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Development of a liquid chromatography–electrospray mass spectrometric method for the simultaneous analysis of benzoxazolinones and their degradation products☆

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Abstract

A new method for the simultaneous analysis of some benzoxazolinones, aminophenoxazinones and malonamic acids was developed based on liquid chromatography (LC) coupled to mass spectrometry (MS), using electrospray ionization (ESI) and operating in positive mode. Different ESI-MS parameters, such as fragmentor voltage, capillary voltage, drying gas flow, nebulizer gas pressure and drying gas temperature, were optimized in order to obtain structural information and to achieve maximum sensitivity. Chromatographic separation was performed by a reversed-phase LC column using a linear gradient of water and methanol. Quality assurance of the developed method was assessed by measuring parameters as linearity, sensitivity, repeatability and reproducibility. Quantification method based on the use of internal standard was developed, selecting synthetic 2-methoxy-2H-1,4-benzoxazin-3(4H)-one as internal standard. Good correlations were obtained for all analytes relative to this compound in the range of 0.05–1.5 ng/μL. Instrumental detection limits were between 0.02 and 0.2 ng/μL. Repeatability and reproducibility studies showed acceptable coefficient of variation values.

Keywords: Allelochemicals; Benzoxazolinones; Aminophenoxazinones; Malonamic acids; Liquid chromatography; Mass spectrometry

1. Introduction

Since it has been found that allelochemical compounds and their decomposition products play an important role in the resistance of plant to insect pests and plant pathogenic fungi, it has increased scientific interest for allelopathy meaning a potential for selective biological weed management [1,2]. Chemical family of benzoxazinones is the main active allelophatic compound in different crops such as wheat, rye or maize. Despite the fact that hydroxamic acids are highly contained in these tissues, different studies have documented their rapid conversion to benzoxazolinones and further biotransformation to degradation metabolites. According to literature, the major degradation products of benzoxazolinones are aminophenoxazinones and corresponding malonamic acids [3–6] (Fig. 1).

The broad range of benzoxazinones produced by plants and the further potential metabolites in plant and soil environments result in a complex analyte mixture to be analysed. Up to date many techniques have been used for the determination of benzoxazinones such as isotopic dilution [7], infrared spectrophotometry [8], fluorometry [9], thin-layer chromatography [10], gas chromatography (GC) [11] and liquid chromatography (LC) [12] obtaining limited separation power. However, most of the work reported in the literature used LC methods because this procedure does not require the time-consuming derivatisation step that is needed prior to the GC analyses. Several procedures were developed for the separation and quantification of benzoxazinones in plant extracts using LC [13]. UV-detector was commonly used and it meant a selectivity problem due to the fact that any compound containing a benzene ring would response to its work-

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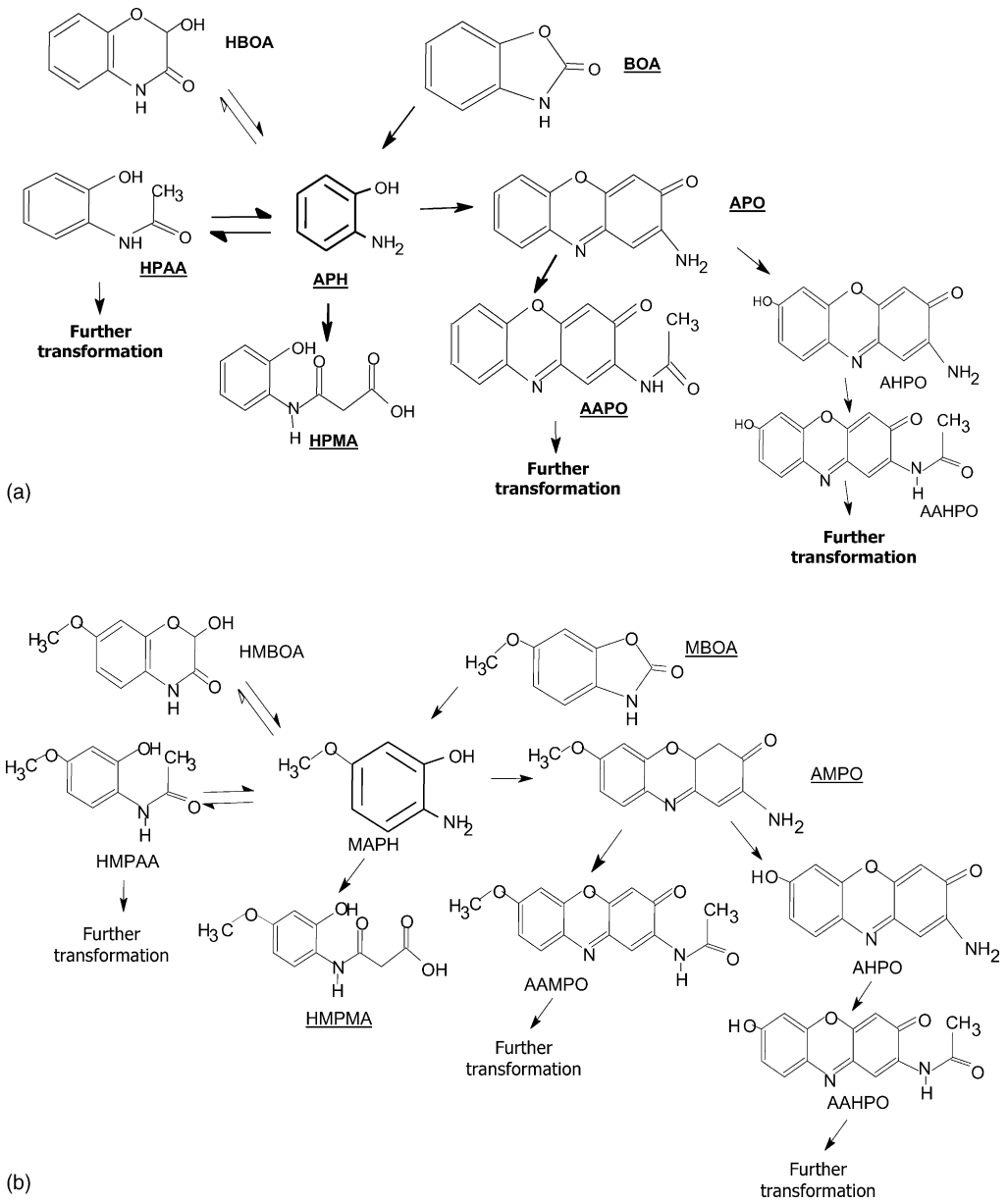


Fig. 1. Degradation pathways of main active benzoxazolinones: (a) benzoxazolin-2(3H)-one (BOA) and (b) 7-methoxybenzoxazolin-2(3H)-one (MBOA) (according to [3,4]).

ing wavelength range. To overcome the LC–UV limitations, some LC–mass spectrometry (MS) methods have been recently published. Unequivocal identification of allelochemical compounds was recently used by Cambier et al. [5,14] with the application of atmospheric pressure chemical ionisation tandem mass spectrometry (APCI-MS–MS). A new method for the identification and quantification of benzoxazinones was also performed using electrospray ionization tandem mass spectrometry (ESI-MS–MS) [15].

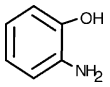
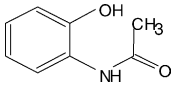
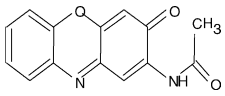
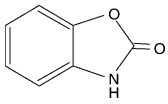
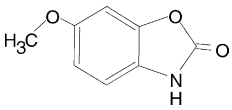
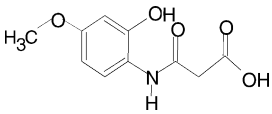
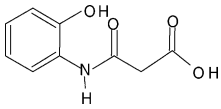
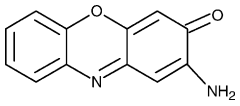
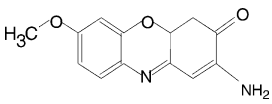
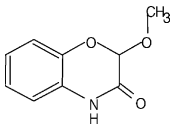
As regards the analysis of degradation products of benzoxazolinones, aminophenoxazinones and corresponding malonamic acids, to our knowledge, no previous studies have described analytical strategies for their analysis. Only Zikmundová et al. [3,4] performed LC–MS analysis but only as a complementary identification technique for biotransformation products of several benzoxazolinones. The aim of this work was to develop a LC–MS method for the determination of naturally occurring 1,4-benzoxazin-3(4H)-one derivatives, including two benzoxazolinones, four aminophenoxazinones and three malonamic acids (Table 1).

2. Material and methods

2.1. Chemicals and materials

The standards were obtained from private and commercial sources as available. Benzoxazolin-2(3H)-one

Table 1
Structure and molecular weight of analysed compounds

Compound	Acronym	M_w	Molecular structure
2-Aminophenol	APH	109	
2N-[2-Hydroxyphenyl]acetamide	HPAA	151	
2-Acetylamino-3H-phenoxazin-3-one	AAPO	254	
Benzoxazolin-2(3H)-one	BOA	135	
6-Methoxy-benzoxazolin-2(3H)-one	MBOA	165	
N-(3-Methoxy-2-hydroxyphenyl)-malonamic acid	HMPMA	225	
N-(2-Hydroxyphenyl)-malonamic acid	HPMA	195	
2-Amino-3H-phenoxazin-3-one	APO	212	
9-Methoxy-2-amino-3H-phenoxazin-3-one	AMPO	242	
2-Methoxy-2H-1,4-benzoxazin-3(4H)-one	2-MeO-HBOA	179	

(BOA), 6-methoxy-benzoxazolin-2(3H)-one (MBOA), 2[N-(2-hydroxyphenyl)acetamide] (HPAA), N-(2-hydroxyphenyl)malonamic acid (HPMA), N-(3-methoxy-2-hydroxyphenyl)malonamic acid (HMPMA), 2-amino-3H-phenoxazin-3-one (APO), 2-acetylamino-3H-phenoxazin-3-one (AAPO) and 9-methoxy-2-amino-3H-phenoxazin-3-one (AMPO) were received from Dr. F. Macias (University of Cádiz, Spain) and the non-naturally occurring synthetic derivative 2-methoxy-2H-1,4-benzoxazin-3(4H)-one (2-MeO-HBOA) from Professor D. Sicker (University of Leipzig, Germany). 2-Aminophenol (APH) was purchased from Sigma Aldrich.

HPLC-grade solvents [water and methanol (MeOH)] and 98% pure acetic acid (HOAc) were purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation

The LC–MS system consisted of a HP 1100 LC with a binary high-pressure pump, a solvent-degassing unit and an automatic sample injector from Hewlett–Packard (Palo Alto, CA, USA). An 1100 series diode array detection (DAD) system was connected in line with a benchtop mass-selective detector for the HP 1100 Series equipped with ESI source. The instrument control and data processing utilities included the use of LC–MSD ChemStation software.

2.3. Preparation of standard solutions

Stock solutions (1 mg/mL) of individual standards were prepared by dissolving accurate amounts of pure standards

in acidified MeOH (1% HOAc). Working standard solutions were obtained by further dilution of stock solutions with MeOH–acidified water (1% HOAc) (60:40). Chromatographic and mass spectrometric conditions were optimized using 1 μg/mL solutions. Mixtures of BOA, MBOA, APH, HPAA, HPMA, HMPMA, APO, AAPO and AMPO (100 μg/mL) were prepared in a range between 0.05 and 5 ppm. These solutions were used to generate the internal standard response calibration curves for subsequent measurements of quality parameters. Internal standard response curves were obtained with mixed solutions spiked with 2-MeO-HBOA at final concentrations of 1 μg/mL each.

2.4. Chromatographic conditions

A Synergi MAX-RP 80A LC column (250 mm × 4.6 mm, 4 μm, Phenomenex) attached to a Phenomenex Guard column was used with a solvent flow-rate of 1 mL/min and an injection volume of 50 μL held at room temperature. Mobile phase consisted of 0.05% HOAc in water as solvent A and 0.05% HOAc in MeOH as solvent B. The solvent gradient adopted was as follows: 0–8 min, 70–30% A; 15.5–17 min, 30–10% A; 19–23 min, 10–70% A; 28 min, 70% A. Total run time was 28 min with the benzoxazinones derivatives eluted over 6–16 min and the final 12 min used for column cleaning and regeneration. The eluent from the first 5 and final 17 min was directed to waste to avoid excessive contamination of the MS source. Elution of the compounds was monitored from 220 to 400 nm.

2.5. Mass spectrometry conditions

ESI in both positive (PI) and negative (NI) modes were assayed. Flow analysis injection (FIA) was performed to achieve major sensitivity for each compound at 50 ng/μl using acidified water–methanol (30:70) as carrier solvent. The optimization of operating conditions was carried out by the evaluation of the area and fragmentation of each analyte in scan mode (*m*/*z* values 100–450). The parameters optimized were: drying gas flow, modifying its value between 8 and 12 L/min (8, 10 and 12 L/min); nebulizer gas pressure, modifying its value between 50 and 60 p.s.i.g. (50, 55 and 60 p.s.i.g.; p.s.i. = 6894.76 Pa); drying gas temperature, modifying its value between 250 and 350 °C (250, 300 and 350 °C); capillary voltage, modifying its value between 3000 and 4000 V (3000, 3500 and 4000 V); and fragmentor voltage, modifying its value between 70 and 250 V (70, 150 and 250 V).

2.6. Stability study

A preliminary study of the stability of selected analytes was performed due to their rapid degradation effect [3–6]. The stability of benzoxazolinones and 2-MeO-HBOA was checked in a previous study [16]. Thus, stability study of aminophenoxazinones and malonamic acids in acidified solution (1% HOAc) was studied here. To determine the stability, spiked solutions were stored at room temperature, 4 °C and −20 °C. The evaluation was performed for 7 days by injections for each temperatured solution by LC–MS developed method.

3. Results and discussion

3.1. Stability study

The stability of MBOA, BOA and 2-MeO-HBOA was previously checked [16], showing that the three compounds are stable (degradation lower than 5%) at the three different temperatures tested. As regards aminophenoxazinones and malonamic acids, Table 2 shows the results obtained from the stability evaluation after 7 days of storage at the three different temperatures. Results clearly demonstrated that significant losses occurred, not only when solution was stored at room temperature but also at 4 °C and −20 °C. APO and AMPO were the most-unstable compounds, with approximately 75–100% of degradation. This degree of degradation was observed after three days of storage. As is described in the degradation pathway scheme by Zikmundová et al. [3,4], APO and AMPO are the main active compounds to further degradation products. Thus, the instability of APO and AMPO was an important fact to consider for standard solution preparation.

Concerning to the rest of the compounds, better stability was observed at −20 °C. At this temperature, AAPO remained stable, whereas HPAA and APH suffered an approximately 20% of degradation. In view of these results and to prevent degradation, storage in acidic conditions at −20 °C was recommended.

3.2. MS method optimization

The objective of this study was to develop an analytical method for the simultaneous determination of some benzoxazolinones, aminophenoxazinones and malonamic acids. Since a previous LC–MS methodology, using ESI, was optimized for the analysis of benzoxazinone derivatives, including BOA, MBOA and 2-MeO-HBOA [15], this ionization technique was selected in this study.

Different ESI-MS parameters were optimized using FIA for all the studied compounds in order to obtain structural

Table 2
Degradation (%) of aminophenoxazinones and malonamic acids stored at different temperatures (room temperature, 4 °C and −20 °C)

Compound	−20 °C	4 °C	20 °C
HPAA	23	14	33
APH	20	25	19
AMPO	81	76	55
APO	100	100	100
AAPO	3	14	27

Table 3
Target compound responses (500 ng injected) of ESI positive and negative mode for fragmentor parameter = 70 V

Compound	ESI positive	ESI negative
APH	11814	35299
HPAA	17218	42492
AAPO	162355	5407
BOA	13231	107751
MBOA	19628	48304
APO	59321	5405
AMPO	52216	3524
HMPMA	17028	36228
HPMA	18271	47718

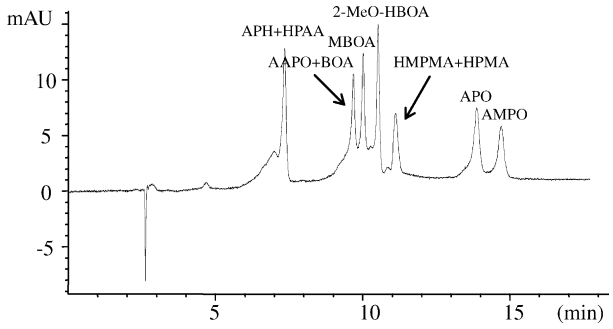


Fig. 2. LC–DAD (280 nm) chromatogram obtained for a standard solution (1 μg/mL) on a RP dodecyl (C_{12}) trimethylsilyl (TMS) end-capped Synergi MAX-RP column.

information and to achieve maximum sensitivity. PI and NI modes were tested at three different fragmentor values. Values of 250 and 150 V of voltage were disregarded for both ionization modes because no additional fragmentation of compounds was obtained. Main information of fragments was obtained at 70 V for both modes. In NI mode, benzoxazolinone and malonamic acid responses were higher than in PI mode. But, it should be pointed that the differences between both polarities were not very high. In contrast, it was clearly observed a more suitable response for aminophenoxazinones in PI mode (Table 3). In order to assume an analytical compromise to obtain the major response for all the selected analytes, PI was selected as polarity ionization. Working under these conditions (ESI, PI and 70 V of voltage), $[M + H]^+$ ions and sodium adduct ions ($[M + Na]^+$) were selected as quantification and confirmation ions for target compounds. Only for AAPO different ions were selected: $[M + H–N(CO(CH_3)]^+$ and $[M + H–N(CO(CH_3)–(C_2H_5OH)]^+$ (Table 4).

For the rest of parameters, such as drying gas flow, nebulizer gas pressure, drying gas temperature and capillary voltage, non-significant differences were detected between the tested values. The selected operating conditions were 13 L/min, 60 p.s.i.g., 350 °C and 3500 V, respectively.

3.3. LC method

No previous studies have described analytical strategies for the separation and quantification of aminophenoxazinones and malonamic acids before. However, benzoxazolinones (BOA and MBOA) were included in Bonnington et al. [15] study. Concerning to the encountered problems in this previous study due to low stability of analytes on the LC columns under the required acidic conditions, retention time shifts and adverse effects on peak intensities due to the coelution of impurities, it was seriously evaluated the application of the dodecyl (C_{12}) trimethylsilyl (TMS) end-capped Synergi MAX-RP. This LC column enhanced the online chromatographic separation through improvements to component resolution, analyte stability, peak shape and the column lifetime. On the basis of these results, the same chromatographic column was selected for our study.

Several gradient programs were assayed with the selected column using acidified H_2O (0.05% HOAc) and acidified MeOH (0.05% HOAc) as mobile phase. The optimal chromatographic separation was achieved using a linear gradient of 70:30–30:70, although slight differences in retention times for selected analytes was observed, and some coelutions (APH with HPAA, AAPO with BOA, and HMPMA with HPMA) could not be resolved. This fact determined that UV-detection method was not an appropriate technique for the simultaneous analysis of target compounds (Fig. 2). In contrast, all compounds were well resolved using LC–MS in the selected ion monitoring (SIM) mode, providing appropriate selectivity to the method (Fig. 3).

Table 4
Retention times and *m/z* ions selected for quantification and confirmation of each selected compound

Compound	Retention time (min)	Quantification ion (*m/z*)	Confirmation ion (*m/z*)
APH	7.66	110 $[M + H]^+$	152 $[M + H + C(OCH_3]^+$
HPAA	7.67	152 $[M + H]^+$	110 $[M+H–C(OCH_3]^+$
AAPO	9.66	198 $[M + H–N(CO(CH_3)]^+$	152 $[M + H–N(CO(CH_3)–(C_2 H_5OH)]^+$
BOA	10.06	136 $[M + H]^+$	158 $[M + Na]^+$
MBOA	10.41	166 $[M + H]^+$	188 $[M + Na]^+$
HMPMA	10.59	226 $[M + H]^+$	248 $[M + Na]^+$
HPMA	10.79	196 $[M + H]^+$	218 $[M + Na]^+$
APO	14.34	213 $[M + H]^+$	235 $[M + Na]^+$
AMPO	15.17	243 $[M + H]^+$	264 $[M + Na]^+$
2-MeO-HBOA	10.95	148 $[M + H]^+$	202 $[M + Na]^+$

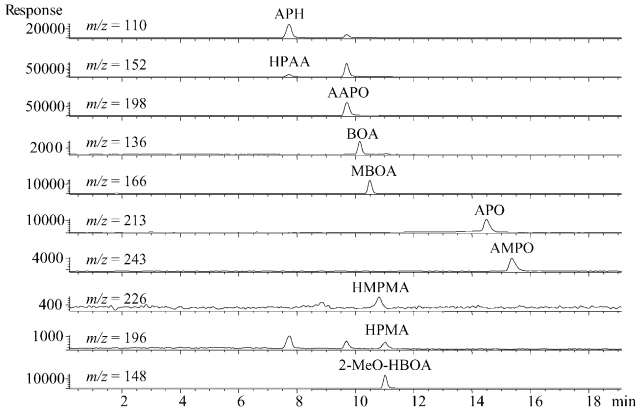


Fig. 3. LC–ESI(+)-MS chromatogram obtained for a standard solution (1 μg/mL). Different *m/z* ions selected for the quantification of each compound are shown.

3.4. Method validation

Quality assurance of the developed method was evaluated by measuring parameters as linearity, sensitivity, reproducibility and repeatability. Quantification was performed by internal standard method. The use of internal standards to aid reliable quantification has not been described previously for LC determinations of aminophenoxazinones and malonamic acids. Here, non-naturally occurring structural analogue of one benzoxazinone (HBOA), 2-MeO-HBOA, with adequate separation from selected analytes, was used as internal standard. The use of internal standard method is very useful in LC–ESI-MS, where matrix induced interference resulted in suppression of signals of target analytes. In this sense, the use of 2-MeO-HBOA could aid to detect any suppression of analyte signals. The linearity of the method was measured in the range of 0.05–1.5 ng/μL. The data were subjected to linear regression analysis and good correlations were obtained for all analytes relative to internal standard, ranging from 0.9879 for HPMA to 0.9997 for HMPMA (Table 5). These results confirmed the applicability of the selected internal standard for quantification.

Sensitivity was evaluated by determining the instrumental detection limits (LOD_{inst}) obtained using LC–ESI-MS in SIM mode. LOD_{inst} were based on the peak-to-peak noise of the baseline near the analyte peak obtained by analyses of a standard solution and on minimal value of signal-to-noise ratio of 3. The applied methodology provided LOD_{inst} in the range between 0.02 and 0.2 ng/μL (Table 5). Aminophenoxazinones showed the lower detection limits (from 0.02 to 0.11 ng/μL), followed by benzoxazolinones (0.09 ng/μL). As regards malonamic acids, they showed the higher detection limit values (from 0.05 to 0.2 ng/μL).

In order to evaluate the repeatability of the developed method, five consecutive injections of a standard solution were performed at the optimum conditions in LC–ESI-MS above described. Relative standard deviations (R.S.D.s) between the five values were calculated for all the selected analytes. R.S.D. values were always below 15% indicating good repeatability (Table 5). On the other hand, five injections were carried out in five different days to establish the reproducibility of the method. The same quantitative analysis used for the repeatability study (internal standard) was applied. As can be expected, the R.S.D.s obtained for reproducibility were higher than those obtained for repeatability (Table 5). R.S.D. values ranged from 2 to 26%, with values higher than 20% only for AAPO.

4. Conclusions

A methodology for chromatographic separation, characterization and quantification of a range of benzoxazolinones and further degradation products based on the use of LC–MS is described for first time. ESI was selected as ionization technique and different ESI-MS parameters (polarity, fragmentor voltage, capillary voltage, drying gas flow, nebulizer gas pressure and drying gas temperature) were optimized by FIA for all analytes as well as for internal standard selected for quantification. Quality assurance of the developed methods was assessed by measuring parameters as linearity, sensitivity, repeatability and reproducibility. The method was lineal in the range of 0.05–1.5 ng/μL, and detection limits were between 0.02 and 0.2 ng/μL. Aminophenoxazinones showed the lower detection limits, followed by benzoxazolinones; finally, malonamic acids showed the higher detection limit values. As regards repeatability and reproducibility, acceptable R.S.D.

Table 5
Quality parameters of the established LC–MS method.

Compound	R^2	LOD (ng/μL)	Repeatability R.S.D. (%, n = 5)	Reproducibility R.S.D. (%, n = 5)
APH	0.9974	0.026	3.0	8.2
HPAA	0.9947	0.046	5.2	17.0
AAPO	0.9955	0.024	6.1	26.3
BOA	0.9941	0.085	9.7	5.1
MBOA	0.9982	0.085	6.9	4.0
HMPMA	0.9997	0.221	13.4	10.5
HPMA	0.9879	0.103	8.8	2.4
APO	0.9991	0.108	2.0	12.5
AMPO	0.995	0.064	11.4	5.2

values were obtained for all selected analytes, with exception of AAPO, which presented R.S.D. of reproducibility higher than 20%.

The advanced analytical method developed could thus be applied to the simultaneous screening and quantification of these allelochemicals in plant and soil materials. However, future research will approach the analysis of real samples in order to assess possible matrix effects and likely influence of interferences.

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