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Characterization of indole-3-acetic acid in cambial zone tissue by ultra-performance liquid chromatography-tandem mass spectrometry

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ABSTRACT

The developmental plasticity of plants according to changes in their growth conditions is mediated by signaling molecules called hormones. The major classes of plant hormones are auxin, gibberellins, abscisic acid, and ethylene. Among these, the most important is indole-3-acetic acid (IAA). Quantification of IAA in an extract of approximately 10 mg obtained from the cambial zone of *Eucalyptus* leaves was performed by ultra-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC/ESI-MS/MS). The leaves were extracted using acetone, and the extract was analyzed using a reverse-phase column (Acquity UPLC BEH C18, 2.1 mm × 100 mm, 1.7 µm) at a flow rate of 0.2 mL/min with gradient elution of an aqueous mobile phase (containing 0.1% formic acid) with methanol. This gradient elution provided an excellent performance in terms of specificity/selectivity, linearity, precision, and ruggedness/stability. In addition, the run time was short (6 min), with excellent linearity ($R^2 > 0.99$) in the range of 10–70 ng/mL. The structure of IAA was elucidated using UPLC/ESI-MS/MS, operating and quantifying in multiple reaction monitoring (MRM) mode.

1. INTRODUCTION

The developmental plasticity of plants according to changes in growth conditions is mediated by signaling molecules called hormones. The major classes of plant hormones are auxin, gibberellins, abscisic acid, and ethylene [1-5]. Among these, the most important is indole-3-acetic acid (IAA). IAA is a key organizer of the growth and development of the vascular cambium [6-8]. In intact plants, polar IAA flow is essential for the initiation of spatially organized patterns of vascular tissues, as well as for the maintenance of vascular cambium. Furthermore, it has been shown that IAA affects many aspects of cambial secondary growth, including cell division, differentiation, cell enlargement, secondary wall thickness, and lignification [9]. In addition, exogenous treatments with IAA, alone or in combination, appear promising for promoting chemical changes in plants such as *Glycyrrhiza uralensis* [10].

The inability to develop a unifying concept for the role of IAA in the regulation of vascular tissue patterns is due to a limited knowledge of both the mechanisms of perception and metabolism of IAA and its transport and final distribution [11]. Detailed knowledge of the transport and distribution of IAA would elucidate its role in signaling position, i.e., would verify its role in acting as a morphogen. Thus, it is necessary to develop accessible and relatively inexpensive techniques to detect IAA at extremely low levels in cambial zone tissues.

Since the identification of IAA as the main auxin in plants, there has been a continuing development of methods to measure its endogenous concentration in plant tissues. One can cite, for example, high-performance liquid chromatography (HPLC) with fluorescence detection [12-14] and immunological techniques [15,16]. However, when applied to plant extracts, which are a complex mixture of multiple components, these methods still require refinement to ensure that the detected response is accurate.

An attractive technique for the detection and quantification of IAA in extracts of approximately 10 mg mass obtained from the cambial zone of *Eucalyptus* is ultra-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (UPLC/ESI-MS/MS). This technique, due to its high degree of information, can be used for quantitative analysis to determine analytes at trace levels in complex matrices. In addition, UPLC/ESI-MS/MS enables the elimination of spectral interference and minimizes the problem of coelution of substances found.

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Given the above, our aim was to develop a methodology using UPLC/ESI-MS/MS capable of quantifying the auxin hormone in small samples of cambial zone tissues through an extraction procedure consisting of a simple and inexpensive method using acetone.

2. MATERIALS AND METHODS

2.1. Chemicals

The standard IAA phytohormone (purity = 99.9%) was obtained from Sigma-Aldrich (Steinheim, Germany). Methanol, formic acid, and acetone (HPLC grade) were used. Ultrapure water was used throughout the analysis, and filter paper (Whatman 12.5 cm, No. 542) and 0.22 µm PTFE filter membranes were also utilized.

2.2. Preparation of Samples for Hormone Analysis

A wood block of about 3 cm × 3 cm × 3 cm, consisting of phloem and xylem tissue, was removed at breast height from hybrid clonal *Eucalyptus grandis* x *Eucalyptus urophylla* trees during September 2015 (growing season in the Southern Hemisphere). The block was immediately frozen in liquid nitrogen, transported on dry ice, and then stored at −75°C.

In the laboratory, the block was carved to dimensions of approximately 5 mm × 15 mm × 20 mm (width × height × depth) with the help of flexible diamond discs with a segmented face (Sorensen, Discoflex). Then, consecutive cryosections from xylem to phloem were obtained using a cryostat (HM 505 E microtome, Laborgeräte Microm, Walldorf, Germany) cooled to −19°C and adjusted to produce sections with a thickness of 30 µm.

2.3. Extraction Method

Cryosections obtained as described above were homogenized in an agate mortar with an agate pestle and posteriorly transferred to Eppendorf vials. Then, 2.0 mL of acetone was added. The vials containing the macerated sample and acetone were closed and subjected to ultrasound treatment for 50 min. Subsequently, the acetone was evaporated in a concentrator (SpeedVac, Thermo Fisher, Waltham, MA, USA) using centrifugation and vacuum at 45°C. Finally, the sample was resuspended by adding 2.0 mL of methanol: Milli-Q water (80:20, v: v) with 0.1% formic acid.

2.4. Preparation of Analytical Solutions

For the preparation of working solutions from stock solution, a diluent solution of methanol, Milli-Q water, and formic acid was prepared. The preparation of the diluent consisted of homogenization of 200 mL of Milli-Q water and 800 mL of methanol in a 1000 mL beaker with lid, followed by the addition of 1 mL of formic acid, homogenization, filtering through a PTFE membrane filter of 0.22 uM, and transferring the solution to a flask with lid.

The standard stock solution was prepared by weighing approximately 10.0 mg of IAA reference standard, transferring it to a 100 mL volumetric flask, adding 50.0 mL of the diluent, and keeping the solution in an ultrasonic bath until complete dissolution had occurred. The solution was then cooled to room temperature, and the flask volume was completed with diluent and homogenized, thus obtaining a solution with a concentration of 100 ug/mL.

The preparation of the linearity stock solution consisted of transferring a 0.1 mL aliquot of standard stock solution to a 10 mL volumetric flask, followed by the addition of diluent to complete the volume. Thus, a linearity stock solution with a concentration of 1 µg/mL was obtained. This solution was diluted, respectively, to obtain working solutions of seven different concentrations (10, 20, 30, 40, 50, 60, and 70 ng/mL). Each working solution was analyzed by UPLC/ESI-MS/MS to construct calibration curves.

2.5. Equipment

The study was performed using a UPLC system (Acquity, Waters) coupled to an electrospray ionization tandem mass spectrometry system (UPLC/ESI-MS/MS). The spectrometer was a triple quadrupole (TQD, Waters). The operating conditions of the mass spectrometer, cone voltage, and collision were optimized in MRM mode.

The main parameters of the UPLC/ESI-MS/MS optimized for determining the test compound are described below.

2.6. Mobile Phases

Solution A was prepared by transferring 900 mL of Milli-Q water to a 1000 mL volumetric flask, adding 5 mL of formic acid, completing the volume of the volumetric flask with Milli-Q water, homogenizing, filtering with a 0.22 µm PTFE membrane filter, transferring to a flask with lid, and finally degassing the solution.

Solution B was prepared by transferring 1000 mL of methanol to a 1000 mL volumetric flask, homogenizing, filtering with a 0.22 µm PTFE membrane filter, transferring to a flask with lid, and finally degassing the solution.

Table 1 shows the optimal gradient elution program for separating components in this study.

Table 2 shows UPLC/ESI-MS/MS conditions used in the study.

2.7. Mass Spectrometer Optimization for Sensitive IAA Detection

A standard IAA solution was introduced into the UPLC/ESI-MS/MS to obtain a mass spectrum in the quadrupole 1 (Q1) scan mode to detect the molecular-related ions of IAA.

2.7. Signal-to-noise Ratio (S/N)

The limit of detection (LOD) and limit of quantification (LOQ) of the method were based on the S/N ratio, and estimates of 3:1 and 10:1 for LOD and LOQ were accepted, respectively. The S/N was calculated by MassLynx software version 4.1 SCN805 of the UPLC (Acquity, Waters) coupled to mass spectrometer TQD (TQD, Waters) equipment.

2.8. Linearity

The linearity of the analytical method was determined by a calibration

Table 1: Chromatographic gradient conditions for the analysis of IAA

Time (min)	Flow (mL/min)	Solution A (%)	Solution B (%)
Initial	0.2	81.0	19.0
2.5	0.2	10.0	90.0
4.5	0.2	10.0	90.0
5.00	0.2	10.0	90.0
5.5	0.2	81.0	19.0
6.0	0.2	81.0	19.0

IAA: Indole-3-acetic acid

Table 2: UPLC/ESI-MS/MS conditions used in the study

Ionization source parameters	
Capillary (kV)	5.00
Cone (V)	25.0
Extractor (V)	3.00
RF lens (V)	0.1
Source temperature (°C)	120
Desolvation temperature (°C)	250
Desolvation gas flow (L/h)	500
Cone gas flow (L/h)	25
Analyzer parameters in MS/MS mode	
LM 1 resolution	10
HM 1 resolution	10
Ion energy 1	0.5
Entrance	1.0
Exit	0.5
Collision	30
LM 2 resolution	10
HM 2 resolution	10
Ion energy 2	0.5
Collision gas flow	0.10
Desolvation gas	Nitrogen
Collision gas	Argon
UPLC	
Column	Acquity UPLC BEH C18 2.1 mm×100 mm, 1.7 µm
Column temperature (°C)	40°C
Sample temperature (°C)	15
Flow (mL/min)	0.2
Run time (min)	6.0

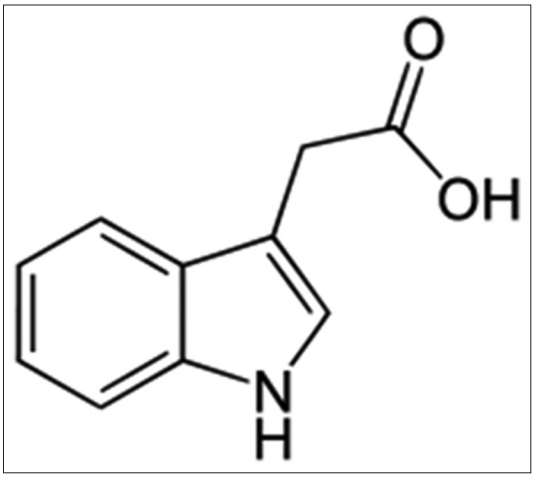

Figure 1: Structure formula of indole-3-acetic acid

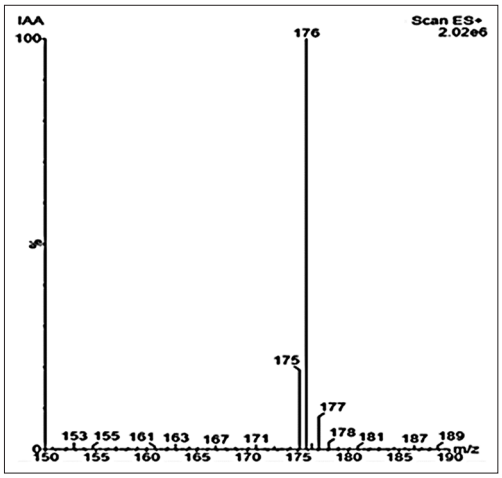

Figure 2: Scan spectrum by electrospray ionization in positive mode (ESI^+) of the IAA standard

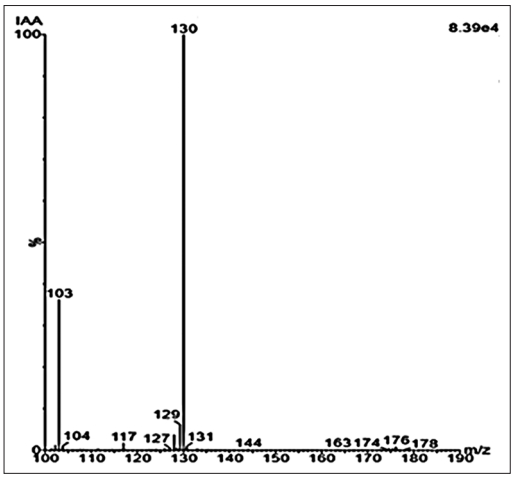

Figure 3: Spectrum of fragmentation of the indole-3-acetic acid standard obtained by collision with argon gas

curve with seven different concentrations of the IAA compound (10, 20, 30, 40, 50, 60, and 70 ng/mL).

3. RESULTS AND DISCUSSION

3.1. Specificity/Selectivity

Specificity refers to the ability of an analytical method to assess unequivocally the analyte in the presence of sample matrix analyte. Figure 1 shows the chemical structure of IAA.

A study published in the literature using an electrospray ionization quadrupole time-of-flight mass spectrometer confirmed that IAA presents the molecular formula $C_{10}H_9NO_2$ with an accurate molecular weight (monoisotopic) of 175.0633 Da, and in the positive mode analysis, there was the formation of ion species $[(M+H)^+]$ with molecular formula $C_{10}H_{10}NO_2$ and measured mass (monoisotopic) of 176.0727 Da [17].

Therefore, when the analysis was performed in the full scan by electrospray ionization in positive mode (ESI^+) using the TQD mass spectrometer equipment, it was possible to obtain a mass/charge ratio of 176 (IAA molecule with addition of a proton). The IAA standard spectrum is shown in Figure 2.

After the ionization was performed, fragmentation of the IAA standard was produced by collision of the analyte with argon gas. The most abundant ion fragment showed a mass/charge ratio (m/z) of 130, which is in accordance with a study stating that a product ion at m/z 130 with

a molecular formula of C_9H_8N and measured mass (monoisotopic) of 130.0674 Da could be observed in the MS/MS mode of IAA due to an electronic conjugation effect between the carbanion and the indole ring [(M+H-HCOOH)$^+$] [17]. Figure 3 shows the the spectrum of fragmentation of the IAA standard obtained by collision with argon gas and electrospray ionization in positive mode (ESI$^+$) using the triple quadrupole mass spectrometer equipment.

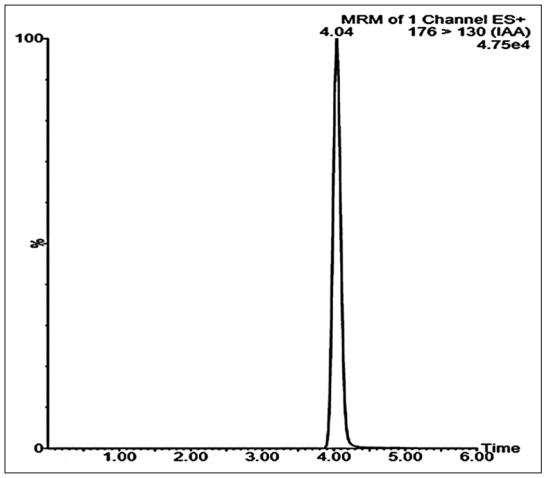

Figure 4: Spectrum of the indole-3-acetic acid standard in multiple reaction monitoring (MRM) mode

For quantitative purposes, we used the multiple reaction monitoring (MRM) mode using the fragment ion m/z 130 of the precursor ion m/z 176 [18-21]. The MRM transitions for detection of the compound were chosen from experiment product ions in the mass spectrometer, selected for the channels of MRM ion greater abundance, as shown in Figure 4.

3.2. Signal/Noise

The LOD and LOQ with S/N ratios of 3:1 and 10:1 were considered. Under the experimental conditions employed, the LOD is the lowest amount of analyte that can be detected, and the LOQ is the lowest amount of analyte that can be quantified.

Figure 5 shows the S/N of hormone extracted from the cambial zone of *Eucalyptus* indicates the adequate sensitivity of the method.

3.3. Linearity

The linear model shown in Figure 6 indicates a high linearity of the method, showing a high linear correlation coefficient (R^2) of 0.9977. Table 3 shows the results of the linearity test.

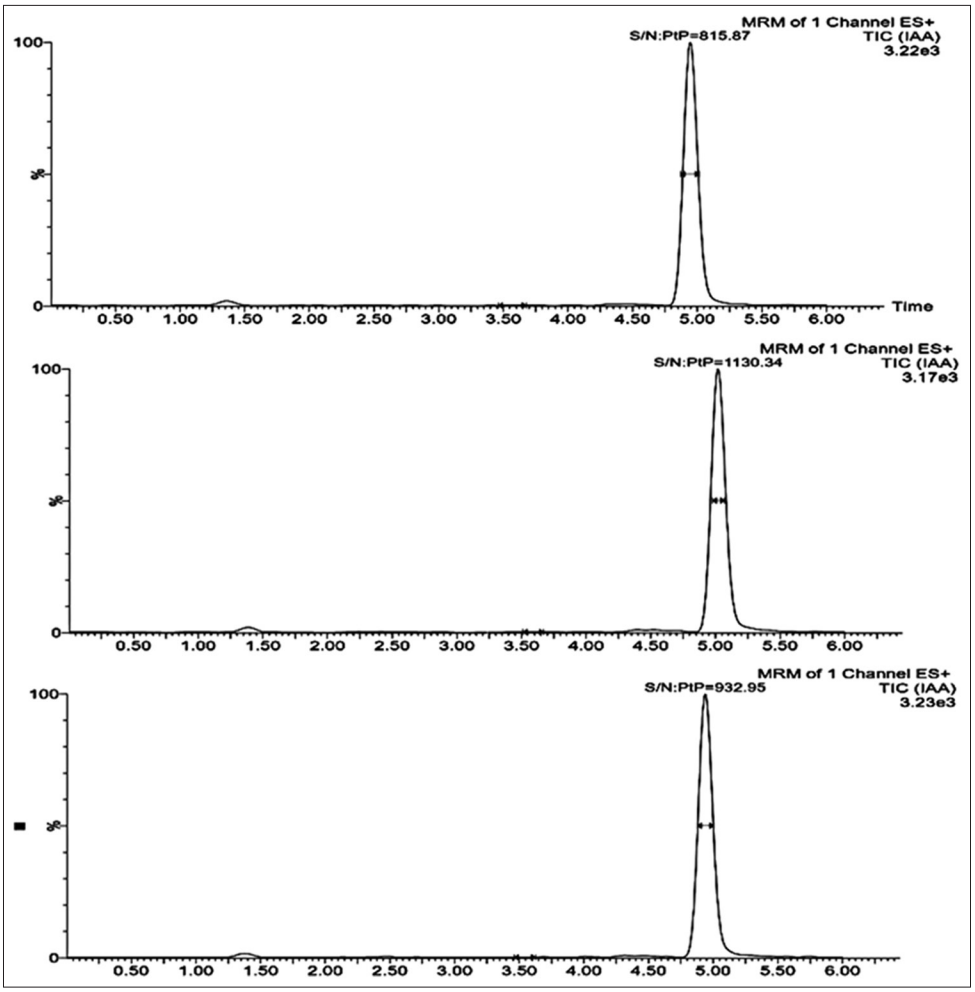

Figure 5: Signal/noise of indole-3-acetic acid hormone extracted from the cambial zone of *Eucalyptus* in multiple reaction monitoring mode (176 > 130)

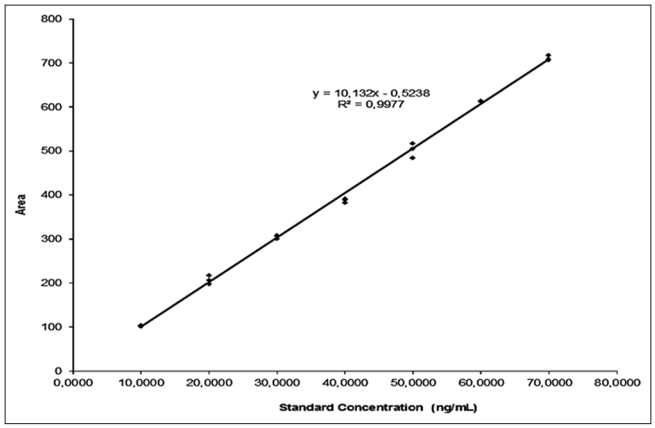

Figure 6: Equation of calibration curve and linear correlation coefficient

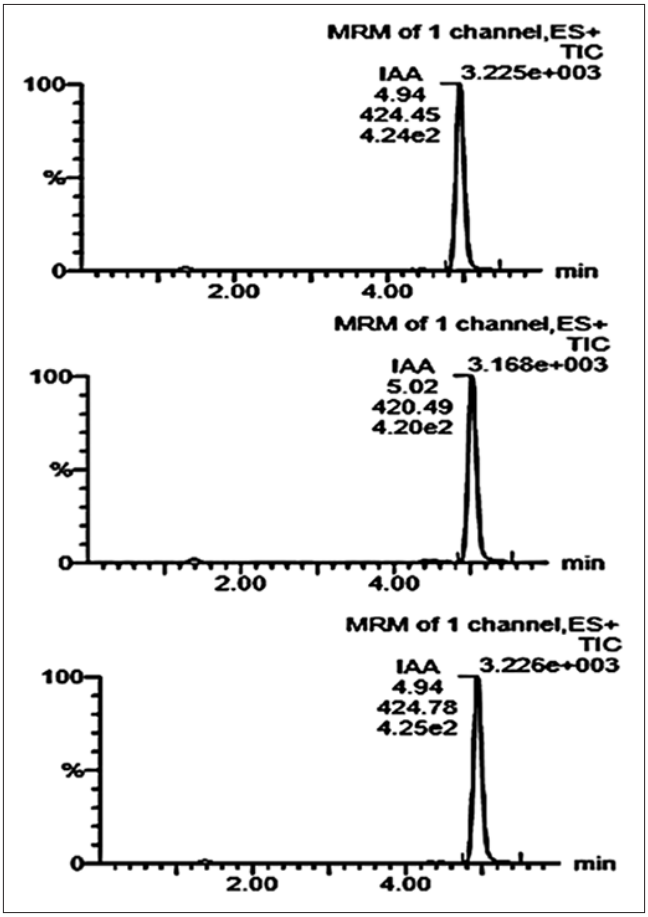

Figure 7: UPLC/ESI-MS/MS chromatograms from the cambial zone of *Eucalyptus*

3.4. Sample Quantification

Figure 7 shows that IAA was detected using the extraction methodology described above from the cambial zone of *Eucalyptus* at the m/z 176→130 transition in MRM positive mode.

The concentrations of IAA used are shown in Table 4.

3.5. Repeatability and Intermediate Precision

The repeatability and intermediate precision were evaluated, and the results are shown in Tables 5 and 6.

Table 3: Results of the linearity test

Theoretical concentration (ng/mL)	Area	Obtained concentration (ng/mL)
9.9900	104	10.3167
	101	10.0206
	104	10.3167
Mean	103	10.2180
Coefficient of variation (%)	1.68	1.67
19.9800	206	20.3842
	218	21.5686
	198	19.5946
Mean	207	20.5158
Coefficient of variation (%)	4.86	4.84
29.9700	308	30.4518
	307	30.3531
	301	29.7608
Mean	305	30.1886
Coefficient of variation (%)	1.24	1.24
39.9600	383	37.8544
	391	38.6440
	389	38.4466
Mean	388	38.3150
Coefficient of variation (%)	1.07	1.07
49.9500	517	51.0804
	505	49.8959
	485	47.9219
Mean	502	49.6327
Coefficient of variation (%)	3.22	3.21
59.9400	614	60.6544
	612	60.4570
	614	60.6544
Mean	613	60.5886
Coefficient of variation (%)	0.19	0.19
69.9300	707	69.8336
	709	70.0311
	718	70.9194
Mean	711	70.2614
Coefficient of variation (%)	0.82	0.82

Table 4: Quantification results obtained from the sample

Injection	Area	Concentration (ng/mL)
1	424	41.9011
2	420	41.5063
3	425	41.9998
Mean	423	41.8024
Coefficient of variation (%)	0.63	0.62

3.6. Ruggedness/Stability

After 24 h, it was possible to verify the stability of the solution, as shown in Table 7.

Table 5: Results of the repeatability test

Injection	Area	Concentration (ng/mL)
1	410	40.5193
2	427	42.1972
3	396	39.1375
4	424	41.9011
5	415	41.0128
6	435	42.9868
Mean	418	41.2925
Coefficient of variation (%)	3.32	3.32

Table 6: Results of the intermediate precision test

Injection	Area	Concentration (ng/mL)
1	410	40.5193
2	427	42.1972
3	396	39.1375
4	424	41.9011
5	415	41.0128
6	435	42.9868
7	437	43.1842
8	436	43.0855
9	420	41.5063
10	433	42.7894
11	430	42.4933
12	426	42.0985
Mean	424	41.9094
Coefficient of variation (%)	2.88	2.88

Table 7: Results of the ruggedness/stability test

Injection	Area	Concentration (ng/mL)
1	383	37.8544
2	391	38.6440
3	389	38.4466
4	352	34.7946
5	351	34.6959
6	357	35.2881
Mean	371	36.6206
Coefficient of variation (%)	5.15	5.15

4. CONCLUSION

Compared to methods applied previously, the method described in this study significantly simplifies the sample preparation and extraction of IAA from the cambial zone of *Eucalyptus*. The introduction of UPLC makes it possible to reduce the separation step of liquid chromatography, and it provides a challenging technique that will become important for applications in the analysis of endogenous plant hormones. The association of UPLC and electrospray ionization tandem MS (UPLC/ESI-MS/MS) allowed higher efficiency associated with high sensitivity, rapid analysis speed, excellent sample recovery, and the versatility of being able to analyze a sample without the step of performing derivatization. Therefore, the present method is simple, fast, and accurate and should be especially useful for routine assays of IAA from the cambial zone of *Eucalyptus*.

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