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Development of a Rapid LC/DAD/FLD Method for the Simultaneous Determination of Auxins and Abscisic Acid in Plant Extracts.

Renato Bosco, Matteo Caser, Francesca Vanara, Valentina Scariot*

Department of Agricultural, Forest and Food Sciences

University of Torino

Via Leonardo Da Vinci, 44 - 10095 Grugliasco (To)

Italy

*Corresponding author Tel: +039-011/6708932 Fax: +039-011/6708798

E-mail: valentina.scariot@unito.it

ABSTRACT

Plant hormones play a crucial role in controlling plant growth and development. These groups of naturally occurring substances trigger physiological processes at very low concentrations, which mandate sensitive techniques for their quantitation. This paper describes a method to quantify endogenous (±)-2-*cis*-4-*trans*-abscisic acid, indole-3-acetic acid, indole-3-propionic acid, and indole-3-butyric acid. The method combines high-performance liquid chromatography (HPLC) with diode array and fluorescence detection in a single run. Hybrid Tea rose 'Monferrato' matrices (leaves, petals, roots, seeds, androecium, gynoecium, and pollen) were used as references. Rose samples were separated and suspended in extracting methanol, after which (±)-2-*cis*-4-*trans*-abscisic acid and auxins were extracted by solvent extraction. Sample solutions were added first to cation SPE cartridges and the eluates to anion SPE cartridges. The acidic hormones were bound to the last column and eluted with 5% phosphoric acid in methanol. Experimental results showed that this approach can be successfully applied to real samples and that sample preparation and total time for routine analysis can be greatly reduced.

KEYWORDS: abscisic acid; auxins; indole-3-acetic acid; indole-3-butyric acid, indole-3-propionic acid, HPLC; fluorescence detection; diode array detection; SPE; *Rosa*

INTRODUCTION

Plant hormones are of vital importance for normal plant functioning. Their minute quantities trigger basic processes that mediate endogenous developmental programs[1] and integrate extracellular signals to regulate and optimize plant growth and performance. They control the balanced response of plants to adverse environmental conditions or biological threats.

To achieve precise regulation of these essential processes, the biosynthetic and catabolic pathways of the different hormonal groups must be highly responsive and adaptable to changing conditions. Comprehensive considerations on hormone biosynthesis, signalling, and control of gene expression have been presented recently.[2] For example, it is not surprising that abscisic acid signalling can act on a target shared with other response pathways for ethylene, jasmonates, gibberellins, and auxins[3,4] among others. These intimate relationships among phytohormones can make accurate analysis complicated due mainly to the minute concentrations (less than 50 ng/g) of secondary metabolites. Generally, traditional hormone determination methods have involved multiple steps of intensive purification and large amounts of plant tissue.[5]

Various analytical techniques have been developed for endogenous and exogenous phytohormone determination: gas chromatography mass spectrometry (GC-MS),[6] liquid chromatography-mass spectrometry (LC-MS),[7,8] capillary electrophoresis with UV (CE-UV)[9] or fluorescence (CE-FLD) detection,[10] high-performance liquid chromatography with UV (HPLC-UV),[11-13] fluorescence (HPLC-FLD) or chemiluminescence (HPLC-CL)[14] detection, and enzyme-linked immunosorbent assay (ELISA).[15] Although these methods have contributed greatly to phytohormone analysis, some limitations persist in their application. For example, when GC or GC-MS is employed, derivatization of phytohormones to more volatile methyl esters is required. The ELISA method exhibits cross-reactivity with structurally-related compounds in the same sample. The LC-MS method is too expensive for the scale needed for real samples. Several new analytical techniques are emerging and are destined to play prominent future roles. At present the preferred method of plant hormone separation and determination is still reversed-phase high performance

liquid chromatography (HPLC), even if complex sample preparation and high costs are limitations for its application.[16-19]

To this end, the present work aimed to develop an effective, selective, sensitive, and inexpensive analytical method for simultaneous quantitation of (±)-2-*cis*-4-*trans*-abscisic acid (**1**), indole-3-acetic acid (**2**), indole-3-propionic acid (**3**), and indole-3-butyric acid (**4**) based on solid phase extraction (SPE) purification and HPLC analysis. Different matrices of Hybrid Tea rose 'Monferrato' have been used to validate the methodology.

MATERIALS AND METHODS

Reagents and Chemicals. Analytical reagent grade chemicals were used unless otherwise indicated. Water (conductivity less than 0.05 μS/cm), methanol, and acetonitrile (Merck) were all of HPLC grade. Acids: **1**, **2**, **3,** and **4 (Figure 1),** cartridges Discovery® SPE DSC-MCAX (bed wt. 300 mg, volume 6 mL; Supelco® Analytical, Bellefonte PA, USA), and Supelclean™ SPE LC-NH2 (bed wt. 300 mg, volume 6 mL; Supelco® Analytical, Bellefonte PA, USA) were all purchased from Sigma-Aldrich (Milano, Italy).

Stock and Working Solution Preparation. Stock standard solutions (1 mg/mL) of **1**, **2**, **3,** and **4** were prepared using methanol as the solvent. All other standard solutions were prepared by dilution of the stock solution to obtain concentrations ranging between 0.0010 and 10 μg/mL for **1** and between 0.00010 and 10 μg/mL for **2**, **3,** and **4**.

Samples and Sample Preparation. Completely opened leaves, unfolded petals, entire roots, mature seeds, androecium and gynoecium in folded flowers, and fresh pollen of the rose hybrid 'Monferrato' were collected in September 2012 (mean temperature of 18.5 °C, mean relative humidity of 67.2 %) in the rose garden of the Experimental Centre of the Department of Agricultural, Forest and Food Sciences of the University of Torino (Italy; 45°03'59.73" N, 7°35'24.72" E), immersed in liquid nitrogen (N_2), and maintained at -80 °C until analysis.

Rose samples were weighed, ground in liquid N_2 and 0.3 g of each homogenized sample was transfer to a 10 mL glass tube, the suspension were extracted with 2 mL of 80% aqueous methanol containing 10-20 mg/L of butylated hydroxytoluene for 16 h at 4 °C in darkness under magnetic stirring.[20] The extract was diluted to 6 ml with water, the pH was adjusted to 2.5 with 1 M aqueous HCl, after which the samples were filtered and eluted. The eluates were then added first to SPE DSC-MCAX cartridges that had been previously washed with 2 mL 100% methanol and equilibrated with 2 mL water. Next, the elutes (6 mL) were added to SPE LC-NH2 cartridges, also previously washed with 2 mL 100% methanol, and equilibrated with 2 mL water. The acidic hormones were bound to the last column and eluted with 1 mL of 5% phosphoric acid in methanol. A total of 20 μL of purified samples were injected into HPLC.

LC/DAD/FLD Conditions. The chromatographic analysis was performed on an Agilent Model HPLC chromatographic system consisting of an HPLC series 1200 (Agilent Technologies, Böblingen, Germany) comprised of the following modular components: a vacuum degassing unit, a quaternary pump, an auto injector, a column oven, a diode array detector (DAD G1315D), and a florescence detector (FLD G1321A). The column used was a 250 mm x 4.6 mm i.d., 5 μm, Zorbax eclipse XDB-C18 (Agilent Technologies, Böblingen, Germany).

Throughout this study, the mobile phases was (A) acetonitrile and (B) aqueous phosphoric acid solution of pH 3.2. The column was equilibrated and the column temperature was maintained at 40 °C +/- 0.1 °C. Separation was carried out by gradient elution with a constant flow rate of 0.5 mL/min. The gradient program was as follows: 5-70% A (0-75 min). After the gradient separation, the column was re-equilibrated with: 70-100% A (75-86 min) for 5 min and 100-5% A in 20 min. An injection volume of 20 μL was used for each analysis.

The standard solution of each acid was prepared in methanol and chromatographed separately to determine the retention time for each **(Figure 2)**. An HPLC Agilent series 1200 fluorescence detector was placed in series with the diode array detector. The signal for **1** was monitored at 265 nm; the excitation wavelength and emission wavelength of the **2**, **3,** and **4** were

measured via fluorimetric detection at 281 nm and 340 nm, respectively. The retention times of the solutes were determined from three different injections. Peak identifications were based on retention times and standard additions to the samples; hormones were quantified according to a calibration curve that was constructed from the measurement data of the matrix matched calibration standards.

Validation. Method validation was performed following the recommendations of the International Conference on Harmonization for selectivity, linearity, extraction efficacy, precision, and accuracy. The limits of detection (LODs) and quantitation (LOQs), as well as analyte stability in the sample and standard solutions were also evaluated.

RESULTS AND DISCUSSION

Method Development. To optimize extraction, a representative quantity of each matrix was used to better understand the process and the influence of sample constituents and secondary metabolites. As a study first step, organic solvents of different polarities (methanol, ethanol, and acetonitrile) were assessed for their relative extraction efficiency. At present, solvent extraction is the most widely used method for plant hormone extraction. Many different procedures and solvents (methanol, methanol/water mixture, acetone, acetone/water, propanol, propanol/water, and neutral or acid buffers) have been developed and broadly used[21-23] for plant hormone extraction. The polarity of the extraction solvent is chosen to closely match that of the target compound; thereby, the ratio of organic solvent to water is defined according to the polarity of hormones. Non-polar solvents such as ether are rarely used to extract plant hormones. Instead, methanol is the preferred solvent as its small size and low molecular weight allow for efficient plant cell penetration during extraction.[24-28] As methanol produced the best recoveries, consequent investigations were performed using methanol with differing proportions of ultra-pure water (80, 70, and 60%).

We found that using 80% methanol as the extraction solvent resulted in the maximum target compound quantities. Butylated hydroxytoluene was added to the solution as an antioxidant.

The optimal efficacy of an extraction procedure is defined as the highest yield of analytes in the shortest time. During this extraction procedure investigation, time values ranged from 1 to 24 h (1, 2, 4, 16, and 24 h). We found that the maximum amount of each analyte was extracted from all sample types at 16 h; additional extraction time showed no increase in extraction efficacy.

Another complication in the process is that some plant hormones are labile during extraction. Auxins, for example, are readily oxidized or degraded when exposed to light, oxygen, and high temperature. In some studies, to protect against degradation, antioxidants are added during the extraction process.[29] The influence of solar light on the efficacy of analyte extraction and the purification procedures was observed in this study. To avoid analyte degradation, all analytical steps of the extraction procedures were performed in the dark at 4 °C with amber glassware and artificial light.

SPE column decision choices should take into account several factors: the sample matrix, analyte physicochemical properties, and the nature of the bonded phase. Combinations of two or more different SPE column types are often employed in plant hormone analysis. Generally, anion-exchange columns and cation-exchange columns are used to extract acidic analytes and basic analytes, respectively.[30-32] The interconversion between the conjugate bases of phytohormones and their acid form is important for anionic SPE-NH2 detention. We used different acids (acetic acid, formic acid, phosphoric acid, and hydrochloric acid) to lower the pH of the sample to 2.5; the best results were obtained with 1 M HCl in H_2O with the SPE DSC-MCAX previously used for preliminary matrix purification. Various mobile phase compositions were tested to identify the optimal chromatographic condition. Each column provided a different combination of hydrophobicity and analyte interaction. The shortest analysis with good resolution and peak shapes with no tailing was observed using Zorbax eclipse XDB-C18.

To obtain good separation and resolution across all analytes, we tested various isocratic solvent systems: methanol/water; methanol/water both to 1 mmol H_3PO_4; acetonitrile/water both to 1 mmol H_3PO_4; and acetonitrile/water with 1 mmol H_3PO_4 only in water and brought to pH 3.2 with

NaOH 1 molar.[33-35] As total analysis time was protracted, gradient elution was employed. The effect of column temperature on analyte separation for the range from 25 to 45 °C was investigated. Good resolution and peak shapes without tailing resulted at 40 °C.

HLPC, which can directly analyze polar compounds, is more suitable for most plant hormones when using a UV detector rather than derivatization methods. The main drawback of using a UV detector is its inferior sensitivity.[36,37] Compared to a UV detector, the sensitivity of a fluorescence detector (FLD) is about 2–3 orders of magnitude higher, making it far more suitable for plant hormone detection.[38] Initially, we expected to use UV detection for all analyte determinations, but auxin levels in selected samples were anticipated as too low for quantitation by the method. Therefore, fluorescence detection was adopted for auxin determination given its superior selectivity and sensitivity. Indole derivates are known to usually contain the key structural characteristics of fluorescent substances: rigid planar structures and a big π-conjugated system. Therefore, **2**, **3** and **4**, all have natural fluorescence properties.

Representative chromatograms of the different rose matrices using DAD are shown in **Figure 3**; the same is displayed in **Figure 4** using FLD. The retention times of **1**, **2**, **3**, and **4** were 39.30± 0.01, 35.85 ± 0.01, 42.07± 0.01, and 46.79 ± 0.01 min, respectively.

Optimized conditions yielded symmetrical and sharp peaks for all four analytes with peak purities higher than 999.1. The peak purities were calculated on the base of the usual standard addition method. Furthermore, the resulting chromatograms revealed that, in spite of the complex matrix of the samples, almost no other components were co-eluted with the compounds of interest.

Method Validation.

Extraction efficacy. The extraction efficacy and procedure reproducibility for **1** (5 μg/mL) and **2**, **3**, and **4** (1 μg/mL) were determined for each representative sample type by comparing the responses from samples spiked before extraction with those from samples extracted and spiked after extraction. All extraction recoveries were relatively high (90–97%), which can be explained by the simple and effective sample preparation procedure. Also of note, is that despite the wide variability

of matrix type and composition, adequate extraction recoveries for all four analytes were achieved in all sample types.

Linearity, LOD, and LOQ. Linearity of **1** was tested in the range of 0.01 to 50 μg/mL, while for **2**, **3,** and **4** linearity was tested in the range from 0.001 to 10 μg/mL. At least nine concentration levels were used in all calibration curves. The obtained correlation coefficients were higher than 0.999, indicating satisfactory linearity of the developed method. The limits of detection and quantitation were determined by injecting a series of dilute solutions with known concentrations. LOD and LOQ were defined as a signal-to-noise ratio equal to 3:1 and 10:1, respectively. Limits of **1** using UV detection were quite low at 0.003 µg/mL (LOD) and 0.010 µg/mL (LOQ). In the case of **2**, **3,** and **4** using fluorimetric detection, the LOD and LOQ values obtained were even lower at 0.0003 μg/mL and 0.0009 μg/mL, respectively. This result highlighted the high sensitivity of the presented method.

Precision. Method precision experiments were performed using spiked assays of each representative sample type (5 μg/mL for **1**; 1 μg/mL for **2, 3** and **4**). The sample preparation procedure and analysis were repeated six times within the same day to obtain the intra-day precision, while inter-day precision was assessed by three replicate analyses on three consecutive days. The precision measurement values, expressed as relative standard deviations (RSDs), were below 1.5% and 3.5%, for intra- and inter-day precision respectively. The data revealed that the proposed method was reproducible.

Accuracy. Accuracy was assessed by the determination of recovery using the standard addition method. Samples were prepared by spiking each representative sample type with three different levels of each analyte, and the entire procedure was repeated three times. The recoveries were calculated based on the ratio of added and obtained amounts. The results show the proposed method to have satisfactory accuracy as the recoveries of all analytes ranged between 90 and 97%.

Stability. As part of method validation, data were also generated to ensure that all analytes were stable at distinct times and temperatures. Stability tests were performed to assess the short-

term and long-term storage, and auto sampler stability. Analyte stability in the spiked representative samples and in the standard solutions, were also analyzed. Short-term stability was assessed at room temperature for 6 h, a time period expected to exceed that of a routine sample preparation. Long-term stability was assessed at -20 °C for 10 days, and the auto sampler stability was tested by storing samples at 4 °C for 24 h. The recovered analyte percentages from samples ranged between 94.4 and 96.6%, indicating that degradation of all analytes was not significant under the chosen conditions.

Analysis of Real Samples. Plant matrix complexity complicates any analysis of phytohormones. Accurate quantitation of trace amounts of these compounds requires robust methods. This work presented and described a highly specific protocol for simultaneous determination of **1**, **2**, **3,** and **4** in plant material. The analysis was conducted in different matrices obtained from the Hybrid Tea rose 'Monferrato'. All samples were collected on the same day and subsequently placed at -80 °C until the day of analysis.

Results displayed in **Table 1** show that all studied phytohormones were present in the collected matrices, which demonstrates that **1**, **2**, **3,** and **4** were quantified simultaneously. Having a method to accurately and readily quantify phytohormones in plant components is highly useful. In this study, **1** content in leaves, petals, roots, seeds, androecium, gynoecium, and pollen was measured at 2.322, 0.670, 0.945, 0.160, 17.053, 6.086, and 3.901 μg/g, respectively. The resulting values agreed with the ranges found in previous studies. The role of **1** in the regulation of seed dormancy is well known.[39] Hormone values differ according to species and seed age. Bo et al.[40] detected 2.410 μg/g **1** in Hybrid Tea rose 'Crimson Glory' and Yambe et al.[41] 1.800 μg/g in Hybrid Tea rose 'Inspiration', while Ueda[42] reported 2.700 μg/g of **1** in the dormant seeds of *R. rugosa* and 0.400 μg/g of **1** in *R. persica*.

Knowledge of **1** concentration in roots and leaves is of particular interest to understand plant response to abiotic stresses. **1** is involved in the regulation of many stress-induced gene-expressions and confers the plant with adaptability toward drought, salinity, cold, and other environmental

239 stresses. A recently conducted study in rose by Arve et al.[43] under dark/light conditions and
240 moderate/high humidity found **1** leaf quantitation levels ranging from ca. 1.00 μg/g to ca. 5.00 μg/g
241 using UPLC-ES-MS/MS. An easy quantitation of the hormone in flower components would be
242 valuable to elucidate the role of **1** in flower senescence. Both Muller et al.[44] and Kumar et al.[45] have
243 observed a direct correlation between **1** and flower senescence; they found that during the stage
244 when petals were completely unfolded, **1** was 0.17 μg/g. Minimal knowledge exists on the role of **1**
245 in rose pollination and flower fertility. Bianco et al.[46] suggested that the androecium might be an **1**
246 source, particularly during pollination and subsequent petal senescence.

247 The effects of auxins in plants are extremely varied. Auxins commonly inhibit root
248 elongation, but high auxin concentrations promote initiation of secondary branches and roots, as
249 well as adventious root formation on stems. Low levels of auxins in the organ have been correlated
250 with abscission, such that auxins have been used to prevent premature fruit drop. **2** is present in
251 lower and higher plants and is reported to represent between **1** and 100 ng/g of fresh weight in
252 plants, **3** and **4** have not been identified in every plant yet, and **4** occurs in only some higher plants
253 such as *Zea mays.*[47]

254 In the present work we were able to quantify **2**, **3**, and **4** at the same time. The lowest
255 concentration of **2** was found in seeds (0.144 μg/g); for **3** and **4**, the lowest levels were found in
256 pollen (0.025 μg/g and 0.012 μg/g, respectively). As expected a very high quantity of **2** (7.615
257 μg/g) was quantified in roots. Limited knowledge on endogenous auxin quantitation in the genus
258 *Rosa*, for comparison purposes, was found in literature. Tillberg[48] obtained a concentration of 0.08
259 μg/g of **2** in mature seeds.

260 In conclusion, we have described a rapid, sensitive, and accurate method to determine the
261 amount of **1**, **2**, **3,** and **4** in different plant matrices based on SPE purification and HPLC separation,
262 coupled with diode array and fluorescence detection. The described procedure allows quantitation
263 of plant hormones in their natural states without any derivatization step prior to analysis. The
264 studied compounds are representatives of two different groups of acidic plant hormones ((±)-2-*cis*-

4-*trans*-abscisic acid and auxins) with several important biological properties. This method opens the possibility to incorporate other plant hormones and related metabolites into a single analysis. The simultaneous determination of phytohormones could support scientific community efforts in precision agriculture, plant functional genomics, and hormone signal transduction.

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ABBREVIATIONS USED

1, (±)-2-*cis*-4-*trans*-abscisic acid; CE-FLD, capillary fluorescence detection; CE-UV, capillary electrophoresis; DAD, diode array detection; ELISA, enzyme-linked immuno sorbent assay; HPLC-CL, high-performance liquid chromatography with chemiluminescence, detection; HPLC-FLD, high-performance liquid chromatography with fluorescence detection; **2**, indole-3-acetic acid; **3**, indole-3-propionic acid; **4**, indole-3-butyric acid; LC-MS, liquid chromatography-mass spectrometry; SPE, solid phase extraction.

ASSOCIATED CONTENT

Supporting information

Table S1. Extraction efficacy of the proposed sample preparation procedure.

Table S2. Method calibration data.

Table S3. Intra- (n = 6) and inter-assay (n = 18) precision of the proposed method.

Table S4. Accuracy (n = 3) of the proposed method.

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Figure legends

Figure 1. Chemical structures of (±)-2-*cis*-4-*trans*-abscisic acid (**1)**, indole-3-acetic acid (**2)**, indole-3-proponic acid (**3)**, and indole-3-butyric acid (**4)**.

Figure 2. Representative chromatograms (LC/DAD in A and LC/FLD in B) of standards of (±)-2-*cis*-4-*trans*-abscisic acid (**1)**, indole-3-acetic acid (**2)**, indole-3-proponic acid (**3)**, and indole-3-butyric acid (**4)**, 1.00 µg/g each.

Figure 3. Representative LC/DAD chromatograms of the rose hybrid 'Monferrato' matrices (A leaves; B petals; C roots; D seeds; E androecium; F gynoecium; G pollen) analyzed with the developed method.

Figure 4. Representative LC/FLD chromatograms of the rose hybrid 'Monferrato' matrices (A leaves; B petals; C roots; D seeds; E androecium; F gynoecium; G pollen) analyzed with the developed method.

Table 1. Phytohormone Concentrations in the Different Investigated Matrices of the Rose Hybrid 'Monferrato'.

		Phytohormones (µg/g)			
Sample type	Sample (n.)	**1**	**2**	**3**	**4**
Leaves	4	2.322 ± 0.44	0.847 ± 0.000	0.114 ± 0.000	0.059 ± 0.000
Petals	8	0.670 ± 0.23	1.081 ± 0.008	0.054 ± 0.000	0.023 ± 0.000
Roots	4	0.945 ± 0.51	7.615 ± 0.048	0.110 ± 0.001	0.027 ± 0.000
Seeds	4	0.160 ± 0.03	0.144 ± 0.000	0.035 ± 0.000	0.074 ± 0.000
Androecium	4	17.053 ± 0.77	7.091 ± 0.037	0.130 ± 0.000	0.032 ± 0.000
Gynoecium	4	6.086 ± 0.14	6.437 ± 0.001	0.078 ± 0.000	0.050 ± 0.000
Pollen	4	3.901 ± 0.03	0.583 ± 0.000	0.025 ± 0.000	0.012 ± 0.000

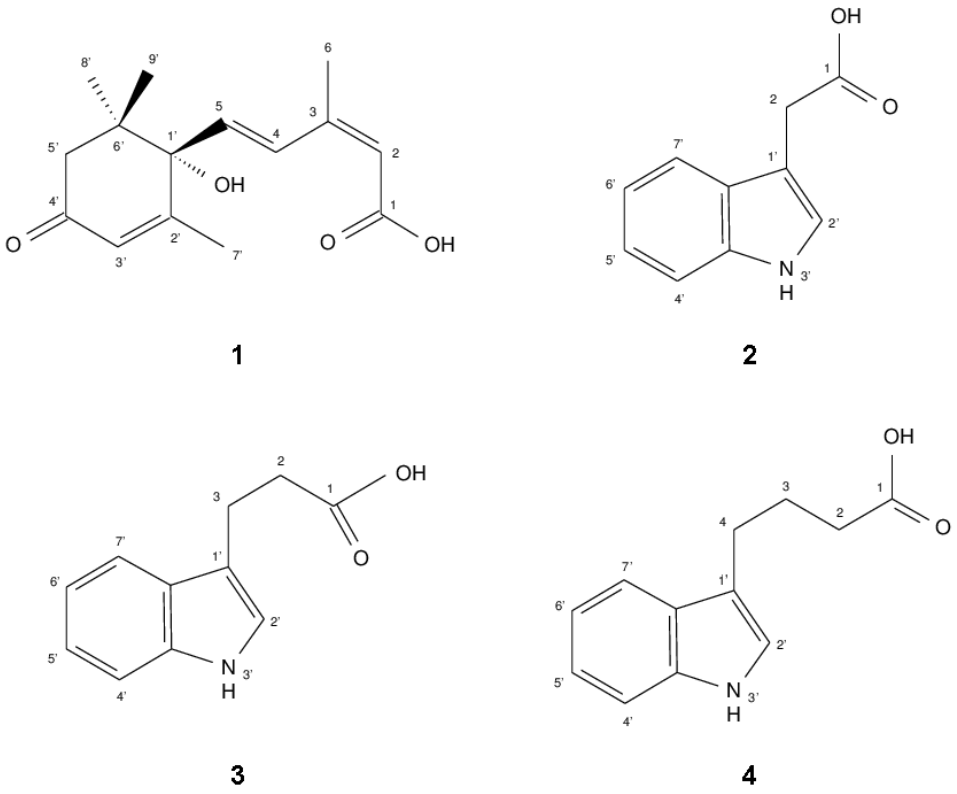
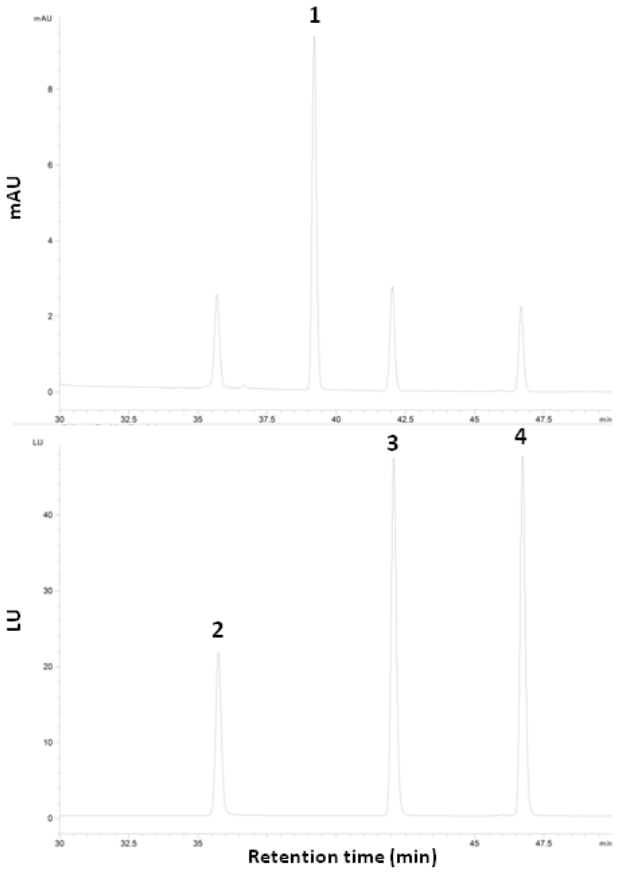


Figure 1


1
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6
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2
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30
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35
37.5
40
42.5
45
47.5
min
3
4
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40
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20
10
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30
32.5
35
45
47.5
min
Retention time (min)

Figure 2

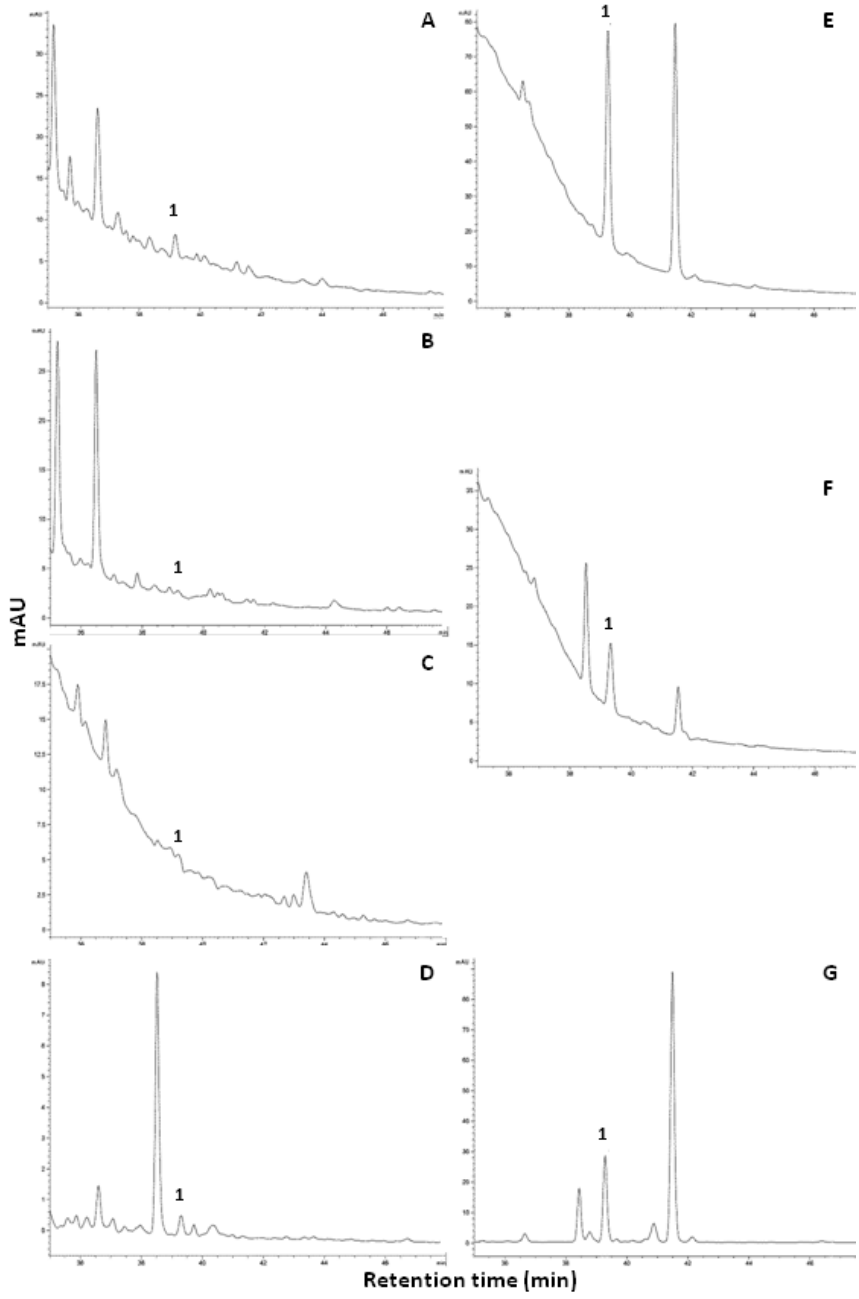

Figure 3

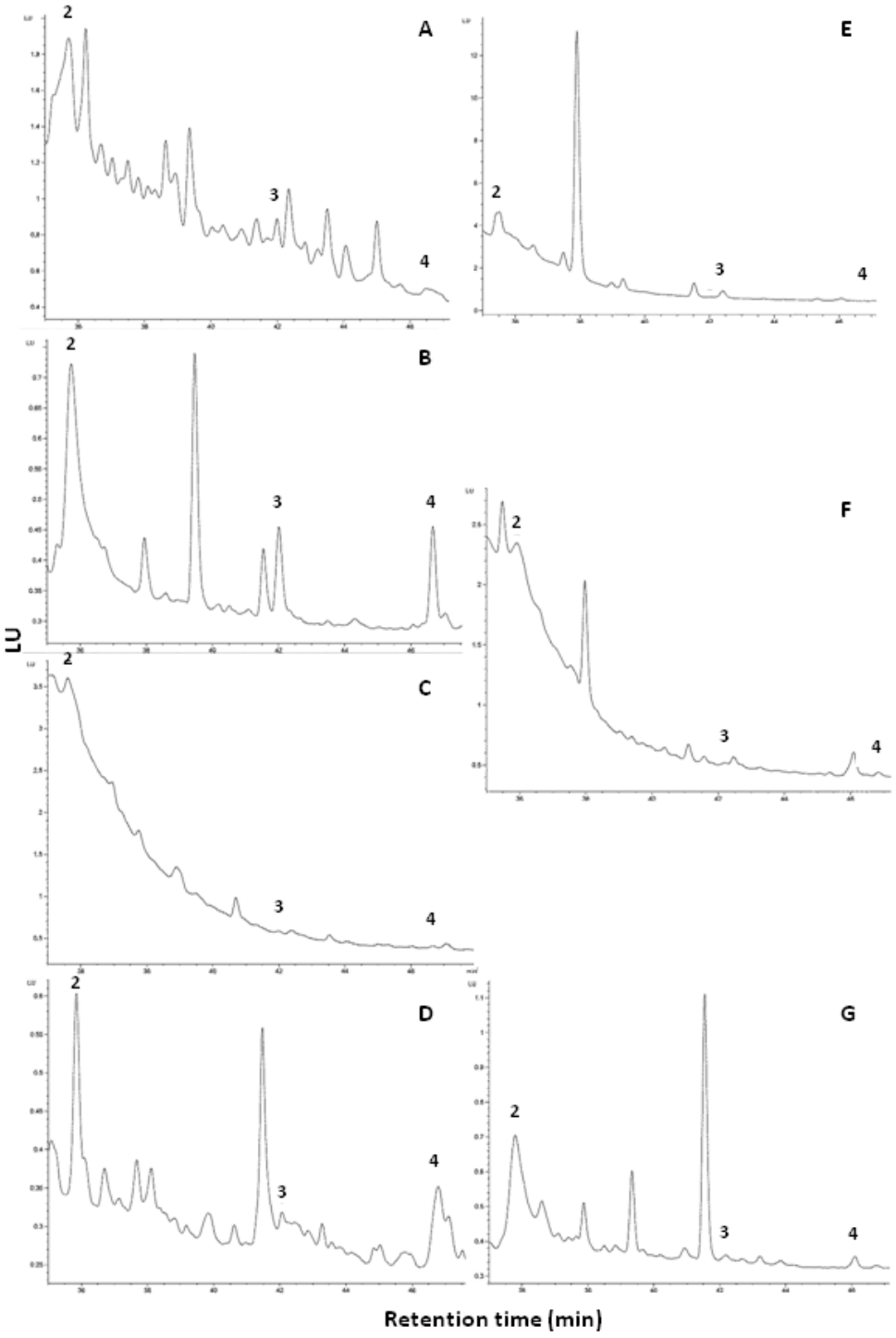

Figure 4