Metodologies analítiques per a l'estudi de compostos al·leloquímics en conreus de blat

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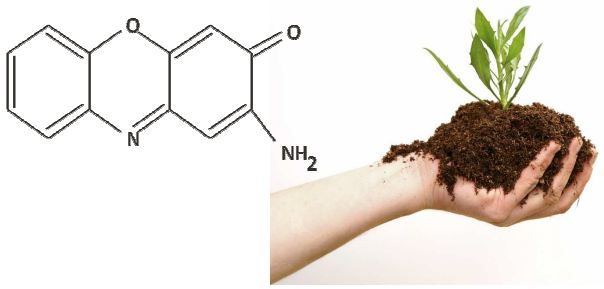
Novembre 2013

CAPÍTOL III.- METODOLOGIA ANALÍTICA EN SÒL



CAPÍTOL III.- METODOLOGIA ANALÍTICA EN SÒL AGRÍCOLA

III.1. INTRODUCCIÓ I OBJECTIUS

En aquest capítol s'exposa el treball realitzat durant la optimització d'un mètode analític per a la determinació i anàlisi de les benzoxazolinones i els seus productes de degradació en el sòl agrícola. Al nostre coneixement i segons la bibliografia consultada, no hi ha estratègies analítiques des de la preparació de la mostra fins la seva anàlisi.

Així doncs, el repte que ens vàrem plantejar va ser el de desenvolupar una estratègia analítica per dur a terme l'anàlisi i quantificació de les benzoxazolines (BOA i MBOA), les aminophenoxazinones (AMPO, APO i AAPO) i els àcids malonàmics (HPAA i HMPA) en mostres de sòl agrícola. El procediment del tractament de la mostra segueix el mateix perfil que el desenvolupat per a les mostres de blat i es composa de tres etapes: homogeneïtzació, extracció per PLE i purificació de l'extracte a través de SPE. En un primer estadi es va dur a terme el desenvolupament d'una metodologia d'anàlisi per LC/MS, però tal i com serà explicat en el transcurs del present capítol posteriorment va ser necessari desenvolupar un mètode basat en LC-MS/MS.

En aquest sentit els objectius plantejats en aquest capítol van ser:

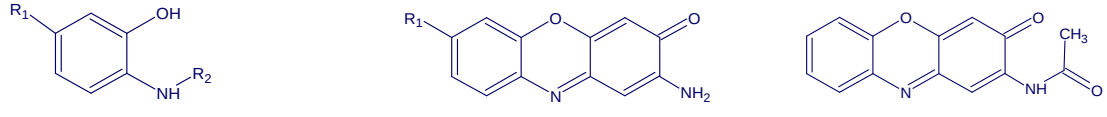
- Optimitzar les condicions per a la ionització dels compostos d'interès i la seva fragmentació, amb el fi de realitzar una anàlisi mitjançant el mode d'adquisició SIM a través de la LC-MS. Posteriorment dur a terme la optimització de l'anàlisi per LC-MS/MS per tal de millorar en sensibilitat i selectivitat.

- Desenvolupar una metodologia analítica ràpida i simple per determinar 2 benzoxazolinones, 3 aminophenoxazinones i 2 àcids malonàmics en mostres de sòl agrícola.

- Dur a terme una avaluació de l'efecte de matriu en els extractes de sòl preparats i aplicar el mètode de dilució de la mostra per tal de minimitzar tal efecte.

III.2 SELECCIÓ DELS ANALITS I DISPONIBILITAT DELS ESTÀNDARDS

Quan els compostos metabolitzats en la planta són alliberats al sòl, majoritàriament a través de l'exsudació per les arrels, els productes de transformació que apareixen després de la hidròlisi de les aglucones són les corresponents benzoxazolinones BOA i MBOA. Aquests analits, com ja hem vist es troben presents en la planta, però també són els productes de transformació primaris en el sòl. La seva transformació forma part d'un procés complex on hi intervenen diferents factors tals com el tipus de sòl, la quantitat de precursors existents, la presència i tipus de microorganismes i l'absorció/adsorció dels analits en el sòl. Els productes de degradació poden presentar una activitat al·lelopàtica més potent que els seus predecessors [1-3], d'aquí la importància del seu estudi. A la Figura III.1 es presenten els compostos objectiu en l'anàlisi dels productes de degradació del BOA i MBOA en el sòl agrícola.



R1=H	R2=$COCH_3$	**HPAA**	R1=H	**APO**	**AAPO**
R1=H	R2=$COCH_2COOH$	**HPMA**	R1=OCH3	**AMPO**	
R1=OCH_3	R2=$COCH_2COOH$	**HMPMA**			
R1=H	R2=H	**APH**			

Figura III.1.- Estructura dels analits d' estudi

De la mateixa manera que els seus precursors, els compostos de degradació de les benzoxazolinones en el sòl tampoc es troben disponibles comercialment. El grup del Dr. Francisco Macías de la Universitat de Cádiz (participant en el projecte FATEALLCEHEM) va dur a terme el desenvolupament de rutes sintètiques per als àcids malonàmics i les aminophenoxazinones i ens va proporcionar els compostos: HMPMA,

HPMA, APO, AAPO, AMPO i AAMPO. D'aquesta manera vàrem poder disposar dels patrons per poder dur a terme el desenvolupament del mètode i l'anàlisi quantitativa de les mostres.

Els àcids malonàmics s'obtenen a partir dels corresponents nitrofenols, el HPMA a partir del 2-nitrofenol i el HMPMA a partir del 5-metoxi-2-nitrofenol [4, 5]. Les seqüències de reacció inclouen diverses etapes com són: la protecció del grup aromàtic hidroxil, la reducció del grup nitro, la introducció de la cadena lateral i finalment la desprotecció del grup hidroxil. Les etapes de protecció i desprotecció s'inclouen en el treball de Macias et al. [6] per tal d'evitar la dimerització de la aminophenoxazinones, obtenint així majors rendiments en la síntesi dels àcids malonàmics (68%), i per proporcionar nous compostos d'origen en la síntesis de nous derivats (Figura III.2).

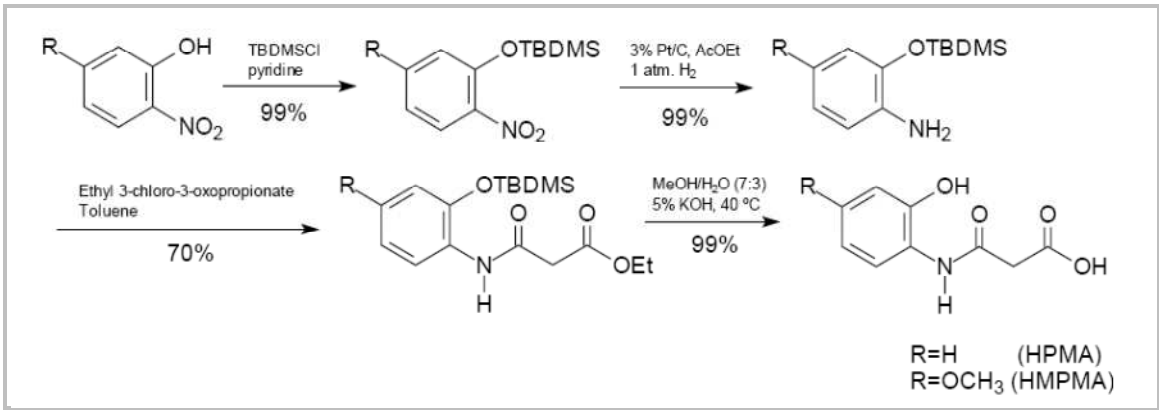


Figura III.2.-Vies d'obtenció dels àcids malonàmics

Através de la dimerització del 2-aminophenols'obté l'APO i amb la posterior acetilació d'aquest s'obté l'AAPO [7]. L'esquelet del 7-methoxyaminophenoxazin s'obté a partir de la dimerització reductiva del 5-aminophenol, donant lloc a l'AMPO amb un rendiment elevat del 70%, posteriorment es dur a terme l'acetilació del AMPO per donar lloc al AAMPO (Figura III.3).

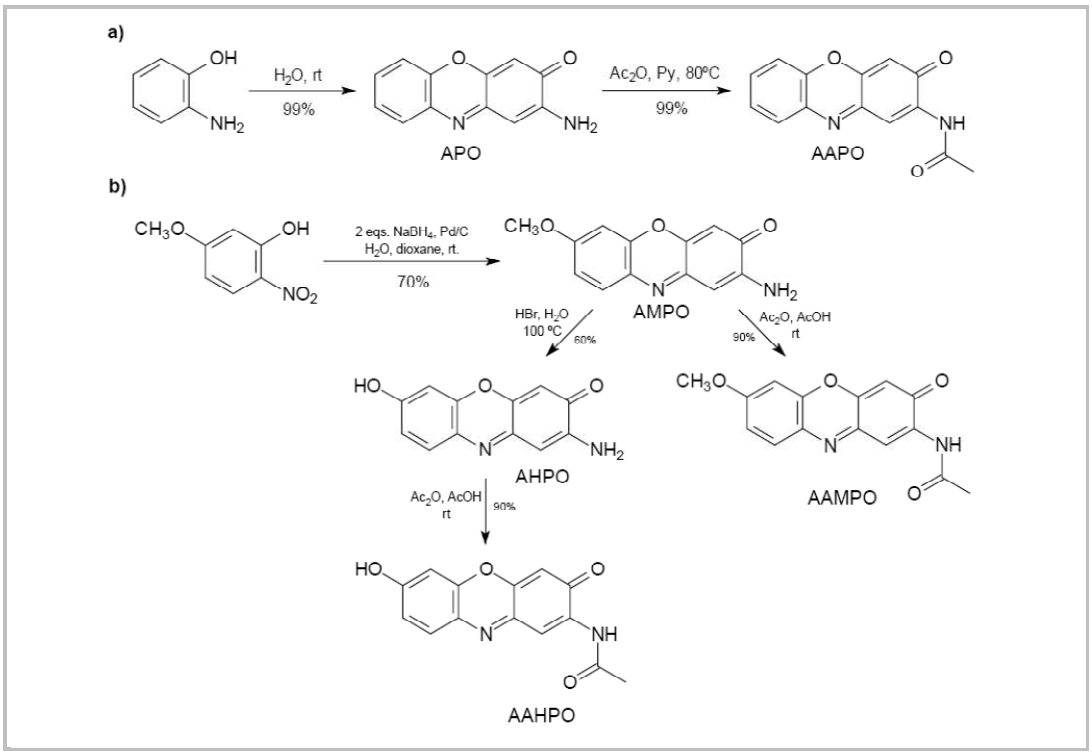


Figura III.3.- Vies d'obtenció de les aminofenoxazinones

L'estabilitat dels analits juga un paper important tant en la validació del mètode com en la quantificació dels analits d'interès. Com s'ha vist en l'anterior capítol, les benzoxazolinones BOA i MBOA van mostrar ser estables durant els 7 dies a la temperatura de -20^{o}C, amb una degradació inferior al 5%. Per determinar l'estabilitat de la resta dels analits, solucions patró d'aquests, es van conservar a diferents temperatures: a temperatura ambient (20^{o}C), 4^{o}C i -20^{o}C en MeOH en medi àcid (1% àcid acètic (HOAc)). Diàriament i durant una setmana es van analitzar per triplicat les solucions de patró conservades a les diferents temperatures mitjançant LC-MS.

A la FiguraIII.4 es mostren els resultats obtinguts als 2, 3 i 7 dies a la temperatura de conservació de -20^{o}C. En vista dels resultats obtinguts en vers l'estabilitat dels analits, per tal d'obtenir resultats fiables, es preparen els patrons i les mostres just abans de la seva anàlisi i no conservant-los més de tres dies des de la seva preparació.

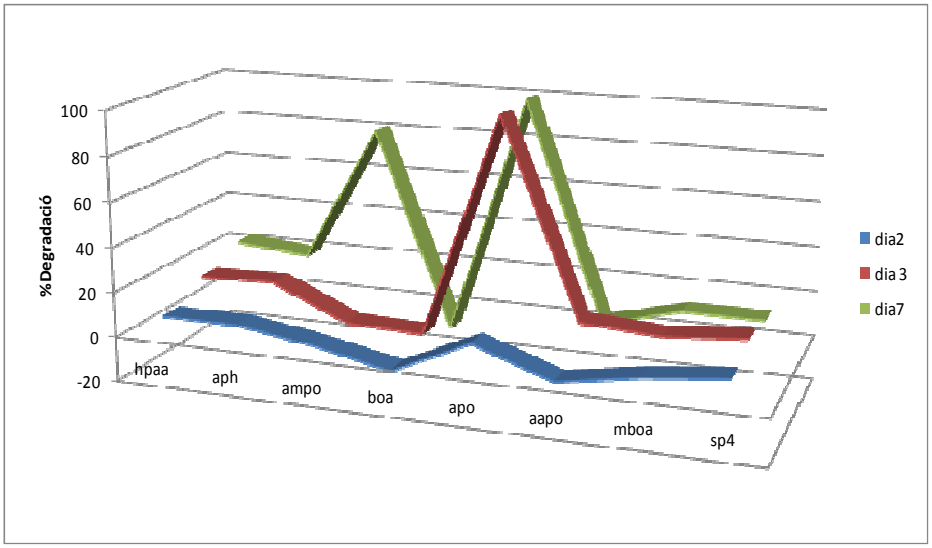


Figura III.4.- Degradació als 2, 3 i 7 dies a la temperatura de -20^{O}C

III.3 PUBLICACIONS CIENTÍFIQUES

L'estudi va donar lloc als dos treballs presentats en aquest capítol. En un primer estadi es va dur a terme el desenvolupament de l'anàlisi instrumental per LC-MS i el treball de referència es troba en la secció III.6.1 d'aquest capítol amb el títol "*Development of a liquid chromatography-electrospray mass spectrometric method for the simultaneous analysis of benzoxazolinone sand their degradation products".* En el segon, que es troba a la secció III.6.2 i porta per títol "*Development of a pressuirized liquid extraction-solid-phase extraction followed by liquid chromatography-electrospray ionization tandem mass spectrometry method for the quantitative determination of benzoxazolinones and their degradation products in agricultural soil*", es presenten també les condicions en el pretractament de la mostra i l'anàlisi instrumental que es va desenvolupar amb un espectròmetre de masses en tàndem.

III.3.1 Desenvolupament i validació d'una metodologia d'anàlisis per LC-MS

Publicació científica#4

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

Per:

Miriam Guillamón, Marta Villagrasa, Ethel Eljarrat i Damià Barceló

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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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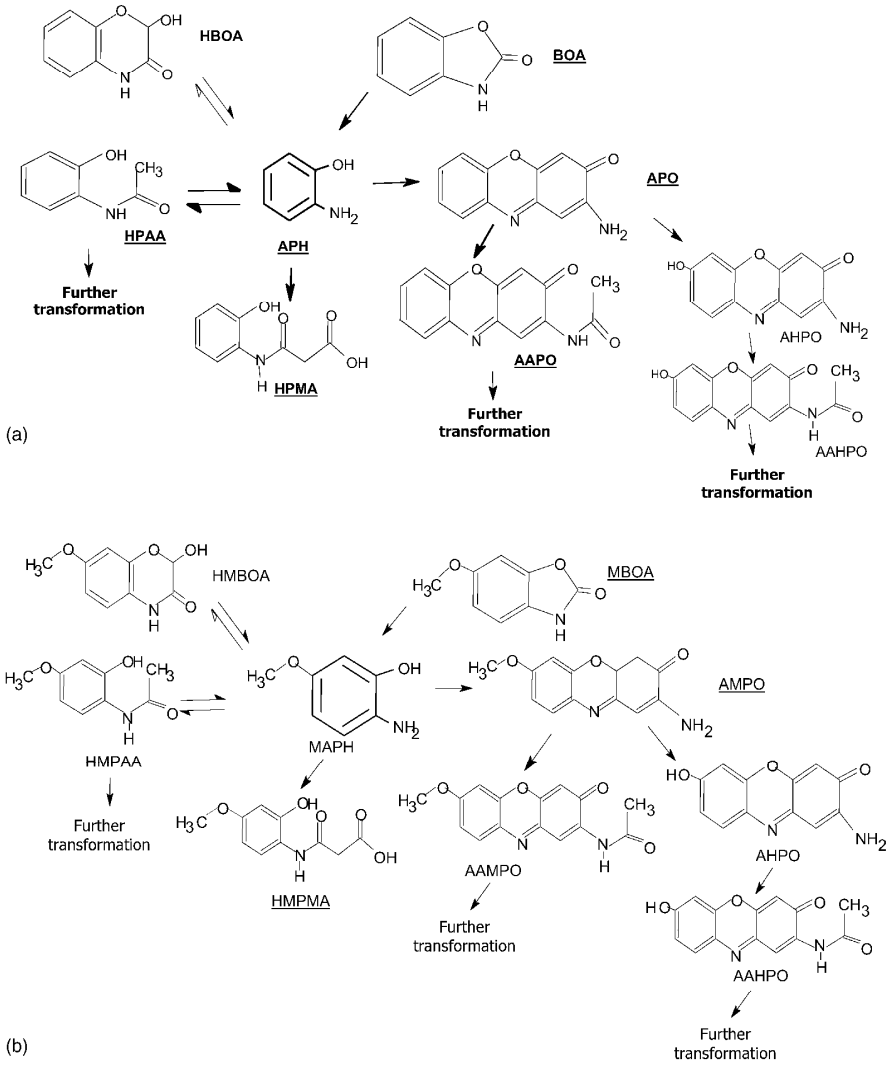


Fig. 1. Degradation pathways of main active benzoxazolinones: (a) benzoxazolin-2(3H)-one (BOA) and (b) 7-methoxybenzoxazolin-2(3H)-one (MBOA) (according to [3,4]).

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].

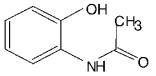
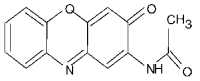
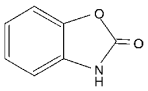
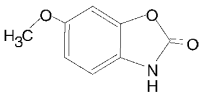
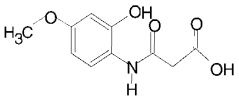
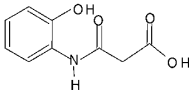
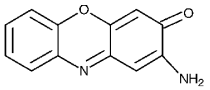
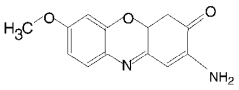
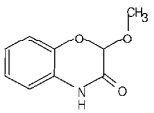
As regards the analysis of degradation products of benzoxazolinones, aminophenoxazinones and corresponding malonamic acids, to our knowledge, no previous studies have described analytical strategies for their analysis. Only Zikmundová et al. [3,4] performed LC–MS analysis but only as a complementary 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

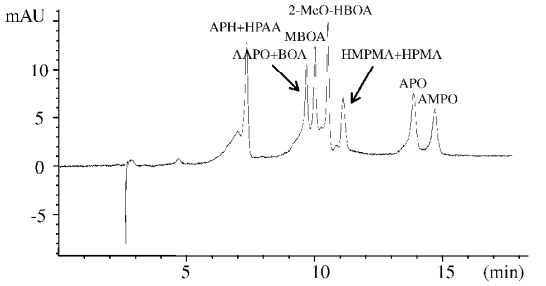


Fig. 2. LC–DAD (280 nm) chromatogram obtained for a standard solution (1 μg/mL) on a RP dodecyl (C_{12}) trimethylsilyl (TMS) end-capped Synergi MAX-RP column.

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 $[M + Na]^+$

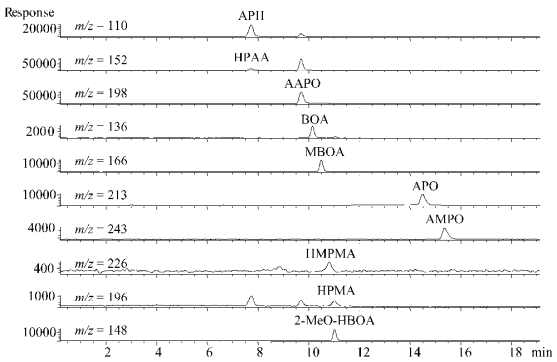


Fig. 3. LC–ESI(+)-MS chromatogram obtained for a standard solution (1 μg/mL). Different *m/z* ions selected for the quantification of each compound are shown.

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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III.3.2 Desenvolupament i validació d'una metodologia d'anàlisis per LC-MS/MS

Publicació científica#5

"Development of a pressurized liquid extraction–solid-phase extraction followed by liquid chromatography–electrospray ionization tandem mass spectrometry method for the quantitative determination of benzoxazolinones and their degradation products in agricultural soil"

Per:

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Development of a pressurized liquid extraction–solid-phase extraction followed by liquid chromatography–electrospray ionization tandem mass spectrometry method for the quantitative determination of benzoxazolinones and their degradation products in agricultural soil

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Abstract

A new analytical method for the quantitative determination of benzoxazolinones and their degradation products in agricultural soils based on the use of pressurized liquid extraction (PLE) followed by solid-phase extraction (SPE) and then instrumental determination using liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI–MS–MS) is described. Using this method, the characterization, separation and quantitative detection of a mixture of two benzoxazolinones, benzoxazolin-2-one (BOA) and 6-methoxybenzoxazolin-2-one (MBOA) and their degradation products, 2-aminophenol (APH), *N*-(2-hydroxyphenyl)malonamic acid (HMPMA), 2-amino-3-H-phenoxazin-3-one (APO), 9-methoxy-2-amino-3-H-phenoxazin-3-one (AMPO), 2-acetylamino-3-H-phenoxazin-3-one (AAPO) and 2-acetylamino-9-methoxy-2-amino-3-H-phenoxazin-3-one (AAMPO) was achieved. The complete LC–ESI–MS–MS precursor–product ion fragmentation pathways for the degradation products of benzoxazolinones are described for the first time. Quantitative analysis was done in the multiple reaction mode using two specific combinations of precursor–product ion transitions for each compound. The optimized method was quality assessed by the measure of parameter as recovery, linearity, sensitivity, repeatability and reproducibility. Recoveries of the analytes ranged from 53 to 123%. The developed method offered improvements to the sensitivity as compared with our previously LC–MS method, with detection limits down to 2.4–21 ng/g of dry weight. This achievement allows us to identify and quantify for the first time degradation products of benzoxazolinones in real agricultural soil samples. Analytes were found in the range of 20.6–149 ng/g dry weight.

Keywords: Allelochemicals; Aminophenoxazinones; Benzoxazinones; Liquid chromatograhy; Mass spectrometry; Soil

1. Introduction

In recent years there has been an increasing focus on the impact on the environment of using pesticides for controlling weeds and also insects and diseases. Allelopathy could be an alternative, and it has increased scientific interest for a selective biological weed management [1,2]. Allelopathy has been defined by the International Allelopathy Society as 'any process involving secondary metabolites (allelochemicals) produced by plants, micro-organisms, viruses and fungi that influence the growth and development of agricultural and biological systems (excluding animals), including positive and negative effects' [3].

The chemicals identified as the most active allelopathic compounds in different crops such as wheat, rye, or maize are of the same chemical family, the benzoxazinones. Hydroxamic acids in wheat are found as β-glucosidases [4]. When plant tissue is damaged, β-glucosidase is enzymatically hydrolyzed to their corresponding aglucones. The aglucones are converted to their corresponding benzoxazolinones MBOA and BOA. When leached to the soil, the aglucones are rapidly transformed to benzoxazolinones as well [5,6]. Moreover, the benzoxazolinones are subjected to additional transformation in soil. Several papers have published the herbicidal, insecticidal, and fungicidal effects of hydroxamic acids and benzoxazolinones [7–11]. The transformation products must be studied because some of

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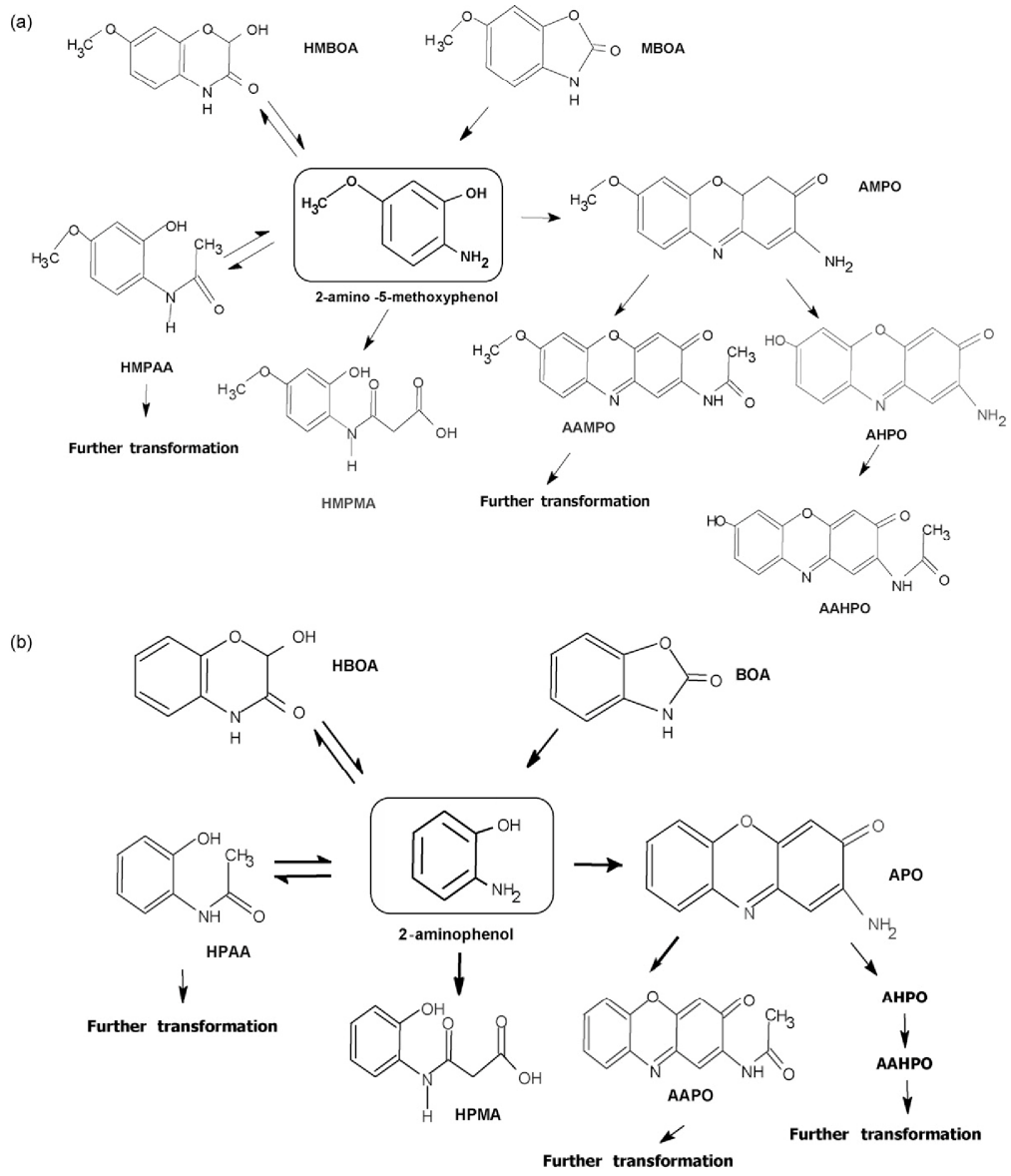
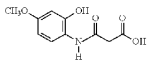
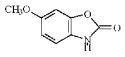
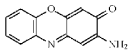
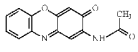
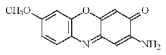
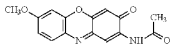


Fig. 1. Degradation pathway of (a) MBOA and (b) BOA, according to Zikmundova et al. [12].

these could be more active than the original ones. According to the literature [12], the major degradation products of benzoxazolinones (MBOA and BOA) are aminophenoxazinones and corresponding malonamic acids. These degradation products were identified from studies carried out in the laboratory by combining soil samples from commercial wheat crops with stock solutions of the substrates to be degraded [5,13]. The degradation pathways of MBOA and BOA have been obtained from these studies and are shown in Fig. 1. But these degradation products have never been detected in real cultivated soil samples.

Several procedures based on GC–MS and LC–UV for the separation and quantification of benzoxazinone derivatives in plant extracts were developed [14]. More recently, the application of liquid chromatography coupled to mass spectrometry (LC–MS) [15] and LC–MS–MS [16] to the analysis of these compounds in plant material was reported, showing better sensitivity and selectivity compared to previous methods. However, and to the knowledge of the authors, there are only two works [13,17], describing an analytical strategy for the analysis of benzoxazolinones and their degradation products in soil. In the first one [17], only an instrumental analysis using LC–MS was developed,

Table 1
MS–MS transitions and optimal conditions used for LC–MS–MS analysis

Compounds	MW	Precursor–product ion fragmentation pathway	Precursor ion		Product ion		Transition	Ratio[a]
			m/z	Cone (V)	*m/z*	Collision (eV)	MRM[b]	Value
Malonamic acids								
APH 	109	110 > 92 [$M+H^+$ > $-H_2O$]	110	30	92	20	MRM 1	–
HMPMA 	225	226 > 208 > 138 [$M+H^+$ > $-H_2O$ > $-C_3H_4O_3$]	226	20	208	10	MRM 1	2
			226	20	138	20	MRM 2	
Benzoxazolinones								
BOA 	135	136 > 108 > 92 [$M+H^+$ > –CO > $-HCOCH_2COOH$]	136	35	108	20	MRM 1	26
			136	35	92	30	MRM 2	
MBOA 	165	166 > 110 > 95 [$M+H^+$ > –2CO > $-CH_3$]	166	35	110	20	MRM 1	0.9
			166	35	95	23	MRM 2	
Aminophenoxazinones								
APO 	212	213 > 185 > 158 [$M+H^+$ > –CO]	213	30	185	20	MRM 1	3.8
			213	30	158	30	MRM 2	
AAPO 	254	255 > 213 > 185 [$M+H^+$ > $-CH_2CO$ > –CO]	255	20	213	16	MRM 1	55
			255	20	185	30	MRM 2	
AMPO 	242	243 > 200 > 172 [$M+H^+$ > –CO; $-CH_3$ > –CO]	243	35	200	30	MRM 1	1.4
			243	35	172	33	MRM 2	
AAMPO 	284	285 > 243 > 228 [$M+H^+$ > $-CH_2CO$ > $-CH_3$]	285	20	243	16	MRM 1	3.6
			285	20	228	30	MRM 2	
Int. standard								
2-MeOH-HBOA 	179	180 > 120 > 120 [$M+H^+$ > $-OCH(OCH_3)$ > –CO]	180	10	120	10	MRM 1	1.2
			180	10	92	20	MRM 2	

[a] Ratio = abundance MRM 1/abundance MRM 2.
[b] MRM 1 = first transition used for quantification; MRM 2 = second transition, used for confirmation.

but no information regarding sample preparation steps (extraction and purification) was reported. The instrumental detection limits obtained in this study were in the range of 1–11 ng. In the second one [13], only two derivatives (BOA and APO) were analysed in spiked soil samples using LC–MS method, with detection limits of the whole method in the range of 9–35 ng/g.

The aim of this study was to develop a rapid and simple method for the determination of eight allelochemicals (Table 1) in agricultural soils. An analytical method based on pressurized liquid extraction (PLE) followed by a clean up using LiChrolut RP C_{18} solid-phase extraction (SPE) cartridges was evaluated. Instrumental determination was carried out by LC–MS–MS method working in multiple reaction monitoring (MRM). To the best of our knowledge, this is the first work that used LC–MS–MS method for the determination of benzoxazolinones, malonamic acids, and aminophenoxazinones in agricultural soils.

2. Experimental

2.1. Standards and reagents

Standards were obtained from commercial and private sources as available. Benzoxazolinones, BOA and MBOA; malonamic acid, HMPMA, and aminophenoxazinones, APO, AAPO, AMPO, and AAMPO, were received from Dr. F. Macias (University of Cádiz, Spain). APH was purchased form Sigma–Aldrich. The non-naturally occurring synthetic derivative 2-MeO-HBOA was purchased from Professor Dr. Sicker (University of Leipzig, Germany).

Stock individual standard solutions (1000 ng/μL) were prepared by dissolving accurate amounts of pure standards in acidified methanol (MeOH) (1% acetic acid (HOAc)). Working standard solutions containing all compounds except internal standard were obtained by further dilution of stock individual solutions with acidified methanol (1% HOAc), and their con-

centrations are of 1, 5, 50 and 500 ng/μL. Working standard solution of internal standard (100 ng/μL) was also prepared by further dilution of stock solution.

HPLC-grade solvents water (H_2O), MeOH and 98% HOAc were purchased from Merck (Darmstadt, Germany). Diatomaceous earth was obtained from Varian. LiChrolut RP C_{18} (500 mg) SPE cartridges were purchased from Biotage.

2.2. Sample preparation

The soil samples come from wheat crops. Three different wheat varieties, Astron (As), Ritmo (Ri) and Stakado (St) were cultivated in Lleida (Catalunya, NW of Spain) under conventional cultivation conditions. After harvest, soil samples were taken in the proximity of cultivated plants. Plants were harvest at ten and twelve days after sowing, corresponding to two different stages. The soil samples were taken at the same time as plants in plastic bags and stored at −20 °C until further manipulation. Excess water was removed from the frozen agricultural soil by liophilization until weight loss was no longer observed. The soil was sieved at 120 μm and preserved at −20 °C until the extraction.

Samples were extracted by PLE using an ASE 200 (Dionex, Idstein, Germany) apparatus, equipped with 11 mL of stainless steel extraction cells. Five grams of soil were taken for the extraction. The sample was mixed with diatomaceous earth (hydromatrix) to ensure good dispersion of the sample and the remaining part was filled with the same material. Hydromatrix was cleaned by ultrasonication with the same solvent of the extraction and dried at 70 °C prior to use. The parameters used were as follow: flush volume, 60%, solvent extractor acidified MeOH, 1% HOAc; temperature, 150 °C; pressure, 1500 psi; static times, three cycles; and cell purge, 60 s.

A purification step prior to instrumental analysis is recommended. A simple cleanup procedure consists of the use of C_{18} SPE cartridge. Organic extracts were concentrated to incipient dryness by rotary evaporation and then were applied to the SPE cartridges. Analytes were eluted using 5 mL of acidified MeOH (1% HOAc). Then, extracts were concentrated to dryness and reconstituted with 2.5 mL of [MeOH/H_2O (0.05% HOAc) (60:40)]. Finally 25 μL of 100 ng/μL of the internal standard, 2-MeO-HBOA, were added to the extract for internal standard quantification.

3. LC–MS–MS analysis

3.1. Chromatographic conditions

LC–MS–MS analyses were performed on a Waters 2690 series Alliance HPLC (Waters, Milford, MA, USA) with a quaternary pump equipped with a 120-vial capacity sample management system. A synergy MAX-RP 80A column (150 mm × 2 mm, 4 μm, Phenomenex) with a solvent flow rate of 0.2 mL/min was used. Our previous work [17] was used as the basis for the chromatographic separation of benzoxazinones. Several gradient programs were assayed using acidified H_2O and MeOH at different proportions of acetic acid. Finally, acidified H_2O (1% HOAc) and MeOH (1% HOAc) were used as the elution solvents A and B, respectively. The sample injection volume was set at 10 μL.

3.2. Mass spectrometry conditions

A benchtop triple quadrupole mass spectrometer Quattro LC from Micromass (Manchester, UK) equipped with a pneumatically assisted electrospray probe and a Z-spray interface was used for the MS–MS analyses. Nitrogen gas (N_2) (99.999% purity) was used as the desolvation and cone gas and argon as the collision gas (3.6×10^{-5} mbar). All data were acquired and processed using Masslynx software.

Since in our previous LC–MS work [17] electrospray ionization was used for the analysis of aminophenoxazinones, this ionization was selected for the analysis in this work. Parameter optimization was performed by flow injection analysis (FIA) for each compound (25 ng/μL) at a constant flow rate 0.2 mL/min. Sensitivity of target compounds was first checked by recording chromatograms in full scan mode in both positive and negative ionization mode. With the aim to select parent ion for each analyte, the analysis was carried out also in full scan mode. Once precursor was selected, product scans were recorded to obtain precursor–product fragmentation, testing different values of cone voltage (modifying it value between 10 and 40 V) and collision energies (modifying it value between 10 and 40 eV). In order to increase sensitivity and selectivity, data acquisition was performed working in MRM mode. Two characteristic MRM transitions were selected for each analyte, MRM 1 for the quantification and MRM 2 for the confirmation. The MS setting parameters were the following: capillary voltage, 3.5 kV; source temperature and desolvation temperature, 150 °C and 350 °C, respectively; extractor, 2 V; rf lens, 0.4 V; cone argon gas flow, and desolvation N_2 gas flow 48 and 600 L/h, respectively; collision gas pressure, 2.52×10^{-3} mbar. The selected transitions and the optimized cone voltages and collision energies for each one are described in subsequent sections.

3.3. Method validation

In order to validate the instrumental method, quality assurance of the instrumental method was assessed measuring parameters as linearity, sensitivity and precision. The linearity was measured in the range of 0.01–2 ng/μL. Sensitivity was evaluated by determining the instrumental detection limits (LODinst) in MRM mode. LODinst were based on the peak-to-peak noise of the baseline on near the analyte peak obtained by analysis of standard solution and on minimal value of signal-to-noise ratio of 3. To establish the precision of the instrumental method an intra-day and inter-day analysis were performed by five consecutive injections of a standard solution at 0.05 ng/μL at the optimum conditions in LC–MS–MS analysis.

On the other hand the whole method (PLE–SPE followed by LC–MS–MS) was also validated with spiked soils at 12.5 ng/g. The method was carried out five times. Different quality parameters were evaluated: recoveries (%R), reproducibility and sensitivity (LODmet).

4. Results and discussion

4.1. LC method optimization

In a previous work, Bonnington et al. [16] evaluated different chromatographic columns with the aim to optimize the separation of different benzoxazinone derivatives. The dodecyl (C12) trimethylsilyl (TMS) end-capped Synergi MAX-RP showed better results. Due to the similarity of benzoxazinone derivatives with their degradation products, the same LC column was selected for our study. In order to accomplish the optimum chromatographic separation, several preliminary experiments were performed, testing different mobile phase consisting of H_2O as a polar phase (A) and MeOH as an organic phase (B), adding different proportions of HOAc (0.05 and 1%). The effect to add more proportion of HOAc into mobile phase improved the MS signals, especially for HMPMA. Therefore, acidified H_2O and MeOH adding 1% of HOAc in each one, was used as mobile phase. The optimum solvent gradient adopted was as follows: 0.0–0.1 min, 40% A; 0.1–8.0 min, 40% A; 8.0–13.0 min, 40–20% A; 13.0–15.0 min, 20–40% A; 15.0–20.0 min, 40% A. Total run time was 20 min with the compounds eluted over 2–10 min and the final 10 min was used for column cleaning and regeneration.

4.2. MS–MS method optimization

In order to optimize the MS parameters, flow injection analysis (FIA) was performed for each compound at 25 ng/μL, using acidified H_2O–MeOH (40:20) as carrier solvent. Sensitivity of target compounds was first checked by recording chromatograms in full scan mode in both positive and negative ionization mode. With the exception of BOA, all the analytes presented higher response in positive than in negative ionization mode. It is important to note that the differences on responses between both polarities were not very high for BOA, and MBOA, while for the rest of the analytes (APO, AMPO, AAPO, AAMPO, APH, 2-MeOH-HBOA, HMPMA) responses in PI mode were considerably higher. This is on agreeing with work of Guillamón et al. [17]. For these reasons, PI was selected as polarity ionization.

Identification of parent ion was also carried out in full scan. Once the parent ions were chosen, selection of optimum cone voltage and collision energy was tested by product ion scan. Cone voltage were selected according to the sensitivity of the precursor ions, and collision energies where chosen to give the maximum intensity of the fragment ions obtained. Maximum abundances were obtained at 10 V for 2-MeOH-HBOA; at 20 V for HMPMA, AAPO, and AAMPO; at 30 V for APH, MBOA, and APO; and at 40 V for AMPO. The complete precursor–product fragmentation pathways observed for the analytes and the optimal conditions for the transitions are given in Table 1. Representative chromatogram of a standard mixture (0.5 ng/μL) is illustrated in Fig. 2. These transitions were selected according to the highest sensitivity for the analyte of interest. The first transition (MRM 1) corresponded to the most abundant and was used for quantification and the second one (MRM 2) for confirmation purposes. The selection of unique transitions for a compound for use in quantitative studies is often ideal in order to avoid interferences caused by possible co-eluting impurities. The precursor ion corresponded in all cases to the protonated molecule $[M - H^+]$. As interpreted in Table 1 the aminophenoxazinones and benzoxazinones with methoxylated group in their structure (AAMPO, AMPO, and MBOA), have common fragmentation involving the loss of methoxy radical and subsequently CO moiety. Regarding malonamic acid (HMPMA), it has a common fragmentation involving the loss of H_2O and the adjacent moiety to the acidic group. APH showed poor fragmentation and only one transition could be monitored.

According to the performance characteristics defined in the EU Commission Decision 2002/657/EC [18] for the confirmation and identification of organic compounds, when using LC–MS–MS as instrumental technique, a minimum of three identification points are required. In our developed method, using two MRM transitions the minimum identification points are accomplished. The MRM ratio, calculated as the ratio between the abundances of two selected precursor–product ion transitions, were also used to confirm the identity of an analyte in the samples (Table 1). The calculated ratio in a sample must be within 20% of the ratio calculated upon the standards.

4.3. Quantification

The use of internal standards to aid reliable quantification has not been described for the quantification of aminophenoxazinones. In this study a non-naturally occurring structural analogue of HBOA (2-MeO-HBOA) benzoxazinone with adequate chromatographic separation from the selected analytes (Fig. 2) was used as internal standard. Good correlations were obtained for all analytes relative to this compound for the 0.01–2 ng/μL range, with correlation values always higher than 0.998. These results confirmed the applicability of selected internal standard for quantification, and this method was used for the quantitative analysis in soil samples.

Matrix induced interference resulting in suppression of signals in LC–MS were well reported [19–22]. Recently, we studied this effect for the determination of benzoxazinone derivatives in plant material [23]. One way to solve or minimize this effect was the dilution of extracts before injection into instrumental setup. In the case of soil samples, we have determined the optimal dilution of the final extract, showing the better results a final volume of 2.5 mL.

4.4. Method validation

Quality assurance of the instrumental developed method was evaluated by measuring parameters as linearity, sensitivity and precision (reproducibility and repeatability). All data are presented in Table 2.

The data were subjected to linear regression analysis and good correlations were obtained for all analytes relative to internal standard, ranging from 0.9980 to 0.9998. Sensitivity was evaluated by determining the LODinst in MRM mode. LODinst ranged from 13 to 77 pg injected, corresponding the lower val-

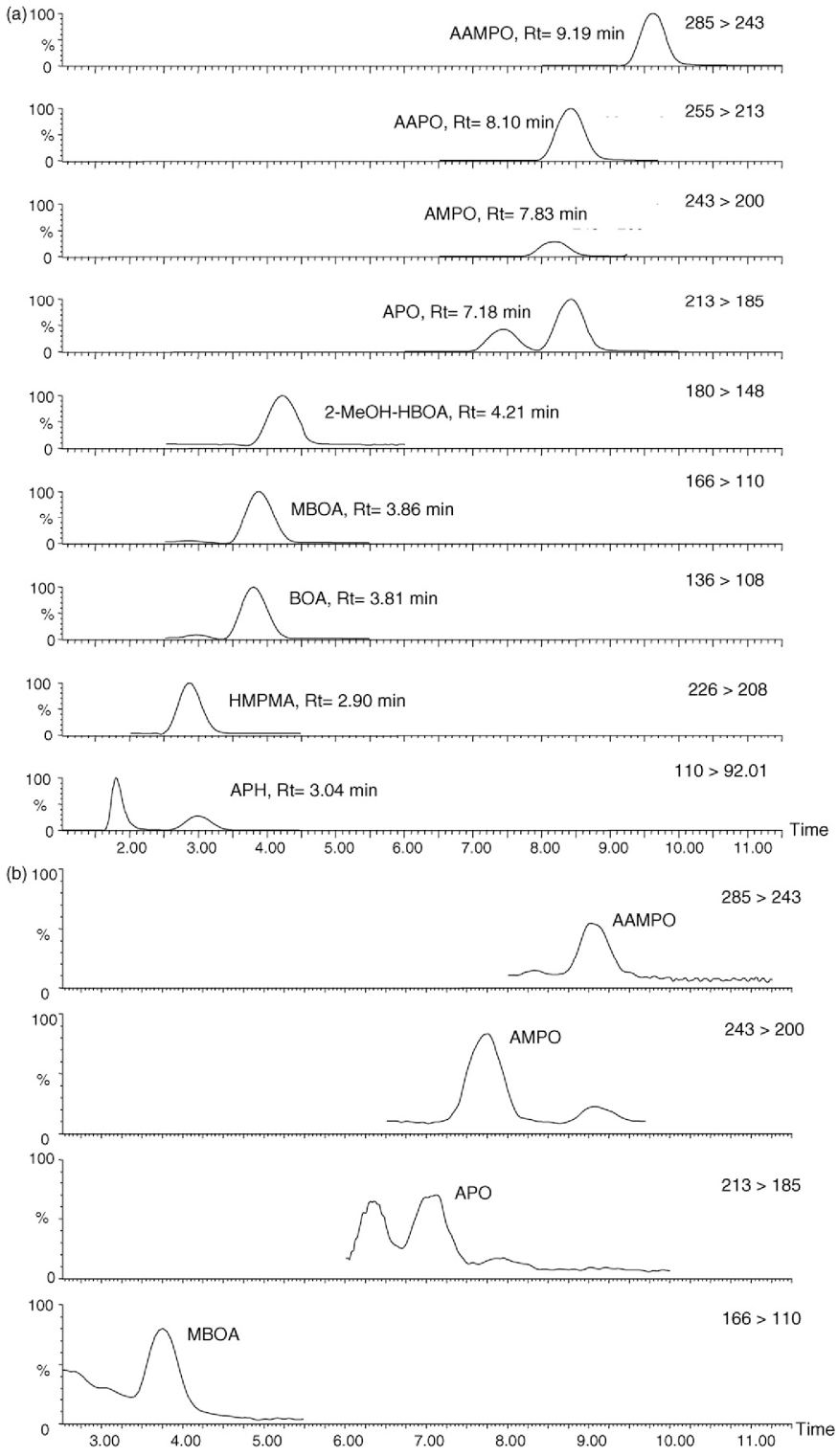


Fig. 2. LC–ESI–MS–MS chromatogram showing the different ion transition used for quantification (MRM 1) of (a) standard mixture (0.5 ng/μL) and, (b) soil sample (Astron variety, twelve days after sowing).

Table 2
Quality parameters of the instrumental LC–MS–MS and LC–MS method as well as of combined PLE–SPE LC–MS–MS method

		APH	HMPMA	BOA	MBOA	APO	AMPO	AAPO	AAMPO
LC–MS–MS method									
Linearity	r^2	0.9986	0.9997	0.9994	0.9981	0.9994	0.9998	0.9986	0.9980
Repetitivity	RSD ($n=5$)	2	1	2	2	3	2	5	1
Reproducibility	RSD ($n=5$)	3	5	3	3	7	9	10	18
Sensitivity	LODinst (pg injected)	14	77	43	27	23	17	24	13
LC–MS[11]									
Sensitivity	LODinst (ng injected)	1.3	11	4.3	4.3	5.4	3.2	1.2	NR[a]
PLE–SPE LC–MS–MS									
Recoveries	%	13	53	96	100	57	85	123	104
Reproducibility	RSD ($n=5$)	2	11	11	7	9	14	1	10
Sensitivity	LODmet (ng/g dry weight)	5.8	21	3.6	4.0	2.4	2.5	2.7	2.4

[a] NR, not reported.

ues to AAMPO, and the higher to HMPMA. If we compare our obtained LODinst using the developed LC–MS–MS method with those obtained previously by LC–MS [17], a great improvement, between 10- and 100-fold, was observed. Using a single LC–MS method, LODinst were in the ng level, whereas with our LC–MS–MS method, LODinst decrease to the pg level. Precision of the method was evaluated by injection of five consecutive standard solutions at 0.05 ng/μL, at the optimum conditions in LC–MS–MS above described. Repeatability (intra-day analysis) was evaluated measuring the relative standard deviation (RSD) between five consecutive injections, showing a precision from 1 to 5%. On the other hand, reproducibility (inter-day analysis) was also evaluated measuring the RSD between five injections in five different days. As it can be expected, RSD values obtained for repeatability were lower than those obtained for reproducibility which ranged from 3 to 18%.

The whole method, including PLE-SPE followed by LC–MS–MS was also validated with spiked soil samples at 12.5 ng/g. The quality parameters evaluated were: recoveries (%R), reproducibility and sensitivity (LODmet). All values are presented in Table 2 and discussed subsequently.

The recoveries (percent of standard added to sample recovered during whole process) obtained ranged from 53 to 123%. Only in the case of the malonamic acid APH, the obtained value was lower (13%). This could be attributed to the low stability of this intermediate product, as it can be seen in the degradation pathway of BOA (Fig. 1). As regards RSD obtained between five replicates, the values were below 14%, indicating good reproducibility of the method.

The sensitivity was evaluated by measuring the LODmet. This value was measured for each compound by a signal-to-noise ratio of 3 in spiked samples. The applied methodology provided a LODmet in the range between 2.4 and 21 ng/g dry weight. As our knowledge only one study reported recoveries and LODmet values using LC–MS analysis [13]. This method was only applied to the study of BOA and APO, with recoveries similar to those obtained in our study (90 ± 10% and 68 ± 20%, respectively). The detection limits achieved by LC–MS [13] were 9 ng/g for BOA and 35 ng/g for APO. These values were about five and ten times higher than those obtained by our developed method, respectively.

5. Application to soil analysis

In order to demonstrate their applicability, the developed method PLE–SPE followed by LC–MS–MS was applied to the analysis of agricultural soils. As our knowledge, this is the first study in which benzoxazinones and their degradation products were detected and quantified in agricultural soils. The MRM ratio of each compound was used to confirm the identity of the analyte. All the MRM ratio calculated were within 20% of the ratio calculated upon standards (Table 3). Quantitative results are summarized in Table 3. Four analytes (MBOA, APO, AMPO and AAMPO) were detected in all the soil sam-

Table 3
Quantification of allelochemicals in agricultural soil samples using PLE-SPE followed by LC–MS–MS (values expressed in ng/g of dry weight)

	First stage						Second stage					
	Astron		Ritmo		Stakado		Astron		Ritmo		Stakado	
	Level	Ratio[a]	Level	Ratio	Level	Ratio	Level	Ratio	Level	Ratio	Level	Ratio
HMPMA	<21	–	<21	–	<21	–	<21	–	<21	–	<21	–
BOA	<3.6	–	<3.6	–	<3.6	–	<3.6	–	<3.6	–	<3.6	–
MBOA	37.8	0.9	40.8	0.9	68.5	1.02	51.0	1.01	35.5	1.04	34.5	0.9
APO	20.6	4.4	27.1	3.4	46.7	3.8	90.6	4.1	43.6	4.2	34.7	3.6
AMPO	31.8	1.7	45.8	2.1	54.1	1.8	149	1.7	81.1	1.7	67.2	1.6
AAPO	<2.7	–	<2.7	–	<2.7	–	<2.7	–	<2.7	–	<2.7	–
AAMPO	<8.1	–	<8.1	–	<8.1	–	<8.1	–	<8.1	–	<8.1	–

[a] Ratio = abundance MRM 1/abundance MRM 2.

ples. Concentration levels ranged from 20.6 to 149 ng/g of dry weight. Maximum levels corresponded to AMPO followed by MBOA > APO > AAMPO, with the exception of two samples (first stage-Astron and first stage-Stakado) where MBOA levels were slightly higher than those of AMPO. As expected, BOA and AAPO were not detected in these soil samples. BOA is the benzoxazolinone derivative obtained from the degradation of DIBOA, which was the major allelochemical in rye; but in the case of wheat, DIMBOA is the predominant derivative, and then MBOA is the main benzoxazolinone obtained from the DIMBOA degradation [15]. It is expected that BOA and AAPO will be found in soil samples coming from rye crops.

The content of benzoxazinoid derivatives in wheat plants showed variations between different species. In our previous results from plant material analyses [23], Stakado was the wheat variety which presented the highest concentration levels. Similarly, higher levels of benzoxazinone and their degradation products were also detected in the soil samples corresponding to Stakado crops.

It is interesting to note that levels of benzoxazolinone derivatives in wheat plants are in the range of mg/g whereas levels obtained in agricultural soils are in the ng/g level. And this is the main reason for the need of a high sensitivity methodology for the determination of these allelochemicals in soil samples. Further presentation and discussion of quantification results will be addressed elsewhere, with correlation between allelochemical levels found in plant material and in cultivated soil.

6. Conclusions

A novel analytical method based on SPE–LC followed by MS–MS was developed, allowing the determination and quantification of benzoxazolinones and their degradation products in soil samples. Recoveries obtained for target compounds were higher than 50%, except for APH due to his instability. Quality assurance of the developed method was assessed by measuring parameters as recovery, linearity, reproducibility, and sensitivity, drawing acceptable data. The application of LC–MS–MS analysis operating in MRM mode, with two transitions (if available) monitored for each compound, improve the sensitivity of a previous LC–MS method [17]. The applicability of the method to the characterization of these allelochemicals has been demonstrated, with the analysis of agricultural soil samples. The low LODmet obtained ranging from 2.4 to 21 ng/g of dry weight, allowed the identification and quantification for the first time of benzoxazolinones and their degradation products in agricultural soil samples at concentration levels ranging from 20.6 to 149 ng/g of dry weight. Moreover, these results confirmed previous laboratory studies in which degradation pathways were proposed, but never before confirmed in real agricultural soils.

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III.4 DISCUSSIÓ DELS RESULTATS

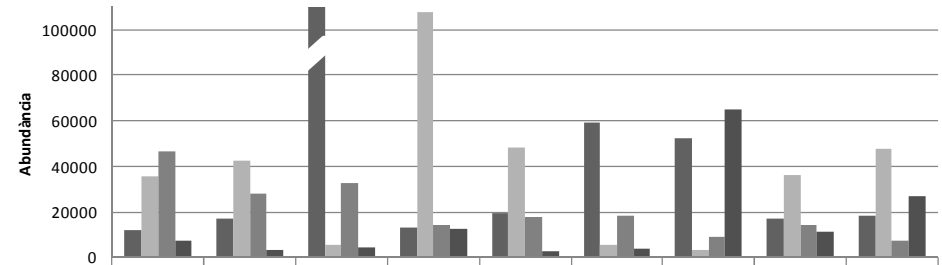
Anàlisi Instrumental

En el moment en que es va desenvolupar la metodologia que presentem en aquest capítol, no hi havia descrits en bibliografia estudis referents a la separació i quantificació de les aminophenoxazinones i els àcids malonàmics mitjançant LC-MS. Tal i com hem vist en el capítol anterior, les benzoxazolinones si que eren incloses en l'estudi de Bonnington et al. [8] on l'estabilitat dels analits i la columna cromatogràfica eren avaluats sota el requeriment de treballar amb fases mòbils àcides, i posteriorment es va desenvolupar el mètode presentat en el Capítol II de la present tesi. Per aquesta raó, la columna per a l'anàlisi dels productes de degradació i les benzoxazolinones BOA i MBOA va ser la dodecyl (C12) trimethyl silyl end-capped Synergi MAX-RP. L'elecció del tipus i quantitat de modificador a afegir a la fase mòbil és un compromís entre el guany en la separació, l'eficiència en la ionització, l'estabilitat dels analits i la columna cromatogràfica. En ambdós treballs presentats en aquest capítol, es van provar diferents gradients d'elució usant H_20 i MeOH i diferents proporcions de modificador (àcid acètic) per tal d'obtenir la millor senyal i separació cromatogràfica dels analits d'interès.

En un primer estadi de l'estudi, la identificació dels analits es va dur a terme mitjançant l'anàlisi per espectrometria de masses en mode de selecció d'ions (SIM) amb la font d'ionització ESI. La optimització dels paràmetres de MS es va dur a terme fent *flow injection analysis* (FIA) per a cada analit a una concentració de 25ng/μL, utilitzant H_20 /MeOH/H^+(HOAc) (60:40) com a solvent de càrrega. Les condicions òptimes van ser escollides en funció a l'abundància i fragmentació de cada analit en el mode d'escombrat (*SCAN*).

Per tal d'obtenir la informació estructural necessària i la màxima sensibilitat, diferents paràmetres van ser optimitzats: pressió del gas de nebulització, de 50 a 60 psi; la temperatura del gas, de 250 a 350^{o}C; i el potencial de fragmentació, de 70 a 150V. A la Figura III.5es mostren les respostes obtingudes pels diferents analits a un potencial de

fragmentació de 70 i 150V en mode positiu i negatiu. Per a la majoria dels analits, amb el potencial de fragmentació de 70V en mode negatiu es van obtenir les majors respostes. No obstant, per a les aminofenoxazinones APO, AAPO i AMPO les respostes obtingudes en mode positiu varen ser considerablement molt superiors a les obtingudes en mode negatiu, raó per la qual es va arribar a un compromís seleccionant la polaritat positiva per dur a terme l'anàlisi. Finalmentes van seleccionar dos ions d'acord amb l'especificitat i selectivitat de cada analit, el primer ió usat per a la quantificació i el segons per la confirmació.



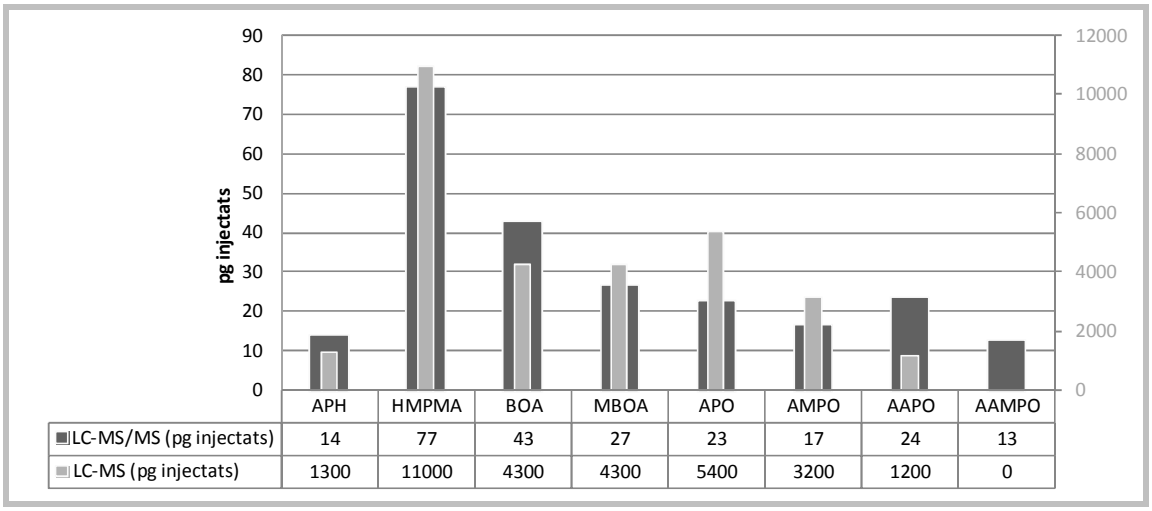
	APH	HPAA	AAPO	BOA	MBOA	APO	AMPO	HMPMA	HPMA
ESI (+) 70V	11814	17218	162355	13231	19628	59321	52216	17028	18271
ESI (-) 70V	35299	42492	5407	107751	48304	5405	3524	36228	47718
ESI (+) 150V	46422	27976	32629	14318	17929	18547	9245	14445	7463
ESI (-) 150V	7615	3104	4451	12641	2861	3809	64816	11110	27164

Figura III.5.- Respostes TIC dels diferents analits per ESI en mode positiu i negatiu.

En un segon estadi i per tal de millorar la sensibilitat del mètode es va desenvolupar una metodologia basada en espectrometria de masses en tàndem. De la mateixa manera els paràmetres van ser optimitzats fent FIA per a cadascun dels analits a una concentració de 25ng/µL, utilitzant H_20 /MeOH/H^+(HOAc) (60:40) com a solvent de càrrega a un flux de 0.2mL/min. L'ió precursor va ser escollit adquirint les dades en el mode full scan i els ions producte a través del productionscan optimitzant diferents paràmetres tals com: el voltatge del con, de 10 a 40V; i l'energia de col·lisió, de 10 a 40eV. Per tal d'obtenir la màxima sensibilitat i selectivitat l'adquisició de les dades es va dur a terme treballant en mode MRM. La identificació i quantificació dels analits d'interès es va dur a terme d'acord amb la normativa de la Unió Europea 2002/657/EC en el que són requerits un mínim de tres punts d'identificació. En el nostre cas es van usar el temps de retenció, dues transicions MRM per a cada compost, la primera per a la quantificació i la segona per a la confirmació i la relació entre ambdues transicions.

Els límits de detecció instrumentals obtinguts per MS/MS ofereix significants millores en quant a la selectivitat i sensibilitat envers el mètode descrit en la publicació #3# mitjançant MS. A la Figura III.6es mostren els límits de detecció instrumentals per ambdós metodologies. La tècnica de MS-MS millora entre 50 i 235 vegades els iLOD obtinguts per MS.

La justificació de perquè primer es va desenvolupar la metodologia per LC-MS és perquè creiem que seria possible analitzar els compostos de degradació mitjançant aquesta metodologia, però al injectar diferents mostres de sòl no vàrem aconseguir detectar cap dels analits tot i incrementar la quantitat de sòl a extreure. Com veurem en capítols posteriors els nivells dels analits en plantes són del rang de mg/Kg mentre que els compostos de degradació es troben a ng/g en el sòl agrícola fet que implica la necessitat de disposar d'una metodologia més sensible com l'espectrometria de masses en tàndem.



	APH	HMPMA	BOA	MBOA	APO	AMPO	AAPO	AAMPO
LC-MS/MS (pg injectats)	14	77	43	27	23	17	24	13
LC-MS (pg injectats)	1300	11000	4300	4300	5400	3200	1200	0

Figura III.6.- Límits de detecció instrumentals obtinguts per LC-MS (gris) i LC-MS/MS (blau clar); nd:no determinat

Així doncs com era d'esperar la MS-MS millora la sensibilitat del mètode tal i com hem vist per a les benzoxazinones, com ara amb els productes de degradació. No obstant, mentre la MS és una tècnica vàlida i amb sensibilitat suficient per determinar les benzoxazinones en plantes de blat, no passa el mateix per a les aminofenoxazinones i derivats en mostres de sòl agrícola. De tal manera que l'ús de la espectrometria de masses en tàndem és necessari i indispensable.

Cal comentar que el mètode desenvolupat per als compostos de degradació en el sòl inclouen les benzoxazolinones BOA i MBOA determinades també en les plantes (Capítol II). En aquest cas, si comparem els iLOD obtinguts mitjançants la LC-MS (Taula III.1), observem que comparats amb el mètode obtingut per LS-MS-MS presentat en aquest capítol hem perdut en sensibilitat (a l'entorn de 40 vegades menys sensible). Aquest fet es deu a que en el mètode desenvolupat per a l'anàlisi de lebenzoxazolinones en la planta la ionització es du a terme en polaritat negativa que és la que ens proporciona una major senyal. No obstant, tal i com es mostra a la Figura III.5, per tal d'arribar a un compromís i poder analitzar les aminofenoxazinones (APO, AAPO i AMPO) es fa necessari treballar en mode positiu i això ens comporta a una pèrdua de sensibilitat per al BOA i MBOA.

Taula III.1.-iLOD expressats com a ng injectats per BOA i MBOA mitjançant LC-MS

Compost	Mètode plantes	Mètode sòls
BOA	0.1	4.30
MBOA	0.1	4.30

Preparació de la mostra

L'etapa de preparació de la mostra segueix el mateix perfil definit en el capítol anterior per a la metodologia de plantes, essent les etapes de liofilització, extracció per PLE, filtració i purificació dels extractes, les necessàries per a l'anàlisi. L'esquema del procediment de la preparació de la mostra i la seva anàlisi és el que es mostra a la Figura III.7.

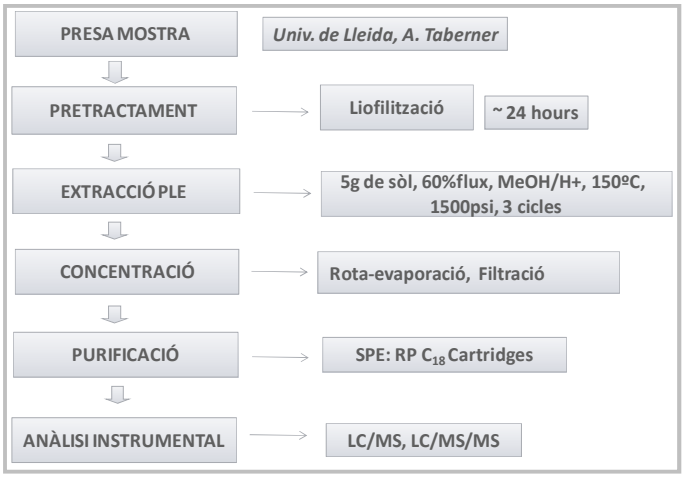
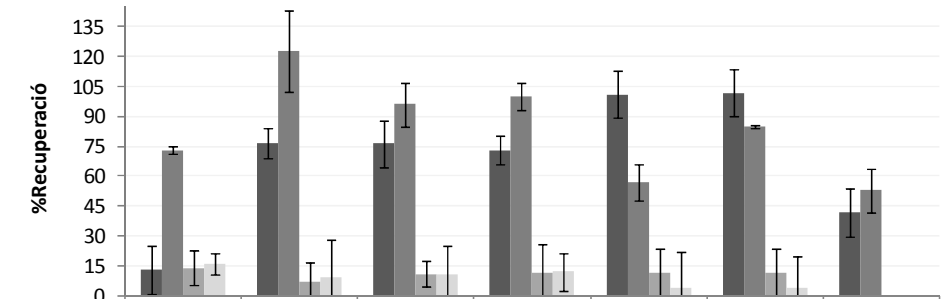


Figura III.7.- Esquema del procés de preparació de la mostra de sòl

Primerament es van dur a terme les proves de recuperació dels diferents analits en cadascuna de les etapes sense la presència de matriu per tal de verificar-ne la seva estabilitat durant el procés i posteriorment es va aplicar el procés complert amb el sòl control.Referent a l'etapa d'extracció per PLE, es van provar diferents solvents: MeOH, MeOH/H^+ (0.1% HOAc), H_2O i H_2O/H^+ (0.1% HOAc), essent l'extracció amb MeOH/H^+ la més efectiva tal i com es mostra a la Figura III.8.



	APH	AAPO	BOA	MBOA	APO	AMPO	HMPMA
MeOH	13	77	76	73	101	102	42
MeOH/H+	73	123	96	100	57	85	53
H2O	14	7	11	11	11	12	0
H2O/H+	16	9	10	12	4	4	0

Figura III.8.- Recuperacions obtingudes per la PLE amb els diferents solvents

En quant a l'etapa de purificació dels extractes, es va optimitzar la seva elució realitzant diferents proves eluint els compostos amb 5 mL MeOH:H_20:H^+ a diferents proporcions: 0:100, 20:80, 40:60, 50:50, 60:40, 80:20, 100:0. Essent la de 80:20 la composició d'elució més òptima (Figura II.9).

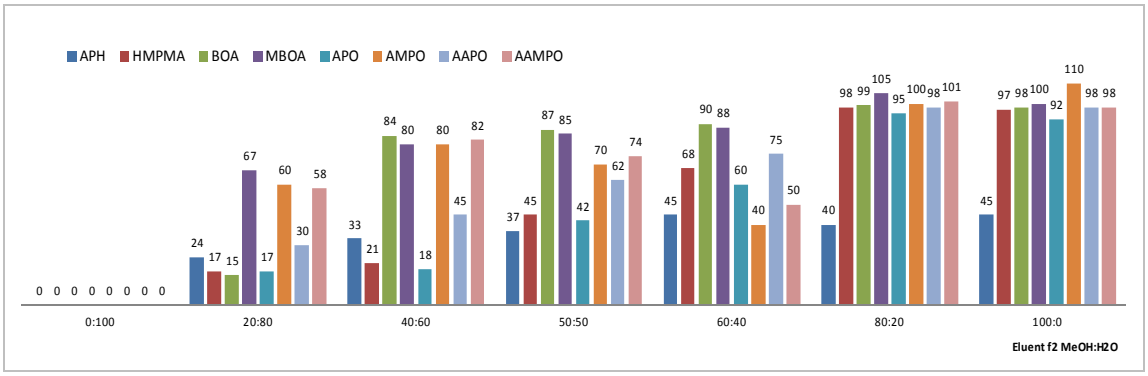


Figura III.9.- Recuperacions obtingudes dels diferents analits en l'etapa de neteja

Estudi de l'efecte de matriu

Un cop optimitzades les etapes de la preparació de la mostra i validat el mètode instrumental per LC-MS/MS, en vista a l'efecte matriu (EM) observat en les mostres de planta, aquest també va ser estudiat en els extractes de sòl agrícoles preparats. En un primer pas es va comprovar si existia EM comparant la resposta obtinguda per a cadascun dels analits en un patró i en un extracte de sòl a la mateixa concentració. Tal i com es pot observar a la Figura III.10, tots els analits presenten una disminució en la resposta obtinguda, és a dir una supressió de la senyal degut a l'EM a excepció del AMPO i APO que presenten un lleuger augment de la mateixa.

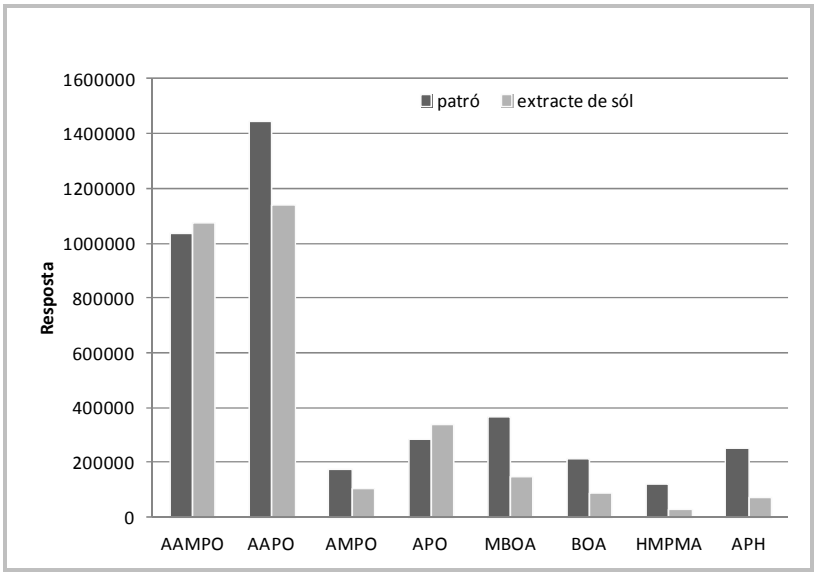


Figura III.10.- Resposta en un patró i un extracte de sòl a la mateixa concentració

En vista dels resultats obtinguts, es va dur a terme l'avaluació de l'efecte de matriu de manera similar a l'estudi realitzat amb els extractes de la planta, prèviament descrit al Capítol II. En primer lloc es va avaluar el mètode de calibratge per patró intern. A tall d'exemple es mostra a la Figura III.11 com es pot compensar l'efecte de matriu en el sòl per l'AMPO usant el 2-MeOH-HBOA com a patró intern. Tal i com es pot veure, es passa d'una disminució del pendent de la recta del 43 al 13%. Cal però, mencionar que l'AMPO és el compost que va proporcionar millors resultats, mentre que per la resta d'analits les millores no van ser el suficientment acceptables com per donar per vàlida aquesta metodologia, com exemple es mostra el cas del BOA.

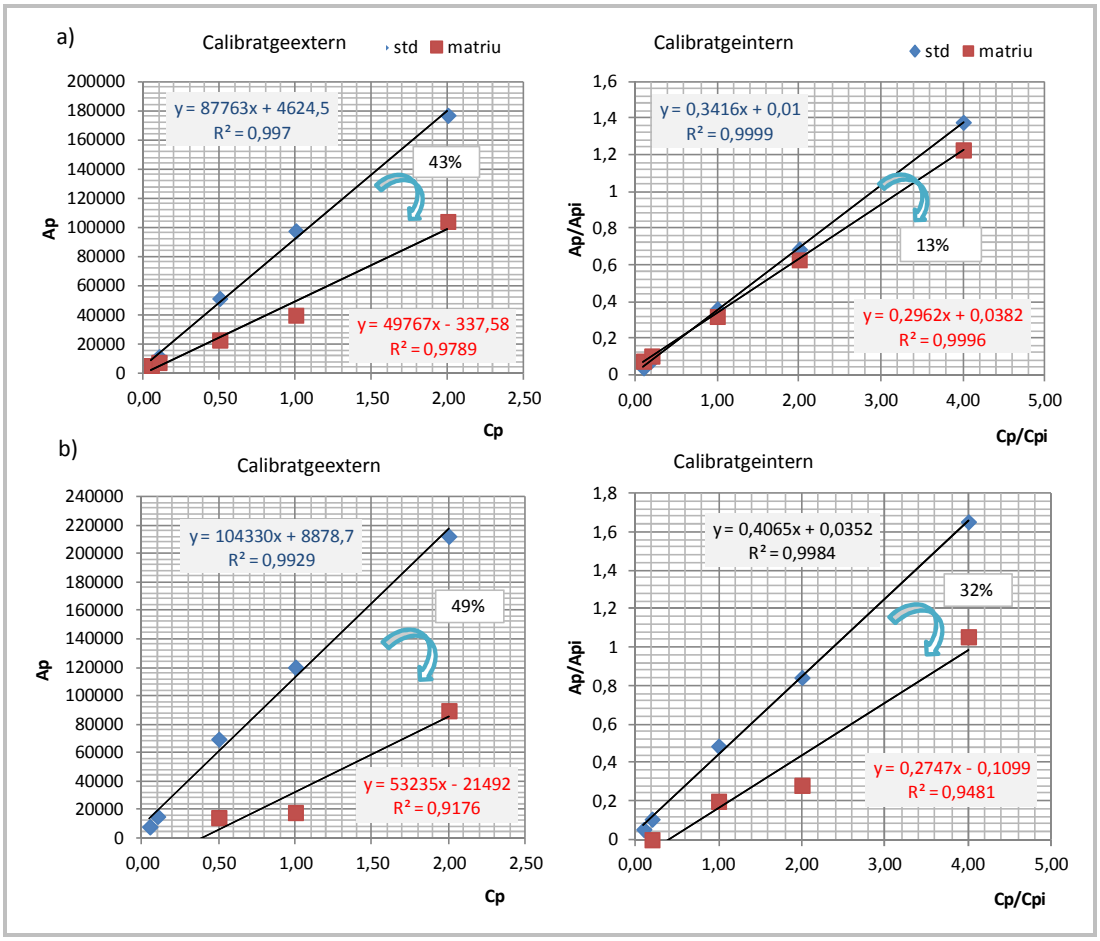


Figura III.11.-Corbes de calibratge preparades en MeOH/H+ (blau) i matriu sòl(vermell) construïdes tant pel mètode de patró extern com intern per a) AMPO i b)BOA

Tenint en compte aquests resultats, que no disposàvem dels corresponents compostos marcats isotòpicament i després de l'experiència en l'estudi de l'efecte de matriu en els extractes de la planta, es va decidir dur a terme una dilució de l'extracte de sòl. A la Figura III.12 es mostren els valors del %EM (calculat segons s'ha exposat al capítol II) de la resposta de cadascun dels analits a una concentració donada a les diferents dilucions testades. Tal i com es pot observar, al anar diluint l'extracte l'EM es va minimitzant, acostant-se els valors al 100%.

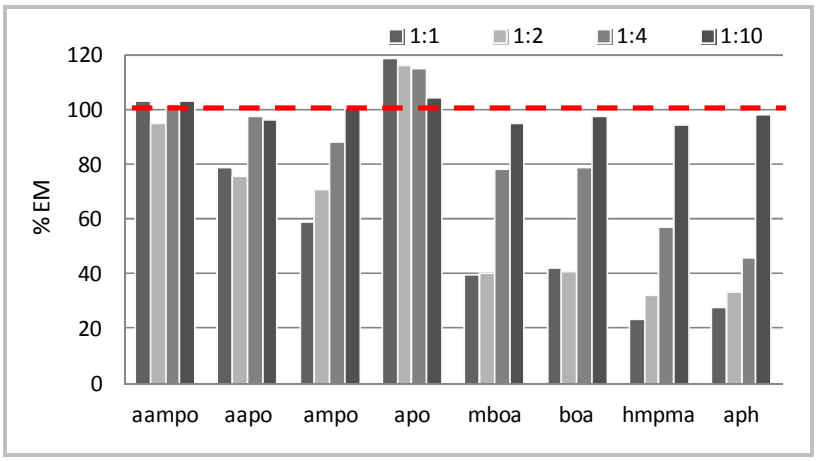


Figura III.12.-%EM dels diferents analits de la resposta dels diferents analitsa les diferents dilucions testades

Segons els resultats obtinguts a les diferents dilucions testades, finalment es va decidir diluir els extractes deu vegades. A tall d'exemple es mostra el resultat de la quantificació d'una extracte de sòl sense i amb dilució de l'extracte (Figura III.13).

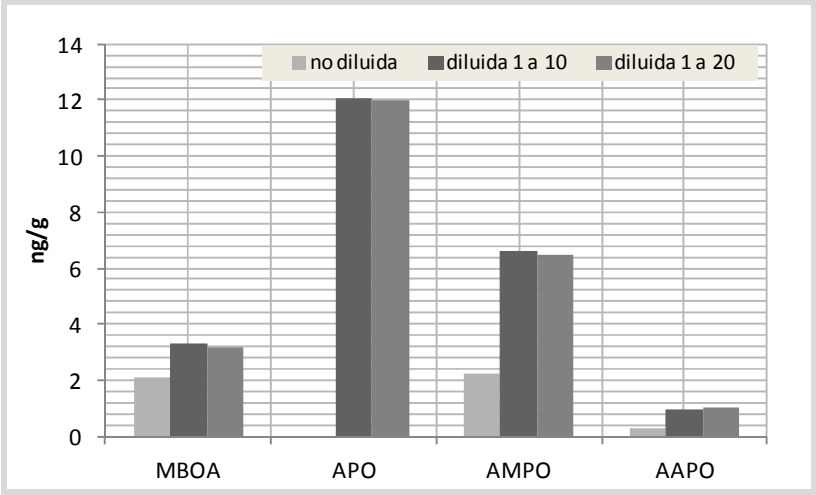


Figura III.13. Quantificaciód'una mostra de sòl pel mètode de patró intern sense dilució i diluït 10 i 20 vegades

Com es pot observar, els nivells de concentració dels compostos MBOA, AMPO i AAPO incrementen amb la millora de l'efecte de matriu a través de la dilució de l'extracte 10 vegades. Cal destacar també el cas de l'APO que passa de no ser detectat a poder ser determinat i quantificat, essent, com veurem en el proper capítol, el metabòlit amb més presència de tots els detectats en les mostres de sòl. També és important destacar que una major dilució de l'extracte, a 20 vegades, no proporciona variacions en els nivells determinats, pel que considerem que la dilució establerta a 10 vegades és

suficient per tal de minimitzar al màxim l'efecte de matriu en els extractes de sòl agrícola.

Valoració del mètode desenvolupat

Un cop avaluats tots els procediments i determinada la dilució òptima en la qual es minimitza al màxim l'efecte de matriu es va passar a fer la validació del mètode complert. Aquesta es va dur a terme per quintuplicat amb una mostra de sòl control a una concentració de 12.5 ng/g. Les recuperacions obtingudes van ser superiors al 50% per a tots els compostos a excepció del APH (Taula III.2). La baixa recuperació del APH pot ser deguda a la seva baixa estabilitat.

Taula III.2.- Paràmetres de qualitat del mètode desenvolupat

	APH	HMPMA	BOA	MBOA	APO	AMPO	AAPO	AAMPO
% Recupeació	13	53	96	100	57	85	123	104
Reproductibilitat	2	11	11	7	9	14	1	10
Sensibilitat (LOD) ng/g	5.8	21	3.6	4	2.4	2.5	2.7	2.4

En el moment en que es va desenvolupar aquesta metodologia hi havia poques dades sobre validació de mètodes analítics descrits en la bibliografia referents a l'anàlisi d'aquests compostos, de manera que era difícil fer una comparació del nostre mètode en aquest aspecte. Es troben dos treballs de LC-MS [9, 10] i un de LC-MS/MS [11], on s'analitzen els compostos de degradació de les benzoxazolinones en mostres de sòl dopades, però on només són analitzats tres dels set analits determinats en el treball dut a terme en la present tesi.

A la Taula III.3 es mostren els paràmetres de qualitat com la recuperació i els LOD dels diferents mètodes descrits a la bibliografia. Comparant les dades obtingudes dels diferents mètodes trobats en la bibliografia amb els paràmetres de qualitat del mètode desenvolupat durant la present tesi, primer de tot podem destacar que cap dels mètodes descrits en la bibliografia analitzen el mateix nombre de metabòlits que els descrits en el nostre mètode i normalment l'anàlisi es du a terme analitzant les benzoxazolinones i les aminofenoxazinones per separat, és a dir fent dues injeccions

per mostra. Aquest fet és degut, tal i com s'ha explicat anteriorment, a que les benzoxazolinones (BOA,MBOA) presenten major sensibilitat quan la ionització es du a terme en polaritat negativa, mentre que la resta d'analits ho són més en positiu. Per a les benzoxazolinones, els treballs de Krogh et al. [9] i Rice et al. [12] duen a terme l'anàlisi en polaritat negativa i presenten mètodes entre 4 i 8 vegades més sensibles per a BOA i MBOA, a diferència del presentat per Gents et al. [10] que resulta ser 3 vegades menys sensible pel BOA respecte al presentat en aquesta tesi. Per altra banda, el nostre mètode és entre 2 i 8 vegades més sensible que el presentat per Understap et al. [11] que també du a terme l'anàlisi a través de la LC-MS-MS en mode positiu. Per a l'anàlisi de les aminofenoxazinones, és el mètode presentat en el present capítol el que mostra millors LODmet excepte per als compostos APO i AAPO, on el mètode de Rice et al. [12] resulta ser considerablement més sensible. Les recuperacions són comparables entre les diferents metodologies.

Taula III.3.- Paràmetres de qualitat dels mètodes descrits a la bibliografia en l'anàlisi de benzoxazolinones, aminofenoxazinones i àcids malonàmics en sòl agrícola (NR: no reportat; NA: no analitzat)

	Gents et al. [10]			Krogh et al. [9]			Rice et al. [12]			Understrup et al. [11]		
	%R	LOD	Tècnica anàlisi	%R	LOD	Tècnica anàlisi	%R	LOD	Tècnica anàlisi	%R	LOD	Tècnica anàlisi
BOA	90	9	LC-ESI-MS	74.9	0.44	LC-ESI(-)-MS	72	0.74	LC-ESI(-)-MS/MS	73	27	LC-ESI(+)-MS/MS
MBOA	NA			95.3	1.18		60	1.05		NA		
APO	68	35	LC-ESI-MS	51.9	0.76	LC-ESI(+)-MS	49	0.003	LC-ESI(+)-MS/MS	60	8	LC-ESI(+)-MS/MS
AMPO	NA			40.0	4.02		NR	NR		NA		
AAPO				94.7	15.9		NR	0.017		66	10	LC-ESI(+)-MS/MS
AAMPO				90.7	2.59		NR	NR		NA		

III.5 REFERÈNCIES

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