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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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

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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-

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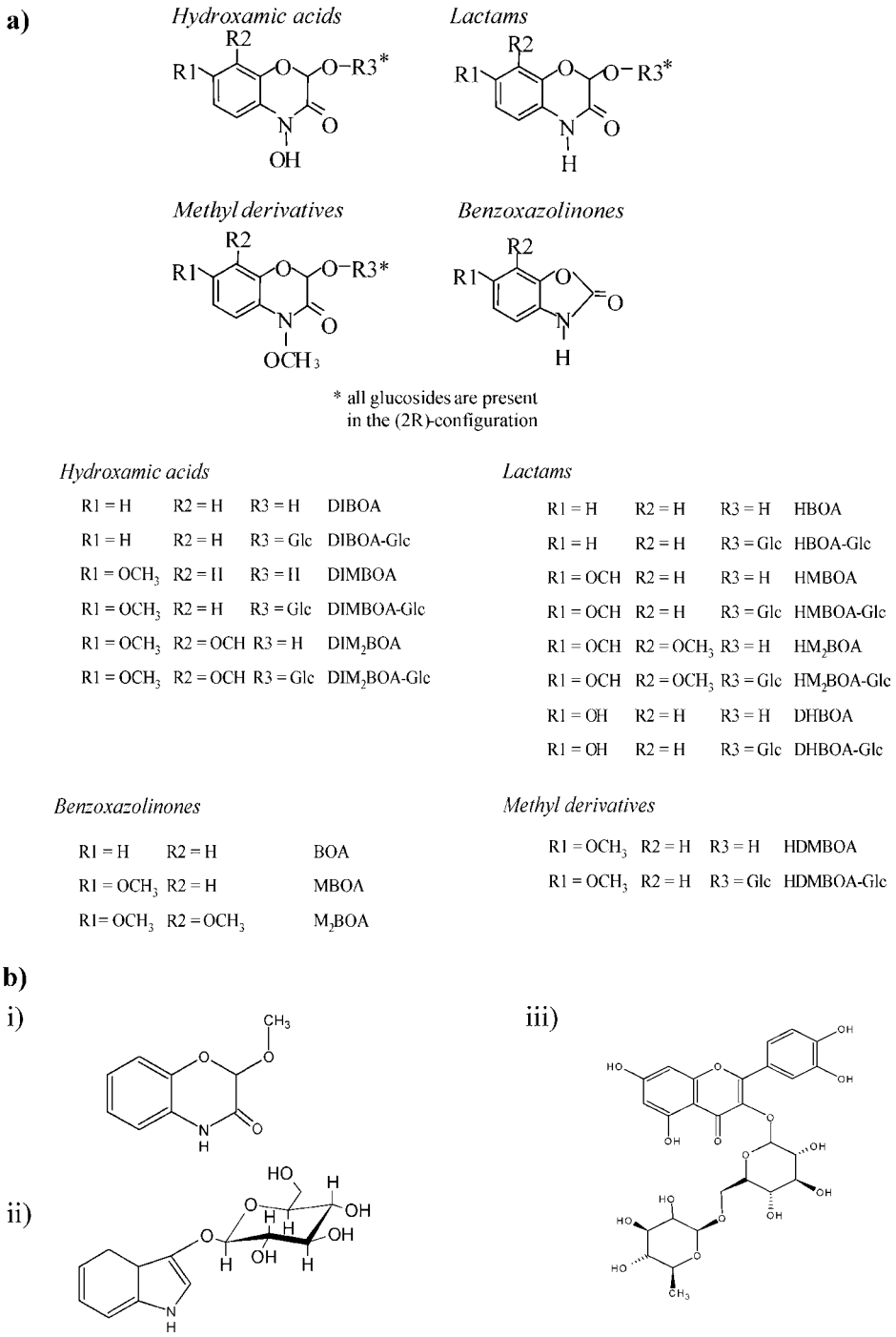

Figure 1. Structures of (**a**) benzoxazinone derivatives [adapted from Cambier et al. (*5*)] and (**b**) internal standards: (i) 2-MeOH-HBOA; (ii) indoxyl-β-D-glucoside; (iii) quercetin-3-*O*-rutinoside.

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.

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

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.

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

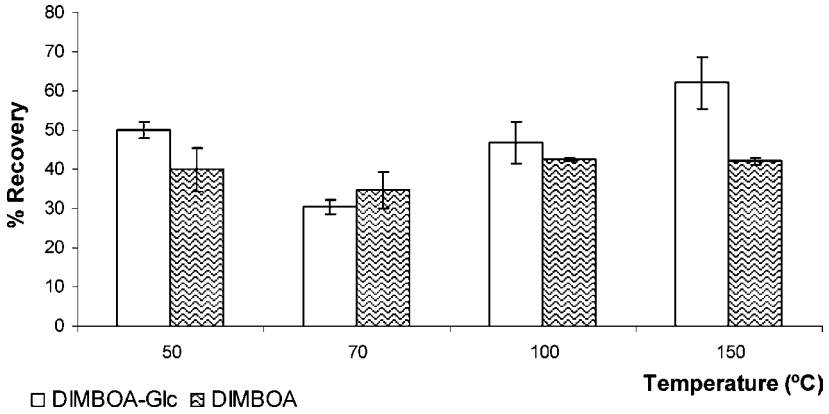

Figure 2. Recoveries and coefficient of variation ($n = 3$) of DIMBOA-β-D-glucoside and DIMBOA at different temperatures tested during PLE.

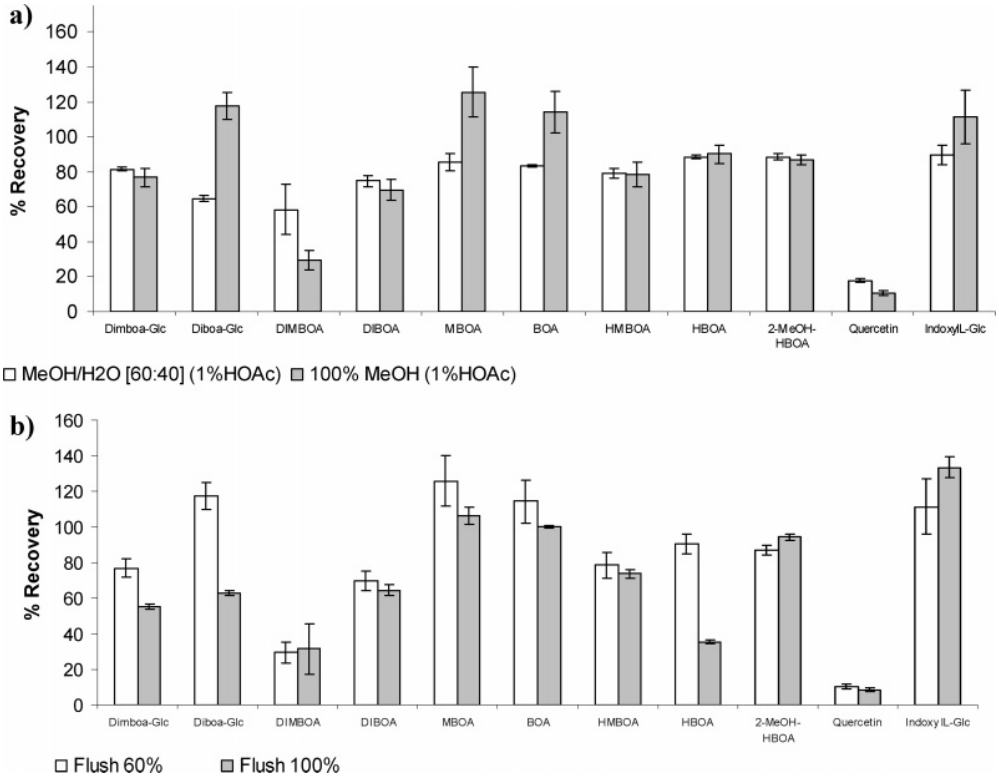

Figure 3. Recoveries and coefficient of variation ($n = 3$) of target compounds after PLE with different (**a**) solvent extractors and (**b**) flush (%).

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 ($\sim$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 (%RSD)	MeOH solution	10	9	2	3	3	4	6	5
	foliage	1	3	6	5	3	9	1	6
	root	6	12	2	5	6	14	5	5
sensitivity LOD (μg/g)	foliage	11	11	9	8	10	8	1	1
	root	6	14	27	2	3	6	2	1
sensitivity LOQ (μg/g)	foliage	36	37	31	27	34	27	3	5
	root	21	47	89	8	9	4	5	3

[a] Analysis of fractions 1 and 2.

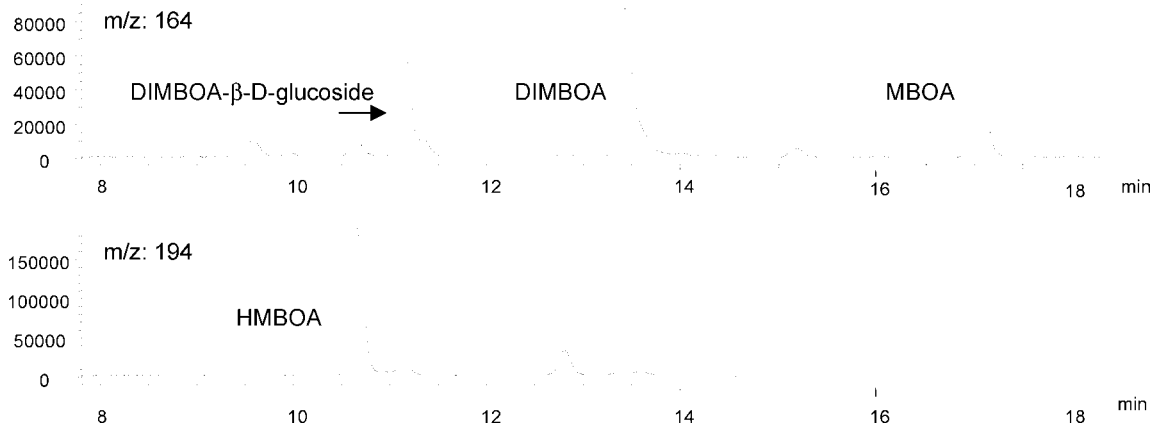

Figure 4. LC-MSMS analysis of root samples (Stakado variety).

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.

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