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Technologies and experimental approaches at the National Institutes of Health Botanical Research Centers

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Technologies and experimental approaches at the National Institutes of Health Botanical Research Centers[1–4]

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ABSTRACT

Many similarities exist between research on combinatorial chemistry and natural products and research on dietary supplements and botanicals at the National Institutes of Health (NIH) Botanical Research Centers. The technologies used at the centers are similar to those used by other NIH-sponsored investigators. All centers rigorously examine the authenticity of botanical dietary supplements and determine the composition and concentrations of the phytochemicals therein, most often by liquid chromatography–mass spectrometry. Several of the centers specialize in fractionation and high-throughput evaluation to identify the individual bioactive agent or a combination of agents. Some centers are using DNA microarray analyses to determine the effects of botanicals on gene transcription with the goal of uncovering the important biochemical pathways they regulate. Other centers focus on bioavailability and uptake, distribution, metabolism, and excretion of the phytochemicals as for all xenobiotics. Because phytochemicals are often complex molecules, synthesis of isotopically labeled forms is carried out by plant cells in culture, followed by careful fractionation. These labeled phytochemicals allow the use of accelerator mass spectrometry to trace the tissue distribution of ^{14}C-labeled proanthocyanidins in animal models of disease. State-of-the-art proteomics and mass spectrometry are also used to identify proteins in selected tissues whose expression and posttranslational modification are influenced by botanicals and dietary supplements. In summary, the skills needed to carry out botanical centers' research are extensive and may exceed those practiced by most NIH investigators. *Am J Clin Nutr* 2008;87(suppl):476S–80S.

KEY WORDS Activity-guided fractionation, bioavailability, isotopic labeling of phytochemicals in plant cell culture, DNA microarray analysis, 2D-gel electrophoresis, peptide mass fingerprinting, tandem mass spectrometry

INTRODUCTION

Under the provisions of the Dietary Supplement Health and Education Act, dietary supplements in use on or before 25 October 1994, may be sold in the United States (1). One provision of the Act was the creation of the Office of Dietary Supplements (ODS) at the National Institutes of Health (NIH) to promote scientific study on dietary supplements. As part of this mandate, the ODS supports several NIH Botanical Research Centers whose purpose is to conduct state-of-the-science research on the efficacy, safety, and mechanisms of action of botanicals and other ingredients used in dietary supplements. Research on botanical supplements in centers therefore represents a particularly complex set of endeavors. The purpose of this presentation is to describe some of the complexities involved in studying botanical ingredients and to show how research in the botanicals centers is being carried out and is setting new standards for all of NIH research.

FOODS, DIETARY SUPPLEMENTS, BOTANICALS, AND DRUGS

The similarities and differences among foods, dietary supplements, botanicals, and drugs must be distinguished. They are all

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xenobiotics. *Foods* are plants, animals, and other previously living forms that are safe although they are not necessary nutritious. *Dietary supplements* are food-derived and are also generally regarded as safe under conditions of use although they are not necessarily nutritious. *Botanicals* and their extracts used as ingredients in dietary supplements are generally associated with alleged improvements in health and risk of disease; they are assumed to be safe although they may also have unanticipated toxic effects when consumed as dietary supplements rather than under conditions of use associated with traditional medicine applications. Unlike vitamins and minerals, botanical supplements are rarely significant sources of nutrients. *Drugs* are designed to be efficacious for specific health uses and safety is evaluated in the context of risk-benefit relations. They are rarely significant sources of nutrients.

THE COMPLEX NATURE OF DIETARY SUPPLEMENTS AND BOTANICALS

Much of NIH-funded research is characterized by a need to understand the molecular or mechanistic basis of disease and of the agents that alleviate or treat the disease. To achieve these goals, an emphasis is placed on reducing complex problems to manageable sizes—a reductionist approach. A criticism often cast at dietary supplement research is that a dietary supplement is not a single compound nor is its composition tightly controlled. However, its complex nature may be its strength (2). It may contain multiple compounds with primary bioactivity as well as adjunct bioactivity (ie, properties that enhance the delivery or activity of the bioactive components). An example of this is (-)-epigallocatechin-3-gallate (EGCG), the most bioactive component of green tea. On its own, EGCG is unstable and readily degraded before it is consumed, well before reaching its biological target. It has greater biological activity in green tea than alone because of the presence of other catechins (3). Investigators outside of botanicals research are familiar with the need to provide carriers for bioactive agents.

FRACTIONATION, BIOACTIVITY, AND BIOAVAILABILITY

In research being carried out at botanical centers, investigators first perform experiments to identify the principal bioactive components by fractionating extracts of the dietary supplement or botanical and then testing the activity of each fraction in a suitable cell culture model or high-throughput assay system. This approach is the same as carried out in drug companies and university research laboratories that examine compounds generated by combinatorial chemistry or from natural inedible products. Once compounds with possible bioactivity have been identified (whether from dietary supplements and botanicals or high-throughput assays), they are subjected to standard absorption, distribution, metabolism, and excretion experiments in small and then larger animals. Investigators also carry out dose-range experiments in the whole animal model and determine whether toxicity occurs with acute or long-term exposure. What distinguishes botanical centers research from other NIH research is that botanicals investigators then examine the activity of the whole dietary supplement at doses that deliver the same amount of the bioactive material as isolated from the supplement. This enables the investigator to assess whether other components of the dietary supplement or botanical matrix have synergistic effects.

TECHNOLOGIES IN THE BOTANICAL CENTERS

Activity-guided chemical fractionation

Fractionation of botanicals and dietary supplements is carried out at the University of Illinois-Chicago (to isolate compounds with estrogenic activity), Iowa State University (to isolate bioactive compounds from *Echinacea* and *Hypericum* species), and Pennington Biomedical Research Center-Rutgers University (to isolate compounds that suppress the metabolic syndrome). Identification of the bioactive compounds requires the use of reverse- and normal-phase HPLC, high-resolution mass spectrometry, ^{1}H- and ^{13}C-nuclear magnetic resonance spectrometry, and other modern analytic methods.

High-throughput analysis

The high-throughput methods used to find the bioactivities of phytochemicals in botanicals are identical to those used to examine synthetic drugs. For instance, at the University of Illinois-Chicago botanicals center, phytoestrogen activity is examined by using an in vitro receptor binding assay and cultured cell approach using a human breast tumor MCF-7 cell line with an alkaline phosphatase or luciferase reporter gene (4). The same center has used ultrafiltration mass spectrometry to assess the binding of phytoestrogens to estrogen receptors (**Figure 1**; 5).

Composition and quality control

Determining the composition of the dietary supplement preparations is vital to botanical centers research. To ensure that research studies use dietary supplements that reflect those available to the public, the National Center for Complementary and

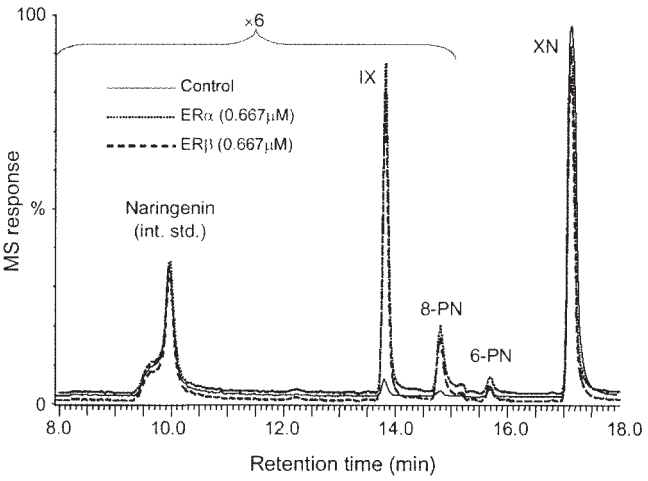

FIGURE 1. Ultrafiltration mass spectrometry (MS) screening of plant phytochemicals for their roles as ligands for estrogen receptor-α (ERα) and ERβ. This is a total ion chromatogram after liquid chromatography–negative ion electrospray ionization MS of ultrafiltrates obtained from incubations of ERα and ERβ with an extract of hops. The control chromatogram was obtained by using denatured ER. Compound XN, although giving strong signals, was bound to ERα and nonspecifically to ERβ. In contrast, compound IX and 8-prenylnaringin (8-PN) were high-affinity ligands. Int. std., internal standard. Reprinted with permission from Overk et al (4). Copyright 2005 American Chemical Society.

Alternative Medicine set up a working group to review the applications it receives (6). Applications that have inadequate analytic methods, do not have a product with known composition, or do not have plans to assess degradation are not approved for funding until these limitations are addressed.

Several of the botanical centers are engaged in clinical trials of botanical agents from the United States and other countries with different medical cultures. The Memorial-Sloan-Kettering Cancer Center specializes in materials used in Chinese traditional medicine. In such cases, much use is made of liquid chromatography–mass spectrometry (LC-MS) to validate the composition of the dietary supplements as well as the concentrations of the bioactive compounds in blood, urine, and other biological fluids and tissues.

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Most methods used to study the bioavailability, pharmacokinetics, and metabolism of specific compounds in dietary supplements and botanicals are based on the qualitative and quantitative use of LC-MS. These studies have been carried out in humans, in small and large animals, and in in vitro models. Richard van Breemen's group at the University of Illinois-Chicago used Caco-2 cells combined with LC-MS to estimate the rate of intestinal uptake and metabolism of specific plant phytochemicals (**Figure 2**; 7, 8). Chao-Chen Wang at the University of Alabama at Birmingham (UAB) applied a microsampling technique with LC-MS to determine the concentrations of genistein and its 7-*O*-β-D-glucuronide in the aqueous humor of rats fed genistein in the diet (**Figure 3**; 9). Elsa Janle at Purdue University applied an automated collection system to obtain blood samples from nonanesthetized, free-moving rats (10).

USE OF ACCELERATOR MASS SPECTROMETRY

The eclectic nature of the botanical centers in some cases allows for unusual combinations of methods. Investigators at the Purdue-UAB botanicals center are applying accelerator mass spectrometry to follow 2 rare radioactive isotopes (^{14}C and ^{41}Ca) in biological experiments. The extreme sensitivity of accelerator mass spectrometry allows for the detection of one ^{14}C or ^{41}Ca atom in 10^{15} atoms of nonradioactive carbon or calcium. Connie Weaver at Purdue University administered a single dose of ^{41}Ca

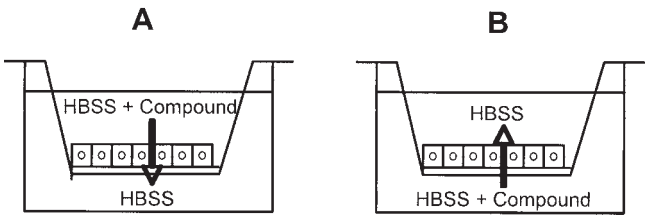

FIGURE 2. Use of Caco-2 cells to monitor the bidirectional intestinal transport of phytochemicals. The cells are first allowed to form a monolayer and tight junctions between the cells. This enables phytochemicals to be introduced on the luminal (A) or the serosal (B) side within the apparatus. The fluid from the opposite side to which the phytochemical is applied is analyzed by liquid chromatography–mass spectrometry to identify and quantify the phytochemical and its metabolites. HBSS, Hank's balanced salt solution. Reprinted with permission from Li et al (8).

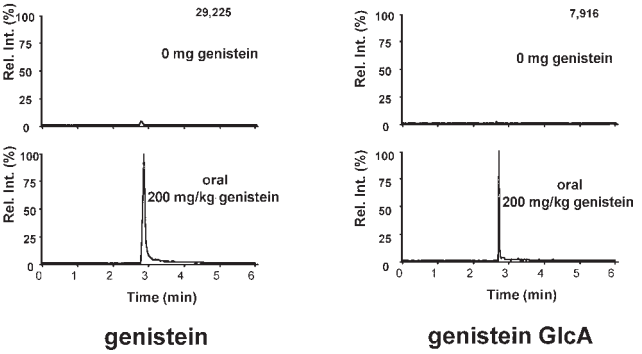

FIGURE 3. Isoflavones in the aqueous humor of the eye. Rats were fed either an isoflavone-free AIN-76A diet or one supplemented with 400 mg/kg genistein. The aqueous humor was removed from the eye under ketamine-xylazine anesthesia and was analyzed by electrospray ionization mass spectrometry with a microfluidics approach. Parent ion-daughter ion transitions were used to detect genistein (*m/z* 269/133) and its 7-*O*-β-glucuronide (genistein GlcA; *m/z* 445/269). The full-scale intensities are given in the upper right corner of the ion chromatograms. Rel. Int., relative intensity. Reprinted with permission from Barnes et al with permission from Elsevier (9).

to postmenopausal women to examine the effects of phytoestrogen dietary supplements on the turnover of calcium in bone; this study has been going on for 5 y in the same subjects (11). Mary Ann Lila, at the University of Illinois-Urbana-Champaign, manufactures ^{14}C-labeled complex phytochemicals such as proanthocyanidins with plant cell culture systems (12). Although these can be used in conventional radiotracer experiments to assess uptake and metabolism, the extreme sensitivity of AMS allows for careful tissue-by-tissue analysis of proanthocyanidin metabolism using only very small doses (≈50 nCi).

PROTEOMICS AND PROTEIN MASS SPECTROMETRY

The combination of dietary supplements and botanicals research with modern proteomics and protein MS for several years seemed to many an oxymoron. However, if dietary supplements are to have biological action, they will affect protein abundance and activity. Helen Kim and colleagues at UAB fed rats a diet containing grape seed extract for 6 wk and identified 14 brain proteins that were changed by grape seed extract (13). They applied a combination of 2D-isoelectric focusing and SDS-PAGE resolution of the brain proteins, image analysis of stained gels, peptide mass fingerprinting using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), and peptide sequencing by LC-tandem MS (**Figure 4**). These studies identified creatine kinase as a candidate brain protein undergoing changes in posttranslational modifications that were induced by grape seed extract.

PROFILING AND IMAGING TECHNOLOGIES

Tissue profiling and imaging are other MS applications being carried out in botanical centers. Stephen Barnes at UAB is examining the spatial changes in protein expression and modification in the lens as part of a project on the role of phytochemicals in the formation of cataracts. The lens proteins are unique in that they are synthesized once and remain in the lens from womb to tomb. Compounds that alter the rate of light-induced singlet oxygen production in the lens may protect or enhance oxidation

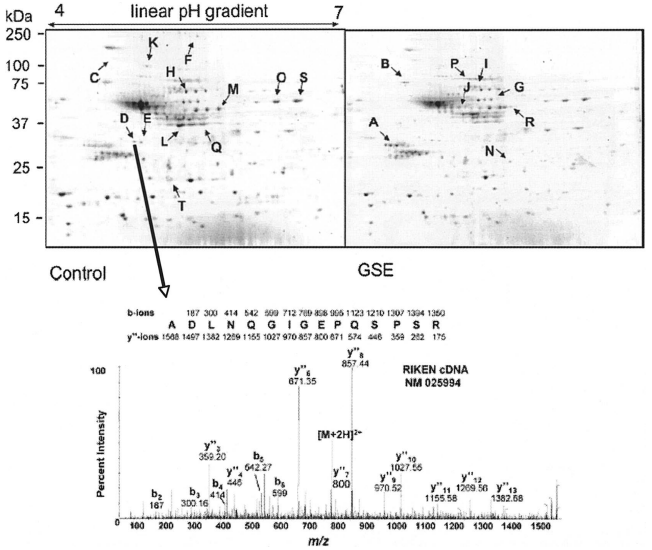

FIGURE 4. 2D-proteomics mass spectrometry for protein discovery. Young female rats were placed on an AIN-76A diet (controls) or an AIN-76A diet supplemented with 5% grape seed extract for 6 wk. Whole brains were harvested and homogenized. After solubilization in 7 mol/L urea–2 mol/L thiourea, clarified extracts were subjected to 2D isoelectric focusing (pH 4–7) sodium dodecyl sulfate–polyacrylamide 10–20% gradient gel electrophoresis. The gels were fixed and then stained with colloidal Coomassie blue. Protein spots were visualized by densitometry. Protein differences (spots A–N) were determined by using BioRad (Hercules, CA) PDQuest imaging software. Individual spots were cut out of the gel and subjected to trypsin digestion. The resulting tryptic peptides from spots D and E were analyzed by peptide mass fingerprinting (not shown) and by tandem MS. Manual interpretation of the tandem MS spectrum confirmed that the protein was one for which there was a matching record in a genomic database. Adapted with permission from Deshane et al (13) Copyright 2004 American Chemical Society.

and oligomerization of the lens proteins and hence cause the loss of their chaperone function that maintains protein solubility. These experiments are being carried out by using MALDI-TOF MS (**Figure 5**) and high-resolution Fourier transform-ion cyclotron resonance MS (**Figure 6**).

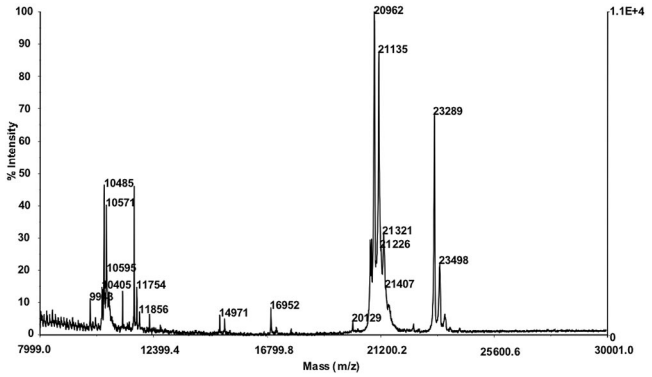

FIGURE 5. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) profiling of proteins from the nuclear region of the lens of an ICR/f rat. A portion of the nuclear region of the lens without visible cataracts was homogenized in 10 μL of 10 mmol/L tris buffer and centrifuged to yield a soluble protein fraction. The solution (1 μL) was mixed with 9 μL of saturated sinapinic acid in 50% aqueous acetonitrile. A 1-μL aliquot was spotted onto a MALDI target plate and allowed to dry. The plate was introduced into the MALDI-TOF MS. Protein ions were generated by use of a pulsed N_2 laser operating at 337 nm and 2–3 Hz. Calibration of the protein molecular weights was carried out by using a carbonic anhydrase standard.

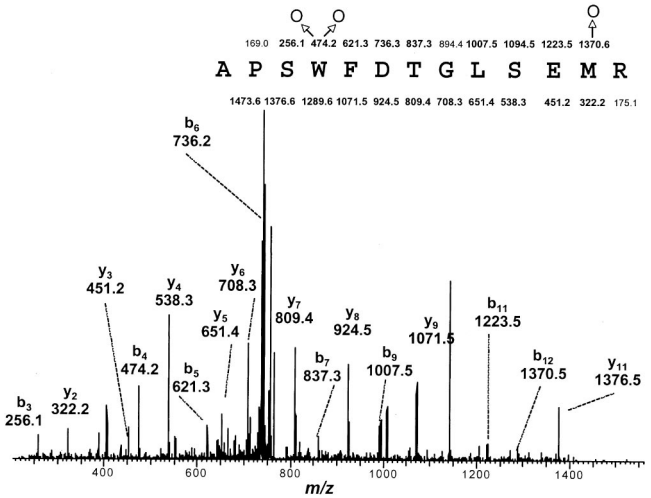

FIGURE 6. Tandem mass spectrum of an oxidized tryptic peptide derived from recombinantly expressed human αB-crystallin. A desalted tryptic digest (1 μL) was resolved by nanoLC (Eksigent, Dublin, CA) on a 15 cm × 75 μm internal diameter reversed-phase C_{18} column with a linear gradient of 5–95% acetonitrile in 0.1% formic acid at a flow rate of 200 nL/min. Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) analysis was performed on a linear quadrupole ion trap (LTQ) FT-ICR hybrid mass spectrometer (Thermo Electron, San Jose, CA). Eluted peptides were electrosprayed at 2 kV. Peptide fragmentation was induced by collision-induced dissociation in the ion trap and fragment ions were analyzed in the ICR cell. The LTQ FT mass spectrometer was operated in a "top 3" data-dependent acquisition mode. The mass spectrometer was set to switch between an FT-ICR MS full scan (200–2000 *m/z*) followed by successive FT-ICR MS single-ion monitoring scans and LTQ tandem MS scans of the 3 most abundant precursor ions in the FT-ICR MS full scan as determined by the Xcalibur software (Thermo Electron).

DNA ANALYSIS

Botanicals and dietary supplements may affect gene expression. DNA microarray analyses are being carried out by Ski Chilton's group at Wake Forest University to examine the effects of n−3 fatty acids in fish oil supplements to determine the biochemical pathways that undergo changes in regulation. As for many other areas of NIH clinical research, the effects of single nucleotide polymorphisms may segregate subjects consuming dietary supplements and botanicals into responders and nonresponders. Similarly, supplements may have their effects epigenetically by altering gene methylation (14) and by modulating acetylation-deacetylation (15, 16) and potentially lysine methylation-demethylation of histones.

SUMMARY

Research in the botanical centers demands the application of the best technologies in current use. The demands brought by the complex nature of the dietary supplements and botanicals are setting new standards that will have impact throughout NIH-funded research.

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Therapeutics Inc. NRF is a consultant to Pharmavite LLC. IR is a consultant for Phytomedics Inc. CMW is on the advisory boards of Pharmavite and Wyeth. She receives grant support from Wyeth and reviews research proposals for the United Soybean Board.

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