



RESEARCH

Listeriosis Research

Listeriosis continues to be an important food-borne disease problem in the United States, causing an estimated 2500 cases and up to 500 deaths per year. Populations that are at risk include the fetuses of pregnant women, the elderly, those with uncontrolled diabetes or kidney disease, and those who are immunosuppressed for a variety of reasons (neoplasia, chemotherapy, HIV infection). Besides the suffering of those afflicted by the disease, recall of contaminated food products causes an estimated \$2 billion dollars in economic losses in the United States annually.

Our laboratory had been involved in a collaborative venture with Dr. John Luchansky and his colleagues at the Eastern Regional USDA Laboratory to investigate further the pathogenesis of *Listeria monocytogenes* in the gastrointestinal tract. We have defined a mouse model (A/J) that allows us to investigate the effects of listerial growth on ready to eat (RTE) meat products affecting its virulence in the g.i. tract. We found that growth of *L. monocytogenes* in packages of RTE meat products such as wieners does not demonstrably affect its virulence in our mouse model. Nor did suspension of *L. monocytogenes* in a slurry of homogenized wiener result in a greater severity of infection in mice. We also have performed experiments with a variety of types of sliced turkey product. In collaboration with Dr. Gaylen Uhlich of the Eastern Regional USDA lab and Dr. Luchansky, we have investigated the effect of the *crp/fnr* family of environmental sensing molecules. *L. monocytogenes* is unusual in that it contains 15 members of this family, at least one of which, (*prfA*), is known to be important for regulating several virulence molecules. We have performed a preliminary screen of transposon mutants for each of the members of the *crp/fnr* family and find that some of them do differ in their virulence in the mouse model. Dr. Uhlich is preparing deletion mutants to better address the role of these molecules in the pathogenesis of *L. monocytogenes*.

We have clearly identified that the *prfA* mutant does not cause systemic infection following gastrointestinal inoculation of mice, although it survives in

the gastrointestinal tract at levels comparable to those of the wild type parent strain. We also occasionally find bacteremia with this mutant, which with other strains we usually see only when there are substantial numbers of organisms present in internal organs. We have confirmed, as recently reported by others, that *L. monocytogenes* can survive and multiply in the gall bladder in mice. It appears to be growing extracellularly within the lumen of the gall bladder, and we do not obtain any evidence of invasion of the gall bladder tissue or an inflammatory response at that site. This finding raises the question of whether the gall bladder might be a site for listerial multiplication between the time of exposure and the onset of clinical disease in humans. Immunizing mice with the *prfA* mutant generates some protection against systemic infection following i.g. challenge with wild type *L. monocytogenes* but has little effect on the numbers of *L. monocytogenes* recovered from either the gall bladder or the gastrointestinal tract.

Finally, we have collaborated with Dr. Brien Neudeck (formerly of the UW-Madison School of Pharmacy, now at Univ. Tenn. Med. Ctr.) to investigate the role of the ATP dependent intracellular pump P-glycoprotein (P-gp) in innate immunity to listeriosis.

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Research In This Issue

Listeriosis Research

LaeA, A Global Regulator of Fungal
Secondary Metabolites (Research on
Control of Aflatoxin)

The FRI Applied Food Microbiology and
Safety Laboratory

Gene Regulation in *E. coli* O157:H7

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P-gp is involved in removal of drugs from mammalian cells; we were interested in whether it might facilitate export of listerial proteins involved in invasion of intestinal epithelial cells. We have evidence that P-gp influences the ability of *L. monocytogenes* to invade intestinal epithelial cells *in vitro* and to translocate across the gastrointestinal tract to the spleen and liver *in vivo*. Over-expression of P-gp diminishes the ability of the organism to invade intestinal epithelial cells, and addition of an inhibitory antibody enhances invasion and multiplication. Likewise, P-gp knockout mice are more susceptible to early invasion by *Listeria monocytogenes*.

These studies suggest that environmental, bacterial, and host derived factors all can influence the pathogenesis of gastrointestinal listeriosis. We will soon embark on a collaborative venture with Dr. Neudeck and Dr. Sophia Kathariou at North Carolina State University, in conjunction with Dr. Luchansky, to investigate the role of surface proteins of *Listeria monocytogenes* serotype 4b on virulence and intestinal cell invasion *in vitro*.

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LaeA, a Global Regulator of Fungal Secondary Metabolites (Research on Control of Aflatoxin)

Research in the Keller lab has resulted in the identification of a transcriptional regulator of secondary metabolism in *Aspergillus* spp. LaeA was identified through complementation of an *A. nidulans* sterigmatocystin mutant (sterigmatocystin is the penultimate precursor in the aflatoxin pathway). Sequence analysis and cellular location of LaeA suggest it to be a nuclear protein methyltransferase with most similarity to arginine and histone methyltransferase, a class of eukaryotic enzymes involved in transcriptional regulation.

Disruption of *laeA* ($=\Delta laeA$) resulted in a strain unable to produce sterigmatocystin. However, examination of the $\Delta laeA$ mutant showed it also was deficient in the production of multiple hyphal metabolites but not in spore pigment production. An identical phenotype was encountered in an *A. fumigatus* $\Delta laeA$ strain where loss of *laeA* resulted in a strain unable to produce gliotoxin in any growth media examined. Dependent on type of growth medium used, *A. fumigatus* $\Delta laeA$ also showed decreased production of several putative mycotoxins including helvolic acids,

fumigaclavines, tryptoquivalines, fumiquinazolines, pseurotins, fumagillin, fumitremorgins, verrucologen and TR-2 (Frisvad, Nielson and Keller, unpublished data). Transcriptional analysis of *A. nidulans* and *A. terreus laeA* mutants showed that LaeA transcriptionally regulated secondary metabolite gene expression so that loss of *laeA* led to a decrease in expression; over expression of *laeA* led to an increase in gene expression and product formation. Both penicillin and lovastatin production was increased in the over expression *laeA* strains. This latter property of LaeA has allowed us to successfully use LaeA mutants to identify novel secondary metabolite gene clusters in *A. nidulans* via microarray analysis.

Further genetic studies of *laeA* in *A. nidulans* showed it to be negatively regulated by two signal transduction pathways, RasA and the cAMP/protein kinase A pathway. Interestingly, the protein is conserved in filamentous fungi but is not present in *Saccharomyces cerevisiae*, a fungus devoid of secondary metabolites. We proposed a conserved role for LaeA in global regulation of secondary metabolite gene clusters in filamentous fungi.

Reference: Bok, J.-W., and N. P. Keller. LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Euk. Cell* 3:527–535 (2004).

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The FRI Applied Food Microbiology and Safety Laboratory

FRI has long recognized the importance of maintaining a dedicated research laboratory for transferring basic research findings to practical solutions for controlling microbial pathogens and toxins in processed food. Since its inception the scientific data generated by the FRI Applied Food Microbiology and Safety Laboratory (AFMSL) has had a worldwide impact on food processing. For example, AFMSL developed the methods used to ensure the safety of process cheese spreads, as well as for ensuring the safety of low-nitrite/no-nitrite bacon. Recent AFMSL research has extended the previous cheese spread work to the important area of low-fat and other nonstandard process cheeses; the findings from this research were unexpected and have led to process and composition recommendations.

Accordingly, AFMSL is an integral part of FRI, and it holds a prominent place in our strategic plans.

The primary objective of the AFMSL is to identify factors that inhibit growth and toxin production of

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pathogens in foods; this assists food manufacturers and regulators in making decisions on safe food production. AFMSL collaborates with food manufacturers to identify combinations of food safety hurdles for formulation-safe foods. These include identifying how food components affect the efficacy of antimicrobials; identifying combinations of intrinsic and extrinsic factors that influence the growth of pathogens in foods; developing models for formulation-safe foods; and verifying food safety systems by screening model food systems, including pathogen challenging of foods.

AFMSL collaborates with federal and state food regulatory officials to evaluate risks. In this way AFMSL provides scientific evidence to support sustaining or in some cases changing food regulations. AFMSL serves as a liaison between food manufacturers and regulators regarding interpretation of regulatory issues and is recognized as a process authority for formulation-safe low-acid canned foods, using the results of sponsored research as documentation.

Funding from government agencies, industry consortia, and individual food companies support AFMSL. In addition to generating dependable research findings, AFMSL provides opportunities for students to gain practical food microbiology skills as they develop their careers in areas such as quality assurance and food safety. A key aspect of AFMSL is its timely responsiveness to sponsors, government agencies, and university faculty in the resolution of special situations and other urgent food safety concerns. AFMSL maintains high visibility for FRI through participation in professional organizations, timely publication of significant research findings and presentations at meetings and workshops.

The AFMSL program is headed by Kathy Glass and overseen by Mike Pariza and Eric Johnson. Specific research projects include direction from FRI faculty members in their areas of expertise.

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Gene Regulation in *E. coli* O157:H7

The identification of cattle feeds and feed supplements that create intestinal tract conditions that are unfavorable to the proliferation, survival, or induction of stress-protection properties in *E. coli* O157:H7 has been an active area of research. Central to the success of this control strategy is an understanding of the signaling of environmental conditions to the internal machinery that regulates the bacterium's response to specific conditions. H-NS (histone nucleoid structuring protein) is one protein that is responsive to environmental conditions and controls over 50 genes in *E. coli*. As a result, H-NS plays an important role in metabolism and cell protection. Identification of the environmental conditions that influence H-NS levels and consequently the genes that it regulates can lead to new insights into persistent shedding of this pathogen in cattle and possible new intervention strategies. To this end, an H-NS mutant of *E. coli* O157:H7 was generated and its growth, metabolism, stress tolerance, and gastrointestinal passage compared to the parent strain.

The *hns* mutant grew slower, exhibited reduced cell size and was non-motile in comparison to the parent strain. Carbon and nitrogen metabolism was significantly altered in the mutant and was incapable of utilizing 42 carbon and 19 nitrogen sources that the parent strain metabolized. The inability of the mutant to utilize several intermediates of the TCA cycle indicates that energy generation is significantly compromised. The mutant was also more sensitive to bile salts and was capable of growth in 6.5% bile salts while the parent strain was able to grow in 15% bile salts. Acid tolerance was also evaluated in log and stationary-phase cells because H-NS is a known repressor of the alternative sigma factor, σ^S , in log-phase cells. Consequently, log-phase cells of the *hns* mutant were significantly more acid tolerant than the parent strain. In contrast, stationary phase cells of the *hns* mutant were significantly more acid sensitive. Mouse inoculation studies determined that the *hns* mutant was recovered at significantly lower frequency than the parent strain. These results collectively demonstrate that H-NS controls growth, metabolism, and survival properties of *E. coli* O157:H7 that influence the ability of this pathogen to survive gastrointestinal passage.

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WORLD LITERATURE

Antimicrobials Active During Refrigeration

One persistent problem for manufacturers of ready-to-eat refrigerated foods, including some vegetables, cheeses and cooked meats, has been contamination with *Listeria monocytogenes*. This bacterium is widespread in the environment and can grow at cold temperatures. If foods are contaminated after heat processing, *Listeria* may survive and even grow during refrigerated storage. While some bacteria produce compounds that inhibit the growth of other competitive bacteria, these compounds and the bacteria producing them are often inactive at cold temperatures.

Where would you look for bacteria that grow in the cold? How about Antarctica? A research group from the UK has done just this. They carefully collected 12 soil samples from three areas in Antarctica: high altitude soils (>500 m), low altitude soils (<500 m), and nutrient rich (ornithogenic) soils from areas where penguins or other birds nested. Samples were stored at -80°C and later incubated in marine broth, dilute tryptone soy broth, and LB broth at 5 and at 10°C.

Relatively high bacterial counts were obtained from all four ornithogenic soils with all media. For low altitude maritime soils, only marine broth supported significant growth, indicating that halotolerant organisms dominated in this soil type. Under most conditions, lowest bacterial counts were obtained from the high altitude soils. Counts of viable bacteria were much lower after incubation at 5°C than at 10°C.

Over 5000 colonies from these soils were picked at random and screened for antimicrobial activity against two spoilage organisms and *Listeria innocua*. Although more bacteria grew from the ornithogenic soils, the best sources of bacteria producing antimicrobial compounds were the low altitude soils. Of the 576 colonies grown at 5°C, only four appeared to have antimicrobial effects during the initial screening. Upon re-screening, none tested positive. Of the colonies grown at 10°C, 74 were positive at the initial screen and 13 of those tested positive after re-screening. Further testing of these bacteria against eight pathogenic or food spoilage bacteria revealed that none was active against *Salmonella enteritidis* or *E. coli* O157:H7. Eight colonies exerted antimicrobial effects against *Listeria innocua* and *Staphylococcus aureus* at 10°C while only four strains were active against *Listeria monocytogenes* at 10°C.

Further characterization of some of the isolates identified two of the *L. monocytogenes* inhibitors as members of the genera *Arthrobacter* and *Planococcus*. Optimal growth temperatures for these bacteria were 21 and 24°C but both also grew at 5 and 10°C in media containing 5 or 10% salt. The inhibitors produced by both of these isolates were not sensitive to catalase, lipase, or α -amylase. One strain produced an inhibitor sensitive to proteases while the inhibitor produced by the other strain was not affected by trypsin or pronase.

Further research is required to determine whether any of these bacteria may be useful in controlling bacterial growth in refrigerated foods. These results do indicate that the Antarctic and other natural cold environments are an untapped source of new antimicrobial compounds.

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PRESENTATIONS & POSTERS

R. K. Bush presented “Clinical responses to low dose food allergen challenges” at the IFT Annual Meeting, July 15, 2004, in Las Vegas.

Amy C. L. Wong presented “Overview of biofilms in food processing environments” in the Symposium entitled “Biofilms in the Food Environment: Current Approaches and Findings” held July 14 at the 2004 IFT Annual Meeting in Las Vegas.

Jaehyuk Yu presented “Negative control of asexual sporulation in *Aspergillus nidulans* at the Gordon Research Conference, Cellular and Molecular Fungal Biology, held at Holderness School, Plymouth, NH, in June 2004.

Poster presented at the 2004 Annual Meeting of the International Association for Food Protection, Phoenix, Arizona, August 8–11, 2004: “Control of *Listeria monocytogenes* in Wiener and Turkey Slurries by Combinations of Antimicrobials,” **Kathleen Glass, Jeffrey Veessenmeyer, Lindsey McDonnell, Patrick Eimerman, and Eric Johnson.**

PERSPECTIVE

Symposium on U. S. Food Law and Regulations

Food Research Institute and the University of Wisconsin Department of Food Science sponsored a two-day symposium in Madison in June 2004 on U.S. food laws and regulations. Food laws are complex, and FDA and USDA frequently issue new regulations refining existing laws. During this meeting food law professionals from the federal and state governments, industry, and academia addressed important issues in manufacturing, product development, and labeling, and presented an overview of the history and structure of food law and regulations to provide a context for understanding current regulations.

Overview of History and Structure of Food Laws.

A brief history of food law in the U.S. was presented by Ann Boeckman (Hogan & Hartson L.L.P.). From the foundation of USDA in 1862, food laws evolved through the "Pure Food Movement," "Poison Squad Experiments," the Pure Food and Drug Act of 1906 to the Federal Food Drug and Cosmetic Act (FD&C Act) of 1938 and the meat and poultry inspection acts which are the basis for current laws. Several major statutes have been enacted and many government agencies, including USDA, EPA, Federal Trade Commission, and Departments of Health and Human Services (including FDA), Commerce, Treasury, Defense, and Homeland Security, regulate food safety with rules addressing adulteration and misbranding. In addition, states also have agencies to protect the safety of foods. Regulations vary depending on whether a product's intended use is as a food or a drug and cover all stages of manufacture: research and development, production, marketing, and post-marketing.

The FD&C Act defines all aspects of food and food components and establishes appropriate safety standards for marketing, procedures for determining safety, burdens of proof, and prohibitions and penalties. Decision-making by FDA regarding the safety of a particular use of a food or food component relies on food laws and scientific principles. Alan Rulis (CFSAN, FDA) discussed this process with regard to food additives, food contact materials, contaminants, carcinogens, dietary supplements, macro-ingredients such as fat substitutes, and biotechnology-derived foods. Safety decisions should be based on an evaluation of data to determine whether there is a reasonable certainty that no harm to health will result.

A comparison of safety evaluation at USDA and FDA was presented by Patricia Kaeding (LaFollette Godfrey and Kahn). Procedures for inspecting food

production plants and animal production facilities were described. If problems are identified, a specific sequence of notification and enforcement activities will result. Responsibilities of these two agencies in dealing with issues related to Bovine Spongiform Encephalopathy and to genetically modified foods were also described.

Advances in technology have created new processing techniques, new food products, and detection methods with enhanced sensitivity. Allen Matthys (National Food Processors Association) discussed the impact of such advances on the application of food laws and regulations. Specific issues considered were regulation of irradiation as a food additive rather than as a processing procedure, methods for rapid detection of allergens, significance of acrylamide and furan in heated foods, validity of GMPs, and the relationship between international laws and requirements and U.S. food laws.

Product Development and Labeling. The Nutrition Labeling and Education Act (NLEA) was passed in 1990 with final regulations published in 1993. Nutrition labeling is required for nearly all processed foods and is voluntary for fresh fruits and vegetables, and raw seafood. According to Mary Brandt (CFSAN, FDA), FDA considers food labels an educational tool that provides information on ingredients, nutrition, health claims and food safety. CFSAN conducts Food Label and Package Surveys (FLAPS) to monitor compliance with regulations.

Regulatory requirements for food label design were described by Mike Dueber (Kraft Foods). These include mandatory information: statement of identity, net quantity, nutrition facts, list of ingredients, and name and place of business of manufacturer. In addition, USDA also requires USDA inspection legend and establishment number. There are position and typography requirements for these different elements. Nutrition and health claims are optional, but if used there are additional requirements both for content and positioning on the label.

Nutrition information on food labels can be an important resource for consumers concerned about nutrition and health. Wendy Reinhardt (International Food Information Council) described results of consumer research done by IFIC indicating that 54% of respondents started purchasing particular products

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PERSPECTIVE CONTINUED

because of nutrition information on a label, and 79% look for and purchase low fat products. Current nutrition issues, including trans fats, low carbohydrate diets, and health claims offer both challenges and opportunities for communication via food labels. Wording of health claims on labels, such as “reduces risk for osteoporosis” vs. “promotes bone health,” may influence effectiveness of message.

Following these presentations, John Norback and Monica Theis (UW Food Science) organized a breakout session where teams of 4–5 people used their knowledge of regulatory requirements to design a label for a newly formulated product that complies with federal law and regulations on format and content.

Manufacturing Issues. Food manufacturing companies must comply with numerous FDA, state, and local regulations designed to ensure production of safe food products. Third-party audits as described by Bill Schwartz (NSF International) can assist companies to register with FDA and to set up facilities and processes that comply with the Code of Federal Regulations (21 CFR 110). In addition companies should make plans for potential recalls, visits from inspectors, and ensuring food security by preventing deliberate acts of sabotage.

USDA, through its Food Safety and Inspection Service, regulates production of meat products, and its inspectors are in plants 24 hours a day. John Weisgerber (Weisgerber Consulting) reviewed some of

the common misconceptions and conflicts arising between facility management and FSIS staff. The goal of both government and industry is the production of safe food with a reasonable profit. Cooperation between companies and federal and state inspectors is necessary.

Any food company may need to recall product at some time. Recalls are generally rare, but plant operators should develop recall plans and strategies and documented standard operating procedures. Mike Barnett (WI Dept. of Agriculture, Trade, and Consumer Protection) discussed important considerations in managing food recalls with reference to some recalls in Wisconsin.

Mark Dopp (American Meat Institute), in a presentation on recognizing and meeting food safety requirements, indicated that there are three important components. There are criminal matters because it is a felony to produce, ship, transport, or sell adulterated products. A felony conviction can severely damage the future of a business and of the persons responsible for the violation. There are regulatory/operational issues including noncompliance records and rules of practice. Finally, there is product liability if consumers are harmed by the defective product.

Summary prepared by Ellin Doyle

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SHORT SUBJECTS

Glass Assumes Presidency of International Association for Food Protection

Kathleen A. Glass, Assistant Scientist at the University of Wisconsin–Madison Food Research Institute, assumed the presidency of IAFP in August. Dr. Glass earned her Doctorate in Food Microbiology and Safety at the University of Wisconsin–Madison, and holds a B.S. in Biology, Secondary Education from the University of Wisconsin–Eau Claire, and M.S. in Microbiology from Northern Illinois University in DeKalb, IL. She joined the Food Research Institute in 1985 where she designs and coordinates microbial challenge studies and assists the food industry in developing formulation-safe foods. Dr. Glass has been a Member of IAFP and its Wisconsin Affiliate (WAFP)

since 1990. She has served on many committees and has organized and chaired numerous Annual Meeting symposia as well as presented technical papers. In addition, Dr. Glass has served as president of WAFP and chair of the Wisconsin Joint Educational Conference.

The International Association for Food Protection is a non-profit educational association of food protection professionals. The Association is dedicated to the education and service of its members, specifically, as well as industry personnel. The Association provides members with an information network and forum for professional improvement through its two scientific journals, *Journal of Food Protection* and *Food Protection Trends*, educational Annual Meeting, and interaction with other food safety professionals.

PERSPECTIVE CONTINUED**Relevant Regulatory and Analytical Challenges for Food and Dietary Supplements**

A second annual symposium was organized by the Food Research Institute and Covance to discuss key scientific and regulatory topics currently facing food and supplement manufacturers. The meetings were held on Aug 31–Sept 1, 2004, and featured speakers from the University of Wisconsin, FDA, USDA, industry, and trade associations.

One driving force behind many new product formulations and supplements is the current obesity epidemic. Susan Nitzke (UW) presented an overview of this problem and discussed proposed changes in dietary guidelines, significance of glycemic index, and the recent trend towards consumption of low carbohydrate diets. Although hundreds of “low carbohydrate” foods have been introduced in the past few years, there are no regulations or standards for labeling these products to indicate relative carbohydrate content. Jeanne Rader (FDA) discussed the current regulatory situation and also analytical methods used for determining carbohydrate content. Another challenge is the definition and determination of “net carbohydrate content” or available carbohydrate minus the indigestible carbohydrate fiber.

Daryl Sullivan (Covance) discussed issues related to accurate carbohydrate analysis and described a new method for the direct determination of “glycemic” or “net” carbohydrates in foods and supplements. Current issues in the analysis of fiber were reviewed by Jon DeVries (General Mills). An updated definition of fiber may be needed because some compounds previously thought to be digestible are now known to be indigestible. New methods and modified methods may be required to accurately measure all types of fiber compounds.

Producers of low carbohydrate foods are eager to have low carbohydrate claims defined by FDA. Mark Nelson (Grocery Manufacturers of America) explained that companies want to respond to customer demands and to have a consistent target for compliance. GMA has proposed specific amounts of carbohydrates that could be used to define terms such as “low in ...,” “a good source of ...,” and “reduced ...” carbohydrates. Proteins may be used as replacements for carbohydrates in some conventional foods. Steve Rittmanic (Future

Beverages Inc.) described considerations for ingredient selection and processing conditions when using different proteins in new formulations.

Another dietary component soon to make its appearance on food labels is trans fatty acid. Gary List (USDA) reviewed the chemistry and structure of fats and fatty acids, the health effects of trans fats and saturated fats, and foods containing trans fats. In response to upcoming labeling requirements, some food products have been reformulated and efforts are underway to minimize trans fat formation during hydrogenation and to find alternatives for trans fats.

A session devoted to dietary supplements was led off by Paul Coates (NIH) who summarized some recent government research on supplements and outlined the proposed list of new supplements to be investigated. Analytical methods and standard reference materials are needed to analyze the estimated 30,000 to 50,000 supplement products now commercially sold.

FDA is close to issuing GMPs for dietary supplement companies which are regulated differently from foods and from drugs. Steven Dentali (American Herbal Products Association) discussed the proposed GMPs with their provisions for ensuring the identity, purity, quality, strength and composition of supplements. Some of these proposed regulations will be an economic hardship for small companies. James Roza (NOW Supplements) discussed compliance auditing in a GMP environment. Impact of the proposed regulations on the industry and difficulties in achieving compliance were considered.

Analyzing supplements is a challenge because active and/or characteristic compounds in many botanicals have not yet been identified. Libby Fitzgerald, a consultant to the supplement industry, discussed analytical methods in terms of validity and robustness. Only a few methods have been rigorously examined by AOAC but a number of other methods have been reported by USP (US Pharmacopeia), AHP (American Herbal Pharmacopeia), and INA (Institute for Nutraceutical Advancement). Another issue to be addressed by future research is the bioavailability of the active ingredients in supplements.

Summary prepared by Ellin Doyle