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

Marta Villagrasa Giménez

,

UNIVERSITAT DE BARCELONA

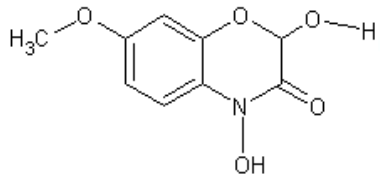
Facultat de Química
Departament de Química Analítica

Institut de Diagnòstic Ambiental i
Estudis de l'Aigua (IDAEA-CSIC)
Departament de Química Ambiental

METODOLOGIES ANALÍTIQUES PER A L'ESTUDI DE COMPOSTOS AL·LELOQUÍMICS EN CONREUS DE BLAT

Marta Villagrasa Giménez
Novembre 2013

CAPÍTOL II.- METODOLOGIA ANALÍTICA EN PLANTES



CAPÍTOL II.- METODOLOGÍA ANALÍTICA EN PLANTES

II.1. INTRODUCCIÓ I OBJECTIUS

Una alternativa a l'ús de plaguicides sintètics pel control de les males herbes i/o plagues podria venir donat per explotar les defenses químiques naturals que presenten els cereals, entre ells el blat. Un dels passos a dur a terme en l'estudi de l'al·lelopatia és identificar quins metabòlits estan implicats en el metabolisme de defensa de les plantes. Tal i com s'ha comentat a la introducció, els àcids hidroxàmics juntament amb les benzoxazolinones podrien ser uns possibles substituts dels plaguicides sintètics en la protecció agrícola. Per aquestes raons es fa necessari el disposar de metodologies analítiques per tal de determinar el contingut d'aquests compostos en cultius, i en concret en la present tesi en el cultiu de blat. La LC amb detecció UV ha estat la tècnica més àmpliament usada en l'anàlisi de compostos al·lelopàtics. No obstant, donada la baixa selectivitat i especificitat d'aquesta tècnica es fa necessari el desenvolupament de noves metodologies més avançades. La GC-MS també ha estat aplicada en l'anàlisi d'aquests compostos però el fet d'invertir força temps en la preparació de la mostra la fa desfavorable per a l'anàlisi d'un nombre elevat de mostres.

El desenvolupament de la LC-MS en l'anàlisi de mostres ambientals pot oferir avantatges tals com la reducció en el temps de preparació de la mostra, millora dels límits de detecció i una major selectivitat i especificitat.Un punt important a tenir en compte en el desenvolupament de les metodologies analítiques és l'estabilitat dels analits. Les benzoxazinones són inestables a la hidròlisi, especialment aquelles que tenen un hemiacetal cíclic en la seva estructura (DIBOA, DIMBOA) i la seva degradació és catalitzada en condicions bàsiques. Per tal d'estabilitzar aquests compostos, la seva extracció i anàlisi es du a terme generalment en medi àcid.

L'altre punt a tenir en compte és com dur a terme l'anàlisi quantitativa. La majoria dels treballs descrits no han usat la incorporació de patrons interns amb la finalitat de determinar la recuperació dels analits, i per tant no aporten informació sobre l'eficiència de la recuperació, pèrdues durant la preparació de la mostra o l'eficàcia de la tècnica analítica emprada. La seva quantificació s'ha dut a terme únicament per comparació amb les corbes de calibratge de solucions de patrons purs i el compost BOA ha estat usat com a patró intern, però el seu ús és desaconsellable ja que pot estar present en les matrius a analitzar.

A continuació es presenten i es discuteixen les metodologies analítiques desenvolupadesdurant el transcurs de la tesi per dur a terme l'anàlisi de compostos al·lelopàtics en plantes de blat mitjançant la LC-MS. En aquest context, els principals objectius plantejats han estat:

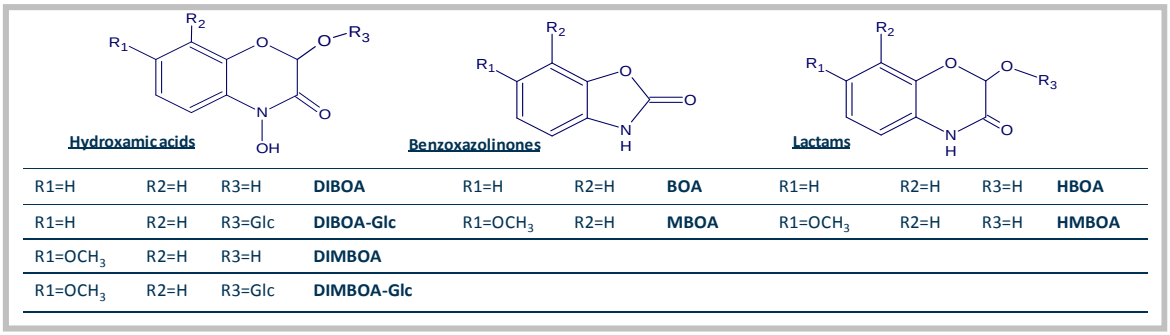
1.- Optimitzar les condicions per a la ionització de les benzoxazinones i la seva fragmentació, amb la finalitat de realitzar una anàlisi mitjançant LC-MS en el mode d'adquisició SIM.

2.- Desenvolupar una metodologia analítica ràpida i simple per determinar les 8 benzoxazinones simultàniament en matrius complexes, com són les fulles i les arrels del blat mitjançant l'extracció amb PLE seguit d'una etapa de neteja amb SPE.

3.- Optimitzar la quantificació de benzoxazinones en matriu per LC-MS, fent una valoració de l'efecte de supressió iònica a través de diferents metodologies com són l'addició estàndard, la dilució de la mostra, i l'ús de patró intern.

II.2. SELECCIÓ DELS ANALITS I DISPONIBILITAT DELS ESTÀNDARDS

En base a la bibliografia consultada [1-6] i els objectius establerts en el projecte FATEALLCHEM, els compostos químics identificats amb més potencial al.leloquímic en el blat, pertanyen a la família de les benzoxazinones. A la Figura II.1 es presenten els analits d'estudi.



R1=H	R2=H	R3=H	**DIBOA**	R1=H	R2=H	**BOA**	R1=H	R2=H	R3=H	**HBOA**
R1=H	R2=H	R3=Glc	**DIBOA-Glc**	$R1=OCH_3$	R2=H	**MBOA**	$R1=OCH_3$	R2=H	R3=H	**HMBOA**
$R1=OCH_3$	R2=H	R3=H	**DIMBOA**							
$R1=OCH_3$	R2=H	R3=Glc	**DIMBOA-Glc**							

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

Un dels punts més crítics associats a l'anàlisi d'aquests compostos és la baixa disponibilitat comercial dels patrons analítics, fet que implica haver de sintetitzar o aïllar de les mateixes plantes els compostos d'interès. Els estàndards que es varen fer servir per dur a terme les anàlisis provenen de fonts naturals i ens van estar subministrats per diferents laboratoris, que han desenvolupat mètodes per aïllar aquests compostos de la planta, principalment del blat de moro, o bé han desenvolupat vies de síntesi per a la seva obtenció. Els derivats metoxilats (Dimboa-Glc, HMBOA, DIMBOA) es poden aïllar de les plantes del blat de moro [7], mentre que la resta (Diboa-Glc, HBOA, DIBOA) s'obtenen de les arrels de l'*Aphelandratetragona* [8].En la Figura II.2 es mostra el procediment bàsic d'obtenció d'aquests analits.

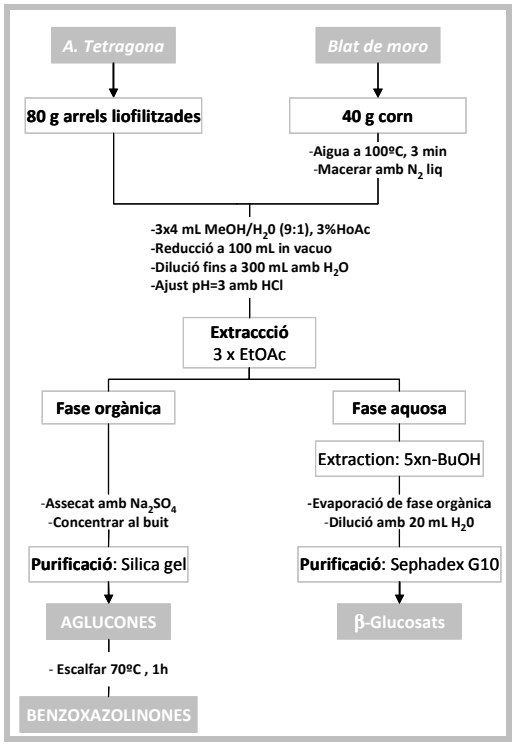


Figura II.2.- Esquema de l'aïllament dels compostos glucosats, aglucones i benzoxazolinones de fonts naturals.

Aquest procediment es basa principalment en: una primera extracció amb MeOH/H_2O en medi àcid seguit d'una extracció amb acetat d'etil. A partir d'aquest moment, es tracten per separat la fase aquosa i la fase orgànica.La fase orgànica es passa per una columna del gel de sílice per tal d'obtenir les aglucones, mentre que la fase aquosa s'extreu amb butanol (n-BuOH) i es purifica en una columna de Sephadex per tal d'obtenir els compostos glucosats. Posteriorment, les benzoxazolinones MBOA i BOA s'obtenen escalfant solucions aquoses de DIMBOA i DIBOA, respectivament, a 70°C durant 1 hora [9]. A banda dels mètodes desenvolupats per a l'aïllament dels analits de les plantes, també han estat desenvolupades diferents rutes de síntesi per a la obtenció d'aquests compostos [10-15].

Degut a l'elevada degradació i/o baixa estabilitat que presenten els analits d'interès [9],es va creure necessari fer un estudi preliminar de la seva estabilitat per tal d'assegurar-ne la qualitat de l'anàlisi. Per determinar l'estabilitat dels analits, solucions patró d'aquests es van conservar a diferents temperatures: a temperatura ambient (20°C), a 4°C i a -20°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. Els millors resultats es van obtenir conservant les mostres a -20°C. A la Figura II.3es mostren els resultats obtinguts als 7 dies a la temperatura de conservació de -20°C.

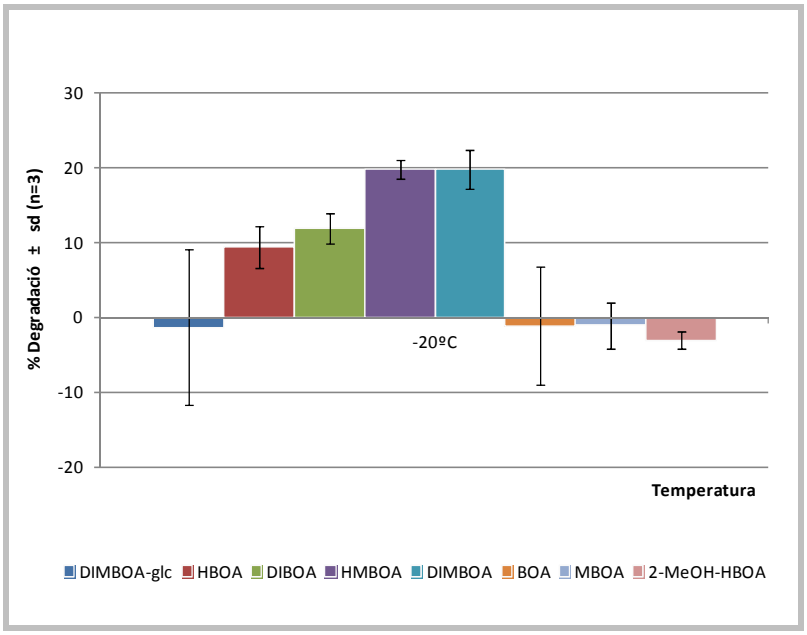
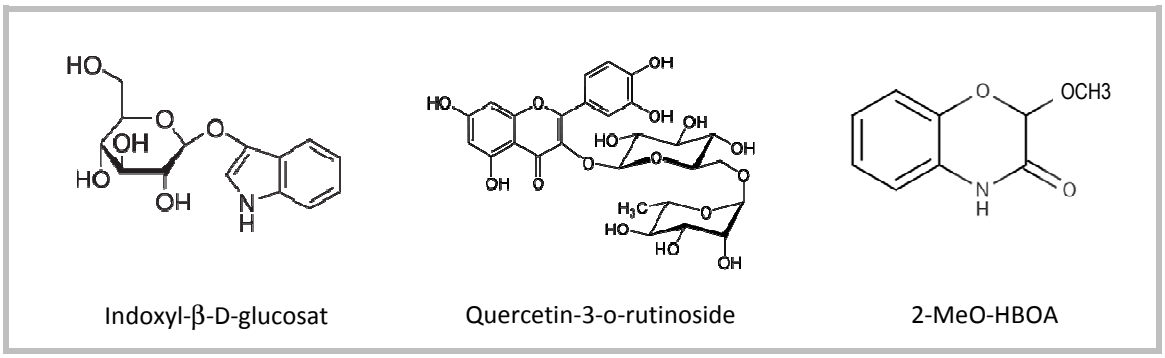


Figura II.3.- Degradació dels analits seleccionats als 7 dies a la temperatura de -20°C.

Com es pot comprovar, els resultats mostren que els compostos Dimboa-Glc, BOA i MBOA romanen estables, els analits HBOA i DIBOA pateixen una pèrdua del 10% i HMBOA i DIMBOA són els menys estables amb una degradació del 20%. Aquest fet ens va implicar haver de treballar de forma molt acurada preparant els patrons i les mostres just abans dela seva anàlisi i conservant-los sempre a -20^{o}C i mai passats 7 dies des de la seva preparació.

Si es vol avaluar l'eficàcia del procediment del tractament en cadascuna de les mostres, és recomanable treballar amb patrons interns (o surrogates). Aquests, són compostos amb característiques químiques similars als analits d'interès i són addicionats en una quantitat coneguda abans del procés de preparació de la mostra. Per tal que sigui viable, ha de tenir un comportament molt semblant als analits objectiu, una similitud en l'estructura química, recuperacions semblants i no ha d'estar present en les mostres a analitzar. Per tot això, el més ideal seria usar patrons marcats isotòpicament, però a la pràctica és difícil poder disposar d'aquests patrons tant per la seva disponibilitat comercial com pel seu elevat cost. En el nostre cas es van provar tres compostos amb característiques similars als analits d'interès: el 2-MeO-HBOA, l'Indoxyl-Glc i Quercetin-3-O-rutinoside, les estructures dels quals es mostren a la Figura II.4.



Indoxyl-β-D-glucosat | Quercetin-3-o-rutinoside | 2-MeO-HBOA

Figura II.4.- Estructures dels compostos químics testats com a patrons interns

II.3. PUBLICACIONS CIENTÍFIQUES

El treball referent a la metodologia analítica en plantes emmarcat en aquest capítol ha donat lloc a dues publicacions científiques. La primera tracta sobre el desenvolupament i validació del mètode d'anàlisi i es troba descrit a la secció II.3.1 del present capítol, amb el títol: *"Determination of Benzoxazinone Derivatives in Plants by Combining Pressurized Liquid Extraction-Solid Phase Extraction Followed by LiquidChromatography-Electrospray Mass Spectrometry*". Mentre que la publicació referent a l'estudi de l'efecte matriu es troba descrit a la secció II.3.2 del present capítol, amb el títol: "*Matrix effect in liquid chromatography–electrospray ionization mass spectrometry analysis of benzoxazinoid derivatives in plant material*".

II.3.1. Desenvolupament i validació d'una metodologia analítica en l'anàlisi de benzoxazinones i els seus derivats en el blat

Publicació científica #2

"Determination of Benzoxazinone Derivatives in Plants by Combining Pressurized Liquid Extraction-Solid-Phase Extraction Followed by Liquid Chromatography-Electrospray Mass Spectrometry"

Per:

Marta Villagrasa, MiriamGuillamón, EthelEljarrat i Damià Barceló

Revista:

Journal of AgriculturalandFoodChemistry

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 1001–1008 **1001**

Determination of Benzoxazinone Derivatives in Plants by Combining Pressurized Liquid Extraction–Solid-Phase Extraction Followed by Liquid Chromatography–Electrospray Mass Spectrometry

MARTA VILLAGRASA, MIRIAM GUILLAMÓN, ETHEL ELJARRAT,* AND DAMIÀ BARCELÓ

Department of Environmental Chemistry, IIQAB, CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain

A new analytical method based on the use of pressurized liquid extraction (PLE) followed by solid-phase extraction with LiChrolut RP C_{18} cartridges was evaluated for the sample preparation, extraction, and cleanup of eight naturally occurring benzoxazinone derivatives, 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one, 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, 2-hydroxy-1,4-benzoxazin-3-one, 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one, benzoxazolin-2-one, and 6-methoxybenzoxazolin-2-one in plant samples. Afterward, liquid chromatography–electrospray mass spectrometry, using the selected ion monitoring mode and internal standard (2-MeO-DIBOA, indoxyl-β-D-glucoside, and quercetin-3-*O*-rutinoside) quantification method was performed. This paper demonstrates the effectiveness of the PLE method, in conjunction with sensitive and specific mass spectrometric detection, for the quantitative recovery of compounds of the benzoxazinone class from plants. The recoveries of the analytes ranged from 66 to 110% with coefficients of variation ranging from 1 to 14%. This method gave detection limits between 1 and 27 μg/g. The method was applied to foliage and roots of three different wheat cultivars, and the analytes were detected in the range of 11–3261 μg/g of dry weight.

KEYWORDS: Benzoxazinone derivatives; wheat; pressurized liquid extraction; liquid chromatography; mass spectrometry

INTRODUCTION

Allelopathy has been defined as "any process involving secondary metabolites (allelochemicals) produced by plants, microorganisms, viruses, and fungi that influence the growth and development of agricultural and biological systems (excluding animals), including positive and negative effects" (*1*). In recent years there has been an increasing focus on the prospects of exploiting allelopathy as an alternative strategy for controlling especially weeds but also insects and diseases (*2, 3*). Weeds can be controlled either by growing a crop with the ability to exude allelochemicals or by incorporating plant residues with a high content of allelochemicals into the soil. Different chemical classes such as tannins, cyanogenic glycosides, several flavonoids, and phenolic acids present allelophatic activity. However, the chemicals identified as the most active allelophatic compounds in different crops such as wheat, rye, or maize are of the same chemical family, the benzoxazinones (*4*). The benzoxazinones include hydroxamic acids and lactams. Benzoxazolinones and methyl derivates of the hydroxamic acids have been described as well (*5*). Hydroxamic acids in wheat are found as β-glucosides (*6*). When plant tissues are damaged, β-glucosides are enzymatically hydrolyzed to their corresponding aglucones (*7*). The aglucones are converted to their corresponding benzoxazolinones 6-methoxybenzoxazolin-2-one (MBOA) and benzoxazolin-2-one (BOA) when heated in aqueous solutions (*8, 9*). When leached into soil, the aglucones are rapidly transformed to benzoxazolinones as well (*10, 11*). The benzoxazolinones are subjected to additional transformation in soil (*10–16*). The main structures of benzoxazinoids are shown in **Figure 1a**.

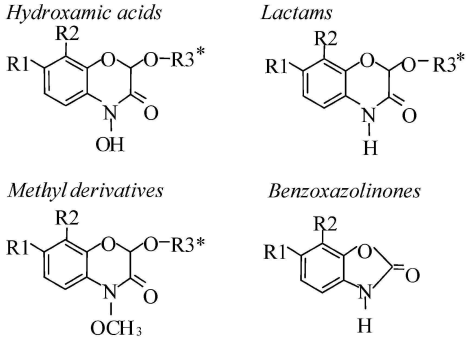
The qualitative and quantitative analysis of the different allelochemicals is of interest because discussions are taking place related to their possible use as substitutes for pesticides in crop protection. Analysis of allelochemicals is usually carried out by an extraction, followed by a cleanup step and, finally, instrumental analysis by liquid chromatography (LC). An overview of aspects such as sample preparation, extraction, purification, and final determination of benzoxazinones was recently published (*17*).

Recovery of metabolites from natural matrices is a complex process and is further complicated in the analysis of the chemical unstable benzoxazinones due to the potential for further conver-

* Corresponding author (telephone +34 93 400 6100; fax +34 93 204 59 04; e-mail eeeqam@cid.csic.es).

10.1021/jf050897p CCC: $33.50
Published on Web 01/24/2006

a)



* all glucosides are present in the (2R)-configuration

Hydroxamic acids

R1 = H	R2 = H	R3 = H	DIBOA
R1 = H	R2 = H	R3 = Glc	DIBOA-Glc
R1 = OCH_3	R2 = H	R3 = H	DIMBOA
R1 = OCH_3	R2 = H	R3 = Glc	DIMBOA-Glc
R1 = OCH_3	R2 = OCH	R3 = H	DIM_2BOA
R1 = OCH_3	R2 = OCH	R3 = Glc	DIM_2BOA-Glc

Lactams

R1 = H	R2 = H	R3 = H	HBOA
R1 = H	R2 = H	R3 = Glc	HBOA-Glc
R1 = OCH	R2 = H	R3 = H	HMBOA
R1 = OCH	R2 = H	R3 = Glc	HMBOA-Glc
R1 = OCH	R2 = OCH_3	R3 = H	HM_2BOA
R1 = OCH	R2 = OCH_3	R3 = Glc	HM_2BOA-Glc
R1 = OH	R2 = H	R3 = H	DHBOA
R1 = OH	R2 = H	R3 = Glc	DHBOA-Glc

Benzoxazolinones

R1 = H	R2 = H	BOA
R1 = OCH_3	R2 = H	MBOA
R1= OCH_3	R2 = OCH_3	M_2BOA

Methyl derivatives

R1 = OCH_3	R2 = H	R3 = H	HDMBOA
R1 = OCH_3	R2 = H	R3 = Glc	HDMBOA-Glc

b)

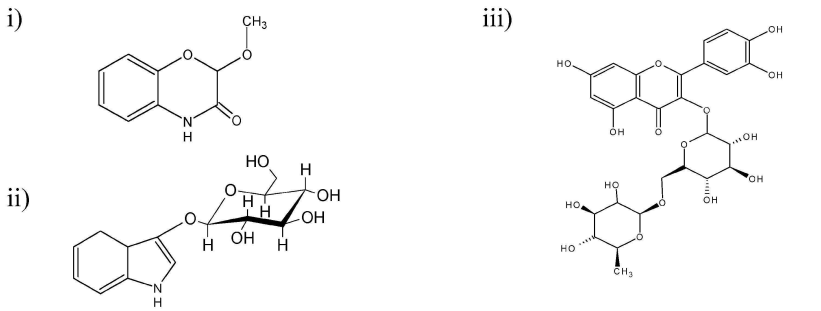


Figure 1. Structures of (**a**) benzoxazinone derivatives [adapted from Cambier et al. (*5*)] and (**b**) internal standards: (i) 2-MeOH-HBOA; (ii) indoxyl-β-D-glucoside; (iii) quercetin-3-*O*-rutinoside.

sion. Extraction methods using ultrasonication, centrifugation, boiling, and stirring in different solvents (H_2O, methanol, ethyl acetate) have been applied to the recovery of benzoxazinoids from plant and soil samples, with some procedures requiring up to 24 h (*18*−*20*). Pressurized liquid extraction (PLE), also called accelerated solvent extraction (ASE), has been demonstrated as an effective new method for sample extraction with low solvent and time requirements, particularly favorable features. Their uses have been reported for several studies in different environmental applications as well as in natural product determinations (*21*). However, the application of PLE to compounds of the benzoxazinone class has not yet been described.

During the 1980s and 1990s, several procedures were developed for the separation and quantification of cyclic hydroxamic acids and their degradation products in plant extracts using LC. Ultraviolet (UV) detection at 288, 280, 263, or 254 nm was most commonly used. The LC-UV method can lead to an erroneous determination of allelochemical contents in plant extracts because of possible coelution of several compounds, and it can be used only when pure reference compounds are available. To overcome the LC-UV limitations, the use of liquid chromatography coupled to mass spectrometry (LC-MS) or tandem MS (MS-MS) has clear advantages. LC-MSMS is better in terms of selectivity and sensitivity (*22*), but LC-MS is preferred for routine analyses.

The aim of this study was to develop a rapid and simple method for the determination of different allelochemicals, especially 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-β-D-glucoside), 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-β-D-glucoside), 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), 2-hydroxy-1,4-benzoxazin-3-one (HBOA), 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA), benzoxazolin-2-one, and 6-methoxybenzoxazolin-2-one in wheat samples. An analytical method based on PLE followed by a cleanup using LiChrolut RP C_{18} solid-phase extraction (SPE) cartridges was evaluated. The instrumental determination was performed by liquid chromatography coupled to mass spectrometry using electrospray ionization (LC-ESIMS) in selected ion mode (SIM). The use of non-naturally occurring compounds (2-MeO-DIBOA, indoxyl-β-D-glucoside, and quercetin-3-*O*-rutinoside) as internal standards was checked for a more reliable quantification. Finally, the developed method was applied to the analysis of wheat samples.

MATERIALS AND METHODS

Chemicals and Materials. The benzoxazinoid standards were obtained from commercial and private sources as available: DIBOA-β-D-glucoside and DIMBOA-β-D-glucoside from Prof. Dr. Hajime Iwamura (Kyoto University), Prof. Dr. Lisbeth Jonsson (Södertörn University College), and Dr. F. Macías (University of Cadiz); DIMBOA from Dr. Scott Chilton, University of North Carolina; HBOA, HMBOA, DIMBOA, MBOA, and BOA from Dr. F. Macías (University of Cadiz); DIBOA and the non-naturally occurring synthetic derivative 2-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (2-MeO-HBOA) from Dr. Sicker (University of Leipzig); indoxyl-β-D-glucoside and a natural flavonoid (quercetin-3-*O*-rutinoside) from Sigma-Aldrich

Stock individual standard solutions (1000 mg/L) were prepared by dissolving accurate amounts of pure standards in acidified methanol (1% HOAc). Working standard solutions containing all compounds except internal standards were obtained by further dilution of stock individual solutions with acidified methanol (1% HOAc), and their concentrations were 1, 5, 50, and 500 mg/L. Working standard solutions of internal standards (100 mg/L) were also prepared by further dilution of stock solution.

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

In a previous study, the stability of selected compounds was checked using different acidified standard solutions stored at different temperatures (−20, 4, and 20 °C) (*23*). To stabilize the compounds, sample preparation, extraction, and analysis were performed in acidified media (1% HOAc). The standard solutions and extracts of sample were stored at −20 °C.

Sample Collection. Three different wheat varieties, Astron (As), Ritmo (Ri), and Stakado (St), were grown in Lleida (Spain) in conventional cultivation conditions. Ten plants were collected at the Zadocks stages of 10 and 12 days, corresponding to two different stages. The stages were defined by the BBCH scale (a system for a uniform coding of phenologically similar growth stages of all mono- and dicotyledonous plant species). The same day roots were washed under a running water stream. Immediately the samples were frozen and stored at −20 °C until further manipulation.

Sample Preparation Procedures. Plant material involves a complex matrix and, therefore, it was necessary to perform a preliminary study to evaluate the recoveries of the benzoxazinone derivatives in each step of the sample preparation. This study was performed by spiking with target analytes in the absence of plant material.

Lyophilization. The water in the wheat samples was removed by lyophilization until weight loss was no longer observed. Samples of 10 plants were divided into roots and foliage; roots were cut finely, whereas foliage was ground with a pestle in a mortar. A 0.1 g subsample was taken for the extraction.

To verify the stability and recovery of the compounds under the lyophilization step, a standard mixture of all target analytes including internal standards was lyophilized for 15 h. The dried sample was then reconstituted in acidified MeOH/H_2O/H^+ (60:40:1) and analyzed.

PLE. Samples were extracted by PLE using an ASE 200 (Dionex, Idstein, Germany) apparatus, equipped with 11 mL of stainless steel extraction cells. Diatomaceous earth was used to fill the extraction cells, with the matrix and sample thoroughly mixed to ensure good dispersion of the sample. The diatomaceous earth was cleaned by ultrasonication with the same solvent of the extraction and dried at 70 °C prior to use. Default conditions of pressure (1500 psi), static times (three cycles), and cell purge (60 s) were used. Flush volume from 60 to 100%, solvent composition from 100% acidified MeOH (1% HOAc) to 60% acidified MeOH (1% HOAc), and temperatures from 50 to 150 °C were optimized.

The stability of the benzoxazinones and internal standards under the PLE process was investigated. The cells were fully filled with the diatomaceous earth and spiked with a standard mixture of all target analytes including internal standards and extracted by PLE under different conditions.

Extract Purification. In the raw extracts of plants, the broad variety of substances (salts, lipids, glycosides, phosphates, peptides, macromolecules, chlorophyll) can influence the quantification. This fact was neglected in various previously published studies in which the raw extracts were analyzed directly. However, Baumeler et al. (*24*) observed that some peaks (according to their UV spectra probably flavonoids or flavonoid glycosides) eluted with identical retention times as the benzoxazinones. Thus, a purification of the samples prior to instrumental analysis is recommended. A simple prepurification procedure consists of the use of a C_{18} SPE cartridge. The use of this cleanup step has been shown to improve quantification significantly, and this procedure was thus used as the basis for the purification step investigations performed here.

The organic extracts were concentrated to dryness by rotary evaporation and redissolved in 2 mL of acidified water (1% HOAc) prior to the cleanup step. Due to the reconstituted extracts in acidified H_2O resulting in samples in a turbid solution, a filtration step prior to purification was required to prevent clogging of the cartridge. Filtration was performed using 1 μm 25 mm syringe-driven filter units.

The stability of the compounds under this concentration step was verified by dilution of standard solutions in acidified MeOH (1% HOAc); this solution was concentrated to dryness by rotaevaporation and reconstituted in 2 mL of acidified H_2O. Purification was performed via LiChrolut RP C_{18} SPE cartridges activated and preconditioned with 5 mL of acidified MeOH (1% HOAc) followed by 5 mL of acidified H_2O (1% HOAc). The purification step was then applied to the concentrated and filtered aqueous extract. A two-step elution procedure was used: first, 6 mL of acidified H_2O (1% HOAc) and, second, 5 mL of acidified MeOH/H_2O (1% HOAc) at different proportions (0:100, 20:80, 40:60, 50:50, 60:40, 80:20, 100:0) were tested.

Whole Process. After the stability of the compounds in each step of the sample preparation had been verified, the whole process was applied. First of all, recoveries of the whole process were calculated by spiking the diatomaceous earth with 100 μL of a working standard solution of 5 ng/μL and 50 μL of internal standards. After that, the recoveries were evaluated by spiking in the same way the plant material (foliage and root). A blank of the sample (foliage and root) was performed to subtract the natural content of the different compounds in plant material.

INSTRUMENTAL ANALYSIS

Chromatographic Conditions. Analyses were performed on a HP 1100 liquid chromatograph. A Synergi Max-RP 80A (C-12 TMS) LC column (250 × 4.6 mm Phenomenex) with a solvent flow rate of 1 mL/min was used. The sample injection volume was set to 50 μL. Acidified H_2O (0.05% HOAc) and MeOH were used as the elution solvents A and B, respectively. The solvent gradient adopted was as follows: 0−2 min, 100−70% A; 2−19 min, 70−40% A; 19−21 min, 40−5% A; 21−23 min, 5−5% A; 23−25 min, 5−70% A; 25−30 min,

Table 1. Retention Time and *m/z* Ion Selected for Quantification and Confirmation of Each Selected Compound

compound	retention time (min)	quantification ion (*m/z*)	confirmation ion (*m/z*)	LOD_{inst} (ng/μL)
indoxyl-β-D-glucoside[a]	9.1	294	131	
DIBOA-β-D-glucoside	9.9	134	342	0.006
DIMBOA-β-D-glucoside	11.0	164	372	0.003
HBOA	11.8	164	108	0.005
DIBOA	12.3	134	78	0.003
HMBOA	12.7	194		0.010
DIMBOA	13.6	164	149	0.003
BOA	16.1	134		0.002
quercetin-3-*O*-rutinoside[a]	16.5	609	300	
MBOA	17.2	164	149	0.002
2-MeOH-HBOA[a]	18.8	178		

[a] Internal standard.

70−100% A. Total run time was 35 min with the benzoxazinone derivatives eluted over 8−20 min, and the final 15 min was used for column cleaning and regeneration (*22*).

Mass Spectrometry Conditions. MS analyses were carried out in a LC-MSD HP 1100 mass selective detector equipped with an atmospheric pressure ionization source. The optimization of MS conditions was carried out using flow injection analysis (FIA) for each analyte at 25 ng/μL, using acidified water/methanol (60:40) (1% HOAc) as carrier solvent. The optimum conditions were selected by the evaluation of the area and fragmentation of each analytes in scan mode. The APCI and ESI ionization modes in negative mode were tested for all compounds, and in both cases, different MS parameters were optimized for all of the studied compounds to obtain structural information and to achieve maximum sensitivity: nebulizer pressure (from 50 to 60 psi), gas temperature (from 250 to 350 °C), capillary voltage (from 3000 to 4000 V), and fragmentor (from 70 to 250 V). Two ions for each analyte were selected, according to specificity and sensitivity, with the primary ions used for quantification and the secondary ion providing confirmation (**Table 1**).

Quantification. In this study, quantification by internal standards was tested. The use of internal standards to aid reliable quantification has not been described for the quantification of benzoxazinones. The only quantitative method describing the use of an internal standard for the analysis of these compounds used the naturally occurring degradation product BOA as internal standard (*25*). As this product can potentially occur in samples it cannot be considered as an appropriate internal standard. Different internal standards were tested in the current study: a non-naturally occurring structural analogue of HBOA (2-MeO-HBOA) benzoxazinone as internal standards for aglycones and benzoxazolinones, a natural flavonoid (quercetin-3-*O*-rutinoside, and indoxyl-β-D-glucoside for glucosides (**Figure 1b**).

Method Validation. After the optimum conditions for each step had been obtained with spiked experiments, the whole method was also validated with standards in the absence of samples (methanol solution) and afterward in foliage and roots. To evaluate the different quality parameters, the method was carried out in triplicate. The quality parameters evaluated were linearity, recoveries, reproducibility, and sensitivity.

RESULTS AND DISCUSSION

The procedure adopted for the sample preparation involved lyophilization, extraction, concentration, filtration, and SPE purification. Recoveries of the benzoxazinones at each step of the sample preparation procedure were determined by spiking experiments with standard solutions. Results for lyophilization, concentration, and filtration are presented in **Table 2**. Results for the extraction and purification are explained in the following subsections.

The compounds were quantitatively recovered following lyophilization of the standard solutions, ranging from 81 to 103% with standard deviations between 1 and 11%, except DIMBOA and HMBOA with higher deviations of 25 and 20%, respectively, probably due to their instability (*23*). The recoveries were also acceptable for the concentration and filtration steps, ranging from 83 to 102% and from 65 to 111%, respectively, with acceptable deviation ranging from 2 to 6% for the concentration and from 1 to 8% for the filtration step.

Table 2. Recoveries and Relative Standard Deviations ($n = 3$) of Benzoxazinones during Lyophilization, Concentration, and Filtration Steps

	lyophilization		concentration		filtration	
compound	recovery	RSD (%)	recovery	RSD (%)	recovery	RSD (%)
DIBOA-β-D-glucoside	81	6	102	2	99	7
DIMBOA-β-D-glucoside	105	5	98	2	69	5
HBOA	103	1	88	2	110	7
DIBOA	99	8	99	3	111	5
HMBOA	99	20	95	4	100	7
DIMBOA	103	25	99	3	65	8
BOA	85	11	96	6	76	2
MBOA	98	9	100	4	76	1

PLE Extraction. To determine the amenability of the benzoxazinone derivatives to the high-temperature and -pressure conditions of the PLE process, control experiments were performed with standard solutions in the absence of matrix.

An increase in extraction temperature, assuming that the stability of the compounds is not compromised, is known to improve extraction efficiencies. Improved recoveries of heteroaromatic analytes from soil matrices with increased extraction temperature have been described for supercritical fluid extraction (SFE) conducted at constant pressure (*26*). The effect of extraction temperature in PLE analyses was studied here, with experiments at 50, 70, 100, and 150 °C. These experiments were carried out using 60% of flux and MeOH (1% HOAc) as a solvent extractor. Results for DIMBOA-β-D-glucoside and DIMBOA are shown in **Figure 2**. In general, a significant improvement in extraction efficiency of the glucoside compound was achieved by raising the extraction temperature, and similar results were obtained for the corresponding aglucone. However, no explanation was found for the decrease of the recoveries from 50 to 70 °C.

The extraction solvent was also studied. Experiments were carried out with an acidified mixture of MeOH/H_2O (60:40) (1% HOAc) and an acidified MeOH (1% HOAc) solvent. The results obtained using both solvents were similar or slightly better with MeOH (1% HOAc), except for DIMBOA, for which the use of acidified MeOH (1% HOAc) yielded lower recoveries (**Figure 3a**). However, due to the long time required for rotary evaporation of the acidified mixture MeOH/H_2O (1% HOAc) solvent, the selected solvent extractor was acidified MeOH (1% HOAc).

The influence of the solvent used to rinse the cell after the static extraction step (flush percentage) was also studied, modifying its value between 60 and 100%. This experiment was carried out at 150 °C and used MeOH (1% HOAc) as a solvent extractor. In this case the low percentage of flush (60%) provided the best results (**Figure 3b**).

After the study of the different PLE parameters, the optimal extraction conditions for benzoxazinone extraction in spiked hydromatrix were as follow: pressure, 1500 psi; flush volume, 60%; cell purge, 60 s; solvent extractor, MeOH (1% HOAc); temperature, 150 °C; three static cycles of 5 min and no N_2 purge during the cell preheat. The recoveries under the extraction step ranged from 70 to 126% for all compounds except for the

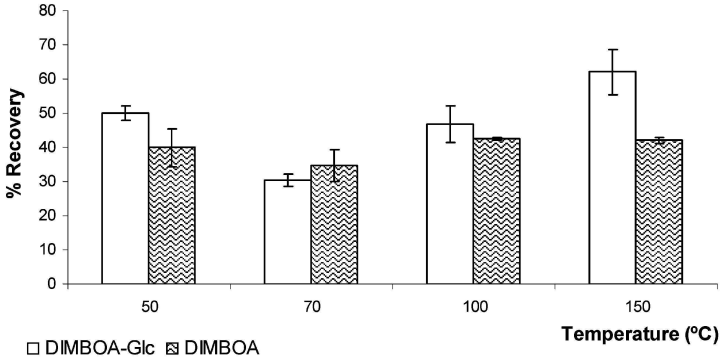


Figure 2. Recoveries and coefficient of variation (n = 3) of DIMBOA-β-D-glucoside and DIMBOA at different temperatures tested during PLE.

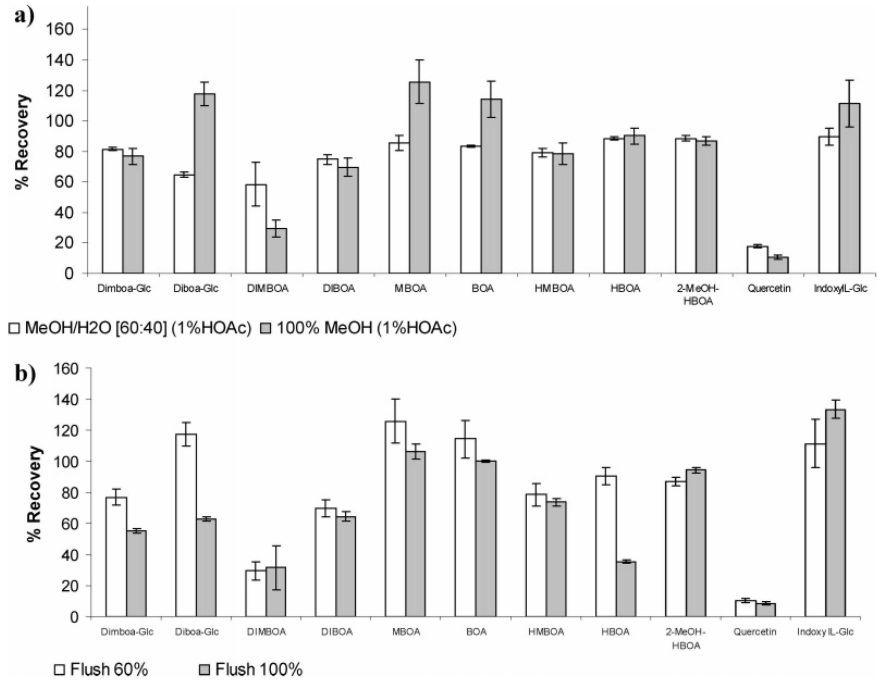


Figure 3. Recoveries and coefficient of variation (n = 3) of target compounds after PLE with different (**a**) solvent extractors and (**b**) flush (%).

most unstable benzoxazinone derivative (DIMBOA) and internal standard quercetin-3-*O*-rutinoside with lower recoveries of 29 and 11%, respectively (**Table 3**), whereas the standard deviation ranged from 3 to 19%. For this reason, quercetin-3-*O*-rutionside was discarded as surrogate.

SPE Purification. The cleanup method proposed by Baumeler et al. (*24*) was used as the basis for the purification step investigations performed here. However, modifications were found to be necessary to obtain complete recovery of the benzoxazinone derivatives. In the cited publication, 10% EtOH was used for analyte elution. The target glucosidal analytes may have been completely recovered under these conditions due to the higher polarities; however, no information was provided to confirm this, nor were recoveries of less polar compounds described.

The standard spiking solutions containing all compounds in acidified MeOH (1% HOAc) were concentrated to dryness and reconstituted in 2 mL of acidified H_2O. The solution was then filtered and applied to the preconditioned LiChrolut RP C_{18} cartridges. Fraction 1 (6 mL of acidified H_2O) used for sample transferral and washing of the filter and cartridges was applied. The eluted fractions (fraction 2) using different composition solvents were carried out. Quantitative analysis showed that a minimum of 60% MeOH was required for complete elution of the compounds. The recoveries obtained for each of eight target analytes and three internal standards from the SPE cartridges are shown in **Table 4**, using 6 mL of acidified H_2O (1% HOAc) as eluent of fraction 1 and 5 mL of acidified MeOH/H_2O (60:40) (1% HOAc) as eluent of fraction 2. Naturally occurring DIMBOA-β-D-glucoside, HMBOA, DIMBOA, BOA, MBOA, and synthetic derivative 2-MeOH-HBOA were quantitatively recovered (recovery up to 60%), showing standard deviations between 2 and 10%. However, analysis of the first fraction obtained from the loading and washing of the cartridge with acidified H_2O (1% HOAc) (fraction 1) showed partial elution of the more polar benzoxazinone derivatives (DIBOA-β-D-glucoside, DIMBOA-β-D-glucoside, HBOA, and DIBOA), indicating that this fraction could not be discarded. For these

Table 3. Recoveries and Relative Standard Deviations ($n = 3$) of Benzoxazinones under the Extraction Process

	PLE extraction	
compound	recovery	RSD (%)
indoxyl-β-D-glucoside[a]	111	14
DIBOA-β-D-glucoside	117	6
DIMBOA-β-D-glucoside	77	7
HBOA	90	6
DIBOA	70	8
HMBOA	78	9
DIMBOA	29	19
BOA	114	11
quercetin-3-*O*-rutinoside[a]	11	10
MBOA	126	11
2-MeOH-HBOA[a]	87	3

[a] Internal standard.

Table 4. Recoveries and Relative Standard Deviation ($n = 3$) of Benzoxazinones under the Purification Step

	fraction 1, 6 mL of H_2O (1% HOAc)		fraction 2, 5 mL of MeOH/H_2O (1% HOAc) (60:40)	
compound	recovery	RSD (%)	recovery	RSD (%)
indoxyl-β-D-glucoside[a]			35	16
DIBOA-β-D-glucoside	60	7	30	2
DIMBOA-β-D-glucoside	40	11	52	3
HBOA	56	8	24	5
DIBOA	69	6	37	5
HMBOA			97	1
DIMBOA			99	3
BOA			96	2
quercetin-3-*O*-rutinoside[a]			25	5
MBOA			94	2
2-MeOH-HBOA[a]			67	4

[a] Internal standard.

analytes, fractions 1 and 2 were mixed, and recoveries ranged from 80 to 106%. Indoxyl-β-D-glucoside with lower recovery (35%) was not a good surrogate for the quantification of glucoside derivatives in fraction 2. It will be a good internal standard for the quantification of more polar compounds.

Mass Spectrometry Conditions. Two different ionization techniques were tested for the determination of benzoxazinone derivatives, APCI and ESI. For all analytes, a significant improvement of sensitivity was observed working in ESI mode (~1 order of magnitude). Moreover, it should be pointed out that glucoside compounds (DIBOA-β-D-glucoside and DIMBOA-β-D-glucoside) were not detected with APCI, and then ESI conditions are mandatory for the determination of these analytes. Once the ionization technique was selected, different parameters were optimized. The optimized ESIMS method was operated in negative ion mode with the following instrument settings: nebulizer pressure, 5 V; gas temperature, 350 °C, capillary voltage, 3500 V; fragmentor, 0−15 min, 250 V, and 15−35 min, 70 V. Under the developed methodology, the instrumental detection limits (LOD_{inst}) were in the range between 0.002 and 0.010 ng/μL (**Table 1**).

Quantification. In this study, quantification by internal standard was carried out. The non-naturally occurring structural analogue of HBOA (2-MeOH-HBOA) seems to be the only appropriate internal standard for quantification of six allelochemicals (HBOA, DIBOA, HMBOA, DIMBOA, and BOA) and indoxyl-β-D-glucoside for the glucoside compounds (DIBOA-glucoside and DIMBOA-glucoside).

Method Validation. In quantitative analysis using LC-ESIMS one of the major problems is the suppression or, less frequently, the enhancement of the analyte signals in the presence of matrix components (*27*). To solve the matrix effect due to the complex matrix of plant material, the foliage and root extracts were diluted to 1:10 and 1:5, respectively, before the LC-MS analysis (*28*).

Quality assurance of the developed method was evaluated by measuring parameters such as linearity, sensitivity, recoveries, and reproducibility. The quality parameters of the whole methods are shown in **Table 5**.

Linearity. Calibration curves were determined for all benzoxazinones by LC-ESIMS. The linear calibration range used was from 0.01 to 2 ng/μL. Good correlations were obtained within the interval studied. The correlation coefficients ranged between 0.9994 and 0.9999.

Recoveries and Reproducibility. The recoveries (percent of standard added to sample recovered during whole process) and reproducibilities (relative standard deviation for triplicate analysis) of the benzoxazinone derivatives during the PLE-SPE and LC-ESIMS were determined by the analysis of spiked samples. To obtain the stability of the benzoxazinone derivatives during the whole process, the method was first validated by adding a mixture of standard to hydromatrix in the absence of plant material (MeOH solution), and afterward the method was applied, spiking with standard solution to the foliage and root of the wheat plant. The recoveries of the most polar compounds (DIBOA-Glc, DIMBOA-Glc, HBOA, and DIBOA) were determined by the analysis of fractions 1 and 2, whereas the recoveries of the rest of compounds (HMBOA, DIMBOA, BOA, and MBOA) were determined in fraction 2. The recoveries obtained with spiked experiments in methanol, foliage, and roots, ranged from 67 to 110%, from 61 to 108%, and from 87 to 107%, respectively. Only in the case of the aglucone DIMBOA

Table 5. Quality Parameters of the Developed Methodology (PLE-SPE and LC-ESI-MS)

		DIBOA-Glc[a]	DIMBOA-Glc[a]	HBOA[a]	DIBOA[a]	HMBOA	DIMBOA	BOA	MBOA
% recovery	MeOH solution	88	67	80	92	66	37	73	110
	foliage	90	90	80	83	61	22	103	108
	root	110	90	97	105	83	27	87	97
reproducibility	MeOH solution	10	9	2	3	3	4	6	5
(%RSD)	foliage	1	3	6	5	3	9	1	6
	root	6	12	2	5	6	14	5	5
sensitivity LOD	foliage	11	11	9	8	10	8	1	1
(μg/g)	root	6	14	27	2	3	6	2	1
sensitivity LOQ	foliage	36	37	31	27	34	27	3	5
(μg/g)	root	21	47	89	8	9	4	5	3

[a] Analysis of fractions 1 and 2.

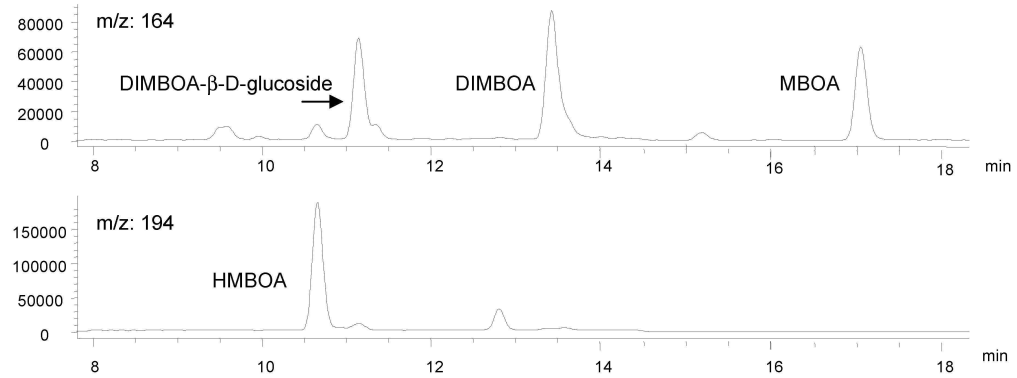


Figure 4. LC-MSMS analysis of root samples (Stakado variety).

Table 6. Levels (Micrograms per Gram of Dry Weight) of Benzoxazinone Derivatives in Wheat Foliage and Root Samples

	DIBOA-Glc	DIMBOA-Glc	HBOA	DIBOA	HMBOA	DIMBOA	BOA	MBOA
				First Stage				
foliage								
Astron	<11	<11	<9	<8	270	833	<1	552
Ritmo	<11	<11	<9	<8	489	1775	<1	942
Stakado	<11	<11	<9	<8	326	3261	<1	695
root								
Astron	46	155	<27	<2	59	303	11	476
Ritmo	<6	318	<27	<2	34	124	<2	290
Stakado	27	329	<27	<2	83	860	<2	420
				Second Stage				
foliage								
Astron	<11	<11	177	66	56	<8	<1	30
Ritmo	<11	<11	<9	<8	161	58	<1	94
Stakado	<11	<11	<9	8–27	126	51	<1	91
root								
Astron	<6	14–47	<27	<2	3–9	62	<2	34
Ritmo	6–11	134	<27	<2	3–9	57	<2	57
Stakado	<6	<11	<27	<2	3–9	55	<2	103

was the recovery obtained lower than 40% in all cases, due to its instability (*23*). The relative standard deviation between three values was always below 15%, indicating good reproducibility.

Sensitivity. The sensitivity was evaluated by determining the method detection limits (LOD_{met}). The detection limit of the method is defined as the minimum amount of analyte that can be detected with reasonable certainty for a given analytical procedure. The LOD_{met} was calculated for each compound by a signal-to-noise ratio of 3 in spiked samples. The methodology applied to foliage produced LOD_{met} values in the range between 1 and 11 μg/g, and the methodology applied to the root produced LOD_{met} values in the range between 1 and 27 μg/g. The method quantification limits (LOQ_{met}) were also determined. The LOQ_{met} were defined as the lowest concentration of a residue that can be identified and quantitatively measured in samples using a developed methodology. LOQ_{met} values were based on the peak to peak noise of the baseline near the analyte peak obtained by analysis of spiked samples and on a minimal value of signal-to-noise ratio of 10. The applied methodology to foliage provided a LOQ_{met} in the range between 3 and 37 μg/g, and the applied methodology to root provides a LOQ_{met} in the range between 3 and 89 μg/g.

The method offers significant improvements to valuable reductions in sample preparation and analysis time and also improvements to the quality of qualitative and quantitative data obtained.

Analysis of Real Samples. The developed method PLE-SPE and LC-ESIMS was applied to the analysis of wheat samples and tries to assess the content of benzoxazinone derivatives in foliage and root of three wheat varieties (Astron, Ritmo, and Stakado) in two growing stages. All compounds were identified by matching the retention time and mass spectrum with authentic standards. Quantification by internal standard was carried out, and the results were corrected by recovery.

Figure 4 shows an example of LC-MS analysis of root sample (Stakado variety), with the detection of DIMBOA-Glc, DIMBOA, HMBOA, and MBOA.

The levels of benzoxazinones in wheat foliage and root samples are presented in **Table 6**. In the first stage, the total content of allelochemicals ranged from 1655 to 4282 μg/g of dry weight in foliage and from 766 to 1050 μg/g of dry weight in root. In the second stage the levels ranged from 268 to 329 μg/g of dry weight in foliage and from 96 to 248 μg/g of dry weight in roots. The quantities obtained in wheat foliage samples showed higher levels for the Stakado variety, followed by Ritmo > Astron. With regard to the wheat root samples, Stakado was also the variety with higher levels, followed by Ritmo > Astron. The major metabolites detected in wheat foliage were DIMBOA, MBOA, and HMBOA. In root extracts, the major metabolites were also DIMBOA and MBOA, and there was the presence of DIMBOA-β-D-glucoside in all cases. In contrast, HBOA and DIBOA were detected only in two foliage extracts, and BOA was only quantified in one root extract, whereas DIBOA-β-D-glucoside was detected in the root extracts of the three varieties in the first stage. These results were consistent with those presented by Cambier et al. (*5*) in foliage and roots of maize

following different treatments prior to extraction. Their findings, using a treatment similar to that used in this study, showed that MBOA and HMBOA were the major analytes detected, whereas DIBOA and BOA were not found.

ACKNOWLEDGMENT

We thank Prof. D. Sicker (University of Leipzig), Prof. S. Chilton (University of North Carolina), Dr. F. Macías (University of Cadiz), Prof. Dr. Hajime Iwamura (Kyoto University), and Prof. Dr. Lisbeth Jonsson (Södertörn University College) for kindly providing standards, and Dr. V. Cambier (Université Catholique de Louvain) and Dr. C. Werner (University of Zurich) for helpful discussions. We greatly appreciate the generous gift of the Phenomenex Synergi MAX-RP HPLC column from Daniel Lopez (JASCO Analytica, Spain) and technical assistance provided by D. Fanjul and R. Chaler (IIQAB-CSIC). Finally, we thank Merck for supplying the SPE cartridges.

LITERATURE CITED

(1) Torres, A.; Oliva, R. M.; Castellano, D.; Cross, P. Introduction. A science for the future. *Proceedings of the First World Congress on Allelopathy*, Cadiz, Spain, Sept 16−20, 1996; p 7.

(2) Einhellig, F. Allelopathy: current status and future goals. In *Allelopathy. Organisms, Processes and Applications*; Inderjit, Dakshini, K. M. M., Einhellig, F., Eds.; American Chemical Society: Washington, DC, 1995; pp 1−24.

(3) Rizvi, S. J. H.; Hauqe, H.; Singh, V. K.; Rizvi, V. In *Allelopaty. Basic and Applied Aspects*; Rizvi, S. J. H., Rizvi, V., Eds.; Chapman and Hall: London, U.K., 1992; pp 1−8.

(4) Wu, H.; Pratley, J.; Lemerle, D.; Haig, T. Crop cultivars with allelopathic capability. *Weed Res.* **1999**, *39*, 171−180.

(5) Cambier, V.; Hance, T.; de Hoffmann, E. Non-injured maize contains several 1,4-benzoxazin-3-one related compounds but only as glucoconjugates. *Phytochem. Anal.* **1999**, *10*, 119−126.

(6) Wahlroos, O.; Virtanen, A. I. The precursors of 6-methoxybenzoxazolinone in maize and wheat plants, their isolation and some of their properties. *Acta Chem. Scand.* **1959**, *13*, 1096−1908.

(7) Hofman, J.; Hofmanova, O. 1,4-Benzoxazine derivatives in plants: absence of 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one from injured *Zea mays* plants. *Phytochemistry* **1971**, *10*, 1441−1444.

(8) Virtanen, A. I.; Wahlroos, O. Absence of 6-methoxybenzoxazolinone in uninjured maize tissue. *J. Pharm. Sci.* **1963**, *52*, 713−714.

(9) Bredenberg, J. B.; Honkanen, E.; Virtanen, A. I. The kinetics and mechanism of decomposition of 2,4-dihydroxy-1,4-benzoxazin-3-one. *Acta Chem. Scand.* **1962**, *16*, 135−141.

(10) Macias, F. A.; Oliveros-Bastidas, A.; Marin, D.; Castellano, D.; Simonet, A. M.; Molinillo, J. M. G. Degradation studies on benzoxazinoids. Soil degradation dynamics of 2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and its degradation products, phytotoxic allelochemicals from Gramineae. *J. Agric. Food Chem.* **2004**, *52*, 6402−6413.

(11) Macias, F. A.; Oliveros-Bastidas, A.; Marin, D.; Castellano, D.; Simonet, A. M.; Molinillo, J. M. G. Degradation studies on benzoxazinoids. Soil degradation dynamics of (2*R*)-2-*O*-β-D-glucopyranosyl-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA-Glc) and its degradation products, phytotoxic allelochemicals from Gramineae. *J. Agric. Food Chem.* **2005**, *53*, 554−561.

(12) Fomsgaard, I. S.; Mortensen, A. G.; Carlsen, S. C. K. Microbial transformation products of benzoxazolinone and benzoxazinone allelochemicals—a review. *Chemosphere* **2004**, *54*, 1025−1038.

(13) Fomsgaard, I. S.; Mortensen, A. G.; Idinger, J.; Coja, T.; Blümel, S. Transformation of benzoxazinones and derivatives and microbial activity in the test environment of soil ecotoxicological tests on *Poecilus cupreus* and *Folsomia candida*. *J. Agric. Food Chem.* **2006**, *54*, 1086−1092.

(14) Gents, M. B.; Nielsen, S. T.; Mortensen, A. G.; Christophersen, C.; Fomsgaard, I. S. Transformation products of 2-benzoxazolinone (BOA) in soil. *Chemosphere* **2005**, *61*, 74−84.

(15) Krogh, S.; Menz, S. J. M.; Nielsen, S. T.; Mortensen, A. G.; Christoffersen, C.; Fomsgaard, I. S. Fate of benzoxazinone allelochemicals in soil after incorporation of wheat and rye sprotus. *J. Agric. Food Chem.* **2006**, *54*, 1064−1074.

(16) Understup, A. G.; Ravnskov, S.; Hansen, H. C. B.; Fomsgaard, I. S. Biotransformation of 2-benzoxazolinone to 2-amino-phenoxazin-3-one and 2-acetylamino-phenoxazin-3-one in soil. *J. Chem. Ecol.* **2005**, *31*, 1205−1222.

(17) Eljarrat, E.; Barceló, D. Sample handling and analysis of allelochemical compounds in plants. *Trends Anal. Chem.* **2001**, *20*, 584−590.

(18) Gagliardo, R. W.; Chilton, W. S. Soil transformation of 2(3*H*)-benzoxazolone of rye into phytotoxic 2-amino-3*H*-phenoxazin-3-one. *J. Chem. Ecol.* **1992**, *18*, 1683−1691.

(19) Mwaja, V. N.; Masiunas, J. B.; Weston, L. A. Effects of fertility on biomass, phytotoxicity, and allelochemical content of cereal rye. *J. Chem. Ecol.* **1995**, *21*, 81−96.

(20) Petho, M. Occurrence and physiological role of benzoxazinones and their derivatives: II. Decomposition of 7-methoxybenzoxazinone and change in its physiological activity. *Acta Agron. Hung.* **1992**, *41*, 167−175.

(21) Kaufmann, B.; Christen, P. Recent extraction techniques for natural products: microwave-assisted extraction and pressurized solvent extraction. *Phytochem. Anal.* **2002**, *13*, 105−113.

(22) Bonnington, L.; Eljarrat, E.; Guillamon, M.; Eichhorn, P.; Taberner, A.; Barcelo, D. Development of a liquid chromatography−electrospray-tandem mass spectrometry method for the quantitative determination of benzoxazinone derivatives in plants. *Anal. Chem.* **2003**, *75*, 3128−3136.

(23) Eljarrat, E.; Guillamon, M.; Seuma, J.; Mogensen, B. B.; Fomsgaard, I. S.; Oliveros-Bastidas, A.; Macías, F.; Stochmal, A.; Oleszek, W.; Shakaliene, O.; Barcelo, D. First European interlaboratory study on benzoxazinone derivatives in plants: phase I. *J. Chromatogr. A* **2004**, *1047*, 69−76.

(24) Baumeler, A.; Hesse, M.; Werner, C. Benzoxazinoids—cyclic hydroxamic acids, lactams and their corresponding glucosides in the genus *Aphelandra* (Acanthaceae). *Phytochemistry* **2000**, *53*, 213−222.

(25) Cambier, V.; Hance, T.; de Hoffmann, E. Variation of DIMBOA and related compounds content in relation to the age and plant organ in maize. *Phytochemistry* **2000**, *53*, 223−229.

(26) Camel, V.; Tambute, A.; Caude, M. Influence of ageing on the supercritical fluid extraction of pollutants in soils. **1995**, *693*, 101−111.

(27) Stüber, M.; Reemtsma, T. Evaluation of three calibration methods to compensate matrix effects in environmental analysis with LC-ESI-MS. *Anal. Bioanal. Chem.* **2003**, *378*, 910−916.

(28) Villagrasa, M.; Guillamon, M.; Eljarrat, E.; Barcelo, D. Matrix effect in liquid chromatography-electrospray ionization mass spectrometry analysis of benzoxazinone derivatives in plant material. In preparation.

Received for review April 19, 2005. Revised manuscript received July 25, 2005. Accepted December 6, 2005. This work was financially supported by the program "Quality of life and management of living resources (1998 to 2002)" of the European Union, FATEALLCHEM Contract QLK5-CT-2001-01967. Funding provided to IIQAB-CSIC (AGL2001-4952-E) by the Ministerio de Ciencia y Tecnologia is gratefully acknowledged.

JF050897P

II.3.2. Avaluació de l'efecte matriu en l'anàlisi de benzoxazinones

Publicació científica #3

"*Matrixeffect in liquid chromatography–electrospray ionization mass spectrometry analysis of benzoxazinoid derivatives in plant material*".

Per:

Marta Villagrasa, EthelEljarrat i Damià Barceló

Revista:

Journal of Chromatography A

Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography A, 1157 (2007) 108–114
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Matrix effect in liquid chromatography–electrospray ionization mass spectrometry analysis of benzoxazinoid derivatives in plant material

M. Villagrasa, M. Guillamón, E. Eljarrat *, D. Barceló

Department of Environmental Chemistry, IIQAB-CSIC, c/Jordi Girona 18-26, 08034 Barcelona, Spain

Received 15 February 2007; received in revised form 8 April 2007; accepted 19 April 2007
Available online 21 April 2007

Abstract

Despite the increasing success of liquid chromatography (LC) coupled to mass spectrometry (MS), matrix effects have limited the ESI applications. Matrix effects result from co-eluting residual matrix components affecting the ionization efficiency of target analytes and can lead to erroneous results. The present work evaluates the LC–electrospray ionization (ESI)-MS behaviour of benzoxazinoid derivatives in wheat plant material as a case where endogenous matrix components were found to interfere with the ionization of target analytes. When LC–MS was applied to the analysis of foliage and root extracts, significant signal suppressions were observed. To compensate for this matrix signal suppression, different methods were applied: the use of internal standards, the application of standard dilution method and the dilution of the extracts before instrumental determination. The results obtained indicated that evaluation of matrix effects should become an integrated part of quantitative LC–ESI-MS method development and validation.

Keywords: Allelochemicals; Benzoxazinoid derivatives; Benzoxazinones; Benzoxazolinones; Electrospray ionization; Ion suppression; LC–MS; Matrix effect; Wheat

1. Introduction

The allelopathic ability of members of the cereal family towards pests and competitors, exhibited for example, as inhibition of feeding and reproduction of aphids and reduced germination of other plants, has long been recognized [1,2]. Several chemical classes have been associated with allelopathic control, including alkaloids, cyanogenic glucosides, fatty acids, flavonoids, tannins, terpenoids and phenolic acids [3]. However, in maize, rye and wheat plants, compounds belonging to the benzoxazinoid class in particular were involved.

During the 1980s and 1990s, several procedures for the separation and quantification of benzoxazinoid derivatives in plant extracts were developed [4]. To date liquid chromatography (LC) coupled with ultraviolet (UV) detection has been the most broadly applied technique for the analysis of benzoxazinoids. However, the low sensitivity and lack of specificity of this method demands a more advanced analytical tool. To overcome the LC–UV limitations, the use of liquid chromatography coupled to mass spectrometry (LC–MS) has clear advantages [5]. Recently, this approach was tested for determining benzoxazinoids in maize and wheat [6–8].

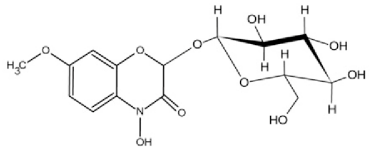
Despite its enormous potential for the determination of benzoxazinoids, the application of LC–MS has some characteristic obstacles. Matrix effects may impair quantification of target analytes in complex samples, especially, when using electrospray ionization (ESI). Large amounts of endogenous species may potentially co-elute with target analytes. While these co-eluting species do not appear at the chromatographic trace when only selected ions of the analyte are monitored, they may, however, significantly affect the efficiency and reproducibility of the ionization process. The ion suppression was described by Kebarle and Tang [9], who showed that ESI responses of organics decreased with an increase in the concentrations of other organics. Matuszewski et al. [10] stated that coeluting matrix components may reduce the ionization efficiency of the analytes and cause poor reproducibility and accuracy. Thus, response factors obtained from a standard solution and in a matrix-loaded sample may differ significantly and matrix effects must be eliminated or compensated to obtain quantitatively accurate results.

* Corresponding author. Tel.: +34 934006100; fax: +34 932045904.
E-mail address: eeeqam@cid.csic.es (E. Eljarrat).

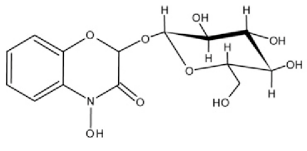
0021-9673/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2007.04.040

Table 1
Structure and acronyms of selected benzoxazinoid derivatives, as well as of selected internal standards

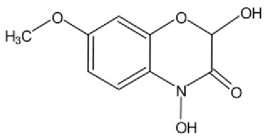
Benzoxazinoid derivatives



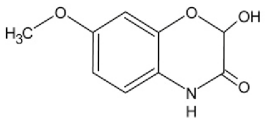
DIMBOA-β-D-Glc



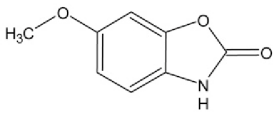
DIBOA-β-D-Glc



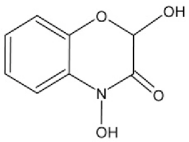
DIMBOA



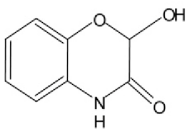
HMBOA



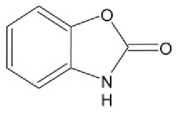
MBOA



DIBOA

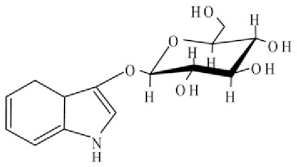


HBOA

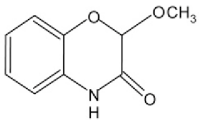


BOA

Internal standards



Indoxyl-β-D-Glc



2-MeOH-HBOA

Some key reports have also highlighted this phenomenon in bioanalytical [11,12], environmental [13,14] and food analyses [15].

Little is known about the matrix effect by coeluting substances in plant extracts. In this paper, the ionization behaviour of eight selected benzoxazinoids, including DIBOA-β-D-glc (2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one glucoside), DIMBOA-β-D-glc (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one glucoside), DIBOA (2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one), DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one), HBOA (2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one), HMBOA (2-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one), BOA (1,3-benzoxazol-2(3*H*)-one) and MBOA (6-methoxy-1,3-benzoxazol-2(3*H*)-one) (Table 1) in foliage and root materials is presented. The characterization of this effect and methods to overcome these interferences are described. The more usual methods used to overcome ion suppression effect are: internal standard method, dilution standard method and standard addition method.

The results obtained for the first and second method are described below in Section 3. Concerning to the third method, no experimental results were obtained. This method involves quantifications by standard addition into each sample and with each analyte investigated; resulting in three to six analyses of each sample instead one analysis. Moreover, each sample has to be calibrated and quantified separately. Nevertheless, this approach provides reliable and firm quantitative data. Though standard addition can correct for accuracy losses by matrix compounds, it cannot avoid a loss of sensitivity. In order to reduce the high number of samples to be analysed when standard addition method is applied and if one is confident of having a homogeneous matrix within a series of samples, calibration can be performed by standard addition of only one sample of a series and applied to the whole series. However, this requires the avail-

ability of a control matrix that is similar to the sample matrix and that does not contain the target analytes. As we are analysing natural products in wheat plants, in our case it was difficult to found a sample without benzoxazinoid derivatives levels. Obviously, all samples presented at least some of the target analytes, especially DIMBOA-β-D-glucoside, DIMBOA, HMBOA and MBOA [16].

2. Material and methods

2.1. Chemicals and materials

The benzoxazinoid standards have been obtained from commercial and private sources as available. DIMBOA-β-D-glucoside, DIBOA and the non-naturally occurring synthetic derivative 2-MeO-HBOA (2-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) were purchased from Prof. Dr. Sicker (University of Leipzig, Germany). DIBOA-β-D-glucoside, HBOA, HMBOA, DIMBOA and MBOA were received from Dr. F. Macias (University of Cádiz, Spain). BOA and indoxyl-β-D-glucoside were purchased from Sigma–Aldrich. LC-grade solvents (water (H_2O) and methanol (MeOH)) and 98% pure acetic acid (HOAc) were purchased from Merck (Darmstadt, Germany).

2.2. Sample preparation

Wheat plant samples were frozen and stored at −20 °C until further analysis. Excess water was removed from the frozen plants by lyophilization for 24 h. The benzoxazinoid derivatives were isolated from the foliage and root samples using pressurized liquid extraction (PLE) followed by solid phase extraction (SPE) cleanup. A more detailed description of the method is given elsewhere [8]. Briefly, 0.1 g of lyophilized root or foliage was extracted by PLE using an ASE 200 (Dionex, Idstein, Germany) apparatus, equipped with 11 mL stainless steel extraction cells. Diatomaceous earth was used to fill the extraction cells, with the matrix and sample thoroughly mixed to ensure good dispersion of the sample. Default conditions of pressure (1500 psi), flush volume (60%) and cell purge (60 s) were used. Extraction conditions for benzoxazinoids were as follows: solvent composition, MeOH (1% HOAc); temperature, 150 °C; three 5-min static cycles; cell preheat, 5 min with no N_2 purge. Purification and concentration was performed via LiChrolut® RP C_{18} (500 mg) SPE cartridges (Merck). The benzoxazinoids have been eluted with 5 mL of MeOH/acidified H_2O (60:40).

2.3. Instrumentation

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 detector (DAD) was connected in line with a bench top 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.

2.4. Chromatographic conditions

A Synergi Max-RP 80A (C-12 TMS) LC column (250 mm × 4.6 mm Phenomenex) with a solvent flow rate of 1 mL/min was used. The sample injection volume was set 50 μL. Mobile phase consisted of acidified H_2O (0.05% HOAc) as solvent A and acidified MeOH (0.05% HOAc) as solvent B. The solvent gradient adopted was as follows: 0–2 min, 100–70% A; 2–19 min, 70–40% A; 19–21 min, 40–5% A; 21–23 min, 5–5% A; 23–25 min, 5–70% A; 25–30 min, 70–100% A. Total run time was 35 min with the benzoxazinone derivatives eluted over 8–20 min and the final 15 min were used for column cleaning and regeneration (Fig. 1) [8].

2.5. Mass spectrometry conditions

MS analyses were carried out in selected ion monitoring (SIM) mode. The ESI-MS was operated in negative ion mode with the following instrument settings: nebulizer pressure, 5 V; gas temperature, 350 °C, capillary voltage, 3500 V; fragmentor, 0–15 min 250 V, 15–35 min 70 V. Two ions for each analyte were selected, according to specificity and sensitivity, with the primary ions used for quantification and the secondary ion providing confirmation [8].

3. Results and discussion

To investigate the influences on the MS responses by coeluting substances originated from foliage and root extracts, a standard calibration and addition standard curves (matrix-matched standards) were prepared. The standard calibration curve was prepared adding a standard solution containing a mixture of the eight selected benzoxazinones in MeOH/H^+ (1%HOAc). Whereas the addition standard curve was prepared by adding the same standard solution into foliage and root extracts. The slopes of standards curves constructed in MeOH/H^+ (1%HOAc) and in the extracts may serve as an indicator of the absolute matrix effect. Both the calibration curves in the standard solutions in MeOH/H^+ (1%HOAc) and in the foliage and root extract solutions were linear in the concentration range between 0.01 and 2.00 ng/μL (Fig. 2). The absolute matrix effect was calculated by comparing the slope of matrix-matched standard curve with the slope of the standard calibration curve. The values of benzoxazinones added to the root extract solutions

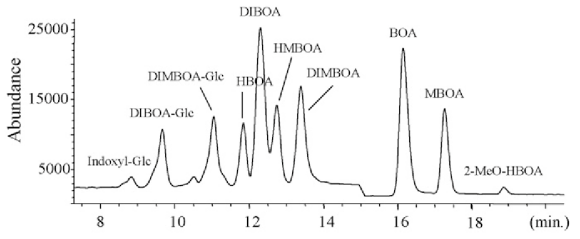


Fig. 1. LC–ESI-MS (TIC) chromatogram obtained for a standard solution mixture (1 μg/mL) on a RP dodecyl (C12) trimethylsilyl (TMS) end-capped Synergi MAX-RP® column.

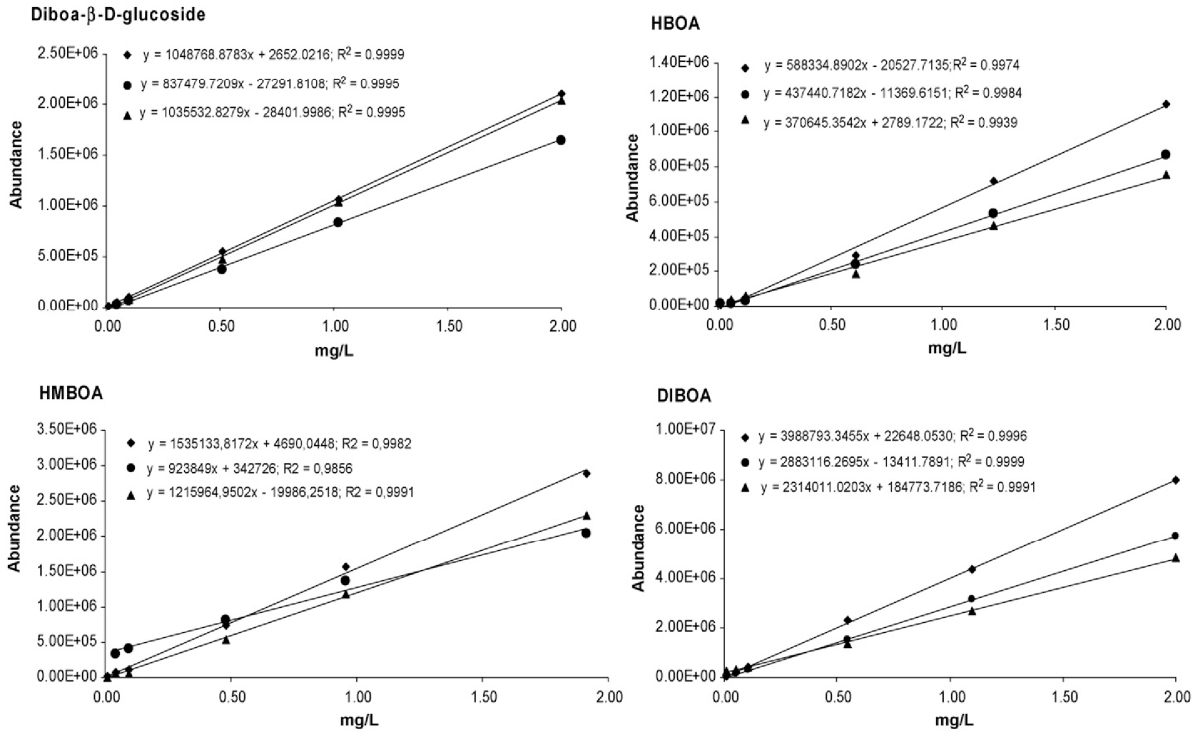


Fig. 2. Calibration curves for benzoxazinoids in the standard solutions in methanol (♦) and the standard solutions in root (●) and foliage extract solutions (▲).

were about [3–73]% lower than those in the standard solutions in MeOH/H^+ (1%HOAc). In the same way, the values of benzoxazinoids added to the foliage extract solutions were about [−3 to 36%] lower than those in the standard solutions in MeOH/H^+ (1%HOAc) (Table 2). The results show that co-eluting matrix substances may reduce the ion intensity of the target compounds and cause signal suppression. The use of simple external standard calibration method may produce erroneous results on the quantification of benzoxazinone derivatives in wheat plants. In the quantitative analysis by LC–MS, several strategies will be appropriate in order to diminish the signal suppression of the analyte. In this study, two different methods such as use of internal standards and external dilution were considered to improve the accuracy of quantification were considered.

3.1. Correction by using internal standards

The special problem of matrix effects in LC–MS stems from the fact that the sample matrix may be subjected to the chromatographic separation, resulting in a different and in each case unknown matrix for each of the analytes in a multicomponent analysis. Thus, one internal standard cannot compensate for these effects but a chemically similar and co-eluting standard compound is required for each analyte. An approach is

Table 2
Absolute matrix effect calculated comparing the slope of matrix-matched standard curve with the slope of the standard calibration curve (expressed in %)

	Root			Foliage		
	External		Internal[a]	External		Internal[a]
	Undiluted	Diluted ×5	Undiluted	Undiluted	Diluted ×10	Undiluted
DIBOA-glc	15	−0.8	10	10	−4.3	23
DIMBOA-glc	43	3	7	33	3	44
HBOA	23	7	18	33	0.1	43
DIBOA	21	7	17	36	2	45
HMBOA	38	10	36	21	1	32
DIMBOA	73	5	72	9	0	22
BOA	3	3	12	−3	−4	3
MBOA	15	−1	−3	23	17	7

[a] Internal standard calibration obtained using indoxyl-β-D-glucoside for DIBOA-β-D-glucoside and DIMBOA-β-D-glucoside calculations, and 2-MeO-HBOA for the rest of compounds.

the addition of isotopically labelled standard. The use of these substances is useful for the correction of the signal deviation because they have the same chemical properties and the same retention times as non-labelled substances. However, isotopically labelled internal standards may not be available for some analytes due to difficulties associated with synthesis and/or cost. It would be difficult to obtain ^{13}C-substituted or ^{2}H-substituted benzoxazinoids, so this correction method would not be practical. It is important to point out that the standards and obviously the isotopically labelled standards are not commercially available. Standards must be obtained using efficient isolation and synthetic methodologies [17].

In this work, we have checked two different compounds as potential internal standards for the correction of ion suppression effects. First, we have selected a glucoside compound, the indoxyl-β-D-glucoside (Table 1), as possible standard for glucoside analytes DIBOA-β-D-glucoside and DIMBOA-β-D-glucoside. The selected standard has some structural similarities with the analytes; moreover, their chromatographic retention time is very close to those of glucoside benzoxazinoids (Fig. 1). On the other hand, a non-naturally occurring synthetic derivative of HBOA, the 2-MeO-HBOA, was selected for the rest of benzoxazinoid derivatives (DIBOA, DIMBOA, HBOA, HMBOA, BOA and MBOA). This internal standard elutes at the end of the chromatogram, near to BOA and MBOA (Fig. 1).

To study the influences of the use of internal standards in the correction of ion suppression effects, foliage and root extracts were spiked with a standard solution containing a mixture of the analytes at different concentrations (from 0.01 to 2.00 ng/μL), including the internal standard at the same concentration in order to construct additional internal standard calibration curves. The slopes of these curves were compared with those obtained from the internal standard calibration (Table 2). The slope values from root and foliage extract solutions were lower than those obtained by the internal standard calibration curves in MeOH/H$^+$ (1%HOAc), indicating that ion suppression still exists. It seems that the use of the internal standard indoxyl-β-D-glucoside for the quantification of glucoside compounds reduces the ion suppression effect in root extracts. Nevertheless, in foliage extracts the signal decreases but the absolute matrix effect still been higher than 20%. The use of the internal standard (2-MeO-HBOA) for the quantification of the rest of the analytes did not reduce the ion suppression effect. Nevertheless, for MBOA analyte a considerable reduction of ion suppression effect was achieved. This fact could be explained because the internal standard eluted close to MBOA. Thus, we can conclude that quantification using selected internal standards may correct some ion suppression effects. However, the structural differences between analytes and standards avoided a more effective reduction of matrix effects. For this reason and because the difficulty to obtain isotopically labelled internal standards, this method was discarded as the method for the correction of the matrix effects in this work.

3.2. Correction by the dilution of the extracts

Decreasing the amount of injected sample by sample dilution cannot only lead to a reduction of matrix effects [18], but also

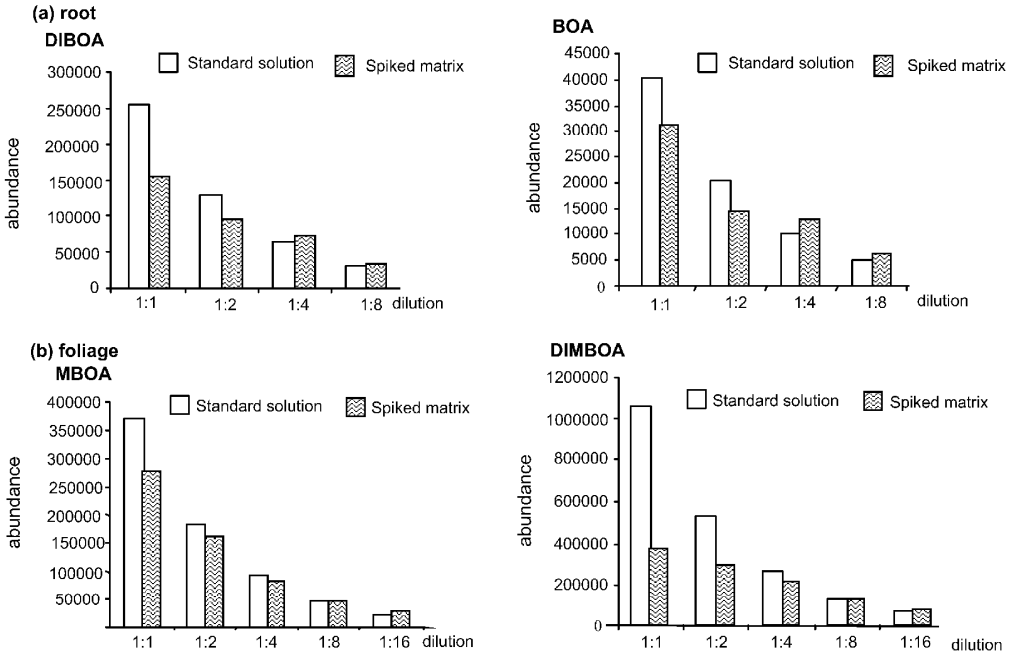


Fig. 3. Comparison between signals obtained in a standard solution and in extracts at different dilutions: (a) DIMBOA-β-D-glc and DIMBOA in foliage extracts; and (b) DIBOA-β-D-glc and DIBOA in root extracts.

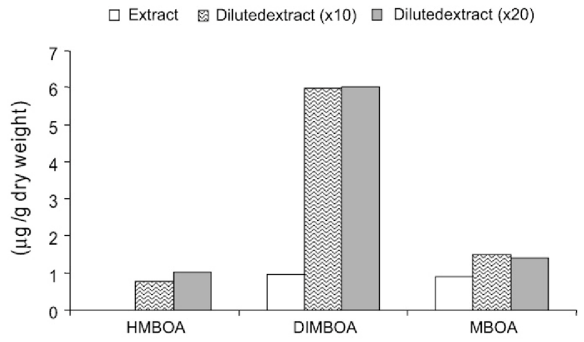


Fig. 4. Quantitative results obtained for a foliage sample by external standard method. Data corresponded to extract without dilution and diluted extracts at 10 and 20 times.

causes decreasing sensitivities. In this work, we have studied the most appropriate dilution needed for each matrix (foliage and root) in order to minimize the ion suppression effect. For this purpose, sample extracts were spiked at 0.1 ng/μL of each analyte, and a standard solution at the same concentration was prepared. The response obtained of each analyte on the spiked extract was compared with the response of the same analyte prepared in MeOH/H^+ (1%HOAc). When ion suppression occurs, the signal of analyte in spiked matrix was lower than the signal in standard solution. The above-mentioned effect is observed in Fig. 3. The signal abundance of the analyte in the standard solution is higher than the signal abundance of the same analyte at the same concentration on the spiked matrix. Next, the extracts and the standard solution were diluted 1:2 consecutively until both signals were equivalent (Fig. 3). The optimal dilution values were established for each matrix, in order to remove or minimize the matrix effects. A 1:5 dilution was needed for root extracts (Fig. 3a) and 1:10 dilution for foliage extracts (Fig. 3b). Using these dilutions, the signal decrease observed for the different analytes ranged from −1 to 10% in root extracts and between 0 and 17% in foliage extracts (Table 2). Once each optimal dilution was established for each matrix by consecutive dilution of the same extract, these values were confirmed by the analysis and further quantification of different samples. For example, Fig. 4 shows the quantification results obtained for a foliage sample by external standard method. As can be seen, concentrations obtained for the extract without dilution were lower than those obtained for diluted extracts. This example showed that extract dilution at 10 times is sufficient to remove or minimize the ion suppression effect. Special attention must be paid on the HMBOA determination: this analyte was not detected in the extract without dilution, whereas a concentration level around 1 μg/g dry weight was obtained by the dilution of the extract (Fig. 5). It is worth it to clarify that the sensitivity could decrease with the dilution of the extracts. Nevertheless, the limit of detection (LOD) obtained with the non-diluted extracts and the diluted extracts were similar, because the ion suppression effect has been reduced. In the case of foliage, the LOD ranged from 1 to10 μg/g and 2 to 11 μg/g of non-diluted and diluted extract, respectively. And in the case of root, the values ranged

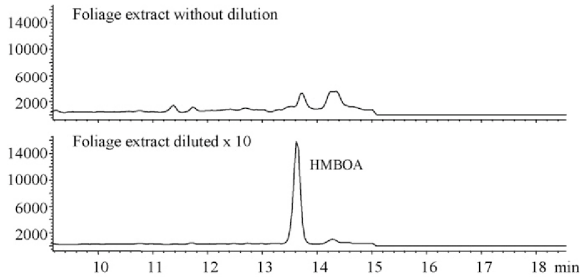


Fig. 5. LC–ESI-MS (m/z = 194) chromatogram obtained for a foliage extract without dilution and the same extract diluted 10 times.

from 1 to 10 μg/g and 1 to 27 μg/g of non-diluted and diluted extract, respectively.

It is important to note that ion suppression effect could vary depending on the analytical instrumentation used for the analysis. Therefore, the optimal dilution values obtained in our study are related to the LC–MS equipment used in this work and these will serve as indicator. But, a new optimization of these values will be required if a different instrumentation will be used.

4. Conclusions

LC–MS is suitable for analysis of benzoxazinoids because of high selectivity and sensitivity. However, it is clear that the suppression of the ionization efficiency occurs due to coeluting substances jointly with the target compounds. This fact causes variation of responses of the analytes in the LC–MS analysis and the benzoxazinoids were difficult to quantify by using the standard solutions in MeOH/H^+ (1%HOAc).

The absolute matrix effect was calculated by comparing the slope of matrix-matched standard curve with the slope of the standard calibration curve. The slope values of benzoxazinoids in plant extracts were 3–73% lower than those obtained with standards in methanol.

One of the most effective methods to reduce the ion suppression will be the addition of standard method. A control matrix similar to the sample matrix is needed to carry out this method. In our case, it was difficult to found a sample without target analyte levels and we could not apply standard addition method for the quantification of the benzoxazinoid derivatives in wheat plants.

In order to improve the analysis and quantification of benzoxazinones in wheat plants, two different methods were studied for the correction of the signal suppression: use of internal standards and extract dilution. Due to the difficulty to obtain isotopically labelled internal standards, the use of internal standard method was discarded. Dilution of extracts seems to be practical in the case of analysis of benzoxazinoid derivatives in plants. Different dilutions were applied and the optimal dilution was chosen taking sensitivity into account. Application of this corrective approach showed that quantitative results by external standards were (−) 1–17% lower than those obtained by dilution of extracts. The optimal dilutions were 5 and 10 times for root and foliage extracts, respectively.

Acknowledgments

This research project was made possible by funding provided by the European Commission (5th Framework programme) under sustainable agriculture initiatives of the Quality of Life and Management of Living Resources (Fate and toxicity of allelochemicals (natural plant toxins) in relation to environment and consumer, Project number QLK5-2001-01967). Ministerio of Ciencia y Tecnología for funding provided to IIQAB-CSIC (AGL2001-4952-E) is also gratefully acknowledged. We would also like to thank Prof. Dr. Sicker (University of Leipzig, Germany), Dr. F. Macías (University of Cádiz, Spain), Prof. Dr. H. Iwamura (University of Kyoto, Japan) and Prof. Dr. L. Jonsson (Södertörn University College, Sweden) for kindly providing benzoxazolinone standards.

References

[1] H.M. Niemeyer, Phytochemistry 27 (1988) 3349.
[2] D. Sicker, M. Frey, M. Schulz, A. Gierl, Int. Rev. Cytol.—Survey Cell Biol. 198 (2000) 319.
[3] T. Haig, J. Chem. Ecol. 27 (2001) 2363.
[4] E. Eljarrat, D. Barceló, TrAC Trends Anal. Chem. 20 (2001) 584.
[5] E. Eljarrat, M. Guillamon, J. Seuma, B. Bugel, I. Formsgaard, W. Oleszek, A. Stochmal, O. Shakaliene, D. Barcelo, J. Chromatogr. A 1047 (2004) 69.
[6] V. Cambier, T. Hance, E. de Hoffmann, Phytochemistry 53 (2000) 223.
[7] L. Bonnington, E. Eljarrat, M. Guillamon, P. Eichhorn, A. Taberner, D. Barcelo, Anal. Chem. 75 (2003) 3128.
[8] M. Villagrasa, M. Guillamon, E. Eljarrat, D. Barcelo, J. Agric. Food Chem. 54 (2006) 1001.
[9] P. Kebarle, L. Tang, Anal. Chem. 65 (1993) A972.
[10] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[11] H. Mei, Y.S. Hsieh, C. Nardo, X.Y. Xu, S.Y. Wang, K. Ng, W.A. Korfmacher, Rapid Commun. Mass Spectrom. 17 (2003) 97.
[12] M.W.J. van Hout, H.A.G. Niederlander, R.A. de Zeeuw, G.J. de Jong, Rapid Commun. Mass Spectrom. 17 (2003) 245.
[13] E. Dijkman, D. Mooibroek, R. Hoogerbrugge, E. Hogendoorn, J.V. Sancho, O. Pozo, F. Hernandez, J. Chromatogr. A 926 (2001) 113.
[14] P.L. Ferguson, C.R. Iden, B.J. Brownawell, Anal. Chem. 72 (2000) 4322.
[15] A. DiCorcia, C. Crescenzi, A. Lagana, J. Agric. Food Chem. 44 (1996) 1930.
[16] M. Villagrasa, M. Guillamon, A. Labandeira, A. Taberner, E. Eljarrat, D. Barcelo, J. Agric. Food Chem. 54 (2006) 1009.
[17] F.A. Macias, D. Marin, A. Oliveros-Bastidas, D. Chinchilla, A.M. Simonet, J.M.G. Molinillo, J. Agric. Food Chem. 54 (2006) 991.
[18] B.K. Choi, D.M. Hercules, A.I. Gusev, J. Chromatogr. A 907 (2001) 337.

II.4. DISCUSSIÓ DELS RESULTATS

Les metodologies desenvolupades en aquest capítol, han fet possible dur a terme l'anàlisi simultània dels compostos glucosats (Diboa-Glc, Dimboa-Glc), les aglucones (DIBOA i DIMBOA), les benzoxazolinones (BOA, MBOA) i les lactames (HBOA i HMBOA). En ambdós estudis, inclosos en aquesta secció, es van aconseguir optimitzar i validar les condicions de preparació, anàlisi i quantificació dels compostos objectiu permetent la seva determinació en mostres de fulla i arrel de blat, mitjançant protocols d'anàlisi senzills i robustos.

Anàlisi Instrumental

L'anàlisi instrumental es va dur a través de la LC-MS. L'elecció de realitzar les anàlisis per LC ve donada per la polaritat i la baixa volatilitat dels compostos. L'acoblament a MS ens va permetre obtenir una major sensibilitat i selectivitat respecte a les anàlisis descrites en la bibliografia que basen les seves anàlisis principalment a través de LC-UV.

Per altra banda, en el nostre laboratori ja s'havia desenvolupat una metodologia d'anàlisi utilitzant la LC-MS/MS [16], aportant major selectivitat i sensibilitat envers la LC-MS. Però donat que en aquell moment aquestametodologia era d'elevat cost, hi havia un nombre elevat de mostres a analitzar, i els nivells presents en la planta ens permetien usar la MS simple, es va decidir desenvolupar una nova metodologia basada en aquesta tècnica.

Les fases mòbils més comunament usades per l'anàlisi de les benzoxazinones han estat mescles d'H_2O amb modificadors àcids (HOAc o fosfòric (H_3PO_4)) o solucions tampó (Na_2HPO_4) com a solvent aquós i MeOH com a solvent orgànic. El treball de Bonnington et al. [16] ens va servir com a base per a la optimització cromatogràfica i la fase mòbil usada va ser H_2O/H^+ (0.05% HOAC) i $MeOH/H^+$ (0.05%).

L'anàlisi de MS es va dur a terme en mode SIM (*Select IonMonitoring*) on es seleccionen els ions més abundants en la fragmentació de cadascun dels analits per a la seva quantificació i els menys abundants per a la confirmació.Les condicions òptimes en

l'anàlisi de MS van ser escollides en funció a l'abundància i fragmentació de cada analit en el mode d'escombrat (*SCAN*).

L'espectròmetre de masses equipat amb font d'ionització a pressió atmosfèrica (API), va ser usat amb interfase ESI en polaritat negativa.Per tal d'obtenir la informació estructural necessària i la màxima sensibilitat,es van optimitzar diferents paràmetres com la pressió del gas de nebulització, de 50 a 60psi; la temperatura del gas, de 250 a 350^{o}C; i el potencial de fragmentació, de 70 a 250V. Les condicions òptimes del potencial de fragmentació van ser de 250V pels compostos més polars (glucosats, HBOA, DIBOA, HMBOA i DIMBOA) i de 70V per la resta.A tall d'exemple es mostra a la Figura II.5 la senyal obtinguda pels compostos glucosats amb el potencial de fragmentació a 250V que és millor que la obtinguda a 70V. Amb un voltatge de fragmentació de 250V la molècula glucosada es fragmenta i ens proporciona major senyal.

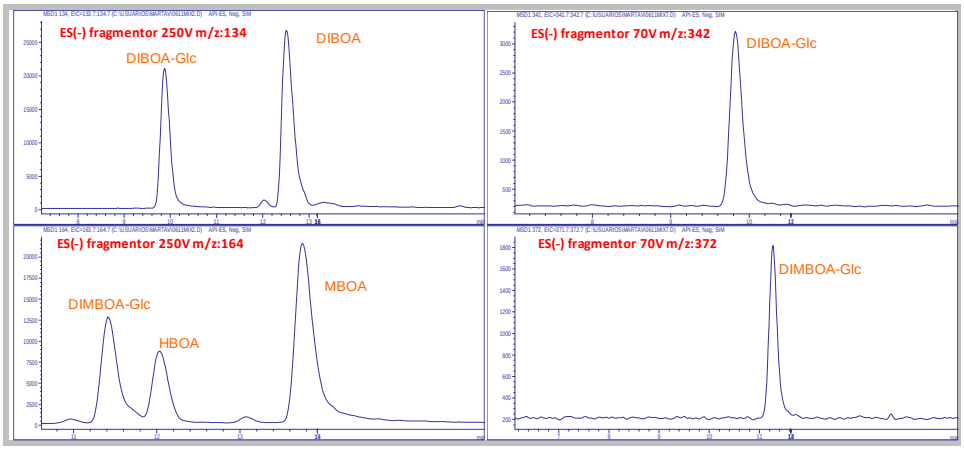


Figura II.5.- Senyal obtinguda a diferents voltatges de fragmentació pels compostos glucosats

La separació cromatogràfica s'engloba en dues finestres cromatogràfiques, una corresponent als compostos que s'elueixen de 0-15 min aplicant el potencial de fragmentació a 250V i l'altre als que ho fan a partir del minut 15 amb potencial de fragmentació de 70V.A part de l'anàlisi per LC-MS els diferents analits varen ser confirmats on-line amb el detector UV Model 996 photodiodeArray en l'interval de 250-440nm (Taula II.1).

Taula II.1.- Taula dels compostos analitzats, el seu pes molecular, el pic corresponent al cromatograma (Figura II.6) i la longitud d'ona a la que són monitoritzats (* Patrons interns).

Compost	MW	Pic	DAD(nm)
Indoxyl-β-D-glucosat*	295	1	229,280
Diboa-Glc	343	2	255,280
Dimboa-Glc	373	3	263,280
HBOA	165	4	229,280
DIBOA	135	5	263,280
HMBOA	195	6	255,280
DIMBOA	165	7	255,263,280
BOA	135	8	229,280
Quercetin-3-0-rutinoside*	610	9	229,280
MBOA	165	10	229,280
2-MeOH-HBOA *	179	11	255,280

A la Figura II.6 es mostra el cromatograma obtingut per a una mescla patró, mitjançant DAD (a) i MS (b). Com es pot observar, per al DAD la separació entre compostos no és la òptima i inclús el BOA i el Quercetin 3-o-Rutinoside coelueixen en un sol pic. No obstant, al treballar en MS en mode SIM la separació és bona per a la totalitat dels compostos.

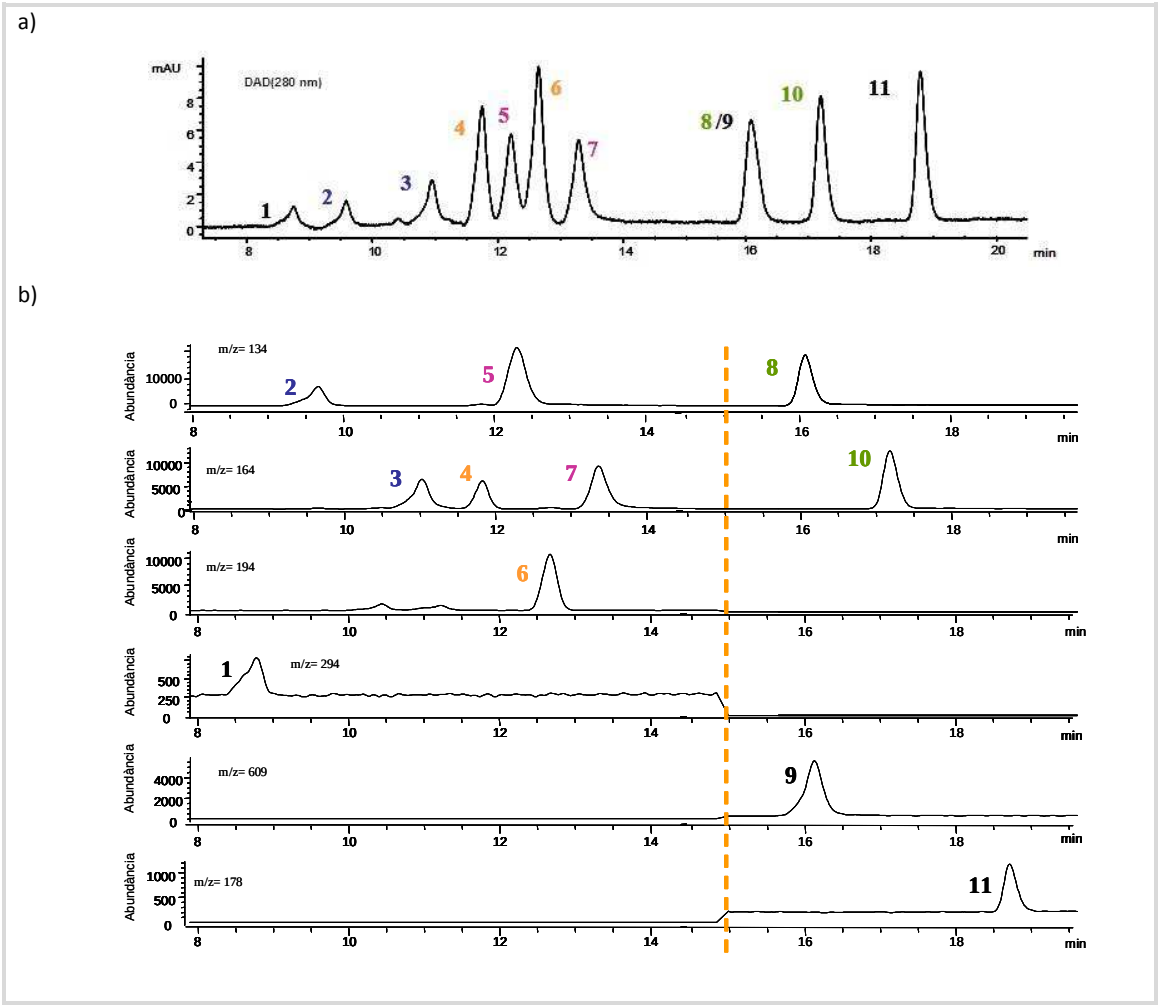


Figura II.6.- Cromatograma corresponent a un patró de 1ng/µL (a) DAD a 280 nm. (b) Ions monitoritzats per analit

Tractament de la mostra

El procediment analític seguit en el tractament de les mostres consta d'una sèrie d'etapes interrelacionades entre si com són: la liofilització del material de la planta,una extracció dels analits per PLE, una etapa de concentració i filtració, seguit d'una etapa de purificació (Figura II.7).

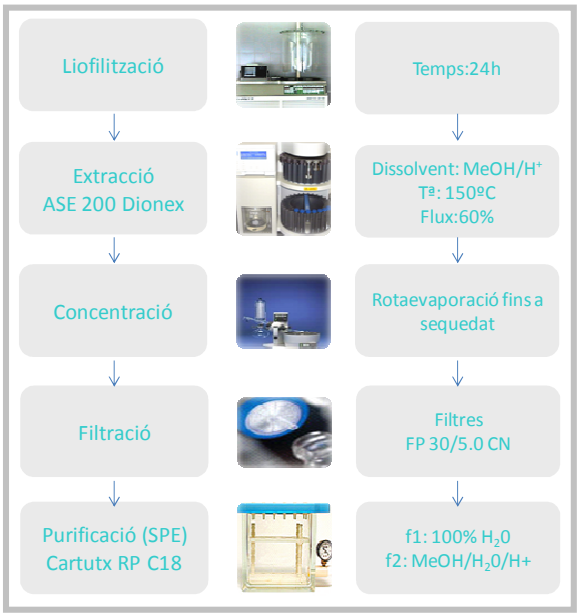


Figura II.7.- Esquema del procés de preparació de les mostres de blat

Tal i com queda descrit en l'article sobre la metodologia d'anàlisi, les etapes més determinants en quant a l'estabilitat i la recuperació dels analits durant la preparació de la mostra van ser l'extracció i el procés de neteja de l'extracte obtingut. Donat l'elevat nombre de mostres a analitzar i la coneixença de la inestabilitat dels analits, es va optar per dur a terme l'extracció per PLE. No s'han trobat referències de l'extracció de compostos al·lelopàtics per PLE, essent per tant, el primer mètode desenvolupat per a l'extracció de benzoxazinones a través d'aquesta tècnica. En el moment en que es va dur a terme la optimització de la present metodologia, la PLE era un mètode d'extracció relativament nou, amb l'avantatge respecte altres tècniques d'extracció, de baix consum de dissolvents i temps d'extracció més curts. Tal i com s'ha comentat a la introducció, els paràmetres més influents per tal d'obtenir un mètode d'extracció per PLE eficaç són, els **dissolvents** d'extracció; la **temperatura**; la **pressió**; els **cicles** en que es repeteix

l'extracció; la quantitat de dissolvent que entra dins la cel·la en cada cicle, expressat en percentatge de volum total de la cel·la (**% flux**); el **temps estàtic** d'extracció; i el **temps** necessari per escalfar la cel·la des de que entra dins el forn fins que assoleix la temperatura desitjada. Així doncs, per a l'etapa d'extracció, es van optimitzar tres dels paràmetres més influents com són la **composició del dissolvent**, la **temperatur**a i el **% de flux**.Coneixent la baixa estabilitat del Dimboa-Glc i el DIMBOA envers la temperatura, es van fer proves addicionant únicament aquests analits i es va dur a terme l'extracció a diferents temperatures per tal de comprovar si durant el procés d'extracció, aquests es degradaven a DIMBOA i MBOA, respectivament. Tot i que les recuperacions obtingudes no són molt elevades, en cap dels casos es va determinar la presència de MBOA en els extractes analitzats.

Una possible explicació a aquest fet, tot i que dur a terme l'extracció a elevades temperatures, podria estar vinculat a una degradació d'aquests compostos, segons el principi de funcionament de la PLE, l'aplicació de temperatures altes redueix la viscositat i la tensió superficial dels solvents i augmenta la difusió solut-dissolvent, millorant així el contacte entre els analits i els solvents d'extracció. També ajuda a trencar les interaccions fortes entre el compostos i la matriu, facilitant l'alliberació d'aquests al medi d'extracció. Treballar a pressió elevada assegura que el dissolvent no excedeixi el seu punt d'ebullició, mantenint-se en estat líquid durant tot el procés i a més a més, ajuda a que les bombolles d'aire que es puguin formar es desfacin, exposant així, la major quantitat de mostra al medi d'extracció. No obstant, hi pot haver l'inconvenient d'extreure altres components que no siguin els d'interès.

Donat que les matrius a extreure són complexes, es va creure convenient aplicar una etapa de filtració i purificació de l'extracte. De la bibliografia consultada, només l'article deBaumeler et al. [8] descriu una etapa de purificació de les mostres i ens va servir de base per a l'etapa de purificació aplicada al nostre mètode. Tot i així, es van dur a terme algunes modificacions per tal d'obtenir recuperacions acceptables dels analits objectiu.No obstant, s'ha de tenir en compte que tot i les millores proposades, els compostos més polars (Diboa-Glc, Dimboa-Glc, DIBOA i HBOA) elueixen principalment enla primera fracció d'elució, essent necessari analitzar les dues fraccions per tal de quantificar-los.

Aquest fet no implica un handicap important en l'aplicació d'aquesta etapa a les mostres de blat, ja que tal i com s'ha comentat anteriorment, els compostos majoritarisen el blat corresponen als compostos amb el grup metoxi en la seva estructura, que elueixen principalment en la segona fracció d'elució.

En resum, en cadascuna de les etapes es varen obtenir bones recuperacions per a tots els analits a excepció de les obtingudes per al **Quercetin-3-O-rutinoside**durant el procés d'extracció i la obtinguda pel **Indoxyl-β-D-glucosat** en el procés de neteja (Taula II.2). Aquest fet fa que l'ús d'aquests analits com a patrons interns (surrogate) no sigui una opció viable. Tot i que l'analit 2-MeOH-HBOA va presentar bones recuperacions en totes les etapes, finalment es va decidir usar-lo posteriorment com a patró intern de quantificació juntament ambl'Indoxyl-β-D-glucosat.

Taula II.2.- Recuperacions obtingudes en les diferents etapes de pretractament de la mostra

Etapa	Rang de recuperació	Compost amb baixa recuperació
Liofilització	81-105	
Concentració	95-102	
Filtració	76-111	
Extracció	70-126	Quercetin-3-O-rutinoside (11) DIMBOA (29)
Purificació	67-106	Quercetin-3-O-rutinoside (25) Indoxyl-β-D-glucosat (35)

Un cop optimitzats tots els paràmetres del procés de preparació de la mostra, es va fortificar una mostra d'arrel i una altra de fulla amb una mescla de patrons. Les recuperacions obtingudes, en el procés sense matriu i per a les dues matrius d'estudi, es troben representades en un diagrama de barres en la Figura II.8. Per a la matriu arrel, a excepció del HMBOA i DIMBOA, les recuperacions dels analits van ser comparables a les obtingudes amb patrons. En el cas de la fulla, els compostos Diboa-Glc, Dimboa-Glc, BOA i MBOA van mostrar recuperacions comparables a les obtingudes amb patrons, mentre que pel HMBOA, el DIMBOA, el HBOA i el DIBOA es van obtenir recuperacions més baixes. En vista d'aquests resultats, es va pensar que les baixes recuperacions obtingudes dels compostos tan en la mostra d'arrel com en la de fulla, podrien ser degudes a l'efecte

matriu, fet que implicaria una afectació molt negativa sobre la quantificació de les mostres.

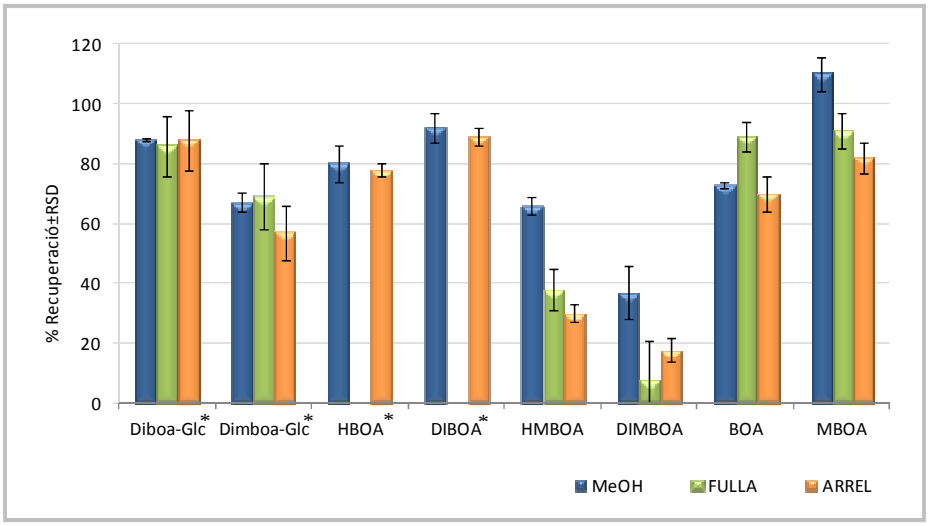


Figura II.8.- Recuperacions del procés complert. *Anàlisi de la primera i segona fracció d'elució

Estudi de l'efecte matriu

La quantificació és un dels aspectes més crítics a l'hora d'assegurar la qualitat dels resultats obtinguts a través de qualsevol tècnica d'anàlisi. D'aquesta manera per tal de poder validar la metodologia desenvolupada es va estimar oportú fer un estudi de l'efecte matriu en les mostres per tal d'intentar minimitzar-lo i obtenir un mètode d'anàlisi i quantificació fiable i reproduïble.

Amb alguns detectors com els basats en UV, és relativament freqüent que els components de la matriu també absorbeixin a la mateixa longitud d'ona que l'analit, especialment en mostres complexes i el mateix pot passar amb analitzadors de quadrupol simple. D'aquesta manera, és possible que en alguns casos, quan es comparen les àrees corresponents als pics del patró amb els d'una mostra fortificada al mateix nivell, es pot trobar falta de concordança entre les mateixes. Això és degut a la presència d'interferents en la matriu que afecten a la ionització dels analits, comportant que la resposta sigui diferent en absència o presència de la mateixa. Normalment el resultat d'aquests efectes es tradueix en una supressió de la senyal de l'analit en presència de matriu, tot i que també es pot donar l'efecte contrari, és a dir, un augment de la mateixa. En tot cas, les

conseqüències de l'efecte matriu es tradueixen en importants errors en la quantificació dels analits d'interès. L'efecte de matriu depèn de les característiques físico-químiques de l'analit, de la interfase usada i especialment de les característiques dels interferents que elueixin de la columna al mateix temps de retenció que l'analit. D'aquesta manera és fàcil pensar que els analits poden presentar un efecte matriu diferent en funció del temps de retenció en els qual elueixen. L'avaluació de l'efecte de matriu (EM) es pot dur a terme comparant la resposta de l'analit en presència i absència de matriu de la següent manera:

$$\%\,EM = \frac{\text{Resposta de l'analit addicionat a matriu}}{\text{Resposta de l'analit en un patró pur a la mateixa concentració}} \text{x}100$$

On:

%EM=100, indica que no hi ha EM

%EM>100, indica que hi ha EM produït per un realç de la senyal

%EM<100, indica que hi ha EM produït per una supressió de la senyal

A la Figura II.9 es mostren els valors de %EM calculats a partir de la resposta dels diferents analits en matriu fulla i arrel respecte a la senyal en el patró pur. Tal i com s'observa en la Figura II.9, l'EM calculat per a tots els compostos és inferior al 100% tan en la matriu arrel com en la fulla, el que ens indica que hi ha un efecte de supressió de la senyal dels analits en matriu respecte la senyal en el patró. Els compostos HBOA, DIBOA i HMBOA són els que es veuen més afectats per l'efecte de matriu especialment en la fulla amb %EM de 8, 22 i 13 %, respectivament.

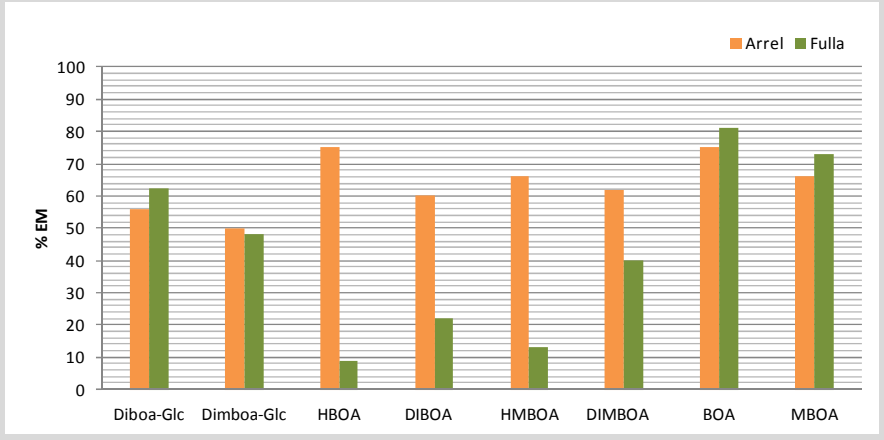


Figura II. 9.- EM calculat en una mostra de fulla (verd) i arrel (taronja)

Degut a la importància d'aquest fenomen i les repercussions que pot tenir en la quantificació, resulta necessari eliminar-lo o bé minimitzar-lo amb la finalitat que els errors comesos siguin acceptables. Hi ha diferents estratègies que poden ser dutes a terme per tal de minimitzar aquest efecte com són: el mètode de calibratge per patró intern, addició estàndard, dilució de l'extracte i un tractament de la mostra més exhaustiu. Algunes d'aquestes metodologies queden recollides en el segon article presentat en aquest capítol. A continuació es detallen els trets més importants d'aplicar-les i els resultats obtinguts.

1.- Optar pel **mètode de calibratge per patró intern** seria una estratègia efectiva per reduir la supressió de la senyal. No obstant, un important requisit és que l'analit i el patró intern presentin característiques químiques similars i per tant temps de retenció el més propers possibles. Malgrat el potencial d'aquesta metodologia, en el nostre cas, no és una tècnica viable donada la manca de patrons marcats isotòpicament. També cal esmentar que en el cas que fossin disponibles, aquests tenen un cost elevat al qual no tots els laboratoris poden accedir. En aquest estudi només disposàvem de dos compostos no marcats isotòpicament per tal d'avaluar-los com a possibles patrons interns i corregir així la supressió de la senyal. A tall d'exemple es mostra la Figura II.10com es pot compensar l'efecte de matriu en la fulla pels compostos glucosats usant l'Indoxyl-β-D-glucosat com a patró intern.

En el cas del calibratge extern pel Diboa-Glc quan comparem les pendents de la recta amb patrons i en matriu fulla, tenim una disminució del pendent del 37%. Aquesta disminució es veu reduïda quan s'introdueix el patró intern fins a un 17%. En el cas del Dimboa-Glc es passa del 62% al 49%. Així doncs, s'observa una reducció en l'efecte de matriu però degut a que el patró intern no és elmés adequat, es segueix observant efecte de matriu. Per aquesta raó aquest mètode va ser descartat.

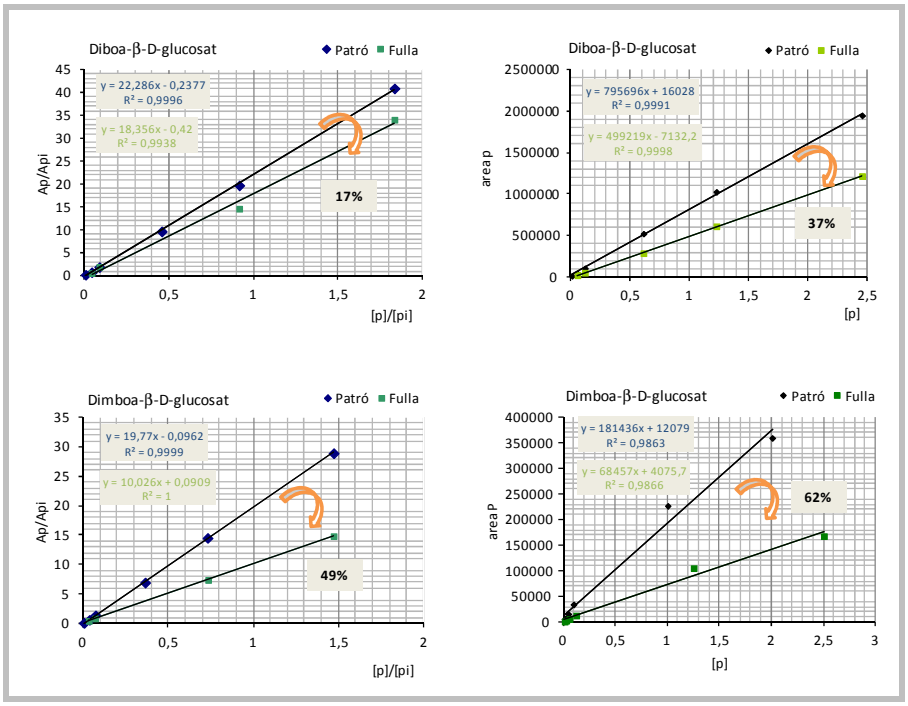


Figura II.10.-Corbes de calibratge pels els compostos glucosats obtingudes per calibratge intern i extern

2.- El **mètode d'addició estàndard** seria el més adient en la correcció de l'efecte de supressió observat, però aquesta estratègia requereix d'un major nombre d'anàlisis on cada mostra ha de ser calibrada i quantificada per separat. Un altre dels inconvenients per poder aplicar aquesta metodologia a les nostres mostres és el de no disposar d'una matriu control, sense presència d'analits, per a preparar les rectes d'addició estàndard i poder quantificar així totes les mostres d'interès.

3.- La tercera aproximació d'estudi va ser la de dur a terme una **dilució de l'extracte** amb l'objectiu de disminuir la quantitat de mostra injectada. La dilució de l'extracte pot reduir l'efecte de la supressió de la senyal, però també pot donar lloc a una disminució de la mateixa. Per tant, s'ha d'arribar a un compromís amb la sensibilitat del mètode. El fet de diluir la mostra ens permet reduir al màxim l'efecte de matriu i dur a terme la quantificació dels analits d'interès amb la recta de calibratge preparada en $MeOH/H^+$.

Tal i com queda descrit en el segon article científic del present capítol, es va dur a terme un estudi de dilució de la mostra successivament a la meitat que va ser comparada amb la senyal d'un patró preparat sense matriu a les mateixes dilucions. El fet de diluir l'extracte de matriu a la meitat indicaria també una reducció de la senyal de l'analit a la meitat. No

obstant, en molts casos el que succeeix és que la senyal obtinguda és superior a la inicial de l'analit sense diluir l'extracte, fet que indica que hi ha hagut una reducció de l'efecte provocat per la matriu. En el moment en que la senyal obtinguda és igual a la senyal teòrica a la dilució corresponent, es considera que s'ha reduït al màxim l'efecte en la supressió de la senyal de l'analit.

Per a la matriu arrel, l'estudi es va fer amb matriu fortificada amb els analits Diboa-Glc, HBOA, DIBOA i BOA (Figura II.11).La dilució que es considera on l'efecte matriu s'ha eliminat o reduït al màxim possible correspon a la de color groc. Per al Diboa-Glc, HBOA, DIBOA i BOA la dilució correspondria a 1:4.

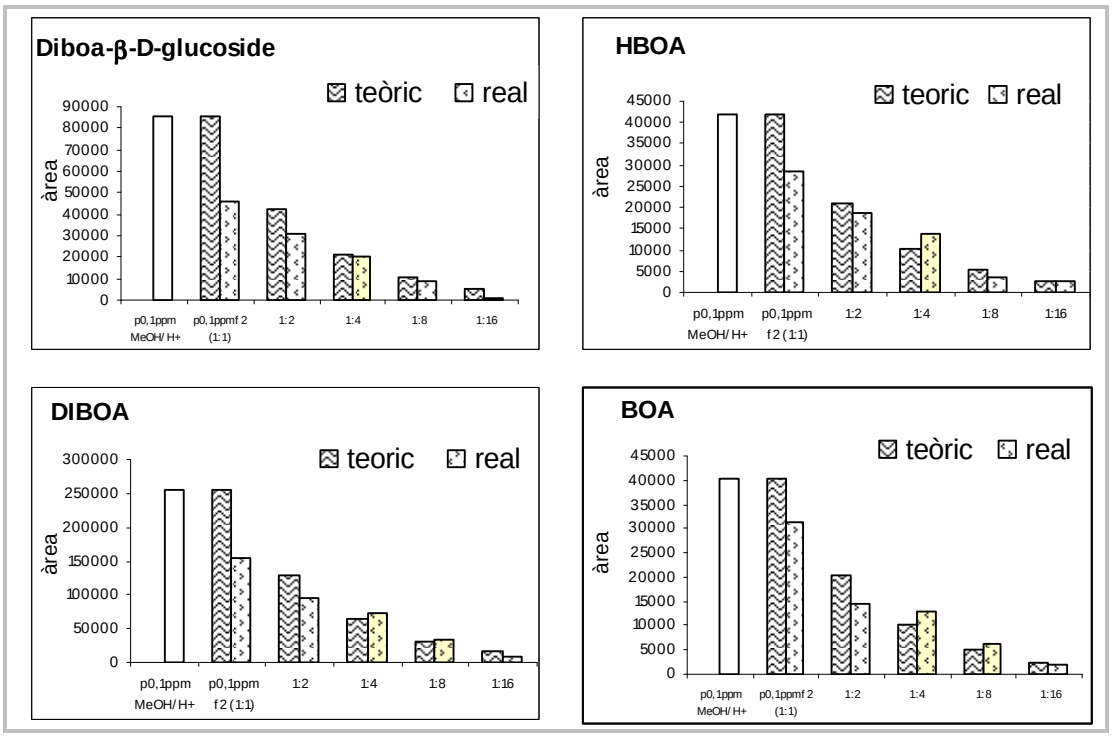


Figura II.11.- Gràfics de la dilució de l'arrel fortificada

Per a la resta de compostos (Dimboa-Glc, MBOA, HMBOA i DIMBOA), donat que els nivells presents en la matriu són elevats, es va fer l'estudi amb la matriu sense fortificar (Figura II.12). Aquests compostos s'han detectat en la matriu sense diluir, però es pot veure que al diluir la mostra a la meitat la senyal obtinguda no es redueix a la meitat. En proporció la senyal és major, fet que ens fa pensar en que hi ha efecte matriu. En el moment en que la senyal és la meitat que l'anterior suposem que ja no existeix efecte matriu o que

aquesta s'ha reduït al màxim. Per aquest grup de compostos la dilució necessària (barra de color groc) correspondria a 1:8, excepte per al DIMBOA on seria necessària una dilució de 1:32. Vàrem concloure que diluir l'extracte d'arrel 5 vegades ,ens permetia poder quantificar les mostres utilitzant la recta de calibratge preparada amb els patrons en $MeOH/H^+$ sense matriu.

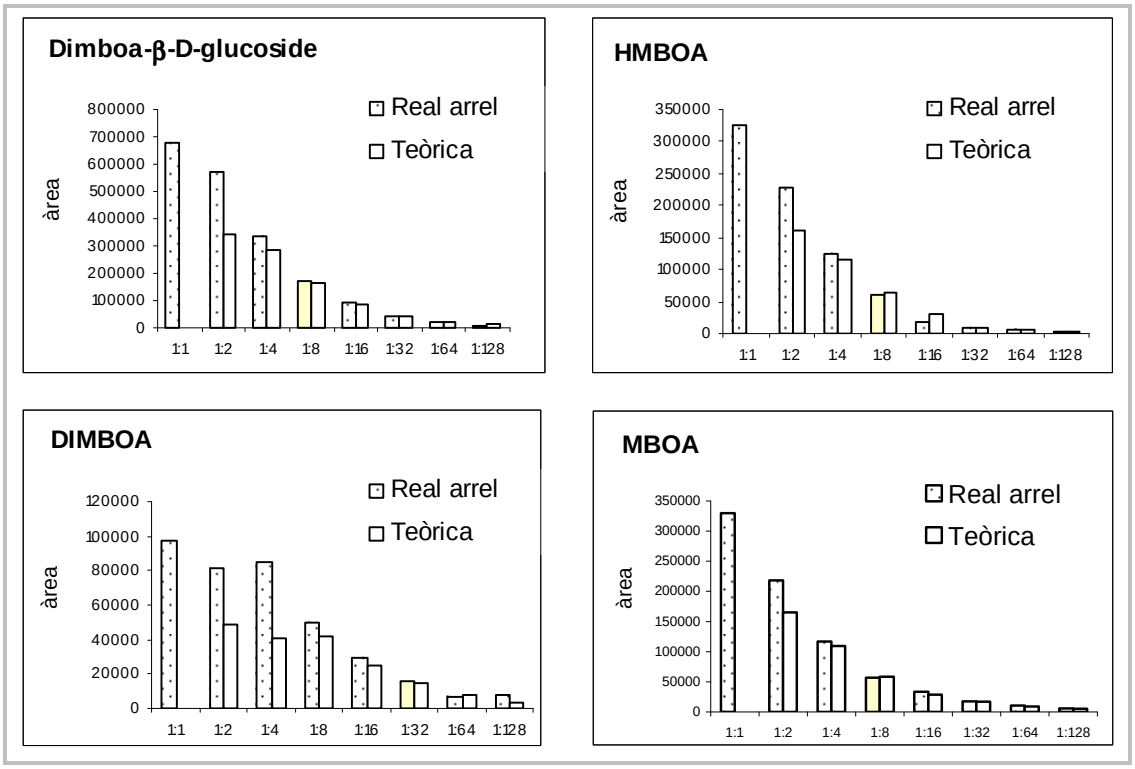


Figura II.12.- Gràfics de la dilució de l'arrel sense fortificar

Els resultats de la matriu fulla també es mostren en forma de gràfic similar als anteriors (Figura II.13). La dilució on es considera que l'efecte matriu s'ha eliminat o reduït al màxim possible en la matriu fulla va correspondreen tots els casos a la dilució 1:8 excepte per al HBOA i HMBOA que és de 1:16. Arribant a un compromís es va decidir diluir les mostres 10 vegades.

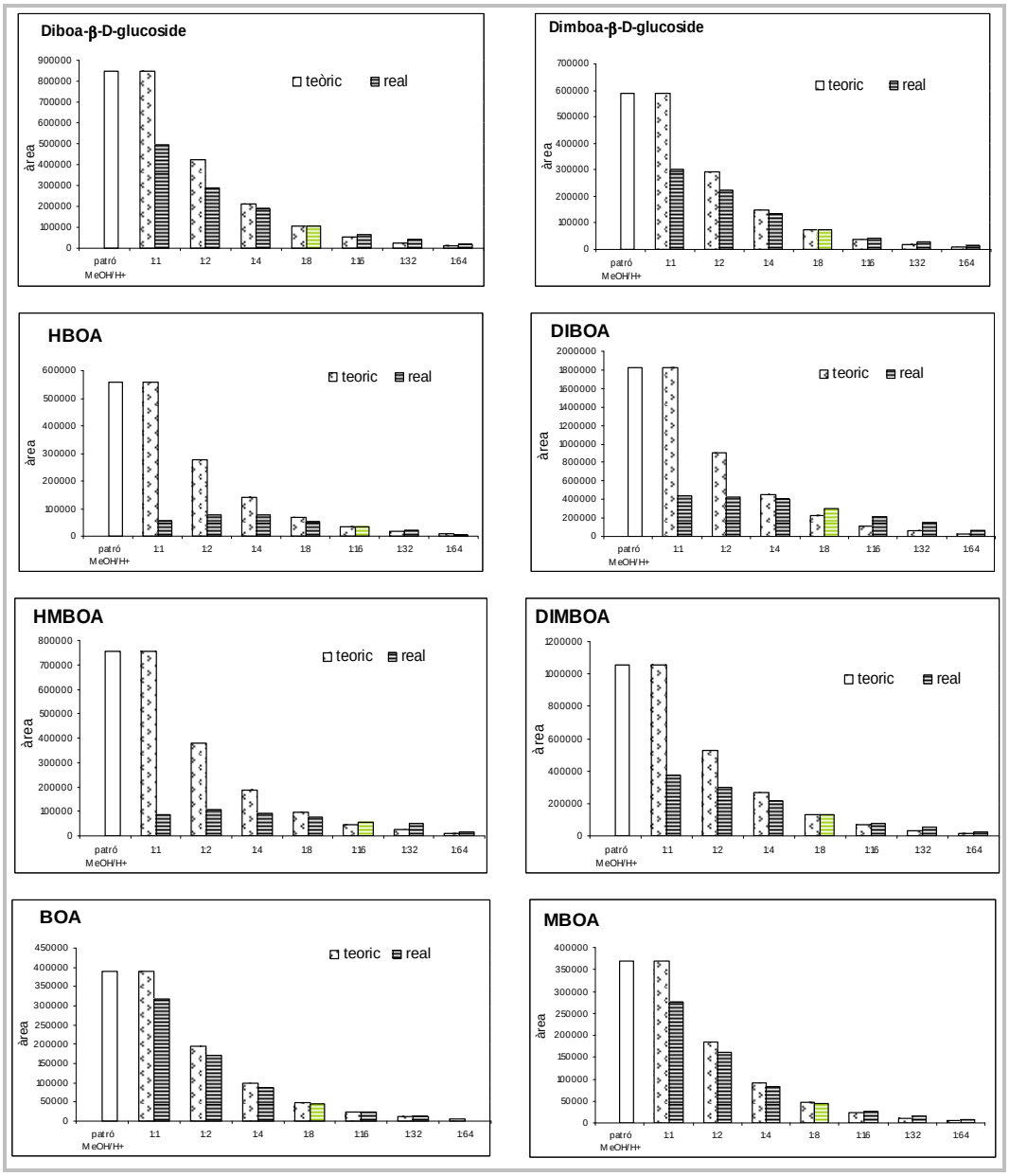


Figura II.13.- Dilució de la matriu fulla fortificada

4.- Dur a terme un **pretractament de la mostra més exhaustiu**, com pot ser l'aplicació d'una etapa de purificació més extensivatambé ens podria aportar a minimitzar aquest efecte. El principals inconvenients derivats d'aquesta acció són el temps que es consumeix, es podria donar una major pèrdua dels analits, una contaminació de la mostra o bé una preconcentració d'un major nombre d'interferents. Així doncs, tal i com queda descrit en l'article presentat en aquest capítol el mètode que ens va aportar millors resultats va ser el d'aplicar la dilució de les mostres.

Valoració del mètode desenvolupat

Un cop optimitzats els diferents paràmetres tant instrumentals com de tractament de la mostra i dut a terme la valoració de l'efecte matriu sobre la ionització dels analits d'interès en fulles i arrels a través de la dilució de la mostra, es va dur a terme la validació del mètode determinant els paràmetres de qualitat com són: els intervals de linealitat, les recuperacions, la reproductibilitat i la sensibilitat amb els límits de detecció i quantificació. Les recuperacions obtingudes varen ser majors del 61% en ambdues matrius per a tots els compostos excepte pel DIMBOA amb recuperacions del 22% en fulla i del 27% en arrel. La baixa recuperació d'aquest compost va ser atribuïda a la seva baixa estabilitat.

Dins del projecte FATEALLCHEM es va dur a terme un exercici intercomparatiu entre dues metodologies d'anàlisi on es va comparar tant el procediment d'extracció com l'anàlisi instrumental realitzat en dos laboratoris diferents. La primera metodologia, desenvolupada al Institute of SoilScienceand Plant Cultivation de Polònia, estava basada en dues extraccions per ultrasons MeOH/H^+ (1%HOAc) intercalades amb una extracció en fred amb un temps de preparació de mostra de més de 16 hores. La segona metodologia, és la desenvolupada en la present tesi, on l'extracció dels analits es va dur a terme mitjançant PLE, amb un temps d'extracció de no més de 30 minuts. La purificació dels extractes va ser la mateixa en ambdós casos i el mètode d'anàlisi es va dur a terme per LC-DAD en el Laboratori de Polònia amb una anàlisi de més de 80 min i per LC-MS en el nostre laboratori. A la Figura II.14 es mostren les concentracions obtingudes dels analits determinats per cadascuna de les metodologies per a una mateixa mosta d'arrel. Tal i com es pot veure, el contingut total d'al·leloquímics determinat per ambdós laboratoris va ser del mateix nivell. Però, si es miren els resultats obtinguts compost per compost, es pot observar que el primer mètode degrada el compost glucosat (Dimboa-Glc) donant lloc a valors de DIMBOA més elevats, mentre que amb el mètode desenvolupat en aquesta tesi, és possible arribar a determinar el contingut de Dimboa-Glci DIMBOA per separat. Aquest fet indica que es tracta d'un mètode més ràpid i sòlid en la determinació del contingut dels diferents compostos al·lelopàtics en les mostres de blat.

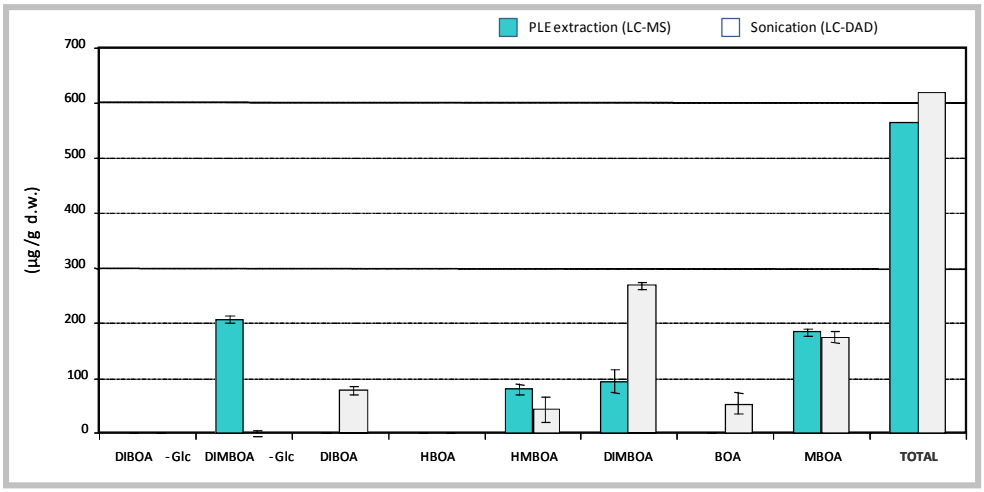


Figura II.14.-Comparació de l'anàlisi dels compostos objectiu obtinguts en ambdós estudis

Per últim, si comparem el mètode de LC-MS amb el prèviament desenvolupat mitjançant LC-MS-MS, es pot observar que la sensibilitat del MS-MS és superior (Taula II.3). Si bé els límits de detecció instrumentals (iLOD) són del mateix ordre de magnitud, quan ens movem a matrius (mLOD), la millora d'aquests és de 2 a 100 vegades usant la LC-MS-MS. No obstant i com es podrà veure amb els resultats de l'anàlisi de les mostres, en el Capítol IV, la LC-MS ens proporciona una sensibilitat suficient per arribar a identificar i quantificar els analits d'interès en les mostres de fulla i arrel de blat.

Taula II.3.- Comparació de la sensibilitat del mètode desenvolupat per LC-MS envers els mètode prèviament desenvolupat per LC-MS-MS

	iLOD (ng/µL)		mLOD (µg/g dw)	
	LC-MS	LC-MS-MS	LC-MS	LC-MS-MS
HBOA	0.005	0.004	9	0.2
DIBOA	0.003	0.023	8	1.1
HMBOA	0.010	0.003	10	0.1
DIMBOA	0.003	0.003	8	0.2
BOA	0.002	0.010	1	0.5
MBOA	0.002	0.002	1	0.1

II.5. REFERÈNCIES

1. **Niemeyer, H.M.** (1988). "Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones) defence chemicals in the Gramineae". Phytochemistry 27: 3349-3358.

2. **Niemeyer, H.M., Pesel, E., Copaja, S.V., Bravo, H.R., Franke, S., et al.** (1989). "Changes in Hydroxamic Acid Levels of Wheat Plants Induced by Aphid Feeding". Phytochemistry 28 (2): 447-449.

3. **Argandona, V.H., Niemeyer, H.M. and Corcuera, L.J.** (1981). "Effect of content and distribution of hydroxamic acids in wheat on infestation by the aphid schizaphis-graminum". Phytochemistry 20 (4): 673-676.

4. **Copaja, S.V., Nicol, D. and Wratten, S.D.** (1999). "Accumulation of hydroxamic acids during wheat germination". Phytochemistry 50 (1): 17-24.

5. **Copaja, S.V., Niemeyer, H.M. and Wratten, S.D**. (1991). "Hydroxamic acid levels in chilean and british wheat seedlings". Annals of Applied Biology 118 (1): 223-227.

6. **Epstein, W.W., Rowsemitt, C.N., Berger, P.J. and Negus, N.C.** (1986). "Dynamics of 6-methoxybenzoxazolinone in winter-wheat - Effects of photoperiod and temperature". Journal of Chemical Ecology 12 (10): 2011-2020.

7. **Whalroos, O. and Virtanen, I.** (1959). "The precursors of 6-methoxy-benzoxazolinone in maize and wheat plants, their isolation and some of their properties.". Acta Chemica Scandinavica 13: 1096-1908.

8. **Baumeler, A., Hesse, M. and Werner, C**. (2000). "Benzoxazinoids-cyclic hydroxamic acids, lactams and their corresponding glucosides in the genus Aphelandra (Acanthaceae)". Phytochemistry 53 (2): 213-222.

9. **Woodward, M.D., Corcuera, L.J., Helgeson, J.P. and Upper, C.D.** (1978). "Decomposition of 2,4-Dhydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one in Aqueous Solutions". Plant Phisiology 61: 796-802.

10. **Honkanen, E. andVirtanen, A.I.** (1960). "Synthesis of precursor II of benzoxazolinone formed in rye plants, and the enzymatic hydrolisis of precursor I, the glucoside.". Acta Chemica Scandinavica 14: 504-507.

11. **Jernow, J.L. and Rosen, P**. (1975). "2H-1,4-Benzoxazin-3(4H)-ones". US Patent 3, 862,180.

12. **Coutts, R.T. and Hindmarsh, K.W.** (1966). "4-Benzoxazine hydroxamic acids and related compounds". Journal of Pharmaceutical Sciences 1: 11-17.

13. **Matlin, S.A., Sammes, P.G. and Upton, R.M**. (1979). "The oxidation of trimethyl sylylated amines to hydroxamic acids". Journal of Chemical Society 10: 2481-2487.

14. **Sicker, D. and Hartenstein, H.** (1993). "A new general approach to 2-hydroxy-2H-1,4-benzoxazin-3(4H)-one skeleton via diisobutylaluminum hydride reduction of 2,3-dioxo-1,4-benzoxazines". Shynthesis: 771-772.

15. **Hartenstein, H. And Sicker, D.** (1994). "α-hydroxylation of cyclic hydroxamic acids by peroxide oxidation: a novel approach to allelochemicals from Gramineae". Tetrahedron Letter 35: 4335-4338.

16. **Bonnington, L., Eljarrat, E., Guillamon, M., Eichhorn, P., Taberner, A., et al.** (2003). "Development of a liquid chromatography-electrospray-tandem mass spectrometry method for the quantitative determination of benzoxazinone derivatives in plants". Analytical Chemistry 75 (13): 3128-3136.