodies, which showed 100% cross-reactivity to HT-2 toxin. The toxins were labeled with 1-anthroylnitrile prior to detection by LC-FLD. LODs were 5 and 3 ng/g for T-2 and HT-2, respectively, and recoveries averaged 70–100%, with RSDs of <8%.

LC/MS continues to find favor in the analysis of trichothecenes. An LC/MS method for both type A [diacetoxyscirpenol (DAS), T-2 and HT-2 toxins] and type B [NIV, DON, fusarenon-X (F-X), and 15-ADON] trichothecenes in wheat flour was reported (148). Sample extracts were cleaned up by commercial charcoal-alumina columns. LOQs ranged from 10 to 20 ng/g and recoveries from 70 to 120%. An LC/MS (TOF) method for the analysis of trichothecenes (and ZON and aflatoxins) was applied to a survey of corn, wheat, cornflakes, and biscuits. The method used atmospheric pressure chemical ionization (APCI) with real-time reference mass correction (30). The reported LODs ranged from 0.1 to 6 ng/g. An LC/MS/MS method for the determination of the type B trichothecenes NIV, DON, F-X, 3-ADON, and 15-ADON in maize was reported and applied to a survey of 78 samples (149). LODs ranged from 2 to 12 ng/g and recoveries from spiked maize (100 ng/g) ranged from 79 to 97%. The method was extended to include 3 other trichothecenes and fumonisins and ZON (105). Another LC/MS/MS method for trichothecenes in food and feed samples employing a commercial charcoal-alumina cleanup column was reported (150). Recoveries ranged from 65 (NIV) to 96% (DON). An LC/MS/MS method using commercial cleanup columns was also reported for the determination of 12 trichothecenes in cereal products (151). Recoveries were reported between 65 and 104%, and LODs were between 0.3 and 5 ng/g. LC/MS/MS was also used for the analysis of rice cultures for 6 trichothecenes: NIV, DON, F-X, 3-ADON, 15-ADON, and T-2 (152). The toxins were extracted and diluted with acetonitrile–water and detected in ESI and MRM modes. An LC/MS/MS method for multiple mycotoxins in milk included T-2 and HT-2 toxins, T-2 triol, DAS, 15-monoacetoxyscirpenol (15-MAS), DON, 3-ADON, 15-ADON, and deepoxy-deoxynivalenol (DOM-1; 153). Extracts were cleaned up by SPE; the type B trichothecenes were detected in negative-ion mode, while the type A trichothecenes were detected in positive-ion mode, and 2 transition products were monitored for each toxin. An LC/MS/MS method for the analysis of trichothecenes (and other mycotoxins) on cellulose filters used in the investigation of sick-building syndrome was described (31). The method used ESI, MRM, and DOM-1 as an internal standard.

The synthesis of stable isotope labeled 15-d(1)-DON permitted development of an isotope dilution LC/MS/MS method for DON and 3-ADON in cereal products (154). DOM-1 was used as an internal standard and was reported to

reduce matrix effects in an electrospray LC/MS/MS method for 12 trichothecenes (155). The method, involving extraction with acetonitrile–water and cleanup by charcoal-alumina columns, reported LODs from 0.2 to 5.0 ng/g and was applied to a survey of wheat and oats.

An array biosensor using a competitive immunoassay was developed and applied to the determination of DON in foods (cornmeal, cornflakes, wheat, barley, and oats) and indoor air samples (156). Samples were extracted with methanol–water and the DON was determined without cleanup or preconcentration. The LODs ranged from 4 ng/mL in the aqueous effluent of an air sampler to 50 ng/g in oats.

The occurrence of mycotoxins in human diets has long been, and remains, a concern due to associated health risks. An excellent review of mycotoxins in foods includes a discussion of the toxicity of DON, methods of analysis, and food processing techniques (157). In a recent Japanese study of industrial processing, the milling of grain to flour was reported to result in average losses of 74 and 63% of the DON and NIV, respectively (158). Sieving, washing, and chemical treatment processes to reduce the DON content of barley were studied (159). LC, ELISA, and a bioassay were used to study the effect of extrusion processing upon the DON content and the loss of toxicity of corn grits (160). Losses of DON were estimated to range from 21 to 37%, and the bioassay result correlated more closely to the LC than the ELISA result.

More recently, the occurrence of bound mycotoxins in plants and foods has also generated interest. In plants, these mycotoxins are conjugated to polar substances such as sugars, amino acids, or sulfates, and may release their toxic parent compound upon hydrolysis in the gastrointestinal tract, thereby contributing to the toxic load. These bound mycotoxins are stored in cellular vacuoles, and may escape detection by routine methods. To establish the potential risk of bound mycotoxins, reliable methods for their detection and quantitation are required. One such method used a trichloroacetic acid treatment in the analysis of *Fusarium*-infected plants for bound DON (161). Nonextractable DON was reported to be 13–63% of the total DON.

A survey of 125 German-grown cereals and cereal byproducts (wheat, oats, corn, corn byproducts, corn plants, and corn silage) collected during 2000 and 2001 was recently reported (162). Samples were analyzed for 13 trichothecenes by GC/MS. DON was the most frequently detected trichothecene (94%). The same publication reported that DON was also the most frequently detected trichothecene (14%) in 95 samples of nongrain feedstuffs (hay, lupines, peas, soy meal, rapeseed meal, and other oilseed meals). A survey of DON in 105 cereal grains (wheat, rye, and barley) from Poland and East Slovakia found only 8 wheat samples that exceeded the European food limit of 750 ng/g (163). A similar survey of South African wheat and wheat-based products confirmed DON as the most prevalent contaminant in wheat, and low but persistent contamination of DON in wheat-based products sold to the public (164). A survey of 80 commercial corn samples from São Paulo, Brazil, detected

NIV in 4 samples, while DON and NIV co-occurred in another (165). T-2, HT-2, and DAS were not detected. Using GC/MS, the reported LODs were from 10 to 40 ng/g. Corn-based food products were also analyzed (166). Interestingly, only 2 of 78 samples were found to contain quantifiable levels of trichothecenes (T-2 and HT-2), and one contained trace levels of DON and NIV. The reported LODs in this study ranged from 20 to 120 ng/g.

Eleven trichothecenes were included in the First French Total Diet Study (12). Using GC/MS detection, DON was the most frequently detected trichothecene, being found in 31 of 456 composites; NIV was the next most frequently detected, found in just 3 composites.

The role and health significance of trichothecenes in the environment is a topic of continuing interest and concern. The air from 7 buildings with known *Stachybotrys chartarum* contamination, from 4 control buildings, and from outdoors, was sampled and analyzed using an ELISA specific to macrocyclic trichothecenes (167). The control buildings demonstrated a statistically lower level of airborne trichothecenes, while levels of trichothecenes in the test buildings increased with sampling time and with periods of air disturbance. Another study concluded that the quantity of mycotoxins aerosolized into air was not strictly proportional to levels found on contaminated materials (168). The concentration of DON (and sterigmatocystin and OTA) in indoor air was measured in this study with an LOD of 60 pg/m³.

Zearalenone

Topic Advisor Rudolf Krska, University of Natural Resources and Applied Life Sciences, Department for Agrobiotechnology-IFA-Tulln, Center for Analytical Chemistry, Christian Doppler Laboratory for Mycotoxin Research, Konrad Lorenz Str. 20, A-3430 Tulln, Austria, Tel: 43-2272-66280-401, Fax: 43-2272-66280-403, E-mail: rudolf.krska@boku.ac.at. Krska summarized selected information on ZON appearing in the last year's scientific literature. In 2001, the European Commission set maximum levels for certain contaminants in foodstuffs, e.g., aflatoxins among others (Commission Regulation 2001/466/EC; 169). In June, 2005, an amendment (Commission Regulation 2005/856/EC; 170) was published that regards *Fusarium* toxins and specifies a tolerable daily intake of 0.2 µg/kg body weight/day for ZON. Additionally, Commission Directive 2005/38/EC (171) was published, laying down the sampling methods and the methods of analysis for the official control of the levels of *Fusarium* toxins in foodstuffs. Both amendment and directive entered into force as of July 1, 2006.

Two useful review articles about mycotoxin analysis that include discussions about ZON have been published (29, 140). The review of Krska et al. (140) covers the latest activities in mycotoxin analysis and the advances of its respective quality assurance. Besides the development of highly efficient, mostly multitoxin methods based on chromatographic separation and MS detection, research interests have focused on the development and improvement

of rapid and innovative detection methods based on antibodies (ELISA, immunological biosensors), molecular imprinted polymers, or spectroscopic screening. Significant improvement has been achieved regarding the quality of analytical data for ZON determination due to extensive interlaboratory exercises in the past years and the recent availability of maize certified reference materials (CRM), e.g., BCR-716 (ZON <5 µg/kg) and BCR-717 (ZON = 83 ± 9 µg/kg, expanded uncertainty, k = 2), as well as a commercial ZON calibrant, ERMAC699 (ZON in acetonitrile, BCR-699 CRM).

The second review (29) summarizes recent advances in mycotoxin determination in food and feed by hyphenated chromatographic techniques and MS detection. The review considers ZON and other mycotoxins such as PAT, aflatoxins, OTA, trichothecenes, and fumonisins.

While screening last year's ZON literature, it became evident that recent mycotoxin method development activities have focused mainly on multitoxin methods based on chromatographic separation and MS detection. Only 4 relevant articles were published that dealt with method development for ZON and analogs, exclusively. A new method for the determination of ZON in edible oils with size exclusion chromatography (SEC) followed (LC/MS/MS) as well as LC-FLD was developed and validated (172). By using LC/MS/MS, no further cleanup step is necessary after SEC. From the 77 edible oils analyzed, 38 corn germ oils were tested positive with a mean average value of 169 µg/kg. The maximum level was 921 µg/kg.

Ramirez et al. (173) developed an electroanalytical method for the quantification of ZON in maize samples. The determination is based on an electro-oxidation reaction of ZON that takes place on the surface of glassy carbon electrodes with an aqueous mixture of HClO₄ and acetonitrile as reaction medium. The electro-oxidation of ZON was studied by using squarewave voltammetry. The method exhibited a linearity range from 20 to 3184 µg/kg and an LOD of 30 µg/kg [signal-to-noise (S/N) ratio 3:1]. Recovery rates for spiked maize samples were slightly higher than 100%.

An isotope dilution method was applied to the quantification of ZON in maize flour (174). The method is based on an alternate reaction of the analyte in a matrix and in a standard solution with isotope-labeled or unlabeled derivatizing reagents. A relative response factor for the analyte/internal standard ratio of the sample can be directly calculated. This approach allows quantification of ZON in maize flour without the need for a calibration curve. LOD (S/N = 3:1) and LOQ (S/N = 10:1) of the method were determined by spiking toxin-free flour samples at the appropriate level and were found to be 5 and 10 µg/kg, respectively. Determination of ZON in spiked samples within a concentration range of 10–1000 µg/kg and in a Food Analysis Performance Assessment Scheme CRM showed recovery rates significantly above 100% (107–141%). The method exhibits excellent repeatability of 2–5% (for the concentration range of 100–1000 µg/kg). The analytical approach presented here avoids errors due to instrumental and

matrix effects, yields accurate results with minimal sample preparation, and exploits all the advantages of isotope dilution methods in a low-cost, easy-to-perform procedure, although 2 independent experiments have to be performed for each sample analyzed.

A method based on GC/MS with negative chemical ionization was described for the determination of ZON and other resorcylic acid lactone analogs [zearanol, taleranol, α - and β -zearalenol, zearalanone (ZAN)] in urine samples (175). Method validation was performed according to the latest EU performance criteria (Commission Decision 2002/657/EC; 176). LODs were between 0.06 and 0.60 $\mu\text{g/L}$ (ZON: 0.35–0.60 $\mu\text{g/L}$) and within-laboratory reproducibilities between 11 and 54% (ZON: 27%), depending on the mycotoxin. Stability tests showed that all investigated compounds were stable in urine at -20°C for at least a year. Part of the validation program was the organization of a small proficiency testing study and a correlation study with an LC/MS/MS method developed in another laboratory. The study showed good agreement between the results of both laboratories.

An interlaboratory study was performed on behalf of the UK Food Standards Agency to evaluate the effectiveness of an affinity column cleanup with subsequent LC-FLD for the determination of ZON in a variety of cereals and cereal products at proposed European regulatory limits (177). Barley, wheat and maize flours, polenta, and a maize-based baby food naturally contaminated, spiked, and blank (very low level) were sent to 28 collaborators. Participants were asked to spike test portions of all samples at a ZON concentration equivalent to 100 $\mu\text{g/kg}$. Average recoveries ranged from 91 to 111%. Based on results for 4 artificially contaminated samples (blind duplicates) and one naturally contaminated sample (blind duplicate), the RSD for repeatability ranged from 6.9 to 35.8%, and the RSD for reproducibility ranged from 16.4 to 38.2%. The method showed acceptable within- and between-laboratory precision for all 5 matrixes, as evidenced by HorRat values <1.7 .

More than 10 papers on multitoxin methods that include ZON have been published in the past year. Biselli et al. (178) describes a method for the determination of several *Fusarium* mycotoxins (ZON, and type A and B trichothecenes) in cereal-based samples using LC/MS/MS. To achieve optimal MS detection, ESI and APCI were compared. Best results were obtained with ESI by implementing a 2-period switching for the ionization polarity. The LOQ differed for each individual substance within the range of 1–10 $\mu\text{g/kg}$. Mean recoveries using a standardized cleanup procedure ranged 54 to 93% (ZON: 92%). Further multitoxin methods based on a similar analytical approach using LC-ESI-MS/MS were published from the same authors (150, 179). The methods were applied to the determination of *Fusarium* mycotoxins, including ZON, in various food and feed samples. The sample preparation based on a standard extraction step was followed by 2 different kinds of solid-phase cleanups: A multifunctional MycoSep cleanup for trichothecenes, and an immunoaffinity purification, which combined antibodies for

ZON, aflatoxins, and OTA. LODs ranging from 0.02 to 10 $\mu\text{g/kg}$ depending on the mycotoxin were reported. For quantification of ZON an internal standard (ZAN) was used, whereas for trichothecenes a recovery standard (verrucarol) was applied. Average recoveries ranged from 65–96% (ZON: 89%).

Another sensitive multitoxin method based on LC-ESI-MS/MS for the detection of some macrocyclic lactones (ZON, α - and β -zearalenol), trichothecenes, and fumonisins in maize was evaluated and applied to field samples (105, 149). An MRM program was developed in which fumonisins and trichothecenes (except NIV and DON) are acquired in positive ESI mode, and all other compounds in negative mode. Method LODs were in the range of 2–14 $\mu\text{g/kg}$, with the exception of NIV (27 $\mu\text{g/kg}$), DON (40 $\mu\text{g/kg}$), and 15-acetyldeoxynivalenol (30 $\mu\text{g/kg}$). Good accuracy (recoveries: 81–104%) and precision (RSDs: 4–11%) were obtained by calibration with spiked analyte-free sample extracts. Sample cleanup was performed with cartridges filled with graphitized carbon black (Carbograph-4). Random maize samples were analyzed both by the ELISA-based methods specific for ZON and DON and by the developed LC-ESI-MS/MS method for type B trichothecenes and macrocyclic lactones. Results were comparable for ZON ($R^2 = 0.982$), but disagreed for DON.

Tanaka et al. (30) performed simultaneous determination of ZON, trichothecenes, and aflatoxins in foodstuffs using LC with APCI and TOF-MS detection (LC-APCI-TOF-MS). A real-time reference mass correction technique was developed. Detection of the mycotoxins was carried out in exact mass chromatograms with a mass window of 0.03 Th. Samples were cleaned up with a MultiSep No. 226 column. Calibration curves were linear from 2 to 200 $\mu\text{g/L}$ for trichothecenes and ZON, and 0.2 to 20 $\mu\text{g/L}$ for aflatoxins, with a 20 μL injection volume. LODs ranged from 0.1 to 6.1 $\mu\text{g/kg}$ (ZON: 0.2–0.8 $\mu\text{g/kg}$) in foodstuffs such as corn, wheat, cornflakes, and biscuits.

Delmulle et al. (31) developed a fast and simple LC-ESI-MS/MS method for the simultaneous detection of 16 mycotoxins, including ZON, that are possibly related to the “Sick Building Syndrome”. Fungi-surface sampling used in “Sick Building Syndrome” studies is preferably done by scraping off fungal material and vacuuming onto cellulose filters. Therefore, the 2 media (filters and fungal cultures) were used as samples and spiked with the mycotoxins. De-epoxy-deoxynivalenol was used as internal standard. The samples were extracted with organic solvents and the different mycotoxins were determined with an LC-ESI-MS/MS system using MRM. LODs varied from 0.009 to 50 $\mu\text{g/L}$ for filter samples and from 0.04 to 75 $\mu\text{g/L}$ for fungal culture samples.

A new SPE cleanup using a Bond Elut Mycotoxin column, originally developed for the determination of 12 type A and B trichothecenes in cereals and cereal-based food, has also been tested for its suitability for ZON detection (151). After a standard extraction of the toxins from cereal samples, the extract was purified by means of a Bond Elut Mycotoxin column and analyzed with LC-ESI-MS/MS. LODs were

calculated for the matrix wheat and ranged from 0.3 to 5 µg/kg, depending on the toxin. Average recovery rates in 7 cereal-based matrixes ranged from 65 to 104% for the trichothecenes and from 95 to 116% for ZON (around 50% without internal standard correction with ZAN). RSDs of the complete method ranged from 2.7 to 20.0% (ZON: 2.7–7.5% with internal standard correction).

Four articles have been published in the past year assessing the occurrence of ZON among other mycotoxins in grains, foods, and feedstuff. Natural occurrence of 16 *Fusarium* toxins, including ZON, in grains and feedstuffs in Germany was investigated (162). ZON and α - and β -zearalenol were analyzed by LC-FLD/UV, while the other *Fusarium* toxins were determined by GC/MS. LODs ranged from 1 to 19 µg/kg depending on the mycotoxin (ZON: 8 µg/kg). A total of 220 samples comprising cereals, cereal byproducts, corn plants, and corn silage as well as nongrain-based feedstuffs were analyzed. ZON was among the most predominant mycotoxins, besides HT-2 and T-2 toxin, 15-acetyldeoxynivalenol, NIV, and DON.

To investigate the exposure of livestock and farm workers to fungal spores and mycotoxins, a multitoxin method for 6 mycotoxins, including ZON, was developed (180). After SPE, the mycotoxins were quantified by LC/MS. An experimental study of fungal species and mycotoxins was conducted in corn silage (Normandy, France) during 9 months of monitoring. The investigated material exhibited significant amounts of ZON (23–41 µg/kg) besides 3 other toxins (aflatoxin B₁: 4–34 µg/kg, citrinin: 4–25 µg/kg, and DON: 100–213 µg/kg). This suggested a possible chronic exposure to low levels of mycotoxins.

Labuda et al. (181) studied the incidence of trichothecenes and ZON in poultry feed mixtures from Slovakia. A total of 50 samples of poultry feed mixtures of Slovakian origin were analyzed for 8 toxicologically significant *Fusarium* mycotoxins, including ZON. Reversed-phase LC-FLD was used for ZON determination. The A-trichothecenes and the B-trichothecenes were analyzed by LC/MS/MS and GC with electron-capture detection (GC-ECD), respectively. The most frequent mycotoxin detected was T-2 toxin, which was found in 90% of the samples in relatively low concentrations ranging from 1 to 130 µg/kg (mean 13 µg/kg), followed by ZON in 88% of the samples with concentrations from 3 to 86 µg/kg (mean 21 µg/kg). In as many as 44% of the samples, a combination of 4 simultaneously co-occurring mycotoxins, e.g., ZON, T-2 toxin, HT-2 toxin, and DON, was revealed.

Preliminary results of a 5-year investigation to assess the level of *Fusarium* mycotoxin contamination in the United Kingdom wheat production were presented at the British Crop Production Council (BCPC) International Congress: Crop Science & Technology in 2005 (182). The study started in 2001, and each year 300 samples of wheat from fields of known agronomy were collected and analyzed for 10 trichothecenes and ZON. Results from the first 4 years have shown that the incidence of mycotoxins was generally low. Only 5 mycotoxins (ZON, HT2, and T2 toxin, DON, and NIV) were detected in more than 5% of the samples tested, with DON being the

predominant mycotoxin found. Results from the completed project will be used to determine “Good Agricultural Practice” to minimize mycotoxin contamination of wheat.

Three articles dealt with the characterization of ZON-producing *Fusarium* species with special regard to ZON biosynthesis, identification of major ZON producers, and detection of *Fusarium* species on infected material. Kim et al. (183) found that 2 different polyketide synthase genes (ZEB1 and ZEB2) are required for synthesis of ZON in *Gibberella zeae*. ZEB1 is responsible for the chemical conversion of β -zearalenonol to ZON in the biosynthetic pathway, and ZEB2 controls transcription of the cluster members. Furthermore, it was suggested that ZON or β -zearalenonol might be involved in transcriptional activation of the gene cluster required for ZON biosynthesis in *G. zeae*. Llorens et al. (184) characterized 44 *Fusarium* species morphologically, physiologically, and genetically. Physiological characterization was based on their ability to produce ZON and type B trichothecenes. ZON was determined by LC with ion-trap MS and trichothecenes by GC/MS. The results indicate that *F. graminearum*, *F. culmorum*, and *F. cerealis* isolates were high ZON and type B trichothecene producers. A polymerase chain reaction (PCR) detection assay for trichothecene and ZON-producing *Fusarium* species was developed for early detection and control of these species in order to prevent toxins entering the food chain (185). The specific primers were designed on the basis of IGS sequences (Intergenic Spacer of rDNA). The IGS sequence is a multicopy region in the genome that enhances the sensitivity of the assay in comparison with PCR assays based on single-copy sequences.

Recommendations

General Referee Recommendations

(1) Appoint Rudolf Krska, Center for Analytical Chemistry, Department IFA-Tulln, University of Natural Resources and Applied Life Sciences, Tulln, Austria, as the Topic Advisor for ZON.

(2) Appoint Mary Trucksess, U.S. Food and Drug Administration, as Topic Advisor for Ochratoxins.

(3) Continue to investigate and develop methods for mycotoxins in all commodities relevant to human health.

Topic Advisors' and Study Directors' Recommendations

(1) *Sampling and subsampling for mycotoxins.*—Topic Advisor Thomas B. Whitaker. Continue study.

(2) *Aflatoxin M₁.*—Topic Advisor Hans P. van Egmond. Continue study.

(3) *Aflatoxin.*—Topic Advisor Gordon S. Shephard. Continue study.

(4) *Alternaria toxins.*—Topic Director Michele Solfrizzo. Continue study.

(5) *Citrinin.*—Topic Advisor David Abramson. Continue study.

(6) *Cyclopiazonic acid*.—Topic Advisor Joe W. Dorner. Continue study.

(7) *Ergot alkaloids*.—Topic Advisor George M. Ware. Continue study.

(8) *Fumonisin*s.—Topic Advisor Chris Maragos. Continue study.

(9) *Mycotoxins in botanicals*.—Topic Advisor Bruce Malone. Continue study.

(10) *Ochratoxin*s.—Topic Advisor Mary Trucksess. Continue study.

(11) *Patulin*.—Topic Advisor Myrna Sabino. Continue study.

(12) *Trichothecenes*.—Topic Advisor Gary Lombaert. Continue study.

(13) *Zearalenone*.—Topic Advisor Rudolf Krska. Continue study.

(14) **2001.04** *Determination of Fumonisin B₁ and B₂ in Maize Flour and Cornflakes by LC*.—Study Director Angelo Visconti, National Research Council, Institute of Toxins and Mycotoxins, Viale Einaudi 51, Bari 70125, Italy, Tel: 39-080-548-6013, Fax: 39-080-548-6063, E-mail: visconti@area.ba.cnr.it. Continue study.

(15) **2004.10** *Determination of Ochratoxin A in Green Coffee by Immunoaffinity Column Cleanup and LC*.—Study Director Eugenia Vargas, Laboratory for Mycotoxin Analysis, Avenida Raja Gabaglia, 245 Cidade Jardim, Belo Horizonte 30380-090, Brazil, Tel: 55-31-250-0398, Fax: 55-31-250-0399, E-mail: gena@cldnet.com.br. Continue study.

(16) **2005.08** *Liquid Chromatographic Analysis of Aflatoxin Using Post-Column Derivatization (Modification of 991.31, 999.07, and 970.45)*.—Study Director Arthur Walkling, Walkling Associates, 482 Rock Rd, Glen Rock, NJ 07452. Study Director submitted validation data. This study has been adopted as First Action. Continue study.

(17) *Determination of DON in Corn, Wheat, Barley, and Malted Barley, ELISA*.—Study Director Mark Mozola, Neogen Corp., 620 Leshner Pl, Lansing, MI 48912-1595, Tel: 517-372-9200, Fax: 517-372-0108, E-mail: mmozola@neogen.com. Study has been completed and report has been submitted to AOAC for review. Continue study.

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