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Streamlining Plant Sample Preparation: The Use of High-Throughput Robotics to Process *Echinacea* Samples for Biomarker Profiling by MALDI-TOF Mass Spectrometry

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Several species in the genus *Echinacea* are beneficial herbs popularly used for many ailments. The most popular *Echinacea* species for cultivation, wild collection, and herbal products include *E. purpurea* (L.) Moench, *E. pallida* (Nutt.) Nutt., and *E. angustifolia* (DC). Product adulteration is a key concern for the natural products industry, where botanical misidentification and introduction of other botanical and nonbotanical contaminants exist throughout the formulation and production process. Therefore, rapid and cost-effective methods that can be used to monitor these materials for complex product purity and consistency are of benefit to consumers and producers. The objective of this continuing research was to develop automated, high-throughput processing methods that, teamed with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis, differentiate *Echinacea* species by their mass profiles. Small molecules, peptide, and proteins from aerial parts (leaf/stem/flowers), seeds, and roots from *E. purpurea* and *E. angustifolia*; seeds and roots from *E. pallida*; and off-the-shelf *Echinacea* supplements were extracted and analyzed by MS using methods developed on the ProPrep liquid handling system (Genomic Solutions). Analysis of these samples highlighted key MS signal patterns from both small molecules and proteins that characterized the individual *Echinacea* materials analyzed. Based on analysis of pure *Echinacea* samples, off-the-shelf products containing *Echinacea* could then be evaluated in a streamlined process. Corresponding analysis of dietary supplements was used to monitor for product composition, including *Echinacea* species and plant materials used. These results highlight the potential for streamlined, automated approaches for agricultural species differentiation and botanical product evaluation.

Key words: *Echinacea*, MALDI-TOF MS, botanical products, automation, automated sample preparation, ProPrep.

Consistent product quality, botanical authentication, and identification of adulterants are ongoing challenges facing the dietary supplement industry. Botanical products are developed from cultivated and wild-crafted plant materials, which can vary greatly in the content of putatively active components, due in part to growing conditions and plant developmental stage.[1,2] Final product chemistry is determined not only by the quality and consistency of the starting material, but the subsequent handling, extraction and formulation.[3] The introduction of other botanical[4] and nonbotanical[5] contaminants can occur throughout this process.

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Species in the genus *Echinacea* (primarily *E. purpurea* [L.] Moench and to a lesser extent *E. angustifolia* DC) are used in literally thousands of dietary supplements. Product quality and species identification are generally determined using high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). Less utilized assays such as near-infrared spectroscopy (NIR) have also been developed for *Echinacea* species identification[6] and correlation to phytochemical concentration.[7,8] All of these techniques have particular strengths, although in general these methods are low throughput and require sampling and drying of relatively large portions of leaf or root.

Based on the growing maturity and success of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) for determination of biomarkers in numerous plant and animal species,[9,10]

TABLE 1

Study Materials and Formulation of the Selected Herbal Supplements

Plant material*		Rexall Sundown *Echinacea*	Nature's Resource Products *Echinacea*
E. purpurea	Leaf/stem/flower	X	X
	Seed		
	Root		
E. angustifolia	Leaf/stem/flower		X
	Seed		
	Root		
E. pallida	Seed		
	Root		

*All *Echinacea* samples were provided and authenticated by the American Herbal Pharmacopoeia (AHP; Scotts Valley, CA).

ongoing research has been conducted to evaluate this technique as a higher-throughput assay using relatively small amounts of fresh and dry plant tissue. For botanical product quality control, MALDI-TOF MS has the potential to address the most challenging issues of species and adulterant identification and product consistency, while also having the potential to support breeding programs and rapid plant screening for phytochemical quantity and quality.

A vital component of higher-throughput method development is sample preparation prior to MALDI-TOF MS analysis. Ideally, the sample extraction, handling, and spotting methods should be simple, reproducible, rapid, and automated. Not all current robotic systems are capable of streamlining sample dilution, transfer, and deposition on the MALDI plate, leading to more cost-effective, rapid, and reliable data generation. Therefore, there is a need for a user-friendly robotic system that allows for method development and method deployment flexibility. The objective of this continuing research was to develop automated, high-throughput methods to process samples and differentiate *Echinacea* species by their MALDI-TOF mass profiles. Based on analysis of *Echinacea* samples, off-the-shelf products containing *Echinacea* could then be evaluated in a streamlined process.

MATERIALS AND METHODS

Materials

Dried *Echinacea* materials including *E. purpurea* (L.) Moench, *E. pallida* (Nutt.) Nutt., and *E. angustifolia* (DC) seeds, roots, and aerial parts used in this study were donated by the American Herbal Pharmacopoeia. Off-the-shelf products included *Echinacea* supplements from Nature's Resource Products (Mission Hills, CA) and Rexall Sundown, Inc. (Boca Raton, FL). MALDI matrixes α-cyano-4-hydroxy-cinnamic acid (CHCA) and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA) were purchased from Fluka (Sigma-Aldrich Switzerland). Solvents included HPLC grade acetonitrile obtained from Honeywell Burdick & Jackson and sequencing grade triflouroacetic acid (TFA) from Fluka. A Milli-Q gradient water system provided molecular-grade water. Tris buffer and phenylmethanesulfonylflouride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO).

Botanical Sample Preparation

Three types of plant tissues—seed, root, and aerial parts (leaf/stem/flower)—and two commercial *Echinacea* supplements were analyzed in this study. The commercial supplements are compositions of the various *Echinacea* tissues analyzed, as indicated in Table 1. Samples were prepared in triplicate. Approximately 100 mg of each material was weighed out in 2.0 mL conical screw cap micro tubes. Roots were shaved prior to weighing. Samples of *Echinacea* supplements from Nature's Resource Products and Rexall Sundown, Inc., were extracted from capsules.

One 6.35-mm stainless steel ball (BioSpec Products, Inc., Bartlesville, OK), 500 μL of 50 mM Tris buffer (pH 8.3), and 1 μL of 1 mM PMSF were added to each sample tube. Tubes were sealed with o-ring caps and placed in a pre-chilled (–20°C) bead-beater sample block. Samples were extracted for 2 min using a mini-Bead Beater (BioSpec Products, Inc.) and centrifuged at 25,000 *g* at 4°C for 10 min.

Liquid Transfers and Sample Deposition

Following compound extraction and centrifugation, sample handling was performed on the ProPrep liquid handling robotic system (Genomic Solutions, Ann Arbor, MI)[11–14] and included supernatant extraction, liquid

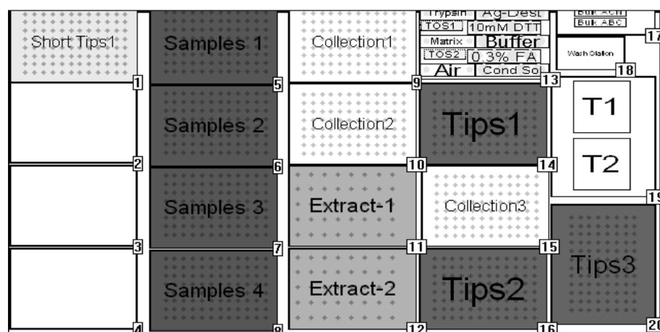

FIGURE 1

ProPrep Workstation configuration. Each position on the work table is assigned a defined type of plasticware. The name given to the plasticware in each position is used by the method as a position reference. This layout is designed for preparation of samples from two bead-beater sample blocks, extracts 1 and 2. Collection blocks 1, 2, and 3 are 96-well microtiter plates. MALDI sample plates are shown as T1 and T2. Tubes and reservoirs for solutions are also indicated.

transfer, mixing, and sample deposition onto MALDI target plates. The ProPrep workstation used in this procedure is presented in Figure 1. The position of sample well blocks, 96-well plates, MALDI plates, and solutions used during sample preparation are defined in two-dimensional space within the workstation diagram. Aspirating and dispensing of liquids was performed using the system's four permanent needle tips. To prevent cross-contamination, disposable tips were used for transferring the botanical samples. Chemical solution transfers were performed with bare tips. The sample-handling method used during sample preparation is outlined in Figure 2.

After centrifugation, samples were returned to the sample block and placed on the ProPrep system stage (Figure 1, Extract-1 configuration). A total of 30 μL of material extract supernatant was pipetted from each sample solution and transferred to a 96-well, polypropylene, conical bottom microtiter plate (Figure 1, Collection 1).

MALDI matrix solution (16 μL, 10 mg/mL CHCA in 50/50/0.1 acetonitrile/water/trifluoroacetic acid) was transferred into a 96-well plate (Figure 1, Collection 3). A sample aliquot, 4 μL, was sequentially transferred from each used well in Collection 1 to a unique well in Collection 3 containing MALDI matrix. The sample/matrix solution in the Collection 3 plate was mixed within the individual wells by aspirating and dispensing 10 μL of the solution (2X).

Sample/matrix solution (1.2 μL) was sequentially transferred from each used well in the Collection 3 plate to an individual well on the MALDI target plate (Figure 1, T1). The target plate was dried under ambient conditions and inserted into the mass spectrometer for analysis.

A second sample preparation procedure was performed using SA (20 mg/mL in 50/50/0.1 acetonitrile/water/tri-

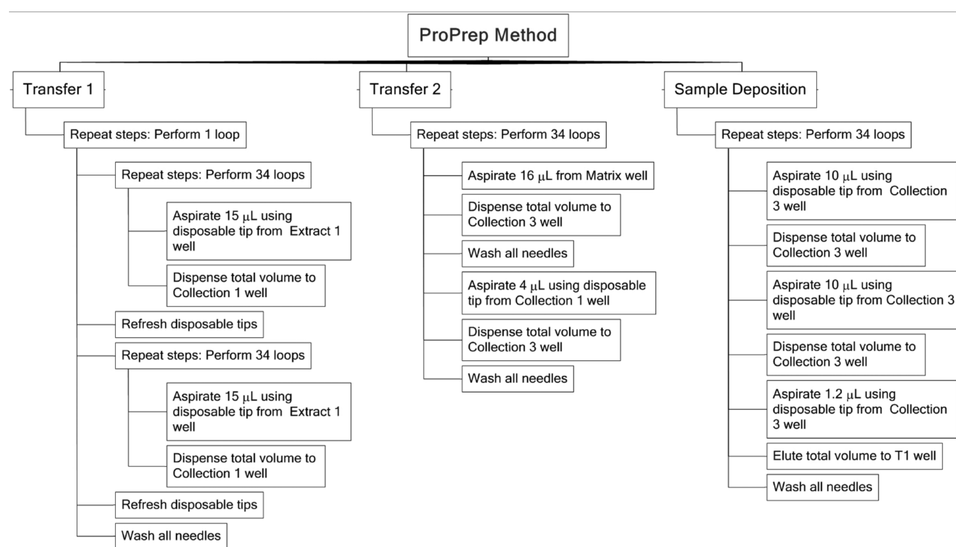

FIGURE 2

ProPrep sample preparation method. Transfer 1: Material extract supernatant, 30 μL, is pipetted in two steps from each sample solution and transferred to a 96-well, polypropylene, conical bottom microtiter plate (Collection 1). Transfer 2: MALDI matrix solution, (16 μL) is transferred into a 96-well plate (Collection 3). A sample aliquot, 4 μL, is sequentially transferred from each Collection 1 well in to a unique MALDI matrix containing well in Collection 3. Sample deposition: The sample/matrix solutions in Collection 3 are mixed in the individual wells by aspirating and dispensing 10 μL of the solution (2x). Sample/matrix solution (1.2 μL) is sequentially transferred from each used Collection 3 well to a unique, sequential MALDI target plate well.

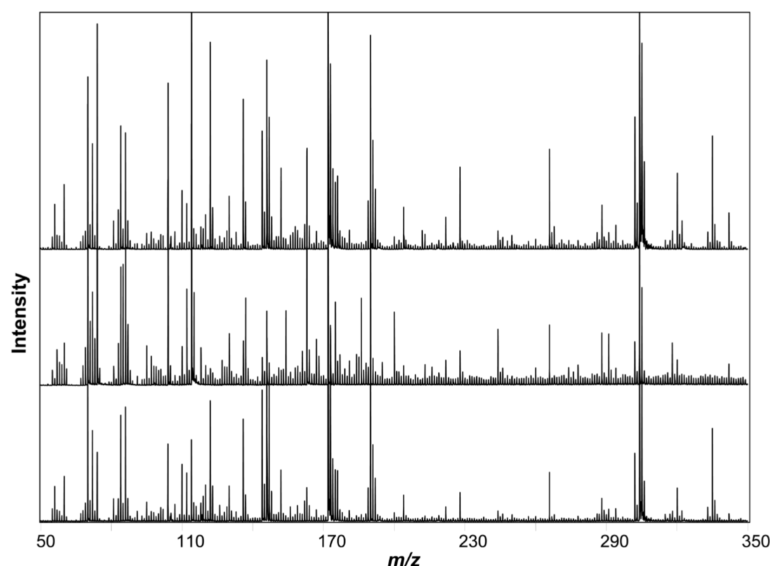

FIGURE 3

MALDI-TOF MS analysis reproducibility. Low-molecular-weight profiles, expanded view for 50–-350 Da, for *E. pallida* root replicates. Visual comparison of sample profiles indicates significant pattern reproducibility with small signal-intensity differences.

fluoroacetic acid) as the MALDI matrix. These samples were deposited on the previously prepared MALDI target plate below the CHCA prepared samples.

MALDI-TOF MS Analysis

MALDI-TOF MS analysis was performed on a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA). Analysis of CHCA prepared samples was performed in reflector mode, over the mass ranges 50–600 (300 shots/sample) and 600–5000 Da (500 shots/sample). Samples prepared with SA were analyzed in linear mode, over the mass range 2000–60,000 Da (500 shots/sample). External mass standards were used to calibrate the data.

RESULTS

Streamlined Sample Preparation and MS Analysis

Small molecules, peptides, and proteins from aerial parts, seeds, and roots from three *Echinacea* species (*E. purpurea*, *E. pallida*, and *E. angustifolia)*, and two off-the-shelf supplements composed of specific *Echinacea* tissues (Table 1) were extracted using streamlined, automated instrumentation for downstream sample profiling by MALDI-TOF MS. Samples were homogenized using a mini Bead-Beater and the supernatant was collected, diluted, and deposited onto a MALDI target plate using a ProPrep liquid-handling system. Automated sample analysis was then performed on the mass spectrometer, resulting in profile-rich spectra for the individual samples analyzed. This approach allowed for a systematic sample-preparation method that significantly improved sample throughput, preparation reproducibility, and data reliability.

Using these procedures, sample-preparation time was reduced by half. The ProPrep performed sample preparation and deposition tasks for 32 plant extracts and 2 blanks in approximately 30 min, compared to a manual preparation time of over 60 min. The system platform's plate configuration and the liquid-handling steps are presented in Figures 1 and 2. The ProPrep liquid-handling steps included: (1) transferring supernatant from the homogenized samples (Extract-1) to a clean microtiter plate (Collection 1), (2) transferring MALDI matrix (CHCA or SA) from the solution vial to a second microtiter plate (Collection 2), (3) pipetting an extract aliquot from Collection 1 to an individual well in Collection 2, (4) mixing the extract/matrix solution in Collection 2, and (5) depositing an aliquot of the extract/matrix solution onto a clean MALDI target plate.

Robust and reproducible sample preparation and MS data were obtained using these procedures. Final MALDI sample deposition performed by this instrument produced reproducible, well formed droplets, properly located and without overlap. MALDI-TOF MS analysis over the 50–600 *m/z* range resulted in spectra with over 200 small-molecule compounds detected. Sample analysis performed in triplicate demonstrates the reproducibility of the sample-preparation and MS analysis techniques used. Triplicate analysis of *Echinacea* samples indicates high reproducibility, as shown in Figure 3. Similar mass spectra are observed for the individual preparation and MS analyses performed for the *E. pallida* root (Figure 3) replicates. Small signal-intensity differences can be identified between the triplicate analyses; however, the similarity in molecular profile patterns can be easily distinguished. Similar results were seen for analysis of the various plant

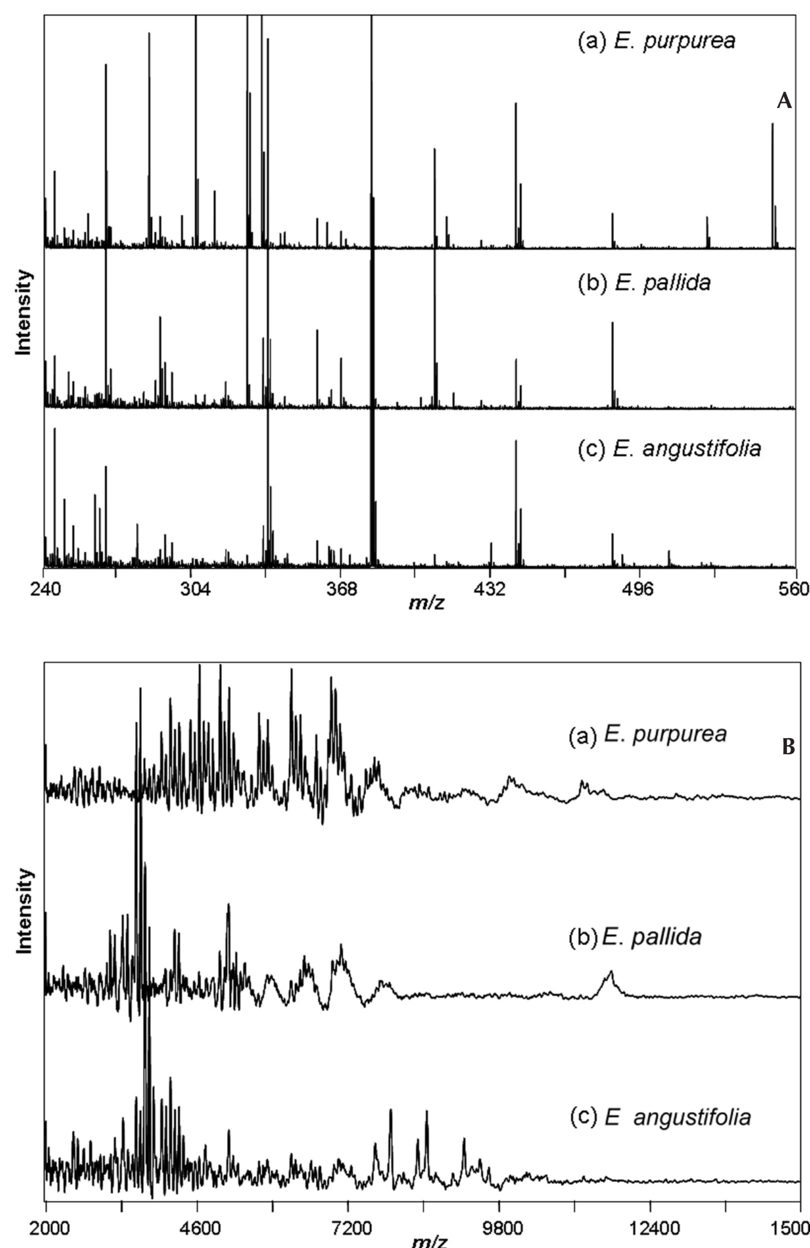

FIGURE 4

MS profile species comparison. **A:** Low-molecular-weight profiles, expanded view for 240–560 Da. **B:** High-molecular-weight profiles, expanded view for 2,000–15,000 Da, for (*a*) *E. purpurea*, (*b*) *E. pallida* and (*c*) *E. angustifolia*. General MS fingerprints characteristic of the *Echinacea* genus, as well as species-specific signal markers, can be easily distinguished within the two mass ranges.

extracts over the mass ranges 50–600, 600–5000, and 2000–60,000 Da (data not shown).

Distinguishing Marker Patterns

MS analysis of plant materials for a variety of *Echinacea* species demonstrates the power of MALDI-TOF MS to generate complex, sample-specific profiles. Comparison of MS profiles collected from the different plant tissues for a single *Echinacea* species (data not shown) or similar plant materials for the three species characterized (Figure 4) highlights the species specificity of the profiles generated. Figure 4A and 4B shows the low molecular weight (expanded view, 240–560 Da) and high-molecular-weight (expanded view, 2,000–15,000 Da) profiles, respectively, for seed extracts from the three *Echinacea* species analyzed. While, MS fingerprints characteristic of the general *Echinacea* genus can be discerned, species-specific signal markers can be easily distinguished within the two mass ranges.

The unique fingerprints generated from the individual plant materials and *Echinacea* species analyzed can be used to evaluate off-the-shelf *Echinacea* products. Detected MS patterns can be compared between samples to potentially further characterize or confirm sample composition. As an example, the mass spectra for leaf materials from *E. angustifolia* and *E. purpurea*, and the Rexall Sundown herbal supplements, of which *E. purpurea* is a component, are shown in Figure 5. Specific signals characteristic of *E. pur-*

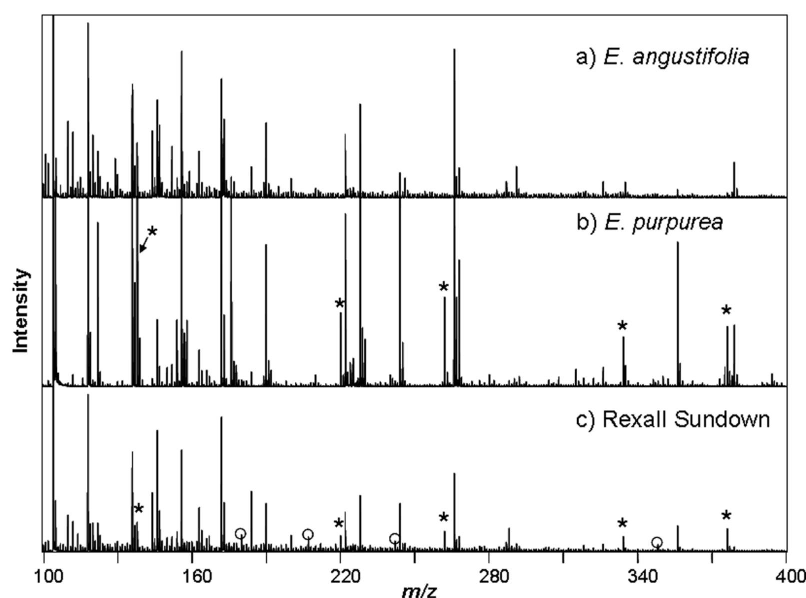

FIGURE 5

Herbal supplement characterization. Low-molecular-weight profiles, expanded view 100–400 Da, for (a) *E. angustifolia,* (b) *E. purpurea*, and (c) Rexall Sundown (*Echinacea*) herbal supplement. *E. purpurea* is a published component of Rexall Sundown. Visual comparison identified *E. purpurea*–specific MS signals (*) in the MS spectrum from the Sundown product. Low-intensity signals not characteristic of either the *E. angustifolia* or *E. purpurea* profiles are also present within the Rexall Sundown profile (indicated by a *circle*).

purea (indicated by *) are identified by visual comparison in the corresponding spectrum from the Sundown product, along with general *Echinacea* leaf material MS signals (as seen in comparing the *E. angustifolia*, *E. purpurea*, and Rexall Sundown profiles). Comparison of these MS patterns shows that the composition of Rexall Sundown includes *E. purpurea* aerial parts. In addition to these, low-intensity signals not characteristic of either the *E. angustifolia* or *E. purpurea* profiles are also present within the Rexall Sundown profile (indicated by a circle) and may be indicative of additional compounds present in the supplement.

CONCLUSIONS

MALDI-TOF MS fingerprints, characteristic of the complex molecular composition of the samples analysis, have the potential for individual botanical species characterization, including sample composition confirmation, product adulteration testing, and rapid screening of immature plants for phytochemical quantity and quality. Sample preparation and MALDI droplet deposition are the most time- and labor-intensive aspects of MALDI-TOF MS analysis. Therefore, rapid, streamlined methods for sample screening and molecular characterization are vital for the utilization of these techniques for real-time analysis of botanical samples.

This study demonstrates a proof of concept for higher-throughput analysis of *Echinacea* samples by MALDI-TOF MS using automated sample-preparation systems. After initial homogenization by bead-beating, samples were prepared for analysis and archival using a ProPrep liquid-handling robot, and analyzed by MALDI-TOF MS. The reproducibility of the generated MALDI droplets deposited by the robotic system and the resulting MS data are evidence of proper sample handling and preparation. As a result, teaming a liquid-handling system, such as the ProPrep, with MALDI-TOF MS allowed for a higher-throughput approach for sample characterization with minimal manual preparation.

Using these technologies, MS fingerprints could be used to differentiate different *Echinacea* species and, based on the profiles, to evaluate off-the-shelf botanical products in a streamlined process. Fingerprint comparisons for the Rexall Sundown product and *E. purpurea* materials support published product composition based on the fingerprints of known product materials and identified signatures suggestive of additional product components. The development of a larger sample database to include increasing the numbers of plants and plant materials sampled, possible degradants, materials used during supplement composition, and materials and products following stability testing would enhance capabilities to monitor and characterize botanical products.

The ability to analyze large numbers of samples quickly using these technologies makes them ideal tools for botanical product analysis and biomarker discovery. Fingerprint comparisons, based on a library of analyzed materials and by-products, yield the opportunity to characterize *Echinacea* materials and products. The data gathered from large sample sets can be subjected to multi-variant statistical analysis methods for pattern identification. Further analysis can also be performed to identify, characterize, and validate potential biomarkers. The techniques presented show promise for characterizing botantical materials and products for important aspects such as product quality control, including species and adulterant identification, product consistency and stability, as well as the ability to

support plant-breeding programs and early rapid screening for phytochemical quantity and quality.

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