High-performance thin-layer chromatography–direct bioautography combined with chemometrics for the distinction of goldenrod species

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Abstract
Thirteen root extract samples of four goldenrod (Solidago) species present in Europe were investigated by hyphenated high-performance thin-layer chromatography (HPTLC). Only S. virgaurea is native, whereas S. gigantea, S. canadensis, and S. graminifolia have been introduced from North America. The bioactive zones in the Aliivibrio fischeri bioautogram were identified as polyacetylenes, labdane diterpenes, or clerodane diterpenes by HPTLC coupled to high-resolution mass spectrometry, exploiting the two interfaces, heated electrospray ionization, and direct analysis in real time. Principal component analysis of the obtained bioprofiles enabled the discrimination of the Solidago species. Furthermore, chemometrics pointed to the discriminative components, the main bioactive markers of the species: Z,Z-matricaria ester from S. virgaurea, solidagenone from S. canadensis, solidagoic acid A, and a dialdehyde clerodane diterpene from S. gigantea, and Z-dehydromatricaria ester from S. graminifolia.

Keywords HPTLC–Aliivibrio fischeri bioassay · Principal component analysis · Solidago species · Polyacetylenes · Diterpenes

1 Introduction
High-performance thin-layer chromatography (HPTLC) fingerprints of plant extracts can be used and compared to discriminate species, subspecies, varieties, or chemotypes [1–3]. Multiple HPTLC fingerprints can be obtained from the same separation by documenting at ultraviolet (UV for absorbance; FLD for fluorescence detection) and visible light (Vis), after performing chemical derivatizations or (bio)assays that visualize the chemical profiles or bioprofiles, respectively. The combination of multi-imaging HPTLC with pattern recognition using different chemometric tools enables the rapid fingerprinting and classification of the samples [4–6]. However, the usefulness of image processing for distinguishing samples based on their bioprofiles or biochemical profiles (effect-directed classification) has been demonstrated in only a few cases [7–9].

HPTLC combined especially with high-resolution mass spectrometry (HRMS) is an efficient tool for the characterization and identification of the selected biomarker compounds [10, 11]. The most widespread elution head-based coupling interface (elution head of 2 mm × 4 mm) is installed between the pump for eluent delivery and the MS [12, 13]. It enables a targeted MS analysis of zones of interest, while ambient desorption–ionization-based techniques, such as desorption electrospray ionization and direct analysis in real time (DART), allow scanning of the whole plate, however, only from an aliquot of each sample on the surface [14, 15].

The herbaceous perennial goldenrods (Solidago, Asteraceae) with yellow flowers often grow up to 2 m high [16]. The only goldenrod species native in Europe is S. virgaurea (European goldenrod). However, three further invasive, alien goldenrod plants are also widespread in Europe, i. e., S. gigantea (giant goldenrod), S. canadensis (Canadian goldenrod), and S. graminifolia (also known as Euthamia graminifolia, grass-leaved goldenrod). They were introduced about 250 years ago as ornamentals from North America and have become remarkably successful competitive invaders of abandoned fields, forest edges, and river banks in
most European countries. Several goldenrods are known as medicinal plants and the aerial part of *S. virgaurea* and those of *S. canadensis* and *S. gigantea* are listed in the European Pharmacopoeia as Latin names *Solidaginis virgaureae herba* and *Solidaginis herba*, respectively. Aerial parts are used in traditional medicine in the treatment of urinary complaints and as anti-inflammatory agents [17–19]. The decoction of *Solidago* roots is used by Indians against diseases of the urinary tract, diabetes, fever, pain, and inflammation [20].

The fully flowered plants can be distinguished based on their aerial parts. Only *S. canadensis* and *S. gigantea* are very similar, but their distinctive mark is their hairy and bald stems, respectively. Recently, HPTLC profiling of root extracts via post-chromatographic derivatization with vanillin-sulfuric acid reagent has been demonstrated as an efficient discrimination tool, which was confirmed by principal component analysis (PCA). Several antimicrobial root components of these species were identified using a non-target HPTLC–bioassay screening followed by compound isolation and highly targeted characterization. Among them were poly-acetylene matricaria esters from *S. virgaurea*, dehydro-matricaria esters from *S. graminifolia*, three labdane diterpenes from *S. canadensis*, and eight clerodane diterpenes from *S. gigantea* [21–24].

This study investigated the effect-directed classification of the four *Solidago* species in Europe based on their HPTLC–*A. fischeri* bioprofiles from root extracts as well as the assignment and identification of the responsible discriminative bioactive compounds.

2 Experiments

2.1 Materials

HPTLC plates silica gel 60 F254 and methanol (MS grade) were purchased from Merck (Darmstadt, Germany). Further solvents (analytical grade) were from Th. Geyer (Remlingen, Germany) or Sigma-Aldrich (Steinheim, Germany). The bioluminescent marine bacterium *Aliivibrio fischeri* (DSM 7151) was from Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures (Berlin, Germany). The culture medium was prepared as described [25].

| No. | Species     | Collection time | Collection area in Hungary |
|-----|-------------|-----------------|----------------------------|
| 1   | *S. gigantea* | March 2017      | Harta                      |
| 2   | *S. canadensis* | March 2017    | Remeteszőlős               |
| 3   | *S. virgaurea* | March 2017      | Budakeszi                  |
| 4   | *S. graminifolia* | February 2017 | National Botanical Garden, Vácrátót |
| 5   | *S. gigantea* | March 2017      | Dobogókő                  |
| 6   | *S. canadensis* | March 2017      | Budapest, district II      |
| 7   | *S. virgaurea* | March 2017      | Pilisszentkereszt          |
| 8   | *S. graminifolia* | February 2017 | National Botanical Garden, Vácrátót |
| 9   | *S. gigantea* | April 2014      | Harta                      |
| 10  | *S. canadensis* | March 2017      | Érd                        |
| 11  | *S. virgaurea* | June 2014       | Budapest, district II      |
| 12  | *S. graminifolia* | February 2017   | Eger                       |
| 13  | *S. graminifolia* | February 2017 | self-grown*                |

*Plant seed provided from the Botanical Garden of Masaryk University (Brno, Czech Republic) and grown in the greenhouse of Plant Protection Institute, Centre for Agricultural Research, Budapest.

2.2 Sample preparation

Roots of 13 goldenrod plants of four species (*S. virgaurea*, *S. gigantea*, *S. canadensis*, and *S. graminifolia*) were collected between 2014 and 2017 from various parts of Hungary (Table 1). Dried and ground (Bosch MKM6000, Stuttgart, Germany) samples were macerated in ethanol (150 mg/mL) for 24 h. The filtered crude extract was used after dilution (1:10 with ethanol).

2.3 HPTLC–bioassay

Extracts (1–5 µL/band) were applied as 6-mm bands with a 9-mm track distance and 8-mm distance from the bottom onto the HPTLC plate (ATS4, CAMAG, Muttenz, Switzerland). HPTLC separation was carried out with *n*-hexane–isopropyl acetate–acetone (16:3:1, *V*/V) in an unsaturated Twin Trough Chamber (20 cm x 10 cm, CAMAG) up to a migration distance of 70 mm, which took about 20 min [22, 23]. After development, the plate was dried in a cold stream of air, documented with a TLC Visualizer Documentation System (CAMAG). The antibacterial *A. fischeri* bioassay was performed as described [25]. Shortly, the dried chromatogram was immersed into the cell suspension of the
bioluminescent *A. fischeri* and immediately recorded (50 s exposure time, BioLuminizer CAMAG). Active zones were indicated as dark zones on the bioluminescent background (depicted as greyscale image).

### 2.4 HPTLC–HRMS

The plates were pre-washed (methanol‒water, 4:1, *V/V*) and dried (100 °C, 20 min). For HPTLC–HESI–HRMS experiments, the zones were online-eluted with MS-grade methanol at a flow rate of 0.1 mL/min (Ultimate LPG-3400 XRS, Dionex Softron, Germering, Germany) via the TLC–MS Interface (CAMAG) or PlateExpress Interface (Advion, Ithaca, NY, USA), both including an oval elution head (4 mm × 2 mm), into the heated electrospray ionization probe (HESI-II) of the Q Exactive Plus hybrid quadrupole–orbitrap mass spectrometer operated by Xcalibur 3.0.63 software (Thermo Fisher Scientific, Bremen, Germany). The spray voltage was 3.5 kV, the capillary temperature was 270 °C, the sheath gas was 20 arbitrary units, and the auxiliary nitrogen gas 10 arbitrary units. A full scan was recorded in the range of *m/z* 50–750 with a resolution of 280,000 in both negative and positive ionization modes. The automatic gain control target (AGCT) was 3 × 10^6, and the maximum injection time (IT) was 100 ms. For HPTLC–DART–HRMS scanning, a modified DART system [14] (IonSense, Saugus, MA, USA) was coupled to HRMS with the following settings: helium gas (99.999%) with flow rate 3.0 L/min and temperature 500 °C; scanning speed 0.2 mm/s; initial needle voltage 4 kV; grid voltage 50 V; positive ionization mode; full scan in the range of *m/z* 100–750 with a resolution of 35,000; AGCT 5 × 10^4; maximum IT 50 ms.

### 2.5 Multivariate image analysis of goldenrod root extracts

The open-source rTLC software for multivariate data analysis of planar chromatograms (http://shinyapps.ernaehrung.uni-giessen.de/rtlc/) [6] was applied. The HPTLC bioautogram image after the *A. fischeri* bioassay was uploaded in the software. Based on unsupervised pattern recognition, PCA was performed for the categorization of the 13 samples. The grey channel signals and *hRF* 30–90 were selected as variables.

### 3 Results and discussion

The compounds of the 13 root samples from four *Solidago* species were separated by HPTLC with *n*-hexane–isopropyl acetate–acetone (16:3:1, *V/V*). After the *A. fischeri* bioassay, several bioactive zones were observed (Fig. 1). As this mobile phase had already been applied for the separation of *S. gigantea* [23], *S. virgaurea* [22], and *S. canadensis* [22], the bioactive compounds could easily be identified by *hRF* value comparison. Thus, zones Sc1 (hRF 45) and Sc2 (hRF 53) from *S. canadensis* were assigned as solidagenone and the mixture of *R* - and *S* -presolidagenone, respectively. Zones Sv1 (hRF 83) and Sv2 (hRF 92) from *S. virgaurea* were identified as *Z*, *Z* - and *E*, *Z*-matricaria esters, respectively. Six bioactive zones from *S. gigantea* corresponded to eight clerodane diterpenes. Zone Sg1 (hRF 9) represented kingidiol, Sg2 (hRF 31) an epoxy-hemiacetal, Sg3 (hRF 46) three di-terpenes, a hautriwaic lactone, an alcohol, and a hemiacetal, Sg4 (hRF 51) a dialdehyde, Sg5 (hRF 58) solidagoic acid A, and Sg6 (hRF 37) solidagoic acid B.

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**Fig. 1 HPTLC–*A. fischeri* bioautograms of 13 root extract samples (Table 1) from the four *Solidago* species *S. gigantea* (Sg), *S. canadensis* (Sc), *S. virgaurea* (Sv), and *S. graminifolia* (Sgr), developed with *n*-hexane–isopropyl acetate–acetone (16:3:1, *V/V*).**
Based on the latest literature [24], dehydromatricaria esters were proposed to be the main bioactive zones Sgr1 (hR 75) and Sgr2 (hR 87) of S. graminifolia. To verify these preliminary assignments, HPTLC–HRMS experiments were carried out. The HPTLC–HESI–HRMS analysis of compound zones Sgr1 and Sgr2 provided very similar HRMS spectra in the positive ionization mode. For zone Sgr1 (Fig. 2), the sodium adduct ions were dominant at m/z 195.0412, corresponding to the molecular formula C_{11}H_{8}O_{2}Na^{+} (calculated m/z 195.0422). Besides, the sodium adduct of the dimer at m/z 367.0952 (C_{22}H_{16}O_{4}Na^{+}, calculated m/z 367.0946) and the protonated molecule at m/z 173.0593 (C_{11}H_{9}O_{2}^{+}, calculated m/z 173.0603) were detected.

Similarly, the same mass signals were recorded for both compound zones by HPTLC–DART +–HRMS scanning (Fig. 3), namely the protonated molecule at m/z 173.0601 for Sgr1 and m/z 173.0602 for Sgr2. These results confirmed the preliminary assignments of zones Sgr1 and Sgr2 as Z- and E-dehydromatricaria esters, respectively.

The open-source rTLC software was used to perform PCA on the signals obtained from the 13 separated root extracts in the HPTLC–A. fischeri bioautogram (Fig. 4). It was evaluated whether it was possible to distinguish the four Solidago species and to point to the most discriminating bioactive compounds according to the loading plot. The first three PCs accounted for 99.33% of the total variance, in which PC1, PC2, and PC3 referred to 95.59%, 2.71%, and 1.03%, respectively. PC2 showed the best separation among the species, while PC3 enabled their discrimination as well. Thus, the samples were divided into four distinct groups, confirming that PCA allowed the classification of the S. canadensis, S. gigantea, S. graminifolia, and S. virgaurea species based on their root extracts collected in wintertime from their persistent rhizomes. PC2 and PC3 were highly influenced by the compounds Sv1, Sgr1, Sc1, Sg5, and Sg4, which were considered as the most important antibacterial compounds for the discrimination of the goldenrod species.

4 Conclusions

The HPTLC–A. fischeri bioautograms of 13 goldenrod root extracts combined with chemometrics allowed us to distinguish the four Solidago species present in Europe. PCA and HPTLC–HRMS revealed the main biomarkers of the species responsible for their distinction. HPTLC–HRMS was shown as a straightforward hyphenation for the characterization and identification of the bioactive compounds.

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Fig. 4 PCA of 13 goldenrod root extract samples based on their A. fischeri bioautogram signals and PCs score plots (a) and loading plots based on effect-directed signal intensities and positions (b)

Declarations

Conflict of interest The authors declare no competing financial interest.

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