

GENERAL REFEREE REPORTS

Committee on Natural Toxins and Food Allergens

Marine and Freshwater Toxins

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Summary

There have been major developments this past year in the Marine and Freshwater Toxins topic area (formerly Phycotoxins). These include AOAC approval and inauguration of a new AOAC Presidential Task Force on Marine and Freshwater Toxins to accelerate methods validation, and the appointment of several new Topic Advisors. A joint FAO/IOC/WHO group addressing biotoxins in molluscan bivalves is also relevant to this report and to the new Task Force.

The AOAC Presidential Task Force on Marine and Freshwater Toxins is an international group that, in late November 2004, consisted of 90 world experts and stakeholders. Chaired by this General Referee, the group establishes methods priorities based on analytical methods criteria, determines fitness for purpose, identifies and reviews available methodologies, recommends methodologies for validation, and identifies complementary analytical tools. Once appropriate analytical methodology has been identified or developed, the Task Force is able to identify financial and technical resources necessary to validate the methods.

The first two formal meetings of the Task Force were held in Bethesda, MD, on May 19, 2004 and in St. Louis, MO, on September 22, 2004. These meetings were held in conjunction with the XI International IUPAC Symposium on Mycotoxins and Phycotoxins and the 118th AOAC INTERNATIONAL Annual Meeting and Exposition, respectively. The Bethesda meeting served to introduce members of the group to the AOAC Community/Task Force model and to discuss objectives, concerns, general workings, and communications. The meeting concluded on an encouraging note, with a commitment from AOAC to help provide financial resources for the review of nonproprietary methods deemed high priority by the Task Force. This development was seen as an important step toward reaching methods validation objectives.

The terms of reference for the Task Force were approved by the AOAC Board of Directors in late June, 2004. They described the Task Force membership as composed of voting and nonvoting members, with the voting members consisting of 13 members (12 plus the Chair). Voting members comprise of a balance of government regulators, academics, and

industry members. No single agency has more than 2 voting members. Task Force members serve as experts in the field and agree to identify other experts; recommend individuals who can serve on the Task Force and as Chair; develop and prioritize a list of marine and freshwater toxins that need validated methods; assist in identifying existing methods for validation through AOAC validation programs; and recommend to the AOAC INTERNATIONAL Board of Directors policies and procedures necessary to accomplish the mission of the Task Force. They endeavor to actively support the work of the Task Force through garnering of sources of funding (except where prohibited by employer); identifying potential participating laboratories, sample identification and acquisition; and increasing program awareness among stakeholders. They assist AOAC in the identification of study directors and in the development of quality measurement tools by participating in the validation of methods and by identifying venues for members of the Task Group or the community to gather and assist with meeting content.

Prior to the September 2004, AOAC Annual Meeting, the Task Force approved a set of Analytical Methods Selection Criteria, which are critical to the mission of the Task Force. They can be found, along with the Terms of Reference, roster of members, and other information, on the Task Force Web site at http://www.aoac.org/marine_toxins/task_force.htm.

The September 22, 2004 Task Force meeting in St. Louis included discussion of 2 interlaboratory studies, a proprietary kit for domoic acid by enzyme-linked immunosorbent assay (ELISA; Biosense Labs AS, Bergen, Norway) and also a nonproprietary liquid chromatography (LC) method for paralytic shellfish poisoning (PSP) toxins by precolumn oxidation (James F. Lawrence, Health Canada). These 2 methods were recommended by the Task Force for review by AOAC in September 2004. The group also discussed future priority directions, aspects of interlaboratory studies and official methods of analysis, other methods validation issues, future meetings, and funding.

In addition to the Task Force meeting, 2 subgroup meetings were held. One subgroup addressed strategies to replace the mouse bioassay for brevetoxins with alternative modern methods based on ELISA or LC/mass spectrometry (MS). Brevetoxin metabolites, toxicity issues, and extraction conditions as well as future field studies were addressed in detail. The receptor binding assay (RBA)/saxitoxins subgroup addressed several aspects of the methodology, radiolabeled saxitoxin, and comparisons of mouse bioassay and RBA response. Both subgroups were productive and were seen as very useful by the participants. Task Force attendees generally agreed that subgroups are the most effective means of progressing towards validation of new methods and of

ensuring thorough discussions of methods under consideration.

By the time of their next meeting (April 2005) at the "Marine and Freshwater Toxins Analysis: 1st Joint Symposium and AOAC Task Force Meeting" in Baiona, Spain, the Task Force will have several well developed new subgroups in the areas of okadaic acid and dinophysins toxins, yessotoxins, domoic acids, and ciguatoxins. Some of the subgroups will hold face-to-face meetings in Spain and others will meet at future symposia or joint meetings. It is likely that training sessions will be associated with multiple Task Force meetings planned for 2005. Details on these meetings can be found on the Task Force Web site. Although the Task Force has experienced rapid growth, the addition of new members to the group, especially industry and government stakeholders, is encouraged.

Task Force member Michael Quilliam, NRC Canada, provided the information given below on a joint CODEX group of special relevance to the new Task Force. This group met in late September 2004. For more information, see <http://www.who.int/foodsafety/chem/meetings/biotoxin/en/>.

Joint FAO/IOC/WHO Expert Workshop on Biotoxins in Molluscan Bivalves

At its 25th session, the Codex Committee on Fish and Fishery Products (CCFFP) asked FAO and WHO to provide scientific advice on biotoxins in conjunction with its work on Proposed Draft Standards for Live and Processed Bivalve Mollusks. The CCFFP, at its 26th session, suggested the following specific areas to be covered through this advice: Provide scientific advice to the CCFFP to enable establishment of maximum levels in shellfish for shellfish toxins; guidance on methods of analysis for each toxin group, monitoring of biotoxin-forming phytoplankton and bivalve mollusks (including sampling methodology); and information on geographical distribution of biotoxin-forming marine phytoplankton.

To implement this program of work, FAO, IOC, and WHO are convening an ad hoc expert consultation on specific shellfish toxins in bivalve mollusks to evaluate the available information (completed national risk assessments, relevant epidemiological data, prevalence data, etc.) on risk assessment. The ad hoc drafting groups have been established to examine the available relevant information and prepare technical documents.

A number of experts in the field were invited to the first working group meeting, which was hosted by the Food Safety Authority of Ireland in Dublin, Ireland, on March 22–24, 2004. The purpose of the first meeting was to bring together the coordinators of the 3 drafting groups in order to plan and begin the work on each subject. The objectives of the meeting were to define the specific scope, approaches and subjects in each section, and develop outlines for the papers to be prepared; suggest authors (drafting group members) for each subject; review the information currently available for this work and identify the additional information requirements and

the potential sources of this information; to discuss and finalize the division of labor among the members of the drafting groups and begin the elaboration of the papers; identify specific ways in which FAO and WHO can assist the drafting groups in their work; and provide guidance and assistance to authors in the preparation of the working papers and to FAO/IOC/WHO in the conduct of this project and the final the Consultation, planned for Norway in November 2004. The outcome of the Workshop will be used in an expert consultation in Oslo in September 2004. The outcome of the process will then be made available for Member States and the Codex Alimentarius Commission.

Update from General Referee/Task Force Chair: The above information will likely be released by the time of publication of this report. It will be made available, if permissible, via links from the Web site of the Marine and Freshwater Toxins Task Force.

New Topic Advisor appointments have been made in the areas of Azaspiracids by LC/MS, Ciguatoxins by LC/MS, Domoic Acid by ELISA, Electrophysiological Methods for Saxitoxins, Immunochemical Methods for Brevetoxins, LC/MS Detection of Marine Toxins, and Tetrodotoxins.

Selected Study Director Reports

Amnesic Shellfish Poisoning Toxins.—Study Director Michael A. Quilliam, National Research Council of Canada, Institute for Marine Biosciences, 1411 Oxford St, Halifax, Nova Scotia, B3H3Z1, Canada, Tel: 902-426-9736, Fax: 902-426-9413, E-mail: michael.quilliam@nrc.ca. The Study Director attended a QUASIMEME workshop in Galway, Ireland, that reviewed laboratory proficiency studies that were conducted on analytical methods for domoic acid. Study participants used a variety of extraction, cleanup, and detection methods. The latter included LC/UV, LC/MS, and ELISA. Scallop tissues were used as the test samples. Overall, the results showed very good performance between laboratories and methods, with z-scores between -2 and +2 for the majority of reported data. Further studies on different matrixes are scheduled, and laboratories interested in participating may sign up at the following Web site: <http://www.quasimeme.marlab.ac.uk/>. The Study Director will join a subgroup of the Presidential Task Force on Marine and Freshwater Toxins to review methodology options, including existing interlaboratory studies for the LC and LC/MS methods for domoic acid in shellfish. Based on the findings of this subgroup, methodology will be submitted to the Task Force for review and prioritization.

Azaspiracids.—Topic Advisor Ambrose Furey, PROTEOBIO, Mass Spectrometry Centre for Proteomics and Biotoin Research and Safefood Biotoin Research Network Co-ordinator (Food Safety Promotion Board), Cork Institute of Technology, Department of Chemistry, Bishopstown, Cork, Co. Cork, Ireland, Tel: 353-21-4326701, Fax: 353-21-4345191, E-mail: afurey@cit.ie. Furey reports that azaspiracids continue to be the target of intense research. Azaspiracid poisoning (AZP) is a recently discovered toxic syndrome,

which was identified following severe gastrointestinal illness from the consumption of contaminated mussels (*Mytilus edulis*). The first recorded human intoxications occurred in the Netherlands in 1995 following the consumption of mussels (*Mytilus edulis*) imported from Ireland. The symptoms included nausea, vomiting, severe diarrhea, and stomach cramps and although these symptoms were typical of diarrhetic shellfish poisoning (DSP), only trace levels of the toxins, okadaic acid (OA) and dinophysistoxin-2, were detected. Azaspiracids have fused polyether rings in common with other marine toxins, such as dinophysistoxins, yessotoxins, brevetoxins and ciguatoxins. However, a number of unique structural features characterize azaspiracids, including a trioxdispiroketal system fused to a tetrahydrofuran moiety (ABCD ring system), an azaspiro ring fused to a 2,9-dioxabicyclo(3.3.1)nonane (FGHI ring system), a 6-membered hemiketal bridge (E-ring system), a cyclic amine (I-ring), and a terminal carboxylic acid moiety (1–3).

Owing to the unique nature of azaspiracid-1, many attempts have been made to synthesize parts of this molecule (4–13). Buszek et al. (14) accomplished a complete synthesis of azaspiracid-1.

In the Advisor's laboratory, extensive LC/MS/MS studies were conducted to structurally elucidate 10 azaspiracid compounds by determining their fragmentation pathways (15, 16). Structural assignments of the azaspiracids were confirmed using high mass accuracy data generated from a hybrid quadrupole time-of-flight (QqTOF) MS (17). A powerful quantitative LC/MS³ quadrupole ion trap method was developed (18–20), and several other LC/MS methods were reported (21–25). Azaspiracid toxins have been shown to accumulate in bivalve mollusks (26–28), including mussels, oysters, scallops, clams, and cockles, that feed on the toxic microalgae *Protoperdinium crassipes*, a species previously considered to be toxicologically benign (29). Although first identified in shellfish from Ireland, azaspiracid contamination of several types of bivalve shellfish species has now been confirmed throughout the western coastline of Europe (United Kingdom, Norway, France, and Spain; 30, 31). Toxicological studies have indicated that azaspiracids can induce widespread organ damage in mice and that they are probably more dangerous than previously known classes of shellfish toxins (32–34). The exclusive reliance on live animal bioassays to monitor azaspiracids in shellfish failed to prevent human intoxications. This was a consequence of the poor detectability of the assay and the fact that azaspiracids are not exclusively found in the shellfish digestive glands, which are exclusively used for toxin testing (26). Detailed solid-phase extraction (SPE) studies have also been conducted in the Advisor's laboratory to clean up shellfish samples to enable rapid monitoring of azaspiracid toxins by LC/MS (35).

A European Union (EU) limit for bivalve mollusks of 0.16 g/g for the combined levels of AZA1-AZA3 in total tissues has been proposed to be determined by LC/MS methods (36). However, implementation of these new regulations has been hindered in most countries by the lack of availability of AZA standards that are necessary for

implementing LC/MS analysis. Efforts are currently being made to isolate purified azaspiracids from mussels and toxic algae to provide standard materials, which will allow for shellfish testing regimens to be implemented. Update from General Referee: Since the time of writing of this report, EU has formally set the limit at 0.16 g/g for the combined levels of AZA1-AZA3 in total tissues and mouse assay is specified as the reference method.

Bioassays for Phycotoxins.—Topic Advisor Donald J.A. Richard, New Brunswick, E1C 4L8, Canada, Tel: 506-851-2683, alternate: 506-855-4824, E-mail: donr@nb.sympatico.ca, alternate: RichardDJ@inspection.gc.ca. The HABTech 2003 Workshop (37) was held November 26–30, 2003, in Nelson, New Zealand. The workshop was the culmination of an international, multidisciplinary effort to resolve constraints to the international trade of fishery products caused by naturally occurring toxins. Part of that workshop consisted of 2 group discussions on pertinent topics: “What is the future of the mouse in monitoring and regulations?” and “What are the risks and benefits of shifting to new technologies for monitoring? How can we speed up regulatory acceptance of new methods?” The discussion differentiated the AOAC method for PSP toxins from the assorted mouse assays in use for lipophilic toxins. None of the latter has been properly validated and there are considerable variations between procedures used in various laboratories. Many reports of false positives and false negatives in such assays have led to a strong demand for replacement methods such as LC/MS. Although it was recognized that the PSP bioassay method has proven itself over the last 60+ years, there was a consensus that a long-term goal should be to eliminate the use of animals in shellfish testing. However, because of the state of technology and other constraints, a more realistic short- to medium-term objective is simply to reduce the number of animals. Regulators are usually somewhat apprehensive about changing something that has protected the public, essentially without fail, for several decades. Nevertheless, the growing number of additional marine toxins, and analogues, has increased the pressure to reduce the cost of all testing, including the PSP mouse bioassay. One of the workshop presentations (38) reported on some quality control aspects of the AOAC PSP method, including the variability of the method when different but acceptable dilution levels and the ddY mouse strain are used. The same variability problem had previously been reported in CD-1 mice (39). Different degrees of toxin conversion (especially of the gonyautoxin GTX2/3 and GTX1/4 groups) were also noted, even when the extraction specifications of the method were followed.

Last year's report communicated, in detail, a proposed plan for revising Method **959.08** (Paralytic Shellfish Poison, Biological Method). This plan will be submitted to the Presidential Task Force on Marine and Freshwater Toxins in the near future. Continue study.

Capillary Electrophoretic Methods for Marine Toxins.—Topic Advisor Ana Gago-Martinez, Universidad de Vigo, Dpto. Química Analítica y Alimentaria, Facultad de Ciencias, Campus Universitario de Vigo 36200-Vigo-E,

Spain, Tel: 34 986 812284, Fax: 34 986 812382, E-mail: anagago@uvigo.es. Gago-Martinez reports (40–50) that previous studies on the application of high-performance capillary electrophoresis (HPCE) to the analysis of algal toxins showed the potential of this technique as an alternative to the conventional and widely used HPLC methods, especially because of its simplicity and efficiency. In addition, smaller amounts of samples are required for the narrow capillaries used and the sample volume scaling laws (sample volume decreases with the square of capillary diameter). This advantage is significant because of the limited amount of standards usually available for these particular applications. Nevertheless, the same scaling is also responsible for the main disadvantage of this technique: low detectability with those same small sample volumes. To overcome this problem the most recent advances on HPCE have been focused in the enhancement of detectability, and with this aim novel strategies based on stacking, which generally consists of increasing analyte mass load within the sample zone during the electromigration process, are used.

Different stacking modes have been applied with this aim and increased sensitivities were found in applications to amnesic shellfish poisoning (ASP) toxins and yessotoxins (YTX). The detectability achieved by CE/UVDAD under these conditions was similar to that obtained by HPLC methods with UV (and even fluorescence detection in the case of YTX). Preliminary studies on the application of capillary electrochromatography to ASP toxins showed the potential of this technique for this application. Further studies have focused on the optimization of the chromatographic and electrophoretic conditions for the analysis of ASP toxins. In these studies the detectability, selectivity, and efficiency of this technique have been considerably increased. As a result this technique has been successfully applied to the determination of ASP toxins present in complex matrixes naturally contaminated with ASP. The main drawback of this technique is attributed to the lack of robustness which can be explained by the limited stationary phases commercially available for applications involving complex matrixes.

Cell Bioassays for Phycotoxins.—Topic Advisor Ronald Manger, Fred Hutchinson Cancer Research Center, PO Box 19024, Seattle, WA 98109-1024, Tel: 206-667-5838, Fax: 206-667-4182, E-mail: rmanger@fhcrc.org. Manger reports continued examination of their previously developed MTT (tetrazolium dye substrate for colorimetric viability via mitochondrial dehydrogenase) cell-based assays for toxins active at the voltage gated sodium channel (51–53) as well as alternative variations based upon flow cytometry and voltage sensitive dyes. The MTT cell bioassays provide a relatively robust in vitro method for a variety of toxins that either inhibit or enhance the activity at voltage gated sodium channels. Although the MTT-based assays are based on cell death, voltage sensitive dyes are used to follow toxin-modulated changes in nerve cell membrane potential. Significant advancements in cell based assays for marine toxins using voltage sensitive dyes combined with fluorescence detection (54–56) have led us to explore the use of these dyes

coupled with flow cytometry. Our preliminary studies indicate that flow cytometry offers a distinct advantage in minimizing the assay time from hours to minutes in a manner similar to that reported previously by other laboratories (54–56). The major disadvantage in conventional flow cytometry is sample throughput. We are presently examining coupling of the 96-well plate format with flow cytometry to enhance sample throughput. The relative detectability to sodium channel enhancing toxins appears highest in the MTT format (about 1.25 pM for CTX3C, *see* citation 2). However, the recently reported fluorimetric microplate assay developed by Louzao et al. (56) shows excellent detectability (about 10 pM for CTX3C) in preliminary work using toxin standards. Although unproven at the time of writing for the analysis of ciguatoxic fish tissues and other biological samples, this procedure (56) shows good potential for high sample throughput when large numbers of samples are analyzed. In general, all of the above methods offer the potential to detect these toxins at or below the concentrations that would present a risk to the consumer, and are generally available to most cell culture laboratories. Other assays using voltage sensitive dyes, have been described in this case with mouse brain neurosynaptosomes, but these require sacrifice of animals rather than the use of secondary cultures (57).

Ciguatoxins by LC/MS.—Topic Advisor Richard J. Lewis, University of Queensland, St. Lucia, 4072, Qld, Australia, Tel: 617-3346-2984, Fax: 617-3346-2101 E-mail: r.lewis@mailbox.uq.edu.au. Ciguatoxins (CTX) are a family of heat-stable, lipid-soluble, highly oxygenated, cyclic polyether molecules produced by *Gambierdiscus* spp. The CTX have a structural framework reminiscent of the brevetoxins (PbTx-1–10) produced by the marine dinoflagellate *Gymnodinium breve* (= *Ptychodiscus brevis*). Modern nuclear magnetic resonance (NMR) has proved an invaluable technique for the elucidation of structures from the microgram quantities of ciguatoxin typically isolated from fish and *G. toxicus* (58–64). More recently Yasumoto et al. (65) described the use of high energy MS/MS methods to solve the structures of 16 CTX from samples containing as little as a few micrograms of mixed CTX. Many additional minor toxins have been detected using LC coupled to MS (66–69). Several smaller CTX that likely cause rapid deaths in mice (fast acting toxins) were identified by LC/MS (70). LC/MS has also been used recently to monitor metabolism of brevetoxins in shellfish (71). Further studies are required to develop more rapid and efficient extraction procedures for CTX sample preparation to complement developments in LC/MS detectability through new generation MS instrumentation.

Diarrhetic Shellfish Poisoning Toxin, Assay Methods.—Topic Advisor J. Marc Fremy, Agence Française de Sécurité Sanitaire des Aliments, Laboratoire d'Etude et de Recherche sur l'Hygiène et la Qualité des Aliments, 10 Rue P. Curie, Maisons Alfort, F-94704, France, Tel: 33-1-4977-2751, Fax: 33-1-4977-2695, E-mail: j.fremy@paris.afssa.fr. In last year's report, the use of polyclonal antibodies specific to yessotoxin (YTX) and its analogues to develop an ELISA test

for YTX in shellfish was mentioned (72). Based on this work, a commercial immunoassay YTX ELISA was recently developed by Biosense Laboratories (Bergen, Norway) able to detect a wide range of the known YTX analogues such as YTX, 45-OHYTX, homoYTX, and 45-OH-homoYTX. As the limit of quantification for sample extract is estimated to be around 80 pg/mL, the manufacturer has defined that the working range is starting from 0.16 mg YTX/kg shellfish (H. Kleivdal, Biosense Laboratories, Bergen, Norway, personal communication, 2004). The PP2A competitive binding assay described by Serres et al in (73) has been redeveloped into a nonradioactive assay with a user-friendly format: promising results have been presented using fluoroprobe as toxin tracer. The lack of commercial availability of stable PP2A has been overcome by using PP2A-like protein isolated from blue mussel (74). In the same way, a new investigation on the interaction of OA with alkaline phosphatase (ALP) has been performed (75) in view of its protein phosphatase inhibition activity. Kinetic analysis of ALP from *Escherichia coli*, human placental and calf intestinal ALP has shown that OA acts as a noncompetitive inhibitor of ALP. The bacterial enzyme displays a higher affinity for OA (Ki 360nM) than the eukaryotic proteins (human placental ALP, Ki 2.05 M; calf intestinal ALP, Ki 3.15 M).

Domoic Acid by ELISA.—Study Director Hans Kleivdal, Biosense Laboratories AS, HIB-Thormøhlensgate 55, N-5008 Bergen, Norway, Tel: 47-5554 3967, Fax: 47-5554 3771, E-mail: hans.kleivdal@biosense.com. Since the first occurrence of amnesic shellfish poisoning following human consumption of contaminated mussels (76), several groups have described the development of immunochemical assays for domoic acid (DA) to complement the reference method using HPLC-UV. Most of these assays were evaluated for the determination of DA in shellfish (77–80), while others reported the assay performance on other matrixes like mammalian body fluids (81, 82). Garthwaite and co-workers at AgResearch Toxinology group, Hamilton, New Zealand, described the development of an indirect competitive ELISA based on polyclonal antibodies from sheep (77). This assay was shown to be suitable for the determination of DA in several shellfish species and for direct analysis of seawater and plankton samples. Since then, Biosense Laboratories has collaborated with the AgResearch Toxinology group to reformat the research-grade assay into a direct ELISA format suitable for commercialization. The optimized ASP ELISA kit was subjected to a single laboratory validation describing the limit of detection (LOD) at 3 µg DA/kg shellfish, a repeatability precision at 9.4 and 106% recovery (83). A collaborative validation study, comprising 16 laboratories from 10 countries, was organized to determine the interlaboratory assay performance parameters of the ASP ELISA, and to compare with HPLC-UV and LC/MS (84). A total of 256 samples with different DA levels spiked in different shellfish species were analyzed. The ASP ELISA showed very good correlation spike values (r^2 0.99, slope 1.01); the within-laboratory repeatability averaged 15%, and the interlaboratory reproducibility averaged 23%

(HORRAT 1.7). The collaborative study report has recently been submitted to the AOAC Presidential Task Force on Marine and Freshwater Toxins, with the intention to pursue the AOAC OMA program. (GR's update: This method now approved by Task Force for OMA review.)

Electrophysiological Methods for Saxitoxins.—Topic Advisor Benjamin A. Suárez-Isla, Laboratory of Marine Toxins, Program of Physiology and Biophysics, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Av. Independencia 1027, PO Box 70005, Santiago 6530499, Chile, Tel: 562-777-6886, Fax: 562-732-9668, E-mail: bsuarez@med.uchile.cl. The molecular target of saxitoxins, tetrodotoxin, brevetoxins, and ciguatoxins is the voltage dependent Na channel present in neurons, muscle and secretory cells. The electrical excitability of cells in all chordates and many invertebrate species is based on the voltage-dependent entry of Na⁺ ions into cells through Na channels (85, 86). The entry of positively charged ions rapidly shifts the membrane potential from negative values (–70 to –90 mV) to positive values (> +20 mV) within milliseconds. The delayed exit of K⁺ ions brings back the membrane potential to resting values. This wave of depolarization/repolarization is called the action potential. Blockade by PSP toxins [saxitoxins (STX)] of Na⁺ entry abolishes cell excitability and conduction of the nerve impulse. Although techniques that accurately measure sodium currents in several biological preparations were developed in 1950 by Hodgkin and Huxley (87), and have been used extensively since then to unravel the structural basis of sodium channel function, it is still an open problem how to reduce into practice a simple design that uses measurement of sodium currents as the end point signal that reports PSP effects. Ideally, it would be desirable to measure directly the biotoxicity of PSP extracts as a direct block of sodium currents. Extensive efforts have been made to design in vitro methods alternative to the mouse bioassay for PSP (AOAC Method 959.08) that do not use live organisms to be used in regulatory work. These include cell culture bioassays, immunoassays, receptor-based assays (reviewed in ref. 88) and electrophysiological methods that use isolated axons from squid and frogs or cultured cells.

An interesting approach was reported by Cheun et al. in 1998 (89). These authors developed a tissue biosensor for STX and tetrodotoxin (TTX) that consisted of a Na⁺ electrode covered with a frog bladder membrane integrated within a flow cell. Active Na transport that takes place from the internal to the external face was found to be TTX and STX sensitive. This procedure allowed the detection of PSP toxins well below the detection limit of the mouse bioassay but no quality control data was provided in the paper.

Velez et al. (90) reported a very sensitive and functional assay based on the electrophysiological impact (Na channel blockade) of STX on mammalian cells. The assay is directly related to the mechanism of mammalian toxicity and is based on cultured, patch-clamped whole cells (HEK 293, rat skeletal muscle cells) expressing the voltage gated Na channel (91). The procedure provides a far more sensitive (3 orders of

magnitude) alternative to the mouse bioassay (AOAC Method 959.08). In the examination of shellfish extracts the method correlated very well with mouse bioassay ($R = 0.95$, $n = 30$). Current recordings could be recorded from a single patch for 0.5–2 h, and variations between cells do not compromise assay precision (<9% standard error). The assay uses cultured cells, thereby avoiding the ethical problems associated with the mouse bioassay. The electrophysiological method does not require the use of radioactive STX.

Recently, the electrophysiological assay for STX was applied to determine the LOD of the mouse bioassay. Naturally intoxicated shellfish samples obtained during a recent PSP outbreak in the island of Chiloé (southern Chile; January–May 2002) were used ($n = 157$). All samples >60 g STX/100 g were detected by mouse bioassay. In contrast, samples between 40 and 60 g STX eq/100 g had a chance of 80.0% of being detected, a percent that decreased to only 52.6% for the range of 30–40 g/100 g, and to 17.2% for 20–30 g/100 g. This suggested that the operational detection limit for the mouse bioassay should be set at 40 g STX eq/100 g. It was recommended that for regulatory decisions and in view of the uncertainty, values <40 g/100 g as determined by mouse bioassay, should be reported as “below the detection limit” (92).

The Topic Advisor will explore the feasibility of using automated patch clamp instruments for multiple samples.

Immunochemical Methods for Brevetoxins.—Topic Advisor Jerome Naar, Center for Marine Science, University of North Carolina at Wilmington, 5600 Marvin K. Moss Ln, Wilmington, NC 28409, Tel: 910-962-2367, Fax: 910-962-2410, E-mail: naarj@uncw.edu. In recent years new polyclonal (93–95) and monoclonal (96) antibodies raised against brevetoxins were produced and integrated in various immunoassay formats including radioimmunoassay (RIA; 93), enzymatic assay (competitive ELISA; 97), and electrochemical immunoassay (95). An immunohistochemistry technique was also developed by Bossart et al. (98) to assess and quantify brevetoxin exposure in marine mammals. Although these assays have been actively used in many studies, we can distinguish 3 principal practical applications.

(1) *Monitoring tools.*—For ethical, safety, accuracy and precision reasons, replacing the regulatory mouse bioassay for brevetoxin monitoring in shellfish has long been a goal of the scientific and regulatory community. Dickey et al. (99) reported the results of a multilaboratory study of 5 methods including an immunoassay as potential replacements to the mouse bioassay. Aside from the competitive ELISA (99), methods included one instrumental assay (HPLC/MS) and 3 activity-specific bioassays (N2A neuroblastoma cytotoxicity assay and 2 formats of the sodium channel receptor binding assay). Among these methods, the competitive ELISA correlated the most favorably with the actual mouse bioassay and appears to be a good candidate to replace it. However, replacing an assay based on the overall toxicity of an extract (mouse bioassay) with an assay based on the determination of an overall concentration of toxins (competitive ELISA, receptor binding assay) is not

straightforward and will require additional work, especially with regard to the issue of brevetoxin metabolites.

Although implicated by Poli et al. (100) in human intoxications, the toxicity of the different brevetoxin metabolites observed in shellfish is not totally established, and mice administered (orally and intraperitoneally) with high concentrations of a metabolite mixture did not develop signs of intoxication (101). Exact evaluation of the potency on mammals of brevetoxins metabolites is still hampered by the absence of standards of these compounds. Because of their higher polarity, the known brevetoxin metabolites are not extracted from shellfish by the regulatory protocol for shellfish monitoring. Thus, to date, they are not taken into account in the regulatory monitoring for shellfish bed re-openings. Whether to include brevetoxin metabolites in monitoring has direct implications on the protocol to be used and will also have important repercussions on the shellfish industry already jeopardized by the high frequency of red tides, especially in southwest Florida. Several extraction and purification protocols are under investigation to allow a quick and precise determination by competitive ELISA of brevetoxin and brevetoxin metabolites in shellfish.

(2) *Diagnostic tools of human and mammalian exposure.*—Because of the high affinity and specificity of antibodies, immunoassays are excellent tools to diagnose exposure to brevetoxins. Woofter et al. (93) described the use of a radioimmunoassay to measure brevetoxin levels of blood collection cards after exposure in mice. They concluded that the technique presents a better detectability than the receptor binding assay and allows detection of toxins in mice blood up to 2 days post exposure. In 1998, Bossart et al. (98) demonstrated the implication of aerosolized brevetoxins in the manatee mortality of 1996 using an immunohistochemistry staining technique on lung, nose, and throat tissues of dead animals. The competitive ELISA has also been used in several studies (102, 103) to quantify human occupational and recreational exposure to aerosolized brevetoxins during red tide at the beach. More recently, this method was used to implicate brevetoxin in the March–April 2004 dolphin mortality event in St. Joseph Bay, FL. During this study, brevetoxins were diagnosed in numerous dolphin tissues and fluids including urine, blood, stomach contents, kidney, lungs, muscle, cerebellum, and liver (104).

(3) *Research tools.*—In the last few years, immunoassays have been used extensively to study the transfer and metabolism of brevetoxins in food webs.

Poli et al. (100) described the use of a brevetoxin immunoaffinity column to purify brevetoxin and brevetoxin metabolites in urine from people intoxicated after consumption of shellfish contaminated during red tides, while Naar et al. (101) used an HPLC-ELISA technique to identify metabolites in shellfish and the role of PbTx-2 as precursor for their production. Very recently, using the competitive ELISA, brevetoxins were demonstrated to accumulate in fish despite their known ichthyotoxicity (105), and Flewelling et al. (106) used the same technique to demonstrate involvement of brevetoxin associated with seagrass in a large scale manatee

mortality. Competitive ELISA was also used by Bourdelais et al. (107) to identify raphidophytes as a source of brevetoxin in the United States, and by Weidner et al. (108) to show very limited variation in brevetoxin accumulation between individual shellfish from the same bed during red tides.

Immunochemical Methods for Seafood Toxins.—Topic Advisor Joanne F. Jellett, Jellett Rapid Testing, 4654 Route 3, Chester Basin, NS, B0J 1K0 Canada, Tel: 902-275-5104, Fax: 902-275-2242, E-mail: jjellett@ns.sympatico.ca, jjellett@jellett.ca, Web site: www.jellett.ca. In January 2004, the U.S. Food and Drug Administration (FDA) approved the recommendations of the Interstate Shellfish Sanitation Committee (ISSC) to incorporate the Jellett Rapid Test for PSP into the National Shellfish Sanitation Program as a tool for use in the control of PSP in shellfish. The test was accepted for use, with the restriction that they use the currently approved procedure for PSP toxin extraction, although the ISSC expressed interest and Jellett Rapid Testing (the Company) is proceeding to validate its novel cold extraction method known as the Jellett Rapid Extraction. The second restriction from the FDA applied to the exclusive use of the Jellett Rapid Test to screen for shellfish toxins, in which case precautionary closures must be verified by a mouse bioassay within 48 h of the closure based on the Jellett Rapid Test. A negative Jellett Rapid Test result can be substituted for a negative mouse bioassay result to maintain an area in an open status, without any corroborative test with the mouse bioassay. The exact recommendations of the ISSC and the FDA can be seen on the Web site (www.jellett.ca).

As a result of this approval, most of the U.S. states that are affected by PSP are commencing the use of the Rapid Test in their shellfish monitoring programs. Two U.S. states also are evaluating the Rapid Test for ASP for use in phytoplankton monitoring. The Rapid Test for PSP and ASP are both being used with the Rapid Extraction method (which can be used for both tests) in parallel with regulatory methods over a broad area along the west coast of North America. This study involves several native tribes and regulatory agencies in both the United States and Canada. This study is part of the validation of the Rapid Extraction method, which will allow more community-based monitoring, or use of the test in remote and rugged areas.

Because it is believed that different geographic areas have predominant PSP toxin profiles, initial evaluations are proceeding in many countries to look at false-positive rates of the Rapid Test for PSP, which varied between 2 and 15% in the initial validation trials (109). In the validation trials, HPLC showed that the majority of the false-positive samples resulted from the presence of significant amounts of B and C analogs in the sample extract. Unlike the mouse bioassay, which does not recognize these analogs as toxic, the Rapid Test for PSP detects these analogs in nM concentrations in the same range as STX, NEO, GTXs and the other members of this toxin family that are recognized by the mouse bioassay as toxic (110). The B and C toxins are the main forms found in phytoplankton and, therefore, blooms developing offshore and coming onshore are more likely to cause false-positive

Rapid Test results, as the shellfish will contain many undigested toxic cells in their guts. Blooms developing in situ are less likely to cause false-positive Rapid Test results, as shellfish have more time to digest the algal cells, at which time the B and C analogs are converted to the more toxic analogs that are detected in the mouse bioassay. Most of the false-positive Rapid Test results were shown by HPLC in the validation trials to contain between 20 and 40 $\mu\text{g}/100\text{ g}$ PSP toxin, and with many having B and C analogues as the majority of the toxin profile. This has made the Rapid Test for PSP useful in both phytoplankton monitoring and in early bloom detection.

Canada is evaluating the Rapid Test for PSP for use in its shellfish monitoring program across the country. New Zealand has approved the use of the Rapid Test for shellfish monitoring there in agencies and institutes that have performed a validation study involving about 250 samples. One major Southeast Asian country is using the Rapid Test for PSP in their commercial shellfish monitoring, and the Company is developing local distribution networks to assist in the distribution of its tests in this area. The UK Food Standards Agency has completed the validation of the Rapid Test for PSP (111–113) in its Scottish Laboratory, and has proceeded to implement it as of spring 2004. In addition, they have extended the validation in additional UK laboratories with the Scottish Laboratory, and are working on the use of the Rapid Test for ASP in phytoplankton monitoring for ASP. There are 2 other European countries now using the Rapid Test for PSP in their shellfish monitoring.

The planned validation study of the Rapid Test for ASP with the NRCC did not proceed, and the Asian Pacific Economic Cooperation (APEC) funds earmarked for this study were used to assist in the validation of instrumental methods instead.

The Company has developed some low detection limit versions of their Rapid Tests for ASP and PSP which are being used for phytoplankton monitoring in many areas, as it has been shown to be effective in this application (114, 115), and for use with marine mammal urine analysis by the National Oceanographic and Atmospheric Administration (NOAA) laboratory with Frances Van Dolah and for some specialized research applications.

Jellett Rapid Testing personnel attended the HABTech04 meeting in Nelson, New Zealand, in November 2003. Two papers were published in the proceedings, one of which is about the commercialization of technology (116). The second paper showed the lowest detection limit (LDL) of the Rapid Test for PSP in extracts containing pure toxins from the NRCC, which demonstrated the ability of the tests to detect the toxins even in extracts containing only NEO and GTX1/4, which are the analogues most weakly detected by the mixture of antibodies in the Rapid Test (117). Company personnel also recently attended the ICMSS04 in Galway, Ireland, and will be publishing a paper in these proceedings. Other articles from the primary literature about the Jellett Rapid Tests are shown below (118–120).

LC/MS Detection of Marine Toxins.—Topic Advisor Patrick Holland, Cawthron Institute, Private Bag 2, Nelson, New Zealand, Tel: 643-548-2319, Fax: 643-546-9464, E-mail: patrick.holland@cawthron.org.nz. Holland reports that LC/MS has been applied to routine shellfish testing as part of the New Zealand Biotoxin Management Programme. The following toxins are routinely analyzed: domoic acid (DA), gymnodimine (GYM), spirolides (A, D and des methyl C), pectenotixins (2, 1, 11 and 2 seco acid), okadaic acid (and ester forms), DTX-1 and 2 (and ester forms), and yessotoxin and derivatives (45 hydroxy, homo, 45 homo and carboxy). The method has been fully validated in-house (121, 122) and preliminary interlaboratory trials are complete (123, 124). Biotoxin LC/MS results are available within 2 h, no mice are used, and solvent use has been greatly reduced; the LODs are 10–1000 times below action limits, and the use of highly selective multiple reaction monitoring (MRM) ensures that toxins are reliably and accurately detected (125, 126).

An LC/MS/MS method for ASP toxins has also been validated (127). Routine application of the method to shellfish samples has proven that isomeric forms of DA may be present and not detectable by HLC-UV (128).

Cawthron has also completed the validation of a marker compound LC/MS method for neurotoxic shellfish poisoning (NSP) toxins (129). As part of these studies it was proven that the chosen marker compound is present in all species of shellfish. Mouse bioassay (APHA ether) testing using local mussel (*Perna canaliculus*) suggests that application of the standard APHA method to non-oyster shellfish species at best causes standardization problems and at worst fails to detect NSP contamination (130). The application of the LC/MS marker method to areas where there is no historical bloom issue will be useful to screen out large numbers of negative samples and detect low levels of contamination, warranting further study or precautionary closure.

Associated with the development and validation of these new LC/MS methods are 2 crucial and related issues:

(1) *Reference materials.*—All methods for quantitative analysis of biotoxins such as LC/MS are dependent on external calibration. Cawthron has provided crude or partially purified toxins to collaborators (Institute for Molecular Biosciences, NRC, Halifax, Canada; AgResearch, Hamilton; Japan Food Research Laboratory, Tokyo, Japan) which has led to production of pure standards of gymnodimine, PTX2, PTX11, PTX2 seco acid, YTX, 45OH-YTX and carboxy-YTX. Some have now been certified and are available for purchase from IMB-NRC Canada (131).

(2) *Regulatory approval.*—The New Zealand Food Safety Authority has established a rigorous procedure for acceptance of new methods (132). This includes full method validation to the highest international standards and interlab studies where practical. Negotiations for international acceptance of

methods and data are also important, and gaining approval is a lengthy and time-consuming process. Progress with adoption of improved test methods for shellfish quality assurance will continue to be slow until there is a greater degree of harmonization of goals and procedures at both national and international levels. Update by General Referee: More widespread use of LC/MS for detecting marine toxins has followed the pioneering work at Cawthron and other laboratories such as in IMB-NRC Canada. In the European Union, a working group on LC/MS for determination of marine biotoxins has been created. Information on developments such as these will be provided on the Marine and Freshwater Toxins Task Force Web site http://www.aoac.org/marine_toxins/task_force.htm. Also, the General Referee and Patrick Holland acknowledge Paul McNabb, Cawthron Institute, for assistance provided with this TA report on LC/MS Detection of Marine Toxins.

Microcystins and Nodularins.—Topic Advisor Geoffrey A. Codd, School of Life Sciences, University of Dundee, Dundee DD1 4HN, Scotland, UK, Tel: 44-1382-344272, Fax: 44-1382-344275, E-mail: g.a.codd@dundee.ac.uk. Good progress has been made on aspects of the detection and analysis of hepatotoxic microcystins and nodularins. Further reports of the quantification of these toxins in natural and high amenity, controlled waters (drinking and recreational resources) have appeared, e.g., microcystins in German, Argentine, and Algerian lakes (133–135), and nodularin and microcystins in estuarine waters (136). Microcystins and nodularins have also been determined in complex matrixes, e.g., dead flamingo tissues (137) and flounder livers (138). Two new structural microcystin variants have been identified: [D-Asp³, D-Glu (OMe)⁶] microcystin-RR and Seco[D-Asp³] microcystin-RR (139). Linearized and truncated microcystins, with reduced toxicity, have been produced (140, 141). These retain partial inhibitory activity against protein phosphatases, indicating that such peptides, whether naturally occurring or produced during water treatment processes, require analysis. Progress on physicochemical methods includes reversed-phase (RP) LC/MS for microcystin analysis in cyanobacterial blooms under alkaline conditions, a characteristic of intense blooms caused by dissolved CO₂ depletion (142) and RP-LC-electrospray ionization (ESI)-MS for nodularins and microcystins (143). A multipurpose LC/MS method for cyanobacterial hepatotoxins and neurotoxins has been presented (144), although this does not give best performance with microcystins. Analysis of nodularins and related detoxicated conjugates has been described in fish livers. This is as an example of the increasing need for the analysis of aquatic food chain members which have been exposed to cyanobacterial toxins via ingestion (137, 138). Immunoaffinity columns, based on antibodies to microcystin, are finding increasing application,

e.g., for the recovery of microcystins and nodularin-R from surface waters (145, 146).

Molecular biology methods are being applied to the detection of potential microcystin- and nodularin-producing cyanobacteria in laboratory cultures and environmental samples. Genes necessary for microcystin and nodularin synthesis have been characterized, and DNA sequences within these are being used to detect potential microcystin- and nodularin-production in cyanobacterial cells (e.g., *Microcystis*, *Anabaena*, *Planktothrix*, *Nodularia*; 147, 148). Real-time PCR has recently been applied for the quantitative determination of microcystin synthase genes in cyanobacteria in lakes (149). Research is in progress in several laboratories to apply microarray technology (DNA and immuno-) for the analysis of microcystins and nodularins.

The availability and supply of certified quantitative analytical standards for these toxins is a continuing need. Awareness of the existence of national and international regulations regarding transportation and holding of these potent toxins is increasing.

Paralytic Shellfish Poisoning Toxins, Instrumental Methods.—Study Director James F. Lawrence, Health Canada, Bureau of Chemical Safety, Banting Building 2203G2, Ottawa, Ontario, K1A 0L2, Canada, Tel: 613-957-0973, Fax: 613-954-4674, E-mail: jim_lawrence@hc-sc.gc.ca. The Study Director completed and published a collaborative study (150) based on the prechromatographic oxidation LC method (151). The method was successfully able to identify and quantify STX, NeoSTX, GTX2,3, GTX 1,4, C1,2, C3,4, and dcSTX in scallops, mussels, clams, and oysters at concentrations well below the commonly accepted regulatory level of 0.8 g/kg saxitoxin equivalents. The study has been submitted to the AOAC Task Force on Marine and Freshwater Toxins for peer review. General Referee's update: This method now approved by Task Force for OMA review, and CEN (European Standardization Committee) has recently approved a standardized method (CEN method 14526) for the determination of STX and dcSTX, based on this method.

Receptor Assays for Phycotoxins.—Topic Advisor Frances Van Dolah, NOAA-National Ocean Service, Center for Coastal Environmental Health and Biomolecular Research, 219 Fort Johnson Rd, Charleston, SC 29412, Tel: 843-762-8529, Fax: 843-762-8700, E-mail: Fran.Vandolah@noaa.gov. Last year the receptor assay for PSP toxins suffered from the worldwide unavailability of tritiated STX. This year, American Radiolabeled Chemicals, in collaboration with FDA, International Atomic Energy Agency (IAEA), and NOAA, produced an exchange labeled tritiated STX equivalent to the product previously produced by Amersham. This product has proved to be stable for 6 months in an ongoing storage stability trial. This material is now available free of charge to U.S. laboratories interested in using the PSP receptor assay and is being distributed internationally by IAEA through a

Technical Cooperation Project. Following successful completion of informal intercalibration exercises this year, we hope to proceed to a formal collaborative trial of the PSP receptor assay in 2005.

Impediments to the use of the receptor assay for domoic acid also lifted this year. Glutamate decarboxylase (GAD), used to remove endogenous glutamate from samples in the method of Van Dolah et al. (152), became commercially unavailable during 2002–2003. NOAA (Doucette et al., unpublished) now purifies glutamate decarboxylase for use in the assay from a bacterial source. This material is available upon request. Additionally, Baugh et al. (153) developed an alternative method which replaces GAD with glutamate dehydrogenase for glutamate removal; this enzyme is not as effective at removing high levels of glutamate that can sometimes be found in shellfish, but the method works sufficiently well for seawater samples for which it was designed. Also this year, the cloned GluR6 glutamate receptor used in the Van Dolah et al. (152) method is now produced commercially by Cell Trends Inc., making this source of receptor readily available to interested laboratories.

The receptor assay for brevetoxin continues to be used as a research tool for determining brevetoxins in various tissue and seawater matrixes. The assay performed well a multiple method intercomparison coordinated by Dickey et al. (99). The receptor assay does not appear to respond to at least some metabolites of PbTx in shellfish and other animal tissues, as it under-reports relative to ELISA or radioimmunoassay (RIA) methods that recognize the polyether backbone. The implication of this for its use for regulatory purposes (and that of the ELISA and RIA) awaits toxicity measurements on purified metabolites.

Sample Pretreatment Methods for Marine Toxin Analysis.—Topic Advisor Ana Gago-Martinez. Gago-Martinez reports (154–163) that the development of alternatives for sensitive determination, confirmation or even structure elucidation of algal toxins has been considerably increased over the last few years. This has contributed to a better knowledge about the toxins involved in different kinds of toxic episodes in the aquatic environment. Nevertheless little attention has been paid to the improvement of sample pretreatment, a critical step and often a significant source of error in analytical protocols for determining analytes in complex biological matrixes. The sample pretreatment of such matrixes can be a tedious process which often compromises the reliability and efficiency of the analytical technique. Alternative approaches for extraction and cleanup are still required for faster and more efficient sample pretreatment. Techniques such as microwave-assisted extraction (MAE) and accelerated solvent extraction (ASE) have been applied for the extraction of algal toxins, especially PSP toxins. These efforts have allowed faster extractions and minimize the use of organic solvents, resulting in promising alternative extraction

procedures. Solid-phase microextraction has also been successfully applied for the extraction and cleanup of algal toxins such as microcystins from cyanobacteria and more recently to ASP toxins and anatoxins. Furthermore, SPE procedures based on immunoaffinity chromatography have also been applied for microcystin cleanup, and at present are also applied for ASP. These sample pretreatment alternatives will contribute to improved sample extraction and cleanup efficiencies. Better recoveries, the ability to preconcentrate the analyte, and increased detectability will be also achieved; selectivity is improved through efficient removal of interferences in the sample matrix.

Tetrodotoxins.—Topic Advisor Mari Yotsu-Yamashita, Tohoku University, Sendai, 981-8555, Japan, Tel/Fax: 81-22-717-8922, E-mail:myama@biochem.tohoku.ac.jp. We reported ESI-LC/MS of tetrodotoxins (TTXs) in 2001 (164), together with the improved chromatographic conditions for the post-column LC-fluorescent detection (LC-FLD) systems (165, 166) for better separation of the analogs. Because of structural variations, some analogs of TTX, such as 11-deoxyTTX and 5,6,11-trideoxyTTX, are difficult to detect by LC-FLD. In this case, LC/MS was a powerful method to identify such analogs. In our LC/MS method, the same column (Develosil C30 UG-5) and solvent containing heptafluorobutyrate as ion pair reagent in ammonium formate buffer were used as those for LC-FLD, to make it easy to compare the chromatograms between LC-FLD and LC/MS. This LC/MS method enabled us to identify 11 TTX analogs. In the selective ion monitoring (SIM) mode, the detection limit of TTX was 0.7 pmol (S/N = 2). We also investigated major fragmentation ions of 8 analogs of TTX, and proposed 2-aminohydroxyquinazoline skeleton as the structure for the origins of the intense fragment ions of MH⁺ of all TTXs examined. This characteristic fragmentation pattern was applied to LC/MS/MS in the single reaction monitoring (SRM) mode. The sample solution, prepared from the ovaries of *Fugu poecilonotus* by treatment with a charcoal column, was analyzed by this LC/MS in SIM and SRM. In SIM, 11-deoxyTTX and the minor analog, 5-deoxyTTX, were clearly detected with other 4 TTX analogs. In SRM, the major TTXs, TTX and 11-deoxyTTX, were detected. We also applied this LC/MS method to determine the toxin profile of TTXs contained in newts (167), and Pires et al. (168) identified 11-oxoTTX in the frog, *Brachycephalus ephippium* by using our LC/MS method. Regarding other LC/MS approaches, Horie et al. (169) reported a method for routine analysis of TTX in puffer fish in 2002. In this method, separation was performed on a Shodex Rspak NN-414 column with 20mM ammonium acetate–methanol (4 + 1, v/v) as the mobile phase. Extraction of TTX from puffer fish tissue was followed by cleanup on a Bond Elut C18 cartridge, and LC/MS analysis was conducted in SIM mode. By this method, the recoveries of TTX from puffer fish were >78%, and good

correlation between the LC/MS and bioassay was shown. Hayashida et al. (170) reported a column switching LC/MS/ESI method for detecting TTX and aconitum alkaloids in serum in 2003. In this system, the extraction column, Shodex Mspak CX-4A (with distilled water), and the analytical column, Shodex Rspak NN-414 (with 20mM ammonium acetate–acetonitrile, 9 + 1, v/v) were used. For this LC/MS method, 0.1 mL serum sample was directly injected into a precolumn. These methods should be useful for practical determinations of TTXs.

Trends in Marine and Freshwater Toxins

This report will close by briefly addressing trends and other developments not covered in the *Summary*. By following one example of an emerging class of toxins, the azaspiracids, one can observe how 3 very different areas, cytotoxicity assays, LC/MS, and production of certified reference materials, now play a key role in discovery and management of marine toxins.

Although mouse data were also important (171), some of the earliest publications addressing azaspiracids used the cytotoxicity approach (172–174) rather than mouse bioassay. As an alternative biological assay to mouse bioassays, cell bioassays offer the advantage of better detectability and reduced sample requirements, and thus lower mass requirements of limited authentic materials, as well as the advantage of providing observable effects at the cellular level. This report has already reviewed the analytical use of cell assays for detecting the sodium-channel active toxins. In addition to these and the azaspiracid examples cited above, the value of cultured cells has also been shown in their use to study or develop functional assays for a variety of other marine toxins, including the yessotoxins (175, 176). Cytotoxicity assays continue to be valuable tools in the study of new toxins (177), brevetoxin metabolites (178), and in forensic investigations of ciguatera outbreaks (Robert Dickey, FDA, unpublished data).

Another key aspect of the azaspiracid response was the role played by LC/MS.

The LC/MS analysis of azaspiracids has also been reviewed in this report, but to illustrate a typical application of this technology, consider a stepwise approach of LC/MS application that follows the discovery of a new toxin such as the azaspiracids. First, the initial separation and fragmentation study is performed (179), followed by more detailed studies of fragmentation and elucidation of toxin analogs (180), and finally, some level of validation of the developed methodology is studied in the sample matrix (181).

With the discovery of a new toxin class emerges the pressing need for authentic samples and initial calibration standards and eventually, certified reference materials (182, 183). A feasibility study on the preparation

of certified reference materials for the azaspiracids has been investigated (184) and a large scale production of such materials will proceed once more materials and funding can be acquired.

Recommendations

(1) *Amnesic Shellfish Poisoning Toxins*. Study Director Michael A. Quilliam. Continue study.

(2) *Azaspiracids by LC/MS*. Topic Advisor Ambrose Furey. Continue study.

(3) *Bioassays for Phycotoxins*. Topic Advisor Donald J.A. Richard. Continue study.

(4) *Capillary Electrophoretic Methods for Marine Toxins*. Topic Advisor Ana Gago-Martinez. Continue study.

(5) *Cell Bioassays for Phycotoxins*. Topic Advisor Ronald Manger. Continue study.

(6) *Ciguatoxins by LC/MS*. Topic Advisor Richard J. Lewis. Continue study.

(7) *Diarrhetic Shellfish Poisoning Toxins, Assay Methods*. Topic Advisor J. Marc Fremy. Continue study.

(8) *Domoic Acid by ELISA*. Study Director Hans Kleivdal. Continue study.

(9) *Electrophysiological Methods for Saxitoxins*. Topic Advisor Benjamin A. Suárez-Isla. Continue study.

(10) *Immunochemical Methods for Brevetoxins*. Topic Advisor Jerome Naar. Continue study.

(11) *Immunochemical Methods for Seafood Toxins*. Topic Advisor Joanne Jellett. (Note: This topic was broadened from the previously termed *Paralytic Shellfish Poisoning Toxins, Immunochemical Methods*). Continue study.

(12) *LC/MS Detection of Marine Toxins*. Topic Advisor Patrick Holland. Continue study.

(13) *Microcystins and Nodularins*. Topic Advisor Geoffrey A. Codd. Continue study.

(14) *Paralytic Shellfish Poisoning Toxins, Instrumental Methods*. Study Director James F. Lawrence. Continue topic with new appointment. Study Director is retiring.

(15) *Receptor Assays for Phycotoxins*. Topic Advisor Frances Van Dolah. Continue study.

(16) *Sample Pretreatment Methods for Marine Toxin Analysis*. Topic Advisor Ana Gago-Martinez. Continue study.

(17) *Tetrodotoxins*. Topic Advisor Mari Yotsu-Yamashita. Continue study.

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Mycotoxins

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Topic Advisors

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The AOAC Harvey W. Wiley Award Selection Committee selected Mary W. Trucksess as the recipient of the 2004 Harvey W. Wiley Award for her significant contributions to analytical methods for mycotoxins, plant toxins, and food allergens. Trucksess regards the award as recognition of contributions of many people working in analytical method development for natural toxins.

Topic Advisor for Aflatoxins, David Wilson, and Topic Advisor for Trichothecenes, Robert Eppley, will retire in January. The Methods Committee for Natural Toxins and Allergens would like to thank them for the many contributions that they made during their term as Topic Advisors.

A comprehensive secondary fungal handbook was published in 2003 (1). The book is presented in 3 volumes. It contains data on approximately 1200 compounds. Methods of isolation and purification, physical and chemical properties, and biological activities are included. Appropriate references are presented with each metabolite.

Codex Activities

The 36th Codex Committee on Food Additives and Contaminants (CCFAC) meeting was held in Rotterdam, The Netherlands, March 2004. The following documents were forwarded by CCFAC to the Codex Alimentarius Commission (CAC) for adoption at the 27th session in June 2004: (1) Draft Code of Practice for the Prevention and Reduction of Aflatoxin Contamination in Peanuts. This was adopted at step 8. (2) Proposed draft Code of Practice for the Prevention and Reduction of Aflatoxin Contamination in Tree Nuts. This was adopted at step 5.

Other Issues

The 36th CCFAC agreed to (1) Continue work on the elaboration of maximum levels for aflatoxins in tree nuts (almonds, hazelnuts, and pistachios). (2) Discontinue the consideration of the Discussion Paper on Deoxynivalenol in cereals and commence work on the elaboration of maximum levels for deoxynivalenol. (3) Prepare revised discussion paper on aflatoxin contamination in Brazil nuts. (4) Endorse a number of recommendations in relation to the Codex General Standard for Contaminants and Toxins in Foods. (5) Continue work towards establishment of maximum levels for

mycotoxin contamination in sorghum. (6) Hold the maximum level of 5 g/kg for ochratoxin A in raw wheat, barley, and rye at step 7 while placing it on the Priority List for Evaluation by the Joint Expert Committee on Food Additives (JECFA). (7) Reconsider the maximum level for patulin in apple juice and apple juice ingredients in other beverages at a future session. (8) Commence work on the development of sampling plans for aflatoxins in tree nuts (almonds, Brazil nuts, hazelnuts, and pistachios). This was approved by CAC in June 2004.

The XI International IUPAC Symposium on Mycotoxins and Phycotoxins was held in May 17–21, 2004, at National Institute of Health (NIH), Bethesda, MD. There were 6 oral presentations and 42 posters on development and advances in analytical methods for mycotoxins. Methods for multimycotoxins appeared to be of current interest. Fluorescence polarization immunoassay, biosensor, and molecular imprint polymer approaches were also described.

During 2004, the following methods were approved by AOAC as Official Final Action: **2000.02** Determination of patulin in clear and cloudy apple juices and apple puree by LC; **2000.03** Determination of ochratoxin in barley by LC; **2000.08** Aflatoxin M1 in liquid milk by immunoaffinity column cleanup and LC; **2000.09** Determination of ochratoxin in roasted coffee by LC; **2000.16** Determination of aflatoxin B1 in baby food by immunoaffinity column and LC.

Topic Advisor Reports

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

The U.S. almond industry, represented by the Almond Board of California, is currently conducting studies to measure the uncertainty and distributional characteristics among sample test results used to estimate aflatoxin in bulk almond lots. The uncertainty and distributional information will be used to develop a computer model to predict the buyers' risk (bad lots accepted) and the sellers' risk (good lots rejected) associated with specific aflatoxin sampling plan designs. Once the evaluation method is developed, aflatoxin-sampling plans can be designed to minimize misclassification risks when detecting aflatoxin in domestic and export almond shipments.

A similar sampling study, conducted by the Brazil Ministry of Agriculture and the Brazil coffee industry, to measure the uncertainty and distributional characteristics among sample test results used to estimate ochratoxin A (OTA) in lots of bulk green coffee beans has been completed (2). Results of the study were presented to the coffee industry, regulatory

inspectors, and scientists at a Mycotoxin Sampling Workshop in Belo Horizonte, Brazil, June 2004. The evaluation method developed from the sampling study will be used to design OTA sampling plans for green coffee in both the domestic and export markets.

Two studies were completed to determine the uncertainty associated with detecting peanut allergens in several food matrixes using commercial analytical methods (3, 4). The sampling and sample preparation steps were the largest sources of error. The average coefficient of variation across the 4 analytical methods averaged 8.8%. These studies will hopefully give inspectors an appreciation of the complexities of detecting small quantities of peanut protein in foods.

A recent study measured the uncertainty associated with sample preparation of pistachio nuts using wet slurry and dry homogenization methods (5). Theory was developed to predict the sample preparation coefficient of variation based upon particle size distribution, subsample size, and contamination level. An interesting observation was a 32% increase in the mean aflatoxin levels with the wet slurry method as compared to dry comminution.

The 36th CCFA met March 2004 in The Netherlands and agreed to work on the development of sampling plans for aflatoxin in almonds, Brazil nuts, hazelnuts, and pistachios. A working group led by the United States with assistance from Argentina, Brazil, Iran, European Union (EU), and the International Nut Council will prepare sampling plans for aflatoxin in these tree nuts for circulation, comment, and consideration at the next session.

Aflatoxin M₁.—Topic Advisor Hans P. van Egmond, National Institute of Public Health and the Environment, Laboratory for Residue Analysis, Postbak 3, PO Box 1, 3720 BA Bilthoven, The Netherlands, Tel: 31-30-2742440, Fax: 31-30-2744403, E-mail: hp.van.egmond@rivm.nl, reported that 5 poster presentations were given on aflatoxin M₁ related subjects at the 11th IUPAC conference on Mycotoxins and Phycotoxins in Bethesda, MD, May 2004. Three of these described the occurrence of aflatoxin M₁ in goat's milk in Brazil (6), in fresh cow's milk in Tanzania (7), and in fresh cow's milk in Brazil (8). Reported also was the finding of aflatoxin M₁ in breast milk of Egyptian women (9) and a report of analysis of aflatoxin M₁ in milk by immunoaffinity column chromatography followed by thin-layer chromatography (TLC; 10). An example is the collaborative study conducted under the auspices of the International Dairy Federation (IDF), the International Union of Pure and Applied Chemistry (IUPAC), and the International Atomic Energy Agency (IAEA) to validate a method combining immunoaffinity column cleanup with TLC for the determination of aflatoxin M₁ in milk (11). The validation study was carried out on samples of aflatoxin M₁ contaminated milk and milk powder at levels close to 0.5 and 0.05 g/kg, 2 levels frequently applied in the world as regulatory limits (12). The study yielded good results, with relative standard deviations for repeatability (RSD_r) and reproducibility (RSD_R) in the range of 26–54% and 34–53%, respectively. HORRAT values ranged from 0.6 to 1.5, which

also indicates adequate interlaboratory performance. The method will be published as a standard ISO/DIS 14674-IDF 190.

Another validation study using TLC to determine aflatoxin M₁ involved a single laboratory validation of a method based on chloroform extraction, purification over a Florisil cartridge, followed by TLC with visual/densitometric detection (13). Milk samples used in the validation were spiked with aflatoxin M₁ at concentrations of 0.02–0.970 g/L. Average recoveries using visual analysis were 84–99% with an RSD_r of 2.3–9.8%; recoveries using densitometry were 85–105%, with an RSD_r of 3.6–13.9%. The latter is remarkable, as densitometry usually leads to a better precision.

There is an increased interest with regard to mycotoxins in organic agricultural products, including aflatoxin M₁ in milk. In Italy, aflatoxin M₁ was found significantly higher in organic milk than in conventional milk (14). The authors concluded that such aflatoxin contamination appears to be a producers-based problem. In contrast to the Italian findings, a German study revealed that conventionally produced milk contained aflatoxin M₁ more frequently than organic produced milk (15). These controversial results lead to the recommendation to do further research in this field.

In addition to the reports on occurrence presented in poster format at the 2004 IUPAC conference, several surveys were published on the occurrence of aflatoxin M₁ in milk and milk products in India (16), Iran (17), Japan (18), Mexico (19, 20), Portugal (21), and Turkey (22–25).

In the EU, aflatoxin M₁ has been a topic of special interest in 2003/2004. In Italy, a significant increase of aflatoxin M₁ was detected in raw milk in the (late) summer of 2003 in 2 regions with a high density of livestock production (26). It appears that the main source of contamination was maize and maize meal, products which, partly because of the specific growing conditions of last year, have shown a steep rise in the levels of aflatoxin B₁. The Scientific Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA) is of the opinion that aflatoxin B₁ is undesirable in animal feed (27). The Panel's opinion, under the circumstances of maximum exposure from feed materials, is that even though the feed is in compliance with the levels set for feed materials the milk obtained from high yielding cows and other milk producing animals might contain aflatoxin M₁ levels exceeding the present statutory limits in the EU, which is 0.05 g/kg. A lower limit of 0.025 g/kg has been established for aflatoxin M₁ in infant formula (28).

Aflatoxin methods.—Topic Advisor David M. Wilson, University of Georgia, Department of Plant Pathology, Coastal Plain Section, Tifton, GA 31793, Tel: 912-386-3368, Fax: 912-386-7285, E-mail: dwilson@tifton.cpes.peachnet.edu, reported several areas of interest in aflatoxin research this past year. Some of the notable items are briefly mentioned.

The bright greenish yellow fluorescence (BGYF) has frequently been associated with aflatoxin contamination in corn and cottonseed. There was an interesting report of many juvenile stages of food storage insects exhibiting a native fluorescence with excitation at 365 nm (29). Therefore, it may

be possible that some of the confounding factors in using BGYF for screening are the occasional presence of juvenile insects.

Aflatoxin producing fungi were studied using enzyme-linked immunosorbent assay (ELISA) and other analytical methods with the addition of cyclodextrins to the culture (30). There were 2 reports on the use of standard reference materials for aflatoxin analysis (31, 32): one paper addressed the use of peanut butter and the other addressed the problems associated with certified reference materials for mycotoxins. At least 3 papers described immunochemical method development (33–35). The use of ELISA for aflatoxin B₁ is fairly common and the methods are always being improved. A rapid ELISA method was developed to reduce the matrix effects for screening peanuts, corn, pistachio, and soybeans (33) and an immunoaffinity chromatography and ELISA assay was developed for determination of aflatoxin B₁ in chicken liver tissue (34).

A radioimmunoassay (RIA) procedure was used to determine aflatoxin B₁ in agricultural products (35). The problems associated with data variability in a collaborative study were discussed (36). The precision is low and coefficient of variation is high with TLC.

The analytical capabilities of the developed countries is greater than that of developing countries. It may be that the whole concept of aflatoxin management in many parts of the world is unrealistic from the marketing and actual risk standpoints at present. A new method based on solid-phase dispersion and liquid chromatography (LC) was developed for aflatoxin B₁ in peanuts (37). A comparison study of 2 post-column derivatization techniques, the photochemical reactor (PHRED cell) and the Kobra cell, found that results of analyses for aflatoxin B₁, B₂, G₁, and G₂ in the same samples were similar (38).

Alternaria toxins.—Topic Advisor Michele Solfrizzo, Institute of Sciences of Food Production, National Research Council, Bari 70126, Italy, Tel: 39-080-5929367, Fax: 39-080-5929373, E-mail: michele.solfrizzo@ispa.cnr.it, reported that a solid-phase microextraction (SPME) method using a silica fiber with a 60 μm thick polydimethylsiloxane/divinylbenzene (PDMS/DVB) film coupled to LC with diode array UV detection (LC-UV/DAD) has been used to analyze tenuazonic acid (TeA) and other acidic mycotoxins in cornflakes. Samples are extracted with a mixture of methanol/2% KHCO₃; the mixture is evaporated to dryness and reconstituted with phosphate buffer for SPME followed by LC-UV/DAD. A mean recovery of 103–99% was obtained from cornflakes spiked at 200 ng/g. A cornflakes sample naturally contaminated with 25 ng/g of TeA was found (39).

An LC-UV/DAD method for the determination of alternariol (AOH), alternariol methyl ether (AME), altertoxin-I (ATX-I), and TeA in carrots has been developed (40). The sample extract is divided into 2 parts—one that is purified by SPE on a C₁₈ column for the analysis of ATX-I, AOH, and AME and the other which is cleaned up on a polymeric Oasis HLB column for

determination of TeA. Mean recoveries ranged from 69 to 90%, mean repeatability ranged from 4 to 18%, and limits of detection ranged from 5 to 20 ng/g. No *Alternaria alternata* mycotoxins were found in 266 carrot samples produced and stored under organic conditions in 3 European locations.

An evaporative light scattering detector (ELSD) coupled to an LC system has been used for direct detection of *Alternaria* (AAL) toxins (41). The linear response was in the range from 400 to 4000 ng AAL toxin TA with a detection limit of 100 ng. The method was used to check the toxigenicity of the tomato pathogenic fungus *Alternaria alternata*.

Most of the *Alternaria* mycotoxins have been included in the database of 474 mycotoxins and fungal metabolites that have been determined by standardized LC-UV/mass spectrometry (MS) methodology (42). AOH and AME have been reported to be produced by fungi other than *Alternaria*. The production of AOH by *Graphis cognata* has been reported for the first time (43). The production of AME by *Chaetomium globosum* KMITL-N0802 has also been reported (44). TeA showed a protective effect against dimethyl benz(a)anthracene (DMBA) induced skin cancer in mice. Animals topically exposed to DMBA developed skin tumors, whereas 40% of the animals topically protected with TeA were tumor free (45).

Citrinin.—Topic Advisor Dave 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, reported that a simple rapid LC method has been proposed for assaying citrinin in rice cultures inoculated with *Monascus* species (46). These cultures serve as a source of cholesterol-lowering substances and of natural red pigments for food coloration. After a simple extraction into acetonitrile–water (3 + 2, v/v) and centrifugation, the authors claim recoveries of 97% using RP LC with detection at 334 nm. In another study on *Monascus purpureus* in rice (47), the authors evaluated the effects of temperature, water content, and added ethanol on citrinin production for the purpose of minimizing levels of this mycotoxin while increasing production of monacolin K and γ -aminobutyric acid.

In food studies, several trials were conducted to evaluate gamma-irradiation technology for reducing mycotoxin production in various fruits during refrigerated storage (48). Citrinin production was common in naturally contaminated nonirradiated grapes, figs, and pears, but was completely eliminated at irradiation levels of 3.5 kGy. In studies on toxigenic fungi, a gas-phase chemosensor array (electronic nose) was used to analyze various strains of *Penicillium verrucosum* grown on wheat agar. Using principal component analysis, it was possible to identify citrinin-producing strains and to differentiate them from ochratoxin-producing and nontoxic strains (49).

Cyclopiazonic acid.—Topic Advisor Joe Dorner, USDA, ARS, 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, reported

that raw peanuts purchased from retail outlets in Botswana were naturally contaminated with cyclopiazonic acid and aflatoxins (50). Aflatoxin concentrations ranged from 12 to 329 ng/g, whereas cyclopiazonic acid concentrations ranged from 1 to 10 ng/g. A greater percentage of samples were contaminated with aflatoxins (78%) than with cyclopiazonic acid (21%). *Aspergillus flavus* was isolated from many of the peanut samples, and 6 isolates were found to produce cyclopiazonic acid.

A method was reported for simultaneous determination of ochratoxin A and cyclopiazonic, mycophenolic, and tenuazonic acids in cornflakes (39). Samples were extracted with methanol + 2% KHCO₃ and subjected to cleanup by solid-phase microextraction. LC analysis with diode array UV detection is performed with a propylamino-bonded silica gel column using a mobile phase of acetonitrile + methanol + ammonium acetate buffer (78 + 2 + 20, v/v/v). Recoveries ranged from 74 to 103%, and cornflake samples were found to be naturally contaminated with cyclopiazonic and tenuazonic acids at 72 and 25 ng/g, respectively.

Ergot alkaloids.—Topic Advisor George M. Ware, U.S. Food and Drug Administration, 60 8th St, Atlanta, GA 30309, Tel: 404-347-2131, ext. 5215, Fax: 404-347-4225, E-mail: gware@ora.fda.gov, reported the use of ELISA for dihydroergosine (DHES) in sorghum (51). DHES is the principal toxic alkaloid produced by sorghum ergot (*Claviceps africana*). This paper describes a competitive ELISA for measuring the DHES concentration in grains and mixed feeds. The assays were developed using a DHES-specific mouse monoclonal antibody and rabbit polyclonal antibodies raised against DHES conjugated to bovine serum albumin. Recoveries of between 77 and 103% were obtained from spiked grain using a simple, one-step extraction with 70% methanol. Both the monoclonal and the polyclonal assays are capable of detecting DHES concentrations above 0.01 mg/kg, but quantification is most reliable at concentrations of 0.1 mg/kg or higher.

There was an application note on identification and characterization of new ergot alkaloids using LC/MS/MS (52). Three unknowns were found: dehydroergotamine, hydroxyergotamine, and ergoval. They are derivatives of ergotamine. Dehydroergotamine is an oxidized derivative of ergotamine; hydroxyergotamine has an amino acid, serine instead of alanine; and in ergoval the lysergic acid is esterified with valine.

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@mail.ncaur.usda.gov, reported that the field of fumonisin research continued to be active, particularly with regard to the areas related to risk assessment, namely, the prevalence of these compounds and their mechanism of toxicity. Previous studies have indicated that fumonisins occur worldwide and the majority of the recent publications deal with prevalence. Fumonisin or the fungi that produce them were found in maize, or maize

products, in Africa (53–55), Italy (56, 57), Croatia (58), Brazil (59), and Ecuador (60).

Fumonisin was also reported to be produced from *F. verticillioides* in edible pine nuts (61). Residues of FB₁ were also isolated from the tissues of pigs receiving an oral dose of 100 mg FB₁/animal/day for 5–11 days. The highest concentrations were in the spleen (854–2212 g/kg), kidney (833–1329 g/kg), liver (231–163 g/kg), and lung (170–311 g/kg). The muscle also contained FB₁, at an average level of 26–41 g/kg (62). These studies reinforce the belief that fumonisins are found worldwide in maize and in a wide variety of products that potentially include the tissues of animals that have consumed high levels of fumonisins.

The toxicity of the fumonisins and their mechanism of action were also very active areas of research, yet most are outside the realm of this review. The relationship between fumonisins, sphingolipid metabolism, folate transport, and neural tube defects was reviewed (63). A means of assessing human exposure to fumonisins through the analysis of hair was reported by Sewram et al. (64). Fumonisin B₁, B₂, and B₃ were isolated from the hair using strong anion exchange cleanup, and were separated and detected by LC/ESI/MS. Recoveries from spiked hair ranged from 81 to 101%. FB₁ was found at mean levels from 23 to 33 g/kg hairs, and FB₂ was found at mean levels from 5 to 11 g/kg hairs, depending upon the region from which they were collected.

The fumonisins do not appear to require metabolic activation in order to assert their effects upon ceramide synthase. However, the possible biological activity of fumonisin metabolites or degradation products, such as the aminopentol resulting from the hydrolysis of FB₁ (AP₁ or HFB₁) remain of interest. An in vitro model of mammalian toxicity indicated that *N*-acylation enhanced toxicity of AP₁, but not FB₁ (65). Under appropriate conditions of heat and humidity, such as during certain types of cooking, fumonisins may be degraded. Cornmeal that was spiked with *F. verticillioides* culture material (containing fumonisins and other metabolites) and then used to prepare baked cornbread, pan-fried corncakes, and deep-fried fritters was not significantly less toxic to rats after such cooking than before (66).

FB₁ was shown to react with glucose, methyl-D-glucopyranoside, and the amino acid derivatives *N*-acetyl-L-lysine methyl ester and BOC-L-cysteine methyl ester (67). FB₁ formed a stable reaction product through the tricarballic acid (TCA) side chains after heating with methyl-D-glucopyranoside. This suggests that fumonisins may bind to polysaccharides via the TCA side chains under certain conditions. Recovery of ¹⁴C-labeled FB₁ from spiked cornmeal was reduced by 43% after it was used to prepare muffins. However, more ¹⁴C was eluted from an immunoaffinity column than could be accounted for as FB₁, suggesting that additional ¹⁴C-labeled products, related to fumonisins and recognized by the antibodies, may have been present (57). Total fumonisins were reduced in tortillas prepared using a traditional nixtamalization method (68).

Fumonisin concentrations were also reduced during the baking of bread, and with the boiling of contaminated corn in water (69).

The occurrence of fumonisins in foods is affected by factors including host resistance and environmental effects. A genetically diverse collection of maize lines was evaluated for potential sources of resistance to *Fusarium* ear and kernel rot and to fumonisin accumulation; several of the hybrids were associated with low ear rot severity and low fumonisin accumulation (70). Bt maize hybrids that contained the Cry1Ab protein generally had lower fumonisin levels than corresponding controls (71), suggesting such hybrids might be useful in reducing fumonisin occurrence. The degree of insect damage caused by *Sesamia nonagrioides* Lefebvre was also correlated with fumonisin contamination in Italian maize (72).

Detection of fungi using biotechnology continues to advance. A real-time polymerase chain reaction (PCR) method was developed for detection of fumonisin-producing *Fusarium* based upon primers for the biosynthetic gene FUM1 (73). The detection range was from 5 pg to 5 ng genomic DNA per reaction. Five corn samples that tested positive for *F. verticillioides* by traditional microbiological methods were also positive for the FUM1 product. A PCR method based upon FUM5 was also developed for detection of fumonisin producing strains (74). Primers based upon the calmodulin gene sequences were used to detect *F. proliferatum* and *F. oxysporum* in asparagus plants (75).

Instrumental methods for measuring fumonisins continued to be investigated, particularly those using LC. LC with fluorescence detection of the *o*-phthalaldehyde (OPA) derivative remains a popular method. Naphthalene 2,3-dicarboxaldehyde was used as a derivatization reagent in the detection of FB₁, FB₂, and FB₃ in maize silage (76). Fumonisin was isolated from the silage using affinity columns. Of 89 samples, fumonisins were found in 97%. The mean FB₁, FB₂, and FB₃ contents of positive samples were 615, 93, and 51 g/kg, respectively, suggesting fumonisins may be a frequent low level contaminant of corn silage. The extraction of fumonisins from corn-based infant foods was investigated (77). The best extraction solvent examined was 70% aqueous methanol at pH 4, whereas an alkaline methanolic solution at pH 9.2 was poor. Extraction with phosphate buffer mixtures at pH 3 was also examined; however, these extracts resulted in interferences in the chromatography. Screening of culture extracts for 474 mycotoxins and fungal metabolites using LC with UV and MS detectors was reported (42).

Several new antibody-based methods were also recently described, including an array biosensor, fluorescence polarization, and surface plasmon resonance methods. The array biosensor was constructed to detect multiple analytes, including staphylococcal enterotoxin B, ricin, cholera toxin, botulinum toxins, trinitrotoluene, and fumonisins (78). Fumonisin attached to the surface of the array was used in a competitive assay with FB₁ in the sample and a fluorescently-tagged antibody. The pattern of fluorescence on the surface was detected using a charge-coupled device (CCD) camera, with the response inversely proportional to the

fumonisin concentration. Another aspect of fluorescence, polarization, was combined with immunoassay to detect fumonisins, deoxynivalenol (DON), and aflatoxins (79). A flow injection liposome immunoassay (FILIA) was used to detect FB₁ in corn-based food and animal feeds, and results were compared with those of the LC method (80). The FILIA method detected as little as 0.1 ng FB₁ in as little as 11 min. There was good correlation between the FILIA results and the LC ($r^2 = 0.945$).

Surface plasmon resonance (SPR) continues to be investigated for mycotoxin analysis, particularly in Europe. A SPR biosensor for the simultaneous detection of 4 mycotoxins, including fumonisins, was described (81). The samples were extracted with acetonitrile–water and impurities were removed using Mycosep 224 MFC columns. A fixed concentration of mycotoxin-specific antibodies was mixed with diluted samples, and the mixture was passed over the sensor surface, upon which a mycotoxin was immobilized. Binding of antibodies to the sensor surfaces was detected. The 4 toxins could be detected within 25 min using the technique. Application of the technology using a miniaturized SPR device is envisioned.

Ochratoxins.—Topic Advisor Benedicte Hald, Royal Veterinary and Agricultural University, Department of Veterinary Microbiology, Stigbøjlen 4, Friedriksberg C, Copenhagen 1870-DK, Denmark, Tel: 45-3528-2762, Fax: 45-3528-2757, E-mail:vetmi@kv1.dk, reported that an interlaboratory study was performed on behalf of the Food Standards Agency to evaluate the effectiveness of an affinity column cleanup LC method for the determination of ochratoxin A (OTA) at European regulatory limits in a variety of dried fruit. Participants were asked to spike test portions of all test samples at a level equivalent to 5 ng/g OTA. Average recoveries ranged from 69 to 74%. Based on results for 5 naturally contaminated test samples (blind duplicates) RSD_r values ranged from 4.9 to 8.7%, and RSD_R values ranged from 14 to 28% (82).

Five procedures for extraction and/or cleanup for determination of OTA in must, wine and beer by LC-fluorescence detection (FLD) were compared (83): (1) dilution with polyethylene glycol 8000 and sodium hydrogencarbonate solution and cleanup on an immunoaffinity column (IAC); (2) extraction with chloroform and IAC cleanup; (3) SPE on RP C-18; (4) RP phenylsilane; and (5) Oasis HLB cartridges. The same LC-fluorescence determination conditions and concentration ratio were used. Oasis HLB cartridges were usually better than phenylsilane.

Several interesting papers relating to OTA determination in wine were published. A new, rapid, highly sensitive, and reproducible RP-LC-FLD method by directly injecting the sample in the chromatographic system without any extraction or cleanup was developed (84). A liquid–liquid microextraction technique was applied to the extraction of OTA from wine prior to its quantification by LC-FLD (85). Recovery was 77%. The method can be a simple and inexpensive alternative to the use of IAC to quantify OTA levels in wine.

The effectiveness of 3 different sample preparatory methods was evaluated and 84 U.S. domestic wines were surveyed for OTA (86). Randomly selected wine samples were used to evaluate the performance of IA, C18, and SPE columns as safe and efficient sample preparation methods. The recoveries of 0.5 g/L OTA fortifications in different samples were studied because natural OTA contamination in wines predominantly occurs at sub- g/L levels. The data indicate IAC had superior OTA recoveries.

A molecularly imprinted solid-phase extraction (MISPE) was developed for the rapid analysis of wheat extracts for OTA (87). Molecularly imprinted polymer particles were synthesized from *N*-phenylacrylamide and slurry-packed into a micro-column for selective supercritical fluid extraction (SFE) of OTA. The method with fluorescence detection, afforded a detection limit of 5.0 ng/mL for OTA. Recovery from wheat extracts was 103–3%.

A stable isotope dilution assay was published for quantification of OTA by using [H-2(5)]-OTA as internal standard. OTA was quantified in foods by LC/MS/MS after extraction with buffers containing [H-2(5)]-OTA and cleanup by IAC or SFE on silica (88). The method showed a sufficient sensitivity with low detection and quantification limits of 0.5 and 1.4 g/kg, respectively.

A total of 320 samples from the 1999 UK harvest comprising 201 wheat, 106 barley, and 13 oat samples were analyzed for OTA and deoxynivalenol (89). The survey found OTA at below 5 g/kg in 97% of the samples. During the past year there have been reports of occurrence of OTA in green coffee from different origins (90), virgin olive oils of Greek origin (91), wine and grape juice marketed in Rio de Janeiro, Brazil (92), infant cereal foods from the Canadian market (93), and Spanish grapes (94).

Patulin.—Topic Advisor Myrna Sabino, Instituto Adolfo Lutz, Dr Arnaldo 355, 355-CEP, São Paulo 01246-902, Brazil, Tel: 55-11-3068-2921, Fax: 55-11-3085-3505, E-mail: mysabino@ial.sp.gov.br, reported that patulin has been found as a natural contaminant of processed apple products, and it has been suggested that its presence may be indicative of the quality of the fruit used in production. In Iran, a survey for patulin was conducted in 65 locally produced commercial apple juice and apple juice concentrates (95). The mean concentration of patulin in apple juice samples surveyed was slightly lower than maximum level recommended by the Codex Alimentarius. In Belgium, local apple juice, imported apple juice, and cider were examined (96). Patulin was detected in 79, 86, and 43%, respectively, of these samples. The contaminated sample exceeded the safe level of 50 g/L. There was no statistically significant difference in the mean patulin contamination between clear and cloudy apple juices or between manually and industrially prepared apple juice. The effects of apple quality, storage, and washing treatments on patulin levels in apple cider were investigated (97). Patulin was not detected in cider pressed from fresh tree-picked apples (7 cultivars), but was found at levels of 40.2 to 374 g/L in cider pressed from 4 cultivars of fresh-dropped apples. Patulin was not detected in cider pressed from culled

tree-picked apples stored for 4–6 weeks at 0–2 C, but was found at levels of 0.97–64.0 g/L in cider pressed from uncultured fruit stored under the same conditions. Cider from controlled-atmosphere-stored apples that were culled before pressing contained 0–15.1 g/L patulin, whereas cider made from uncultured fruit contained 59.9–120.5 g/L patulin. The washing of ground-harvested apples before pressing reduced patulin levels in cider by 10–100%, depending on the initial patulin levels and the type of wash solution used.

A survey in Italy found no significant differences in patulin levels in organic and conventional apple products (98). Qualitative production of metabolites by *Penicillium expansum* was examined using TLC (260 isolates), LC (85 isolates), and MS (22 isolates; 99). The results showed that none of the 260 isolates produced OTA, penitrem A, or rubratoxin B. Patulin and roquefortine C were produced by 98% of the isolates. Chaetoglobosins and communesins were detected in naturally infected juices and potato pulp, whereas neither patulin nor citrinin was found.

Aqueous extract of the leaves of neem (*Azadirachta indica*) was tested in vitro for antifungal activity against *P. expansum* (100). Patulin production was inhibited during cultivation when concentrations higher than 50 mg/mL neem leaf extract were added to the culture medium. The absorption of the mycotoxin patulin from the rat stomach was studied (101). The enrichment of patulin in the tissue of perfused rat stomach after luminal application and its vascular appearance was quantified by stable isotope dilution assays. Patulin was found immediately in the perfusates after the addition of juices with patulin added at concentrations of 350 and 3.5 mg/L patulin to the rat stomach, and about 26 to 29% of patulin were removed from the gastric lumen over 55 min, respectively. From this quantity, 17 and 2% were transferred into vascular circulation and 3 and 0.06% were detectable in gastric tissue for the high and the low patulin dose, respectively. In another study, patulin was not found in the serum of a volunteer whose blood was drawn shortly after consumption of a juice containing the maximum tolerable amount of patulin (102). The degradation of patulin by reacting it with whole blood was also investigated. After addition of 100 g patulin/9 mL blood, only 6.1% of the mycotoxin was detected after 2 min. It was concluded that even high levels patulin in foods would not cause toxic effects because patulin degraded quickly before reaching tissues other than the gastrointestinal tract.

A PCR method using primers based on the polygalacturonase gene of *P. expansum* was used for identification of *P. expansum* (103). The PCR amplified a 404-bp DNA product from all the *P. expansum* isolates tested, but not in other common foodborne *Penicillium* species and *Escherichia coli*. Experiments to determine the sensitivity of the PCR indicated that it can detect the DNA equivalent from as low as 25 spores of *P. expansum*. The PCR could potentially be used as a rapid tool for screening fruits for the presence of *P. expansum*.

A syringe-cartridge SPE method for the determination of patulin in apple juice was developed (104). A 2.5 mL portion of test sample was passed through a conditioned

macroporous SPE cartridge and washed with 2 mL 1% sodium bicarbonate followed by 2 mL 1% acetic acid. Patulin was eluted with 1 mL 10% ethyl acetate in ethyl ether and determined by RP-LC. Recoveries averaged 92% and the RSD was 8.0% in test samples spiked with 50 ng/mL patulin. The use of atmospheric pressure chemical ionization (APCI) and the recently introduced atmospheric pressure photo ionization (APPI) technique for the LC/MS determination of patulin in clear apple juice was investigated (105). The results indicated that APPI provides lower chemical noise and signal suppression than does APCI. The linear range for patulin in apple juice was 0.2–100 ng/mL. Mean recoveries of patulin in 3 apple juices ranged from 94.5 to 103.2%, and the limit of detection (LOD; S/N = 3), repeatability, and reproducibility were 1.03–1.50 ng/mL, 3.9–5.1%, and 7.3–8.2%, respectively. The total analysis time was 10.0 min.

Trichothecenes.—Topic Advisor Robert M. Eppley, U.S. Food and Drug Administration, 5100 Paint Branch Pkwy, College Park, MD 20740, Tel: 301-436-1951, Fax: 301-436-2644, E-mail: reppey@cfsan.fda.gov. Robert Eppley is resigning as the Topic Advisor since he is retiring from FDA at the end of 2004. He reported that interest in the trichothecenes continues to be strong, especially in methodology, with publication of 9 reports pertaining to analytical procedures for the determination of trichothecenes (89, 93, 106–112).

Three of these reports were surveys (89, 93, 106). In a survey of infant cereal foods for mycotoxins (93), deoxynivalenol (DON) was the most commonly detected mycotoxin. The incidence of detection ranged from 58% (barley-based) to 100% (soy-based) with levels in the range of 32–150 ng/g. Another survey for DON and ochratoxin A in stored grains found DON in 88% of the samples with 83% below the 100 ng/g level, with a maximum level of 600 ng/g (89). Interestingly, in the samples with DON levels exceeding the 150 ng/g, nivalenol was also detected at 50 ng/g or higher. A survey of breads on the German market (106) found incidence levels of DON ranging from 79 to 100% with a median content of 134 ng/g and a high of 690 ng/g. Nivalenol, 3-ADON, T-2, toxin and HT-2 toxin were detected in some of the samples.

The refinement of methods for the determination of trichothecenes continues with both LC/MS and GC/MS studies (107–110). In one of these studies, GC/MS was used for the simultaneous detection of fusaproliferin and 7 trichothecenes from grain samples (107). Two other papers report on the use of LC/MS for the determination of the B-trichothecenes and the de-epoxy-metabolite of DON in pig urine and maize (108), and the detection of DON, 15-ADON, and zearalenone in various matrixes (109). The preparation of the trimethylsilyl derivative of the trichothecenes for GC analysis was optimized with the reagent *N,N*-dimethyl-trimethylsilyl-carbamate (110). The limit of detection was 1 ng for each of the studied trichothecenes. A rapid and sensitive cytotoxicity screening assay for the trichothecenes in cereal samples was noted (111).

Some other reports of interest for the trichothecenes are a review of the diversity of metabolites produced by *Fusarium* sp. (112), trichothecenes in the environment: relevance to human health (113), and *Stachybotrys chartarum*: a fungus of our time (114).

All of the accumulated occurrence data to date indicate that DON is the most prevalent trichothecene found in grain-based human food and animal feed. The amounts of DON present are generally below what is considered toxicologically significant; however, DON is not the only trichothecene produced by *Fusarium*. A number of reports over the last several years have shown the co-occurrences of nivalenol, 3- and 15-ADON, T-2 toxin, HT-2 toxin, and diacetoxyscirpenol especially with the higher levels of DON. Amounts of these toxins present in samples have been generally low in comparison to the levels of DON, but the total toxicity load could be significantly higher. Risk analysts should have the added data on the complete spectrum of trichothecenes present in samples in order to make an informed assessment of the toxicological consequences. There is a need for validated methods to detect and determine this group of trichothecenes in suspect samples. A significant number of recent reports have included these trichothecenes in the analytical methods using either GC/MS or LC/MS as the determining step. The new referee is encouraged to evaluate these procedures and recommend a method or methods for further validation.

The Topic Advisor recommends that the Topic Advisor for trichothecenes be retained and studies on methods for the determination of trichothecenes be continued.

Zearalenone.—Topic Advisor Winston M. Hagler, Jr, North Carolina State University, College of Agriculture and Life Sciences, Department of Poultry Science, Mycotoxin Laboratory, Box 7608, Raleigh, NC 27695-7608, Tel: 919-515-3228, Fax: 919-515-2625, E-mail: winston_hagler@ncsu.edu, summarized selected information appearing in the last year's scientific literature. An LC/MS method was used to detect DON, 15-acetylDON, and zearalenone (ZEN) from fungal liquid culture, maize grain, insect larvae, and pig serum (109). Toxicokinetic studies were performed with maize insect pests reared on ZEN added diets; the results suggested that larvae may be able to degrade ZEN. Results of feeding pigs with DON contaminated diet indicated that DON accumulated quickly in pig blood, and the level declined progressively for 12 h thereafter.

Three multi-mycotoxins methods including ZEN were reported. An immunoaffinity column packed with antibodies for aflatoxins, OTA, and ZEN was used for cleanup of acetonitrile–water extracts of rice and rye. After derivatization with trifluoroacetic acid, the 3 toxins were determined simultaneously by LC with gradient elution and fluorescence detection (115). The limits of determination were aflatoxins 0.25, OTA 0.5, and ZEN 5 g/kg. The recoveries ranged from 86 to 93% when 0.5 g sample was added to the column. Accelerated solvent extraction (ASE) and LC with fluorescence detection were used to determine ZEN and -zearalenol in cereals and swine feed (116). The extraction solvent was methanol–acetonitrile (1 + 1) in an 11 mL ASE

cell, 1500 psi, 50 C, a 5 min static time, and 60% flush volume. Recoveries were 96–98% for ZEN and β -zearalenol at 50–200 ng/g. Fluorescence polarization immunoassay was used for ZEN and related metabolites in maize (117). The assay showed cross-reactivity to the ZEN analogs ZEN, β -zearalenol, β -zearalanol, β -zearalenol, β -zearalenol of 195, 139, 71, and 20%, respectively, relative to ZEN (100%). Recovery of ZEN from spiked maize over the range of 0.5–5 g/g averaged 100%.

Adsorption of ZEN by β -D-glucans in *Saccharomyces cerevisiae* cell wall was determined. Four strains of the yeasts were used and those with higher levels of β -D-glucans were able to complex larger amounts of ZEN (118). The strains with the high chitin content had a lower complex-forming capacity than expected based on their β -D-glucans content.

Recommendations

General Referee Recommendations

(1) Appoint Gary A. Lombaert, Health Canada, Canada, as the Topic Advisor for Trichothecenes.

(2) Appoint Gordon Shephard, Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Tygerburg, South Africa, as the Topic Advisor for Aflatoxins.

(3) Approve the following Methods: **2001.01** and **2001.06** as Final Action Official Methods.

(4) Continue development of methods for mycotoxins in dietary supplements.

Topic Advisors and Study Directors Recommendations

(1) *Sampling and Subsampling for Mycotoxins*: Topic Advisor Thomas B. Whitaker. Continue study.

(2) *Aflatoxin M*: Topic Advisor Hans P. van Egmond. Continue study.

(3) *Aflatoxin*: Topic Advisor David M. Wilson. 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) *Ochratoxin*s: Topic Advisor Benedicte Hald. Continue study.

(10) *Patulin*: Topic Advisor Myrna Sabino. Continue study.

(11) *Trichothecenes*: Topic Advisor Robert M. Eppley. Continue study.

(12) *Zearalenone*: Topic Advisor Winston M. Hagler, Jr. Continue study.

(13) **2001.04** *Determination of Fumonisin B₁ and B₂ in Maize Flour and Cornflakes by HPLC*: 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.

(14) **2003.02** *Determination of Aflatoxin B₁ in Cattle Feed by Immunoaffinity Column Cleanup and LC with Post-Column Derivatization*: Study Director Joerg Stroka, European Commission DG Joint Research Center, Institute for Health and Consumer Protection Food Products Unit, TP 260, I-21020 Ispra, Italy, Tel: 0039-0332-785170, Fax: 0039-0332-785707, E-mail: joerg.stroka@jrc.it. Continue study.

(15) **2001.06** *Veratox for Fumonisin*s: Study Director Mark Mozola (see 18). Approved as First Action in 2001. Recommended for Final Action.

(16) **2001.01** *Determination of Ochratoxin A in Wine and Beer by Immunoaffinity Column Cleanup and HPLC*: Study Director Angelo Visconti (see 13). Approved as First Action in 2001. Recommended for Final Action.

(17) *Determination of Ochratoxin A in Green Coffee by Immunoaffinity Column Cleanup and HPLC*: 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@cdlnet.com.br. Ready for First Action approval.

(18) *Determination of Deoxynivalenol 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.

(19) *Analysis of Aflatoxin by an LC with Activation Using a Photochemical Reactor for Enhanced Detection (PHRED)*: Study Director Arthur Waltking, Waltking Associates, 482 Rock Rd, Glen Rock, NJ 07452. Study Director will submit single laboratory validation data. This study will be evaluated as a matrix extension (modification) of an Official Method.

(20) *Mycotoxins in Botanicals*: Topic Advisor Bruce Malone, Trilogy Analytical Lab, 111 West 4th St, Washington, MO 63090, Tel: 636-239-1521, Fax: 636-239-1531, E-mail: bruce@trilogylab.com, reported that mycotoxins in botanicals requires further study because of the high usage of botanicals in human diets and the potential for mycotoxin contamination. There was little published research in this area for the past year. Analytical methodology for aflatoxins and ochratoxin A are needed along with product surveys to assess the level of mycotoxin contamination. Based on past analytical experience with the analysis of aflatoxins in botanicals, the sample extract purification is the most critical step. Because of the wide variety of botanical products and their complex matrix, combinations of solid-phase and immunoaffinity purification columns are sometimes required. Continue topic.

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