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Original Article

What's in the box? Authentication of *Echinacea* herbal products using DNA metabarcoding and HPTLC

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ABSTRACT

Background: Differences in regulatory policies between countries as well as a lack of appropriate standardized methods for the authentication and quality control of herbal products directly impact their quality and safety. *Echinacea* products are among the top-selling herbal products in Europe and the United States with indications for a broad range of ailments. The increased use of *Echinacea* species has led to concerns about adulterated products resulting from challenges in morphology-based identification, due to overlapping morphological variation, frequent hybridization between species, and deliberate adulteration.

Purpose: This study addressed the need for a novel analytical strategy in the authentication of herbal products.

Methods: A combination of high performance thin layer chromatography (HPTLC) and DNA metabarcoding was employed. Fifty-three *Echinacea* herbal products marketed across Europe were tested to evaluate the accuracy of these methods in plant identification and their potential for detecting substitutes, adulterants and other unreported plant constituents.

Results: HPTLC provides high resolution in the detection of *Echinacea* phytochemical target compounds, but does not offer information on the other species within the product. Alternatively, we showed that the limitation of HPTLC in detecting non-targeted species can be overcome by the complementary use of DNA metabarcoding. Using DNA metabarcoding, *Echinacea* species were detected in 34 out of the 38 retained products (89%), but with a lack of discriminatory resolution at the species level due to the low level of molecular divergence within the *Echinacea* genus. All of the tested herbal products showed considerable discrepancies between ingredients listed on the label and the ones detected using DNA metabarcoding, registering an overall ingredient fidelity of only 43%.

Conclusion: The results confirm that DNA metabarcoding can be used to test for the presence of *Echinacea* species and simultaneously to detect other species present in even highly processed and multi-ingredient herbal products.

Introduction

Legislation and Echinacea

The regulation of herbal products varies globally, and products can be classified as food supplements, medicines, homeopathic products, cosmetics or even biocides depending on specific legislation. The European Medicines Agency (EMA) regularly produces updates of the European Pharmacopoeias (EDQM, 2014) and has in addition produced a number of monographs on quality and authentication of specific herbals providing relevant methodological specifications for their quality assessment. However, the primary legal responsibility for the

Abbreviations: EMA, European Medicines Agency; BLAST, basic local alignment search tool; FTIR, Fourier-transformed infrared spectroscopy; IR, infrared spectrometry; HPTLC, high performance thin layer chromatography; *mat*K, maturase K; MS, mass spectrometry; MOTU, molecular taxonomic unit; nrITS, nuclear ribosomal internal transcribed spacer; Ph.Eur., European Pharmacopoeia; *rbc*L, ribulose bisphosphate carboxylase; TLC, thin-layer chromatography; UV–VIS, ultraviolet–visible

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safety of the marketed products is delegated by default to the manufacturers.

Sales of herbal dietary supplements have seen year-on-year increases of 5–10%, and in 2014 sales reached an estimated total of more than $6.4 billion, with *Echinacea* among the top five top grossing taxa (Smith et al., 2015). *Echinacea* is a genus of composites (Asteraceae) comprising a small number of herbaceous grasslands perennial taxa whose natural distribution is limited to North America (Kindscher and Wittenberg, 2016). Three *Echinacea* species, *E. angustifolia* DC. (Narrow-leafed purple coneflower), *E. pallida* (Nutt.) Nutt. (Pale purple coneflower) and *E. purpurea* (L.) Moench (Purple coneflower), are used in traditional herbal medicine (EDQM, 2014).

Today *Echinacea* is cultivated widely in Europe and North America for use in commercial herbal products in a diverse range of products, including herbal teas, capsules, tablets, powders, tinctures and beverages (Brown et al., 2011). A 2007 survey by the National Centre for Complementary and Alternative Medicine showed that *Echinacea* was the most commonly used herbal medicine among adults and children in United States (Barnes et al., 2008). In Europe, a study on the use of food supplements and medicines showed that *Echinacea* and *Ginkgo* are the most common herbals (EAS, 2007).

Pharmacological use and effects of Echinacea species

Echinacea has been extensively studied for its pharmacological effects in both *in vitro* and *in vivo* studies (Table S1). *In vivo* human clinical studies on the oral administration of *E. purpurea* commercial herbal juice have failed to confirm previous *in vitro* findings (Schwarz et al., 2005, 2002; Sperber et al., 2004). However, a recent study conducted on 68,522 Norwegian women and their children revealed no risk of malformations or adverse pregnancy outcomes associated with the use of *Echinacea* in pregnancy (Heitmann et al., 2016).

Chemical profiles and differences between Echinacea species

The constituents of *Echinacea* species used for medicinal purposes differ qualitatively and quantitatively (Barnes et al., 2005; Binns et al., 2002). Alkamides, polysaccharides, glycoproteins, volatile oils and phenolic compounds have been considered important constituents of the plant (Table S2). *Echinacea purpurea* shows a similar phenolic phytochemical profile (cichoric acid, caftaric acid, chlorogenic acid) in roots, flowers and leaves, but with no cynarine in the aerial parts, whereas *E. angustifolia* roots shows low amounts of cichoric acid and cynarine, and that of *E. pallida* shows both cichoric acid and cynarine. *Echinacea purpurea* aerial parts contain no echinacoside, while echinacoside is a major component in the roots of *E. pallida* and *E. angustifolia* (and also present in small quantities in the flower and leaves. Alkamides are found in the rhizomes and roots of *E. angustifolia,* and less abundantly in the aerial parts of *E. purpurea* and mainly absent in *E. pallida* roots). *E. pallida* contains large amounts of ketoalkenes. Rutoside is a flavonoid present in the leaves of all three species of *Echinacea* (Barnes et al., 2005).

Quality issues of Echinacea herbal products

The increased use of *Echinacea* species has led to concerns about adulterated products resulting from challenges in morphology-based identification, due to overlapping morphological variability and frequent hybridization between species (Flagel et al., 2008). Furthermore, reported adulteration of *E. purpurea* with the roots of *Parthenium integrifolium* L., *Lespedeza capitata* Michx., *Eryngium aquaticum* L., *Rudbeckia nitida* Nutt., *Helianthus annuus* L. or *Liatris aspera* Michx. lead to safety concerns about the herbal products (Zhang et al., 2017). The use of unreported ingredients is a serious safety concern as adverse drug reactions cannot be associated with the ingredients listed on the product label (Gilbert, 2011). Commercially available herbal products contain one or more *Echinacea* species originating from the same or different geographical areas, and the resulting phytochemical diversity across these products can complicate further investigations (Barnes et al., 2005). *Echinacea* secondary metabolites are used for the qualitative identification of species in the industry. Presence of for example, cichoric acid for *E. purpurea and E. angustifolia*, echinacoside for *E. angustifolia* and *E. pallida*, ketoalkene for *E. pallida* were routinely tested (Mistrikova and Vaverkova, 2006). Nevertheless, the presence of these markers does not provide unequivocal identification of the species since, for instance, traces of echinacoside can be also found in *E. purpurea* (Arnason et al., 2002). Presently, the differentiation of species is based on the relative abundances of metabolites by various phytochemical techniques, but marketed herbal products are often highly processed complex formulations with numerous ingredients, and these methods might not enable accurate identification of all plant ingredients, especially if target species are admixed with other species (De Boer et al., 2015).

HPTLC of herbal products and Echinacea

HPTLC is a qualitative analytical technique that provides robust separation and detection of chemical compounds making it appropriate for the quality control of raw materials and finished herbal products (Reich and Widmer, 2008). HPTLC has been proposed for the identification of target species (Reich et al., 2008), as well for adulterants and substitutes in herbal products (Gallo et al., 2011). The HPTLC protocol elaborated by Reich et al. (2008) showed a high identification resolution at species level of *Echinacea* spp. based on alkylamide and phenylpropanoid chemical profiles.

DNA metabarcoding of herbal products and Echinacea

High phenotypic plasticity within *Echinacea* has complicated the taxonomy of the genus and led to misidentifications, confused taxonomies and misapplication of taxa (Kindscher and Wittenberg, 2016). Combined plastid (*trnS* and *trnG*) and nuclear (*Adh*, *CesA*, and *GPAT*) phylogenies found no resolved topologies, suggesting incomplete lineage sorting, as well as the potential for widespread hybridization and backcrossing following secondary contact within the genus (Flagel et al., 2008). Flagel et al. (2008) note that in contrast to the low discriminatory resolution of these molecular markers, previous studies using morphological characters and metabolic profiles found well-resolved and taxonomically supported relationships within *Echinacea*, and suggest the use of markers with more broad genomic coverage. Zhang et al. (2017) recovered the complete plastid genomes from all nine *Echinacea* species, and based on both coding and non-coding regions of the chloroplast genomes, *Echinacea* species were separated into two clades with strong support. In addition, Zhang et al. (2017) report that the core barcoding markers *mat*K and *rbc*L do not differ sufficiently for species-specific identification of *Echinacea* but suggest a combination of nrITS and *trnH-psbA* as the optimal barcoding markers instead. The incongruence in nuclear ribosomal and plastid phylogenies, reported by Flagel et al. (2008), however suggests that neither plastid markers nor nrITS provide an accurate picture of the phylogenetic history of the genus.

In this study, we use DNA metabarcoding to detect species diversity in *Echinacea* herbal products. The use of DNA metabarcoding for the identification of commercialized plant products has evolved with the advances in molecular biology and sequencing (De Boer, 2017; Coghlan et al., 2012; Raclariu et al., 2017b), and is defined as high-throughput multispecies (or higher-level taxon) identification using the total and typically degraded DNA extracted from an environmental sample (Taberlet et al., 2012). Here we test the hypothesis that *Echinacea* species are frequently admixed with other species in *Echinacea* herbal products. We test this hypothesis by analyzing European *Echinacea* herbal products using DNA metabarcoding and HPTLC to authenticate

these using phytochemical constituents, and aim to answer the following research questions: (1) Can DNA metabarcoding be used to test for the presence of *Echinacea* species in herbal products, and to detect the presence of off-label plant species due to substitution or adulteration?; (2) Can HPTLC be used to distinguish *Echinacea* species and to identify their exclusive presence in herbal products?

Materials and methods

Sample collection

Fifty-three herbal products listing different *Echinacea* species, marketed as single (35) and multi-ingredient (18) food supplements consisting of herbal teas (9 loose teas and 8 bagged teas), capsules (16), tablets (13) and extracts (7), were purchased from different retail stores (20), pharmaceutical companies (32) and via e-commerce (1). The countries of origin of these products were Austria (1), China (1), Czech Republic (3), France (1), Germany (4), Italy (2), Macedonia (1), Poland (2), Romania (26), Switzerland (1), United States (7), and four of the products did not specify a country of origin. A list of samples is included as Table S3, but the producer/importer name, lot number, expiration date and any other information that could identify the specific products and producers are omitted. These herbal products were imported into Norway for scientific analysis under Norwegian Medicines Agency license no. 16/04**551**-2.

High performance thin layer chromatography (HPTLC) analysis

Echinacea phenylpropanoids (echinacoside, cynarin, cichoric acid, chlorogenic acid, caffeic acid, caftaric acid) from 53 herbal products were analyzed using HPTLC following recommendations in the specific monographs from the European Pharmacopoeia 8th Edition (EDQM, 2014) and the CAMAG application notes on HPTLC identification of *Echinacea* species CAMAG (2015). Herbal products were processed depending on their pharmaceutical formulation. Capsules (about 300 mg), tablets (400 mg), and teas (500 mg) were homogenized and mixed in a 1:100 ratio with methanol/water (80:20 V/V) in an ultrasonic bath for 15 min, and tinctures were diluted in 1:3 methanol. The obtained solutions were centrifuged and the filtered supernatant was collected and used as sample for further analysis. The following chemical and botanical standards were used. *Reference substances*: caftaric acid (HPLC grade, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), cynarine (min. 98.0%, HPLC/Sigma-Aldrich Co., St. Louis, Missouri, USA), cichoric acid (min. 95.0%, Sigma-Aldrich, St. Louis, Missouri, USA), echinacoside (min. 98.0% HPLC, Sigma-Aldrich Co, St. Louis, Missouri, USA), chlorogenic acid (min. 95.0%, Sigma-Aldrich), caffeic acid (TLC grade, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), verbascoside (min. 99.0% HPLC, Extrasynthese SAS, Lyon, France), ferulic acid (min. 99.0%, Sigma-Aldrich, St. Louis, Missouri, USA). *Solvents*: ethanol, min. 96% V/V, and methanol, 99.3% analytical reagent (Chimreactiv SRL, Bucharest, Romania). *Analytical reagents*: formic acid ACS (Merck KGaA, Darmstadt, Germany), ethyl acetate (min. 99.5%, Sigma-Aldrich Co, St. Louis, Missouri, USA), distilled water prepared in laboratory; Natural Products—polyethylene glycol (NP/PEG) reagent (Honeywell Fluka, Seelze, Germany). *Botanical standards*: United States Pharmacopoeia (USP) analytical reference botanical standards: USP Powdered *Echinacea purpurea* F0D018, USP Powdered *Echinacea angustifolia* G0I377 and USP Powdered *Echinacea pallida* F0I285 (Rockville, Maryland, USA). *Apparatus*: A CAMAG high performance thin layer chromatography system (CAMAG AG, Muttenz, Switzerland), with a Linomat IV sample applicator, a Canon digital camera, and the following software, Reprostar III with winCATS planar chromatography manager software, Digistor II digital system with winCATS software with an Image Comparison Viewer enabling high resolution visualization of multiple samples for comparison of specific compound retention times and the included references.

Samples and references were analyzed on Silica gel HPTLC plates (60 F245 20 × 10 and 10 × 10 cm for tinctures and solutions, Merck KGaA, Darmstadt, Germany). The reference solutions were dissolved in methanol to obtain the following concentrations: caftaric acid 0.006%, cynarine 0.02%, cichoric acid 0.005%, echinacoside 0.018%, chlorogenic acid 0.015%, caffeic acid 0.011%, verbascoside 0.01%, ferulic acid 0.01%, USP Powdered *Echinacea purpurea* F0D018 1.0%, USP Powdered *Echinacea angustifolia* G0I377 2.0% and USP Powdered *Echinacea pallida* F0I285 1.0%. *Echinacea* product sample extracts and reference solutions were applied separately in twin bands, on a maximum of 12 tracks at 4–8 mm distance using different application volumes (4–16 µl for extracts, 12–17 µl for bag and bulk teas, 12–17 µl for tablets, 9–15 µl for capsules and 3–6 µl for standard solutions) with a band length of 8–10 mm and a delivery speed of 8 s/µl using a CAMAG Linomat IV automatic sample applicator (see above). The plates were run for 30 min in the developing box and subsequently dried at 105 °C for 5 min. For phenylpropanoids identification, the plates were inspected at 254 and 366 nm before and after homogenous spraying with the Natural Products-polyethylene glycol reagent (NP/PEG), and subsequently air-dried. For each set of samples, the plates were developed in a saturated vertical-developing chamber at room temperature (20–22 °C) for 30 min with ethyl acetate: formic acid: acetic acid: water = 20:2.2:2.2:5.4 V/V as mobile phase; the development distance was 7 cm; after the development, the plates were air dried at room temperature. Derivatization of the chromatograms were performed by spraying the plates with NP (0.1 g in 10 ml methanol) and PEG400 (0.5 g in 10 ml ethanol), followed by heating of the plates at 105 °C temperature for 15 min. Plates were subsequently imaged using a CAMAG Reprostar 3 with digital video camera at 254 nm for developed plates and 366 nm for derivatized plates. HPTLC densitometry (CAMAG-TLC Scanner 3 with WinCATS Planar Chromatography Manager Software) was done using spectra recording from 200–700 nm, absorption at 254 nm wavelength, slit dimension 8.00 × 0.40 mm, scanning speed 100 nm/s, and peak area evaluation by linear or polynomial regression.

DNA metabarcoding

The total DNA was extracted from small amounts (about 300 mg) of each homogenized herbal product, using the exact method described in Raclariu et al. (2017b). Amplicon DNA metabarcoding using barcoding markers nrITS1 and nrITS2 was done using the method described in Raclariu et al. (2017b), and sequenced on an Ion Torrent Personal Genome Machine (Life Technologies, Thermo-Fischer Scientific, USA). Sequencing read data was processed using the HTS barcode-checker pipeline (Lammers et al., 2014) as described in Raclariu et al. (2017b). A 99% sequence similarity threshold was used for MOTU clustering with a minimum of 10 reads per cluster to reduce the formation of false MOTUs and the potential effects of sequencing bias known to affect the Ion Torrent sequencing platform (Loman et al., 2012). One representative sequence from each MOTU was taxonomically assigned using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) against a reference nucleotide sequence database represented by a local copy of the NCBI/GenBank. BLAST results with a maximum e-value of 0.05, a minimum hit length of 100 bp, and similarity of > 99% against the reference barcode were accepted as species level matches.

Results

High performance thin layer chromatography (HPTLC)

Identification and detection of *Echinacea* species, *E. purpurea, E. angustifolia* and *E. pallida*, from tea, tablets, capsules, and extracts were done using HPTLC with the standard *Echinacea* phenylpropanoid references echinacoside, cichoric acid, caftaric acid, chlorogenic acid, and cynarine, and the botanical standards of *E. purpurea, E. angustifolia, E.*

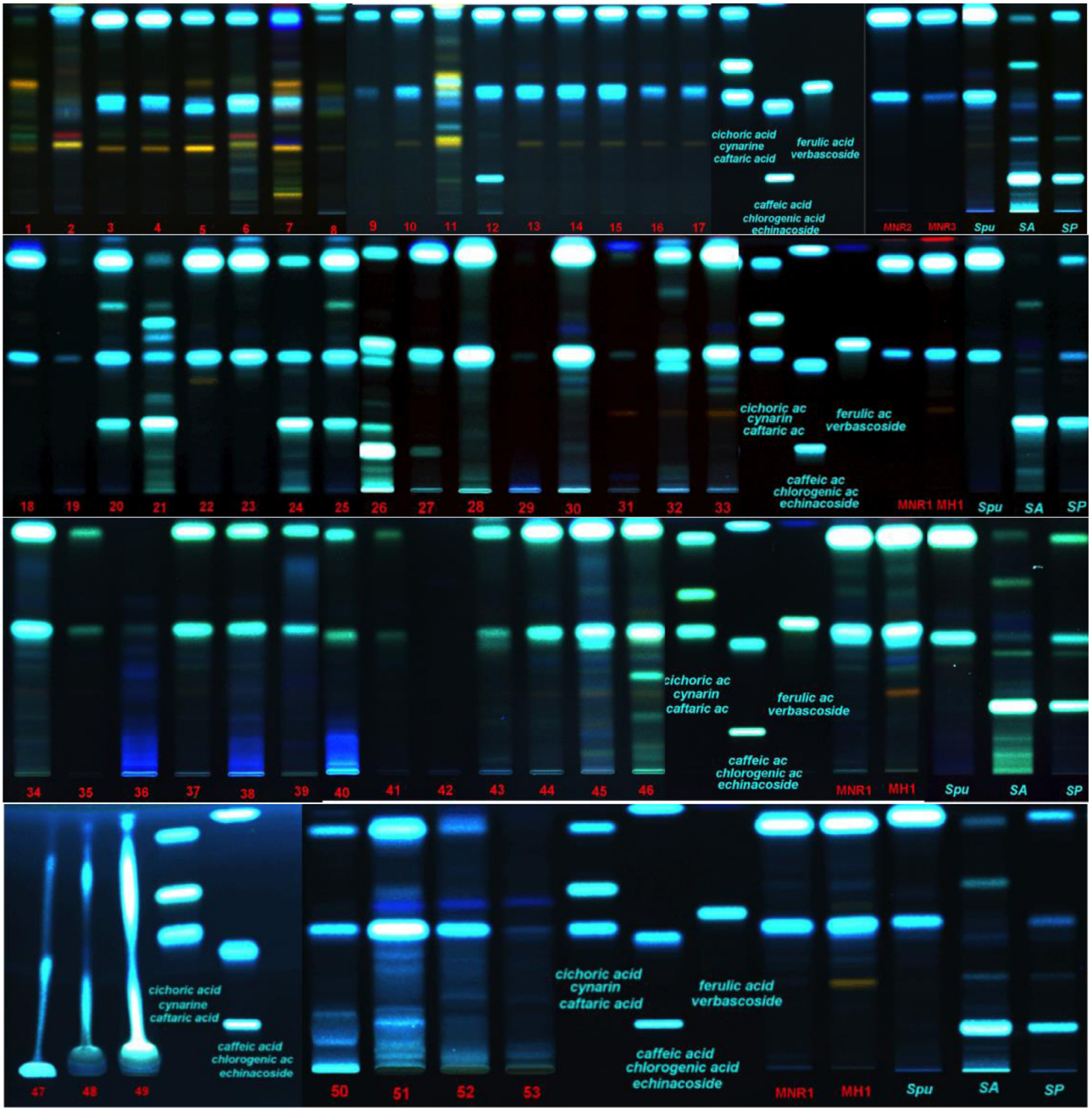

Fig. 1. High performance thin layer chromatogram (HPTLC) based identification and detection of phenylpropanoids from the *Echinacea* sp. herbal products. The track assignment: (1) test solutions (1–53); (2) phenylpropanoids chemical reference solutions in the following order (increasing Rf): echinacoside, cholorogenic acid, caftaric acid, cynarin, cichoric acid, caffeic acid; (3) botanical reference solution of *Echinacea purpurea* (root (MNR1) and aerial parts (MH1)); (4) United States Pharmacopoeia (USP) analytical reference botanical standards (*E. purpurea* (Spu), *E. angustifolia* (SA) and *E. pallida* (SP)). (A) HPTLC chromatograms of herbal teas. (B) HPTLC chromatograms of capsules. (C) HPTLC chromatograms of tablets. (D) HPTLC chromatograms of extracts. The chromatograms were scanned after derivatization in UV 366 nm. Details about the herbal products can be found in Table S3.

pallida. In the HPTLC chromatograms visualized at 366 nm after derivatization, the occurrence of fluorescent blue spots indicates the presence of phenylpropanes (Fig. 1). The phenylpropanoid refraction (Rf) values were: 0.15–0.17 (echinacoside), 0.47–0.49 (chlorogenic acid), 0.51–0.53 (caftaric acid), 0.65 (cynarine), 0.87–0.9 (cichoric acid), 0.96–0.97 (caffeic acid), and other polyphenols (ferulic acid and verbascoside) at Rf = 0.35; 0.46; 0.52; 0.73 and 0.90.

High performance thin layer chromatogram (HPTLC) based identification and detection of phenylpropanoids from the *Echinacea* sp. herbal products. The track assignment: (1) test solutions (1–53) (2) phenylpropanoids chemical reference solutions in the following order (increasing Rf): echinacoside, cholorogenic acid, caftaric acid, cynarin, cichoric acid, caffeic acid; (3) botanical reference solution of *Echinacea purpurea* (root (MNR1) and aerial parts (MH1)) (4) United States Pharmacopoeia (USP) analytical reference botanical standards (*E. purpurea* (Spu), *E. angustifolia* (SA) and *E. pallida* (SP)) A. HPTLC chromatograms of herbal teas. B. HPTLC chromatograms of capsules. C. HPTLC chromatograms of tablets. D. HPTLC chromatograms of extracts. The chromatograms were scanned after derivatization in UV 366 nm. Details about the herbal products can be found in the Table S3.

The results of HPTLC chromatographic identifications of the analyzed herbal teas yielded the following results for the 10 samples

labelled as including *Echinacea* sp. (Fig. 1A and Table S4): Sample #1 does not contain any of the targeted phenylpropanoids specific to *Echinacea* species; sample #8 has caffeic acid and low caftaric acid and content suggesting that only *E. purpurea* is present and in low concentration; sample #12 has very strong zones for echinacoside, cichoric acid and caftaric acid indicating a possible mixture of *E. purpurea* and *E. pallida*; seven samples (samples #4, #6, #9, #10, #11, #13, and #14) show distinct presence of caftaric and cichoric acid indicating the presence of *E. purpurea*. The six samples labelled as including specifically *E. purpurea* yielded the following: in four samples (samples #3, #15, #16, #17) the presence of this species was confirmed by high content of caftaric and cichoric acid; in sample #2 low content cichoric and caffeic acid suggests a low concentration of *E. purpurea*; in sample #7 the presence of caftaric acid and a comparison of the profile with that of the botanical standard *E. angustifolia* (Fig. 1A) indicates a mixture of *E. purpurea* and *E. angustifolia*. In sample #5 *E. purpurea* was identified from the distinct presence of cichoric and caftaric acid.

The results of the HPTLC chromatographic identifications of the analyzed capsules yielded the following (Fig. 1B and Table S4): The three samples labelled as including *Echinacea* sp. (#18, #31, #32) contained caftaric acid and cichoric acid, and this confirmed the presence of *E. purpurea*. In the nine samples labelled as including specifically *E. purpurea* (samples #19, #22, #23, #26, #27, #28, #29, #30, #33) the content of cichoric acid and caffeic acid confirmed the presence of this species, except for sample #29. In sample #21 labelled as including *E. angustifolia* the detection of echinacoside and cichoric acid and the comparison of the profile with that of the botanical standard *E. angustifolia* (Fig. 1B) indicates the presence of a mixture of *E. angustifolia* and *E. purpurea*. In sample #24 labelled as including *E. pallida* the presence of echinacoside and cichoric acid and the comparison of the profile with that of the botanical standard *E. pallida* indicates the presence of a mixture of *E. pallida* and *E. purpurea* (Fig. 1B). In samples #20 and #25 labelled as including mixtures of *E. purpurea* and *E. angustifolia*, the presence of these species was confirmed by the content of echinacoside, caftaric acid, cichoric acid and caffeic acid.

The results of the HPTLC chromatographic identifications of the analyzed tablets yielded the following (Fig. 1C and Table S4): the two samples labelled as including *Echinacea* sp. (samples #36, #46) differ in their chromatographic profiles. Sample #36 differs from that of the *Echinacea* species, and sample #46 contains caffeic acid and cichoric acid indicating the presence of *E. purpurea*. For the eleven samples labelled as including *E. purpurea* (samples #34, #35, #37, #38, #39, #40, #41, #42, #43, #44, #45) the presence of this species was confirmed in eight samples by the presence of caffeic acid and cichoric acid, and the presence of echinacoside, caftaric acid and cichoric acid indicated a mixture of *E. purpurea* and *E. pallida* in sample #40, as well as a low concentration of *Echinacea* in sample #41. In sample #42 no *Echinacea* was identified.

The results of the HPTLC chromatographic identifications of the analyzed extracts yielded the following (Fig. 1D and Table S4): Of the five samples labelled as including *E. purpurea* (samples #47, #49, #51, #52, #53), sample #47 was a glycerinated solution and this hampered an accurate composition estimation, sample #53 contained very weak zones for echinacoside, caftaric acid and cichoric acid that may indicate the presence of *E. angustifolia,* and the presence of cichoric acid and caftaric acid in the other samples confirmed the presence of *E. purpurea*, however sample #49 does not allow an accurate estimation. Sample #48 labelled as including *E. purpurea* and *E. angustifolia* was also a glycerinated solution. Sample #50 labelled as including *E. pallida* confirms the presence of this species.

DNA metabarcoding

All 53 samples had detectable DNA concentrations but the content varied from sample to sample. Fifteen samples had a concentration lower than 0.1 ng/µl, 26 samples ranging from 0.1 to 10 ng/µl, and twelve higher than 10 ng/µl. No correlation between obtained DNA concentration and the substrate type of the extracted product was observed (Table S5).

The raw data before demultiplexing consisted of 12,190,865 sequences, with an average of 115,008 sequences per sample for each marker. After applying our trimming and filtering quality criteria, 38 herbal products (72%) were retained, and they were used for further analysis (Table S6). Fifteen products, including three herbal teas (2, 7, 12) three capsules (19, 21, 29), five tablets (35, 36, 40, 43, 44) and four extracts (47, 50, 51, 53) did not yield reads or MOTUs after applying the quality-filtering criteria and were excluded from further analyses. A total of 305,018 sequences passed the trimming and quality-filtering criteria (2.5% of reads), including 79,918 nrITS1 reads and 225,100 nrITS2 reads (Table S6). The MOTUs were formed using a 99% similarity clustering threshold, and 2,529 MOTUs that contained minimum 10 reads were retained and further identified using BLAST as 83 different species (Table S7). For nrITS1 we detected a total of 60 different species and 37 species on only nrITS2 (Table S7). For both, nrITS1 and nrITS2, the number of species detected per sample ranged from 1 to 19, with an average of 5 species per sample.

Detection of *Echinacea* sp. using DNA metabarcoding within the herbal products per category of pharmaceutical form.

The targeted *Echinacea* species were detected in 34 out of 38 products (89%) of the retained samples (Fig. 2 and Table S3). Twenty-four (89%) out of 27 analyzed products labelled as including *Echinacea* sp. as the single ingredient, contained at least one *Echinacea* species. However only seven products (26%) contained exclusively *Echinacea* species, whereas the other 20 contained several other species, likely contaminants. Ten (91%) out of 11 analyzed products labelled as including a mixture of *Echinacea* sp. and other plant species, contained *Echinacea* sp. but all of them showed considerable discrepancies between ingredients listed on the label and the ones detected. The overall ingredient fidelity, meaning the proportion of the number of species indicated on the product label and the entire species diversity, as detected by DNA metabarcoding, was 89% for single ingredient products and 31% for the multiple ingredient products. The overall ingredient fidelity for all products was 43%.

Per formulation category the DNA metabarcoding yielded the following results (cf. Fig. 2 and Table S3): In thirteen out of 17 samples the presence of *Echinacea* sp. was confirmed, and in the remaining four samples, three did not yield MOTUs that passed the quality filtering criteria. The capsules contained *Echinacea* sp. in twelve out of 16 samples, and identification was not possible in four of the products because these did not yield MOTUs that passed the quality filtering criteria. The herbal tablets contained *Echinacea* sp. in only six out of 13 samples, and in the remaining seven samples five did not yield MOTUs that passed the quality filtering criteria. The extracts contained *Echinacea* sp. in only three out of seven samples, and the remaining four did not yield MOTUs that passed the quality filtering criteria.

Complementary identification

DNA metabarcoding identified *Echinacea* species only at genus level, and it is thus difficult to make an overall comparison of the two analytical methods. We have a clear case with the samples #1, #36, and #42 which we labelled as including *Echinacea* sp., but where chromatographic profiles did not confirm this. This absence was confirmed by DNA metabarcoding where no *Echinacea* sequences were generated.

Fig. 2. Detection of *Echinacea* sp. using DNA metabarcoding within the herbal products per category of pharmaceutical form.

Among the glycerinated samples #47 and #48 that had an indistinguishable chemical profile, we confirmed the presence of *Echinacea* sp. in #48 using DNA metabarcoding.

For the 5 samples (#11, #28, #29, #39, #40) that yielded MOTUs using DNA metabarcoding but in which no *Echinacea* sp. was detected, HPTLC confirmed the presence of *Echinacea* sp. in four samples (#11, #28, #39 and #40).

Discussion

The quality and authenticity of herbal products have direct impacts on their safety. Quality control must include a series of procedures to ensure the identity of the raw materials used and screening of target compounds along the value chain (Raclariu et al., 2017c). Authentication assays in herbal production must discriminate potential adulterants and/or substitutes. However, despite the existence of well-established and widely accepted analytical methods recommended in the regulatory guidelines (EDQM, 2014; EMA, 2006) for herbal product quality assessment, their resolution and efficacy can be impeded by various factors. First, the marketed herbal products are often highly processed, containing numerous ingredients, and even if these analytical methods are accurate in detecting specific target compounds, they have limited efficiency in detecting intrageneric substitution and do not yield any information on other plant ingredients in the products (Rossi Forim et al., 2015). Morphology based taxonomic methods are also impeded by highly processed herbal products as these often consist of finely powdered materials (Zhao et al., 2006).

In this study, we combined HPTLC and DNA metabarcoding. HPTLC is a more automated and reproducible form of thin-layer chromatography (TLC), and with a better separation and detection of the compounds that can be successfully used in quality control of raw materials and finished herbal products (Reich and Widmer, 2008). Reich et al. (2008) summarized a procedure for HPTLC based identification of *Echinacea* and other species, which was applied on 53 herbal products in this study, including herbal tea, capsules, tablets and tinctures. The results showed that each of the three targeted *Echinacea* species have distinguishable chromatograms for most of the herbal products (Fig. 1). The main limitation of the HPLTC assay is the inability to offer insights into the presence of other plant species in the herbal product. HPTLC is less suitable for the analysis of volatile and certain sensitive samples (Morlock and Schwack, 2010). Combination of HPTLC with other methods, such as mass spectrometry (MS), ultraviolet–visible (UV–VIS) and infrared spectrometry (IR) or Fourier-transformed infrared spectroscopy (FTIR) offer further possibilities for analytical refinement in the analysis of herbal products by increasing the amount of qualitative and quantitative information. In summary, HPTLC is not an ideal method for detection of substitution and adulteration within marketed herbal products, but it is a powerful and cost-effective method to identify specific chemotaxonomic markers, and thus applicable in the quality control of the derived herbal products.

DNA barcoding and metabarcoding are not yet validated for use in a regulatory context of quality control (Agapouda et al., 2017), but there are several studies advocating its usefulness for herbal product authentication and pharmacovigilance (Cheng et al., 2014; Coghlan et al., 2012; Ichim et al., 2017; Ivanova et al., 2016; Newmaster et al., 2013; Raclariu et al., 2017b, 2017a). The DNA metabarcoding results in this study show that the presence of *Echinacea* sp. was detected in 34 (89%) out of 38 sequenced and retained samples. However, the results showed a lack of discriminatory resolution at the species level likely due to the low level of molecular divergence (Flagel et al., 2008; Zhang et al., 2017). Furthermore, most of the retained samples revealed a high level of discrepancy with most products not containing all the species listed on the label, but rather other off-label species. For all products, only 43% of the species indicated on the product's label were detected species using DNA metabarcoding. Here we need to mention that the general monograph number 1433 on 'Herbal drugs' of the European Pharmacopoeia allows up to 2% foreign matter (EDQM, 2014), and that DNA metabarcoding is not a quantitative method that can be used to check if the contaminants are within this allowed range. As suggested by previous studies (Ivanova et al., 2016; Raclariu et al., 2017a), the results related to the authentication of herbal products using DNA metabarcoding need to focus primarily on checking the presence of the labeled ingredients and contaminants. The presence of non-listed species may be explained by various factors, including but not limited to the deliberate adulteration and unintentional substitution, that may occur starting from the early stage of the supply chain (*i.e.*, cultivation, transport, storage), to the manufacturing process and the commercialization of the final products. DNA metabarcoding is a highly sensitive method and even traces of DNA, from grains of pollen from anemophilous species, for instance, or from plant dust in the entire manufacturing process that may accidentally contaminate the product, can be detected and identified (Raclariu et al., 2017b, 2017a). Several factors may influence the accuracy of the final results, starting with the first steps in processing the raw material to the final data analysis and interpretation. Extraction procedures, barcoding markers, primers, PCR amplification bias, high throughput sequencing library preparation, sequencing platform and trimming and filtering quality thresholds, clustering, and molecular identification algorithm all influence the final results (Pawluczyk et al., 2015; Staats et al., 2016). Moreover, public sequence reference databases, such as NCBI Genbank, pose significant challenges for reliable taxonomic affiliations, due to incorrectly identified or missing reference sequences and high levels of missing data (Hinchliff and Smith, 2014). However, at least one reference sequence of almost every genus of the known terrestrial plants is represented in NCBI GenBank (Hinchliff and Smith, 2014) and thus the use of DNA metabarcoding should at least be possible for identification at higher taxonomic levels.

As molecular genetics and sequencing technology continue to advance rapidly, the use of DNA metabarcoding is promising for large-scale authentication of herbal products and other mixtures of economic importance. Standardization is required before DNA metabarcoding can be implemented as a routine analytical approach (Agapouda et al., 2017; Staats et al., 2016). Although DNA metabarcoding is beneficial in authenticating species and in studying species diversity in complex mixtures, it does not provide other essential information on target compound presence and concentration or the presence of chemical contaminations such as heavy metals, allergenic dyes and synthetic pharmaceuticals.

Conclusions

The increasing use of herbal medicines needs to be accompanied by an enhanced program of comprehensive quality control that is suited to all the stages of the supply chain, starting from the cultivation of the raw material to the marketing of the herbal product. The results of our study show that HPTLC is a reliable analytical tool for routine use to identify and distinguish *Echinacea* species in herbal products. It allows for better separation and a coarse quantification of chemical constituents, but it has limited resolution in detecting the presence of other species within the product. Here, we show that this limitation can be overcome through the complementary use of DNA metabarcoding that simultaneously confirms both the presence of the target species and of all other species, in even highly processed and multi-ingredient herbal products. Advances in sequencing technology make the use of DNA metabarcoding promising for large-scale authentication of herbal products. Nevertheless, standardization is required before it can be implemented as a routine complementary analytical method for regulatory quality control and herbal pharmacovigilance.

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Conflicts of interest

The authors declare no conflict of interests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2018.03.058. High-throughput sequencing raw data is deposited in DRYAD.

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