Biomarker Profiles of *Echinacea* Species Using Pressure Cycling Technology and MALDI-TOF Mass Spectrometry

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Abstract

Several species in the genus *Echinacea* are beneficial herbs popularly used for many ailments. Of the nine species of *Echinacea*, the three most popular for cultivation, wild collection, and the development of herbal products are: *E. purpurea* (L.) Moench, *E. pallida* (Nutt.) Nutt., and *E. angustifolia* (DC). Product adulteration is an issue of great importance in the natural products industry, where botanical misidentification and the potential for introduction of other botanical and non-botanical contaminants can occur in the formulation and production process. Rapid and cost-effective methods that can be used to determine complex product purity and consistency are of benefit to consumers and producers. The objective of this continuing research was to develop methods to differentiate *Echinacea* species by their MALDI-TOF mass profiles and to evaluate off-the-shelf products containing *Echinacea*. MALDI-TOF has been successfully used to produce protein biomarker profiles for rapid typing of many bacteria species, viruses, and other microorganisms. Pressure cycling technology (PCT) was used for rapid, efficient, and reproducible extraction of *Echinacea* leaves, roots, and products. Current methods for plant tissue extraction rely mostly on mechanical or chemical-based methods that can often cause shearing and stability problems with compound classes such as proteins. During the PCT process, cycles of pressure from ambient to high (35K psi) were used to release the cellular components of tissue and eliminated many of the problems associated with mechanical methods of extraction such as grinding. In these experiments, PCT extraction, followed by direct MALDI-TOF detection of small and large molecule biomarkers from plant tissue extracts, was evaluated as an alternative to more time-consuming and limited separation methods such as electrophoresis and high performance liquid chromatography (HPLC).

Illustration of Rudbeckia pourpre (*Echinacea purpurea* (L.) Moench, Eastern purple, eastern purple coneflower, purple coneflower). Source: http://ridgwaydb.mobot.org/mobot/rarebooks/

Introduction

Product quality, authentication, and adulteration in the botanical and dietary supplement industry are challenging issues. Many products are developed from cultivated plant materials that vary greatly in the content of putative active components. Factors known to alter phytochemical content in the field include growing conditions such as environmental stress and time of harvest coinciding with the plant's developmental stage.[1,2] The final chemical consistency of the product is an issue of importance that can not only affect customer satisfaction, but also possible toxicological aspects and clinical efficacy.

Source: Dean Gray, 2000

Species in the genus *Echinacea* (primarily *E. purpurea* (L.) Moench. and, to a lesser extent, *E. angustifolia* DC) are cultivated or wildcrafted for incorporation into various popular products sold and used as dietary supplements. While product quality and species identification can be determined from HPLC and thin layer chromatography (TLC), these analyses are time-consuming and generally require sampling and drying of large portions of the plant.

Rapid assays using near-infrared spectroscopy (NIR) have recently been developed for *Echinacea* spp. identification[3] and correlation to phytochemical concentration.[4,5] However, these techniques have not yet been proven on immature plants for the development of a predictive model for future phytochemical concentration at plant maturity, or to determine extraordinary cultivars.

Based on the growing success of techniques such as MALDI TOF mass spectrometry (MS) for determination of biomarkers in numerous plant and animal species, a feasibility study is in progress to evaluate this technique as a high-throughput and quality control assay using small amounts of plant tissue. For botanical studies, MALDI MS has potential not only for species identification and product adulteration issues, but also for rapid screening of immature plants for the construction of predictive models of phytochemical quantity and quality.

Study Materials and Instrumentation

All *Echinacea* samples were provided by the American Herbal Pharmacopoeia (AHP; Scotts Valley, CA; www.herbal-ahp.org). All samples were botanically authenticated by AHP staff.

Echinacea purpurea
Seed: Lot #44 (AHP) • Leaf/Stem/Flower: Lot #799 (AHP) • Root: Lot #010904 (AHP)
E. angustifolia
Seed: Lot #68 (AHP) • Leaf/Stem/Flower: Lot #826 (AHP) • Root: Lot #565 (AHP)
E. pallida
Seed: Lot #69 (AHP) • Root: Lot #291 (AHP)

Sundown *Echinacea*, 100 capsules, Lot #941006 01 05, Rexall Sundown, Inc., Boca Raton, FL USA. (*E. purpurea* aerial parts)

Nature's Resource *Echinacea*, 30 capsules, Lot #NK10696 10 06, Nature's Resource Products, Mission Hills, CA USA. (*E. purpurea* and *E. angustifolia* aerial parts extract)

Chemical Information

Tris Extraction Buffer
Contains: 50 mM Tris (using Trizma Pre-Set Crystals) - Sigma No. T-8943, pH 8.3
40 mM 2-mercaptoethanol - Sigma No. M-7154
1 mM Phenylmethanesulfonylfluoride (PMSF) - Sigma No. P-7626
80% Water, ≤ 18 mΩ, – Milli-Q
20% Methanol - Burdick and Jackson, Cat. No. 230-1

MALDI matrix
Contains: α-Cyano-4-hydroxy-cinnamic acid (CHCA) – Fluka No. 70990
Acetonitrile - Burdick and Jackson, Cat. No. 015-1
Water, ≤ 18 mΩ, – Milli-Q
Trifluoroacetic acid (TFA) – Fluka No. 91699

Instrumentation

Pressure Cycler:	Barocycler™ NEP2017 (Figure 1) Pressure Biosciences, Inc. (West Bridgewater, MA) Operated with a circulating chiller (Polyscience, Model 9506)
PULSE™ Tubes:	Pressure Biosciences No. FT00500
Bead beater:	Mini-BeadBeater-96 BioSpec Products, Inc. (Bartlesville, OK)
Vial rack:	1.5- to 2.0-mL tubes , BioSpec Products, Inc. Cat. No. 702ALU
Microtubes:	Screw-cap, conical, 2 mL, QSP, BioSpec Products, Inc. Cat. No. 10832
Beads:	Zirconia Beads, 2.4 mm, BioSpec Products, Inc. Cat. No. 11079110zx Stainless steel beads, 6.35 mm, BioSpec Products, Inc. Cat. No. 1079635ss
MALDI-TOF:	Applied Biosystems Voyager DE-STR

Figure 1. Pressure Biosciences Barocycler

- Pressure-enhanced extraction and purification number
- Rapid, automated, and inexpensive method of biomolecule extraction
- Used for a wide variety of cells and tissues
- Suitable for downstream analytical processes
- Compatible with a variety of solvents and extraction solutions for flexible applications
- Preserves proteins from either enzymatic or chemical degradation

Experimental Approach

Sample Preparation

Three types of plant tissue were studied: leaf/flower pieces, seed, and root. Roots were shaved to fine pieces using a razor blade prior to sample weighing. Samples of *Echinacea* supplements from Nature's Resources and Sundown were taken from capsules. All samples were weighed in either Barocycle PULSE tubes or 2.0-mL microtubes for approximately 7.5 mg of material (bead beating and Barocycler) or 100 mg of material (Barocycler).

Tris-based buffer was used for plant extractions (see "Chemical Information" section). Chilled lysis buffer (4°C) and phenylmethylsulfonyl fluoride (PMSF) were added to plant material in PULSE and 2.0-mL microtubes. PMSF serves to improve the yield of intact proteins by inhibiting endogenous enzymes, such as proteases and phosphatases, known to be present in crude cell extracts. Future work will use MALDI-TOF to examine peptides and proteins extracted from *Echinacea*. Samples were kept chilled (4°C) until extraction.

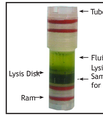
Bead-beating was used in this study for its ability to disrupt plant cells. Zirconia beads measuring 2.4 mm were added at an approximate volume of 100 µL to each microtube containing sample. Samples were loaded into a pre-chilled (-20°C), 48-position vial rack and secured in the Mini-BeadBeater-96. Bead-beating was performed for 2 minutes, then samples were centrifuged at 25,000 Xg, 4°C, for 5 minutes. Extraction of root samples was done with a stainless steel ball rather than zirconia beads. Stainless steel balls were washed with methanol before use.

Pressure Cycling

Pressure cycling was performed using the parameters listed in Table 1. Six samples were processed simultaneously, and the pressure cycling took approximately 10 minutes. Sample supernatants were transferred into 2-mL tubes and centrifuged at 25,000 Xg, 4°C, for 5 minutes. Figure 1 describes the Pressure Biosciences Barocycler NEP2017 that was used for this study. Figure 2 depicts a PULSE tube after the pressure cycling process was completed on ground leaf material.

Table 1. Pressure cycling parameters

Parameter	Value
Temperature	4°C
Pressure	35 Kpsi
Up time	10 seconds
Down time	10 seconds
Number of pressure cycles	3
Iterations per method	5



- Sample is fully contained in closed PULSE tubes.
- Hydrostatic pressure is applied to a closed chamber via piston in liquid medium (60% ethylene glycol).
- Pressure is transmitted uniformly and rapidly throughout the sample.
- Temperature can be controlled using a chiller.

Figure 2: Pressure Biosciences PULSE™ Tube for Leaf Extraction

MALDI-TOF of Small Molecule Fraction

Samples were diluted in extraction buffer 50:50 (v/v), or 20:80 (v/v) (roots only), then diluted 20:80 (v/v) with matrix α-cyano-4-hydroxy-cinnamic acid (CHCA). CHCA matrix solution was prepared as 10 mg/mL in 50:50 (v/v) acetonitrile:water, with 0.3% TFA. Sample/matrix mixture, 1.0 µL, was spotted onto a stainless steel 10x10 MALDI plate and air dried to obtain a homogenous co-crystal. The samples were analyzed using reflectron mode (mass resolution = 5,000) on an Applied Biosystems Voyager-DE STR MALDI-TOF mass spectrometer. Table 2 summarizes key MALDI-TOF parameters used.

Table 2. MALDI-TOF Instrument parameters

Parameter	Reflectron Mode
Extraction mode	Delayed
Polarity	Positive
Accelerating voltage	20,000 Volts
Grid voltage	62%
Mirror voltage ratio	1.12
Extraction delay time	100 nsec
Acquisition mass range	50 - 600 Da
Laser intensity	2250
Laser rep rate	20.0 Hz
Number of laser shots	30/spectrum
Number of spectra averaged	5

Results

Figures 3 and 4 represent the *Echinacea* purpurea and *E. angustifolia* spectra for seeds, leaves, and roots. Extract profiles were obtained using CHCA as the MALDI matrix, and the samples were analyzed in reflectron mode (*m/z* 50 to 500). Some profile variation was observed in the different species in this *m/z* range, although it is unclear at this time whether these variations are due to inter- or intra-species variability due to the small sample size. Of particular note are the similarities between root, leaf, and seed spectra between the *Echinacea* species. For example, a unique mass was observed in the seed spectra at 406.2 for *E. purpurea* and *E. angustifolia*. An additional mass apparently unique to the root tissue for all three *Echinacea* species was observed at *m/z* 175.1.

Figure 5 represents the *Echinacea* root extract profiles. A number of unique masses were observed in these spectra from species to species, including 304.2 and 466.2 for *E. pallida* and 337.2 for *E. purpurea*.

Figures 6 and 7 compare the two dietary supplement products to their corresponding authenticated *Echinacea* materials. The Sundown dietary supplement spectrum was very similar to the corresponding *E. purpurea* leaf spectrum (Figure 6). The spectrum for the Sundown dietary supplement also contained *m/z* 406.2, which was seen exclusively in the seed for *E. purpurea* and *E. angustifolia* (Figure 3 and 4), indicating that the product was probably not composed entirely of leaf tissue. The Nature's Resource dietary supplement spectrum was also very similar to the corresponding *E. purpurea* and *E. angustifolia* spectra (Figure 7). Several masses were observed that indicated the presence of leaf tissue in the dietary supplement product from both *Echinacea* species, in addition to the seed-related mass at *m/z* 406.2.

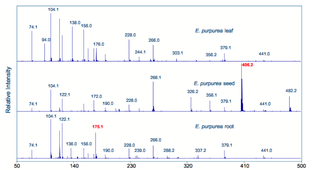


Figure 3. *Echinacea purpurea* leaf, seed, and root extracted with a bead beater.

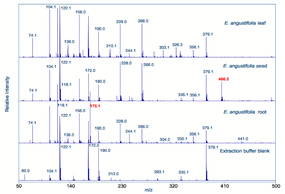


Figure 4. *Echinacea angustifolia* leaf, seed, and root extracted with a Barocycler.

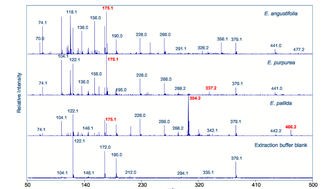


Figure 5. *Echinacea* root extracted with a bead-beater.

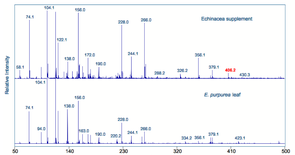


Figure 6. Barocycle-extracted *Echinacea* supplement from Sundown (*E. purpurea* aerial parts) and *E. purpurea* leaf.

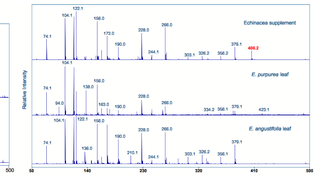


Figure 7. Barocycle-extracted of *Echinacea* supplement from Nature's Resource (dry extract of *E. purpurea* and *E. angustifolia* aerial parts) and leaves of *E. purpurea* and *E. angustifolia*.

Conclusions

This study demonstrated the utility of combining two rapid techniques for obtaining characterization profiles of small molecules: pressure cycling technology and MALDI-TOF mass spectrometry. The results of this study indicate that a range of molecular weight components are effectively extracted from plant tissue using PCT.

Although we are currently evaluating PCT and its application to plant proteomics, PCT has proven to be a rapid and reproducible means of sample extraction for complex plant and finished product matrices. Following PCT extraction, samples can then be directly analyzed using MALDI-TOF with very little further processing necessary. The combined techniques hold promise for use in species differentiation of *Echinacea*, product adulteration, and quality control assays.

Future work will focus on (1) increasing the yield of higher molecular weight components from the extracts for further discriminating power; (2) evaluating a greater number of samples from each species and finished products; and (3) evaluating multidimensional statistical techniques, including principal components and discriminant analyses to differentiate species, organs, and finished products.

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Acknowledgments

The authors would like to acknowledge the American Herbal Pharmacopoeia for donating the plants used in this study. The authors thank James Behnke at Pressure Biosciences, Inc., for loaning MRI the Barocyler used in performing this research.

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