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Development of a Liquid Chromatography-Electrospray-Tandem Mass Spectrometry Method for the Quantitative Determination of Benzoxazinone Derivatives in Plants

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A new method for the quantification of benzoxazinone derivatives in extracts of wheat foliage and root samples using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS-MS) is described. Using this method, the characterization, separation, and quantitative detection of a mixture of six naturally occurring 1,4-benzoxazin-3(4*H*)-one derivatives, including the hydroxamic acids (DIMBOA, DIBOA), lactams (HBOA, and HMBOA), benzoxazilinones (BOA, MBOA), and two synthetic methoxylated variations of DIBOA and HBOA, was achieved. The application of a novel, highly modified reversed-phase LC column, the dodecyl (C_{12}) TMS end-capped Synergi MAX-RP, enhanced the on-line chromatographic separation through improvements to component resolution, analyte stability and peak shape and also to the column lifetime. The complete ESI-MS-MS precursor−product ion fragmentation pathways for the benzoxazinone derivatives are described for the first time and used to deduce a generic fragmentation pattern for the compound class. Characteristic transitions for the benzoxazinones were thus used in the developed analytical method enabling reliable quantification with simultaneous screening for other potentially present derivatives, while eliminating interferences from other coeluting contaminants from the complex plant extract matrix. Quantitative analysis was done in the multiple reaction monitoring mode, using two specific combinations of a precursor−product ion transitions for each compound. The ESI-MS-MS detection method offered improvements to the sensitivity and selectivity, as compared with previously applied LC methods, with detection limits down to 0.002−0.023 ng/μL. The developed method was demonstrated by analyzing foliages and roots of six different wheat cultivars using pressurized liquid extraction-solid-phase extraction clean-up-LC-ESI-MS-MS. The analytes were detected in the range of 0.7−207 μg/g of dry weight.

The allelopathic ability of members of the cereal family toward 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 benzoxazinone class in particular (Figure 1) are implicated. These compounds are present in the plants as the relatively nontoxic glucoside derivatives.[4,5] Upon injury of the plant, enzymatic deglucolysation occurs to release biologically active aglucones.[6] Further conversion of these compounds occurs to give the benzoxazolinones,[7] although it is still unclear as to whether this occurs naturally in the plant in vivo or whether they are experimental artifacts.[8]

During the 1980s and 1990s, several procedures for the separation and quantification of benzoxazinone derivatives in plant extracts were developed. To date, octadecyl-modified reversed-phase (RP-C_{18}) liquid chromatography (LC) coupled with UV detection has been the most broadly applied technique for the analysis of benzoxazinones;[1,9−15] however, the low sensitivity and lack of specificity of this method demands a more advanced

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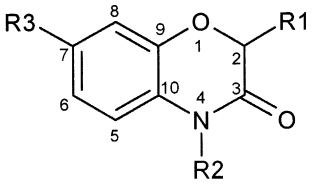

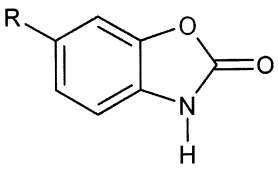

Naturally occurring Derivatives:

Glucosides (R1 = Glc)

R2 = OH R3 = H DIBOA-Glc

R2 = OH R3 = OCH_3 DIMBOA-Glc

R2 = H R3 = H HBOA-Glc

R2 = H R3 = OCH_3 HMBOA-Glc

Hydroxamic acids (R2 = OH)

R1 = OH R3 = H DIBOA

R1 = OH R3 = OCH_3 DIMBOA

Lactams (R2 = H)

R1 = OH R3 = H HBOA

R1 = OH R3 = OCH_3 HMBOA

Benzoxazolinones

R = H BOA

R = OCH_3 MBOA

Synthetic Derivatives:

Hydroxamic acids (R2 = OH)

R1 = OCH_3 R3 = H 2′OMe-DIBOA

Lactams (R2 = H)

R1 = OCH_3 R3 = H 2′OMe-HBOA

Figure 1. Structures of selected benzoxazinone derivatives.

analytical tool. Gas chromatography (GC)-flame ionization detection,[16] GC/MS[17,18] and GC/MS−MS[19] methods have also been applied. The GC/MS approaches have the advantage of high sensitivity and selectivity, and the existence of mass spectra libraries for screening of unknown samples, but derivatization is required prior to analysis. Lengthy sample preparation steps renders the technique unfavorable for analysis of large numbers of samples.

To overcome the LC−UV limitations, the use of LC−MS has clear advantages. This approach was recently tested for determining benzoxazinones in maize.[20] Spectra were acquired using atmospheric pressure chemical ionization in negative ion mode. The application of LC on-line with MS−MS detection methods to the analysis of environmental samples potentially offers many advantages over other quantitative methods in use, such as reduced sample preparation, improved detection limits, and increased specificity and selectivity. Qualitative MS−MS of glucosylated derivatives has been previously reported;[8] however, the described quantification was performed by on-line UV detection and only limited information regarding the quantitative potential of the MS technique was provided. In this paper, the MS−MS method is extended to the complete characterization and quantitative detection of a range of aglucone derivatives, including DIMBOA, DIBOA, HBOA, HMBOA, BOA, and MBOA, and selected synthetic derivatives (2-methoxylated DIBOA (2-MeO-DIBOA) and HBOA (2-MeO-HBOA)). A selective quantitative method using MS−MS was sought with the aim to develop a method that would eliminate interferences from coeluting impurities from plant extracts, provide detection of known analytes, and also act as a diagnostic tool for other potentially present derivatives. This would offer the benefit of reducing sample analysis time, by eliminating the need for additional qualitative scan

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analyses. However, to achieve reliable quantification, it was essential that adequate separation of the analytes also be achieved.

The objective of this work was to develop a reliable and reproducible quantitative LC−MS−MS method, suitable for the analysis of benzoxazinone derivatives from plant extracts. A second objective was to develop an advanced chromatographic separation method capable of resolving of a broad range of derivatives. Several reversed-phase LC columns were thus evaluated for their potential. To our knowledge, this is the first paper that has used a LC−MS−MS method for the quantitative determination of benzoxazinone derivatives in plants.

EXPERIMENTAL SECTION

Standards and Reagents. The benzoxazinone standards were obtained from commercial and private sources as available. DIBOA, HBOA, and HMBOA and the nonnaturally occurring synthetic derivatives 2-MeO-DIBOA and 2-MeO-HBOA were purchased from Prof. Dr. Sicker (University of Leipzig). DIMBOA was received from Dr. S. Chilton (University of North Carolina) and MBOA from Dr. F. Macias (University of Cadiz). BOA was purchased from Sigma Aldrich.

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

Plant Samples. Six different wheat varieties were grown in field experiments in Lleida, Spain, using conventional cultivation conditions. Plants were harvested 47 days after sowing. The same day, roots were washed under running water and the samples were then frozen and stored at −20°C until further manipulation.

Excess water was removed from the frozen wheat plants by lyophilization for 24 h. The benzoxazinone 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.[21] Briefly, 0.1 g of lyophilized root or foliage was spiked with internal standards (2-OMe-DIBOA and 2-OMe-HBOA). Samples were 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. Solvent composition, temperature, number and time of static cycles, cell preheat time, and use of N_2 purge during the cell preheat were optimized. Final optimal extraction conditions for benzoxazinone extraction 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 benzoxazinones were eluted with 5 mL of MeOH/acidified H_2O (60:40). Recoveries of the method were calculated by spiking experiments, and ranged from 72 to 89%.

Chromatographic Conditions. Analyses were performed on a Waters 2690 series Alliance HPLC (Waters, Milford, MA) with a quaternary pump equipped with a 120-vial capacity sample management system. A Synergi MAX-RP 80A LC column (250 × 4.6 mm, 4 μm, Phenomenex) with a solvent flow rate of 1 mL/min was used. The system was incorporated with a zero volume T solvent splitter such that 0.2 mL/min was directed to MS and 0.8 mL/min to the waste (with the on-line UV detection prior to the solvent split). The sample injection volume was set at 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, 70% A; 2−19 min, 70−40% A; 19−21 min, 40−0% A; 21−23 min, 0% A; 23−25 min, 0−70% A; 25−38 min, 100% A. Total run time was 38 min with the benzoxazinones eluted over 5−19 min and the final 19 min used for column cleaning and regeneration. Detection of the analytes was carried out by UV using a Waters model 996 photodiode array detector over the range 250−440 nm.

In the method development phase, the LiChrospher 100, Purospher STAR 100, Superspher 100 RP C_{18} (Lichrocart), Nucleosil RP C_{18} obtained from Merck (Darmstadt, Germany), and Ultracarb RP C_{30} obtained from Phenomenex (Jasco Analytica, Barcelona, Spain) LC columns were also investigated.

Mass Spectrometry Conditions. A benchtop triple quadrupole mass spectrometer Quattro LC from Micromass (Manchester, U.K.) equipped with a pneumatically assisted electrospray probe and a Z-spray interface was used for the MS−MS analyses. Nitrogen gas (99.999% purity) was used as the desolvation and cone gas and argon as the collision gas (3.6×10^{-5} mbar). Parameter optimization was performed by continuous infusion of a standard solution (1 μg/mL) via a syringe infusion pump (Kd Scientific, model 100) at a constant flow rate of 200 μL/min. All data were acquired and processed using Masslynx V3.5 software.

Parameters for the quantitative multiple reaction monitoring (MRM) mode method for the benzoxazinone derivatives were optimized using results from precursor ion and product ion scans in flow injection analysis-electrospray ionization (ESI)-MS-MS mode. The samples were introduced to the analytical system via a syringe infusion pump operating at 0.1 mL/min. The overall solvent flow reaching the ESI interface was supplemented to 0.2 mL/min using a zero-volume T solvent splitter and makeup flow of 0.1 mL/min (50:50 MeOH/acidified H_2O) from the LC pump. Quantitative LC−MS−MS analysis was carried out in MRM mode and was confirmed by on-line UV detection using a Waters model 996 photodiode array detector over the range 250−440 nm. The ESI-MS was operated in negative ion mode with the following instrument settings: capillary voltage, 2.8 kV; source temperature, 150 °C; desolvation temperature, 350 °C; desolvation gas flow rate, 600 L/h; cone gas flow rate, 50 L/h; extractor 7V; rf lens, 0.6 kV; multiplier, 650; ion energy, 1.0 V; entrance, −2; exit energy, 1; resolution, 12.0; interchannel delay, 0.02 s; total scan time, 1.01 s. Two time windows (5−13.5 and 13.5−19 min) for the selected transitions (eight per window), with two transitions for each analyte were selected. Primary transitions were used for quantification and the secondary transitions for confirmation. Optimized cone voltages, collision energies, and dwell times for each transition were used, as described in subsequent sections.

RESULTS AND DISCUSSION

In the analysis of complex environmental samples, such as plant extracts, interferences from coeluting impurities can be significant and can often preclude reliable quantification.[10,19] To alleviate the matrix effects observed in the analysis of samples

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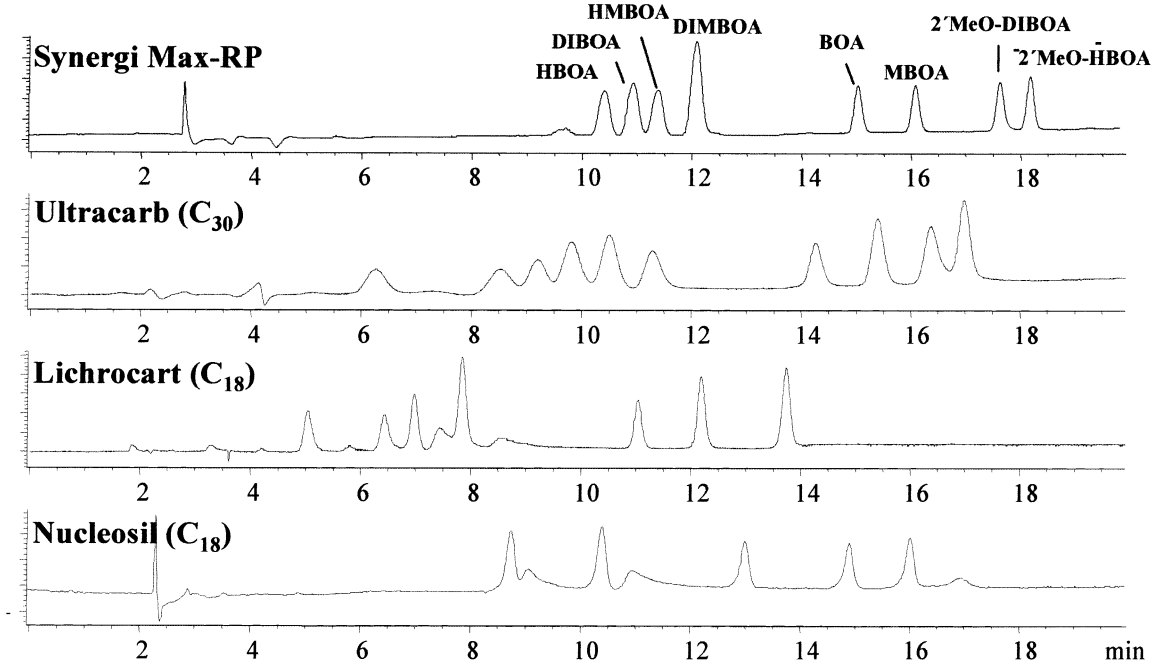

Figure 2. Comparison of LC separation of benzoxazinone derivatives on different RP columns.

by more conventional LC−UV methods, a MRM-MS-MS method was developed. The benzoxazinone derivatives are known to exhibit relatively high chemical instability, and this property also translates to their behavior under MS conditions, with a high level of fragmentation observed. This could be used beneficially in the development of a tandem MS quantification method with high responses obtained for the selected ion transitions.

LC Method Optimization. Previously peer-reviewed published literature was used as the basis for the initial LC method development investigations.[10−15,22−24] However, several problems were encountered due to low stability of the hydroxamic acid analytes on the LC columns investigated, compromised integrity of the LC columns under the thus required acidic conditions, and retention time shifts and adverse effects on peak intensities due to coelution of impurities. These latter two factors could only be diagnosed by use of the selective MS detection method, through which the observed peaks could be correctly assigned for quantification. UV detection is commonly applied to benzoxazinone quantification; however, parallel analysis with MS performed here demonstrated that the unspecific UV detection method was not an appropriate quantitative technique for plant extracts as it was not capable of determining adverse effects from impurities in the matrix. Using MS detection in the selected ion mode, run in parallel to UV detection (280 nm), it was observed that (a) peaks obtained in the extracts were shifted relative to the pure standards and that (b) not all peaks were due to the benzoxazinone standards (data not shown). Such retention time shifts were also observed in the analysis of mixtures of pure standards depending on the mixture composition, indicating the complex interaction of this class of compounds under LC conditions (data not shown) and demonstrating the nonapplicability of nonspecific detection methods for reliable quantification of real samples.

Significant tailing and also degradation of the chemically unstable hydroxamic acid derivatives (DIMBOA, DIBOA) was also observed under the three different Lichrocart (LiChrospher 100, Purospher STAR 100, Superspher 100) C_{18}, Nucleosil C_{18}, and Ultracarb C_{18} columns tested. A more highly modified reversed-phase column, the Synergi MAX-RP LC column (C_{12} hydrocarbon modified silica phase with TMS end-capping of the surface silanols), was thus investigated and found to give much improved results to analyte and column stability, peak shape, and matrix interference. The use of the Synergi MAX-RP column reduced the undesired interactions of the basic analytes with the bonded phase, reducing tailing and analyte degradation, and furthermore, the compatibility with acidic solvents over a broad pH range eliminated the problems with column stability under the acidic conditions required for hydroxamic acid analysis. A comparison of the LC separation obtained for mixtures of benzoxazinone standards on the different RP columns is shown in Figure 2. Using the Synergi MAX-RP column, chromatographic separation of the naturally occurring allelochemicals DIBOA, DIMBOA, HBOA, HMBOA, MBOA, and BOA, the glucosides of DIBOA and DIMBOA (data not shown), and also two synthetic derivatives, 2-MeO-DIBOA and 2-MeO-HBOA, could be achieved enabling the development of a reliable LC−MS−MS quantification method.

MS−MS Method Optimization. The complete precursor−product fragmentation pathways observed for the benzoxazinone derivatives and the optimal conditions for the transitions, as determined by product ion and precursor ion scans, are given in Table 1. The assignments for these transitions are also shown, which was achieved using comparison with ESI-time-of-flight MS results (discussed in further detail elsewhere),[25] comparison between the structural analogues, and application of knowledge of feasible structures for the possible structures. From the observed transitions, several analogies in the fragmentation for the range of derivatives investigated could be elucidated, and this information has been summarized into a generic fragmentation scheme for the benzoxazinones, as presented in Figure 3. In particular, the MS−MS precursor−product ion data results obtained here provided information regarding the sequence of ion

Table 1. MS−MS Transitions and Optimal Conditions As Determined by Product Ion and Precursor Ion Scans

compound (MW)	precursor−product ion fragmentation pathway	precursor ion (m/z)	product ion (m/z)	cone voltage (V)	collision energy (eV)
DIMBOA (211)	194>166>138>123	194	166	15	8
	[M−OH^+>−CO>−CO>−•CH_3]	194	138	15	12
		166	138	20	10
		138	123	25	13
	194>179>164>149>121>93	194	179	15	12
	[Mn-OH^+>−˙O+H>−CH_3>− •CH_3>− CO>−CO]	194	164	15	18
		179	164	30	15
		164	149[a]	15	13
		164	121[b]	15	20
		149	121	35	12
		121	93	50	10
	165>150>122	165	150	15	14
	[M−OH^+ −COH>−CH_3>−CO]	150	122	35	14
DIBOA (181)	134>91 [M−OH^+−CHOH>−CO_2+H]	134	91[b]	35	20
	134>78 [M−OH^+−C^2HOH>−2CO]	134	78[a]	35	18
	108>107 [M−C^2OH−CO−O>−•H]	108	107	40	18
BOA (135)	134>91 [M−H^+>−CO_2+H]	134	91[b]	35	18
	134>78 [M−H^+>−2CO]	134	78[a]	35	18
MBOA (165)	164>149>121	164	149[a]	15	13
	[M−H^+>− •CH_3>−CO]	164	121[b]	15	20
		149	121	35	12
HBOA (165)	164>136>108>107	164	136[c]	15	8
	[M−H^+>−CO>−CO>−•H]	164	108[a]	15	15
		136	108	20	10
		136	107	20	24
		108	107	40	15
	108>91 [M−H^+−2CO>−•OH]	108	91	40	15
	108>90 [M−H^+−2CO>−H_2O]	108	91	40	15
	164>134 [M−H^+>- HC^2OH]	164	134	15	20
	164>118>90	164	118	15	12
	[M−H^+>-H_2O−CO>−CO]	118	90	35	15
HMBOA (195)	194>179>150>122	194	179[b]	15	12
	[M−H^+>−O+H>−2CH_3+H>−CO]	179	150	30	10
		150	122	45	10
		179	122	30	18
	179>123 [M−O^+>−•CH_3−CHCO]	179	123	30	16
	194>138>123>122	194	138[a]	15	13
	[M−H^+>−2CO>− •CH_3>−H]	194	123	15	20
		194	122	15	30
		138	123	30	13
		138	122	30	22
	194>166>123>122	194	166	15	10
	[M−H^+>−CO>−CO− •CH_3>n-H]	166	123	20	16
		166	122	20	25
	194>148>133	194	148	15	11
	[M−H^+>−H_2O−CO>− •CH_3]	194	133	15	15
		148	133	25	12
2-OMe-DIBOA (195)	194>134 [M−H^+>−OCH(OCH_3)]	194	134	15	8
	194>122>92	194	122[b]	20	16
	[M−H^+>−CH(OCH_3)−CO>-NO]	122	92	45	14
	194>118>90	194	118[a]	20	10
	[M−H^+>−O−OCH(OCH_3)>−CO]	118	90	40	15
2-OMe-HBOA (179)	178>118>90	178	118[a]	25	14
	[M−H^+>−OCH(OCH_3)>−CO]	118	90[b]	40	15
	166>134 [M−CH^+>−H−OCH_3]	166	134	40	18

[a] Highest intensity transition. [b] Second highest intensity transition. [c] Highest selectivity transition.

formation and, therefore, structural confirmation. Furthermore, several new fragments were observed, namely, the ions at m/z 179 (5a; R3 = OCH_3, R4 = O•, R5 = CH_3; DIMBOA), 166 (3; R1 = OH, R3 = OCH_3; DIMBOA), 138 (4d; R = OCH_3; DIMBOA), 123 (4d; R = O•; DIMBOA), 93 (6; R = O•; DIMBOA), 164 (1a; R1 = OH, R3 = H; DIBOA), 91 (4a; DIBOA, BOA, HBOA), 78 (6; R = H; DIBOA), 90 (7; HBOA), 166 (5a; R3 = H, R4 = OH, R5 = OCH_3; 2-OMe-HBOA), and 92 (4f; 2-OMe-DIBOA), for which complete assignment has been achieved (see Figure 3 for structures). The 2a → 4a, 4b → 6, 3/5b → 4d, and 1b → 4e → 4f fragmentations were also observed for the first time.

The selected ion transitions used for the quantitative LC-ESI-MS-MS method are indicated in Table 2. These ions were selected according to highest sensitivity where possible but also according to optimal selectivity for the analyte of interest and for the benzoxazinone class in general in order to provide a simultaneous screening procedure for other derivatives that may be present. The selection of unique transitions for a compound for use in

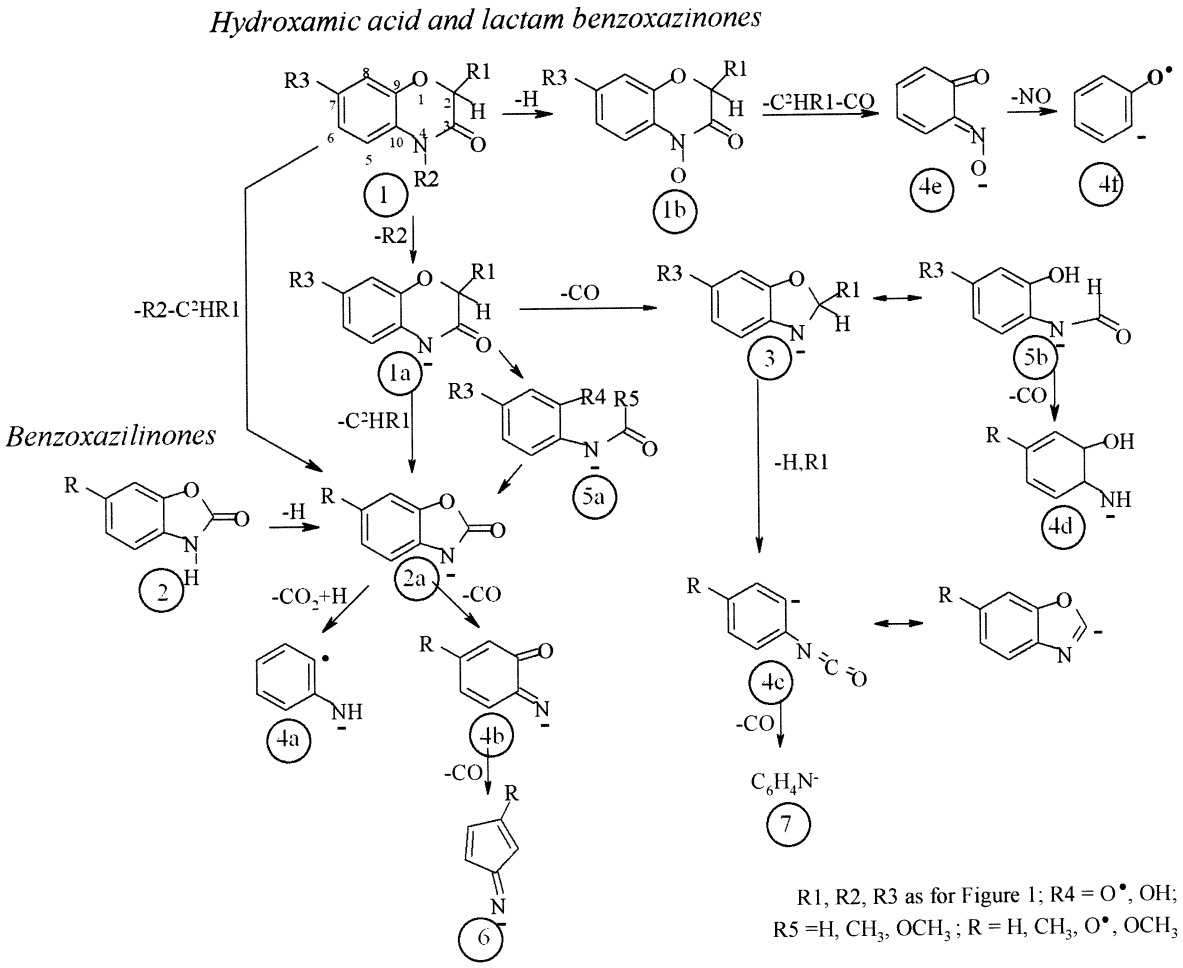

Figure 3. Generic benzoxazinone fragmentation pattern as determined by ESI-MS-MS under negative ionization mode.

Table 2. Transitions and Conditions Used for Quantification by MS−MS (MRM Mode)

compd	MRM first transition (quantification)				MRM second transition (confirmation)				
	precursor → product (m/z)	cone (V)	collision (eV)	dwell (s)	precursor → product (m/z)	cone (V)	collision (eV)	dwell (s)	ratio[a]
HBOA	164 → 136	15	8	0.1	164 → 108	15	15	0.05	4.95
DIBOA	134 → 78	35	18	0.2	134 → 91	35	20	0.2	1.87
HMBOA	194 → 138	15	13	0.05	194 → 179	15	12	0.1	6.08
DIMBOA	164 → 149	15	13	0.05	164 → 121	15	20	0.1	4.36
BOA	134 → 78	35	18	0.17	134 → 91	35	18	0.17	1.81
MBOA	164 → 149	15	13	0.05	164 → 121	15	20	0.05	3.73
2-MeO-DIBOA	194 → 118	20	10	0.12	194 → 122	20	16	0.12	
2-MeO-HBOA	178 → 118	25	14	0.12	118 → 90	40	15	0.05	

[a] Ratio = MRM first transition/MRM second transition.

quantitative studies is often ideal in order to avoid interference caused by possible coeluting impurities. Where possible, unique transitions for compound quantification were sought; however, due to analogous fragmentation patterns for the different derivatives, as demonstrated in Figure 3, and the often weak response of the unique precursor molecular ions ($[M - H]^-$/$[M - OH]$), as evident from the data presented in Table 1, this was not possible for many of the derivatives. As such, generic transitions based on the strongest intensity fragments were selected in most cases. For example, the m/z 164−149 and 164−121 transitions, involving the loss of a methoxy radical and subsequently a CO moiety, are common fragmentations for the methoxylated derivatives DIMBOA and MBOA. The transitions selected for the non methoxylated derivatives DIBOA and BOA—m/z 134−78 and 134−91—were also analogous and correspond to losses of 2CO moieties and CO_2, respectively.

The advantages of using generic fragmentations common to the benzoxazinone class, however, are manyfold. The quantitative procedure thus functions simultaneously as a screening procedure for other potentially present derivatives, while avoiding interference from other coelutants from the matrix, and offers improved detection limits through the monitoring of the more sensitive

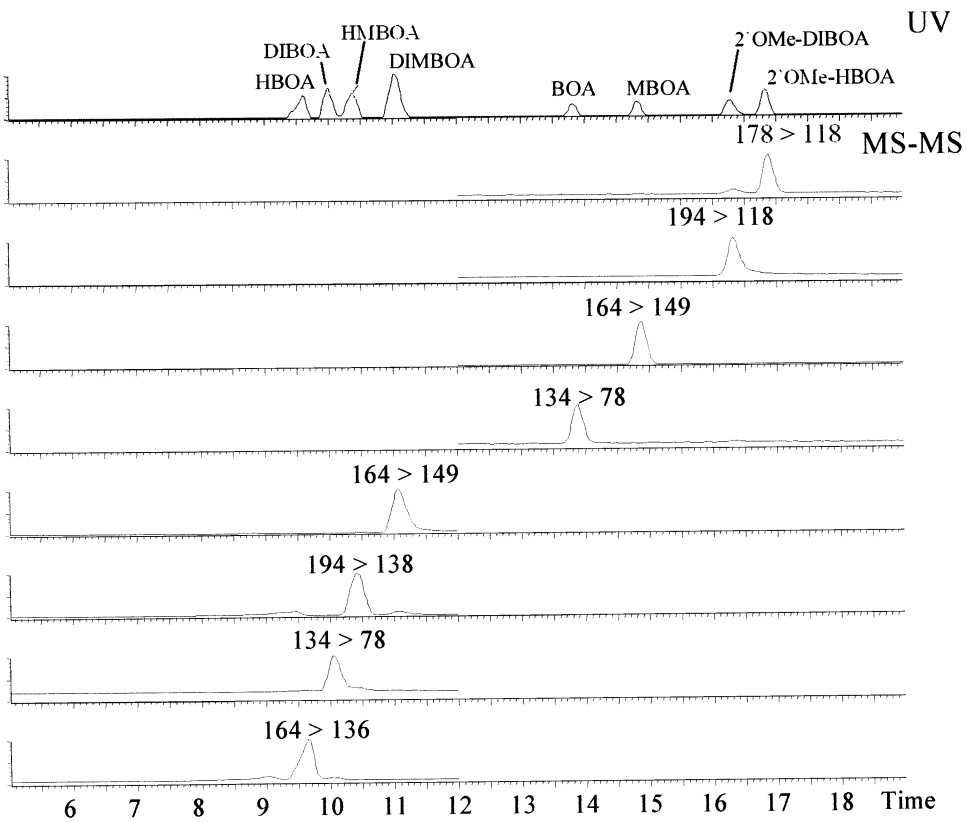

Figure 4. LC−UV (280 nm) and LC-ESI-MS-MS chromatograms of benzoxazinone derivatives showing the different ion transitions used for quantification.

transitions. Furthermore, the use of generic precursor−production fragmentations reduces the need for additional analysis in scan mode for screening purposes and, thus, offers potential savings in time and costs involved in sample analysis. However, to maximize selectivity while maintaining the high sensitivity, an advanced chromatographic separation system was thus required in order to adequately resolve the different analogues.

The sensitivity of the detection method was enhanced by the selection of transitions of high intensity, as discussed earlier. Varying dwell times were also used to increase sensitivity, due to the variation in responses observed for the different transitions and analytes. In particular, the transitions involving the m/z 134 ion (nonmethoxylated DIBOA and BOA derivatives) were given longer dwell times (0.17−0.2 s) due to the higher relative stability of this ion, which thus yielded lower relative responses. In contrast, the m/z 164 ion fragmentations (DIMBOA, MBOA, HBOA) gave higher responses and thus were scanned over shorter dwell times (0.05−0.1 s).

To further increase sensitivity, two time windows for the MRM analysis were also used. The eight selected transitions for HBOA, DIBOA HMBOA, and DIMBOA were monitored over the 5−13.5-min period (retention times (RT) = 10.5, 11.1, 11.5, and 12.4 min, respectively), and those for BOA, MBOA, 2-MeO-DIBOA and 2-MeO-HBOA were monitored over 13.5−19 min (RT = 15.0, 16.1, 17.7, and 18.0 min, respectively) as can be seen in the chromatograms shown in Figure 4.

Quantification Method. Quantification was performed by external and internal standard methods. External standard quantification was performed by comparison of responses with calibration curves obtained from solutions of pure standards, as is the commonly applied method for the LC quantification of these compounds. An example of the calibration curve data obtained for the m/z 164−149 ion transition for MBOA is shown in Figure 5. The data presented spans a concentration range of three powers of magnitude (0.005−5 ng/μL) with high degree of correlation (regression coefficient, $R^2 = 0.998$). Further improvements to the trend line coefficient were also possible using curves generated over smaller concentration ranges (e.g., 0.25−5 ng/μL, R^2 = 0.999), which can be applied in practice according to sample concentration. However, even the responses for the solutions over the four powers of magnitude (0.001−10 ng/μL) also still gave good correlation ($R^2 = 0.992$).

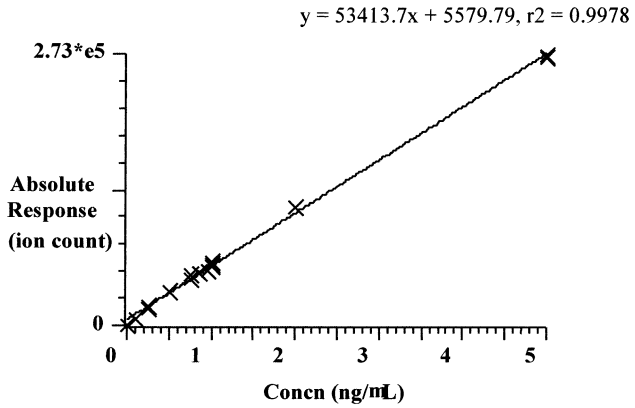

Figure 5. Calibration curve for MBOA as determined by LC-ESI-MS-MS, using the external standard method. (Data obtained for *m/z* 164 to 149 ion transition.)

The use of internal standards to aid reliable quantification has not been described previously for LC determinations of benzox-

Table 3. Instrumental Detection Limits (LOD_{inst}) and Method Detection Limits (LOD_{method}) of Combined PLE-SPE-LC-MS-MS for the Analysis of Target Compounds in Foliage Samples

	LOD_{inst}			LOD_{method} (foliage)			
compound	ng/μL	ng	pmol	μg/g of dw[a]	nmol/g of dw[a]	μg/g of ww[b]	nmol/g of ww[b]
HBOA	0.004	0.2	1.3	0.2	1.3	0.9	5.3
DIBOA	0.023	1.1	6.3	1.1	6.3	4.5	25.1
HMBOA	0.003	0.1	0.7	0.1	0.7	0.6	2.8
DIMBOA	0.003	0.2	0.8	0.2	0.8	0.7	3.1
BOA	0.010	0.5	3.9	0.5	3.9	2.1	15.5
MBOA	0.002	0.1	0.6	0.1	0.6	0.4	2.5

[a] dw, dry weight. [b]ww, wet weight (estimated value calculated according to observed average mass loss of 75% upon lyophilization).

azinones. The only quantitative method reporting the use of an internal standard for the analysis of these compounds used the naturally occurring degradation product BOA as the internal standard.[20] As this product can potentially occur in samples, it cannot be considered as an appropriate internal standard. Here, nonnaturally occurring structural analogues of the benzoxazinones, 2-OMe-DIBOA and 2-OMe-HBOA, with adequate separation from the naturally occurring derivatives, were used as internal standards. Good correlations were obtained for all analytes relative to these compounds. For example, the calibration curve regression coefficients for the primary selected transition of MBOA relative to that of 2-OMe-DIBOA and 2-OMe-HBOA were 0.994 and 0.983, respectively, for the 0.005−5 ng/μL range, with improvements up to 0.998 obtained for the latter (MBOA/2-OMe-HBOA) with selection of narrower concentration ranges (0.2−2 ng/μL). These results confirmed the applicability of the selected internal standards for quantification, and this method was finally selected for the quantitative analyses in plant samples.

Plant extract can be rather complex and coelution may exist. Matrix-induced interference resulting in suppression of signals of target analytes in LC−MS were well reported. In this sense, the developed method included a purification step via LiChrolut RP C_{18} SPE cartridges, allowing the injection of clean extracts in the LC−MS−MS system. Moreover, the use of selected internal standards (2-OMe-DIBOA and 2-OMe-HBOA) could aid to detect any suppression of analyte signals.

Method Validation. Positive identification criteria of the target compounds was based on the following: (a) LC retention time of the analyte compared to that of a standard (±2%); (b) the ratio of abundances of two specific precursor ion → product ion transitions (within 10% of the ratios obtained for the standard).

The instrumental detection limits (LOD_{inst}) and method detection limits (LOD_{method}) obtained using LC−MS−MS in MRM mode are shown in Table 3. The values are presented in different units in order to enable comparison with other LODs reported in the literature. The LODs were based on the peak-to-peak noise of the baseline near the analyte peak obtained by analyses of a standard solution (LOD_{inst}) and spiked real samples (LOD_{method}), respectively, and on minimal value of signal-to-noise ratio of 3. The LOD_{method} refer to the entire PLE-SPE-LC-MS-MS procedures for plant samples.

Values of LOD_{inst} obtained by LC−MS−MS were much improved, between 10- and 1000-fold more sensitive over those reported previously by other methods, although only a limited number of reports have described this parameter for their investigations. The LOD_{inst} reported in the literature include the following (detection methods used indicated in parentheses): 0.5 ng/μL for HBOA, and 1 ng/μL for DIMBOA and MBOA (UV);[14] 0.3 μg for BOA and 0.5 μg for MBOA (UV);[26] 5 nmol for HBOA and DIBOA (GC);[7] 50 pmol for DIBOA, DIMBOA and MBOA (UV);[12] and 200 pmol for DIBOA[27] and DIMBOA (UV).[15,27] The use of GC/MS and GC/MS−MS has been described as improving the sensitivity; however, no specific data were provided.[19]

Regarding the LOD_{method} values obtained in this study, corresponding values described elsewhere are between 50 and 500 times less sensitive and include the following: 1.25 μmol/g of fresh weight for a broad range of glucoside benzoxazinone derivatives (UV)[28] and 1 μmol/g of fresh weight for DIBOA and DIMBOA (UV).[22,29]

A solvent split prior to the MS analysis was incorporated such that only 20% of the sample was directed to the detector. Under this configuration, all samples and standards were well within the range of the MS detection. On-line UV detection was performed prior to the split, thereby increasing the detection limit of the less sensitive UV method. The advantage of this configuration was that both the UV and MS detection could be optimized. Higher concentration solutions were determined by UV and then a lower concentration portion was directed to the more sensitive MS detector, ensuring that samples were in the linear range of the instrument and avoiding unnecessary contamination of the MS source. This latter point is especially important when a large sample throughput is required for extensive investigations. All analytes in real samples thus far analyzed were found at concentrations amenable to the split MS system; however, it is possible that omitting the split system by incorporating a smaller column and lower flow rates may decrease the detection limits for lower level analytes and this could be incorporated in future investigations if necessary.

Application to Plant Analyses. The optimized method for MS−MS analysis was applied to samples of wheat foliage and root extracts. At the scale of sampling used in this study, the protocol

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Table 4. Method Precision ($n = 3$) of Wheat Foliage Sample As Determined by PLE-SPE-LC-MS-MS[a]

	HBOA	DIBOA	HMBOA	DIMBOA	BOA	MBOA
mean value	3.27	6.37	7.68	8.01	3.74	82.46
SD	0.168	0.994	0.134	1.390	0.266	4.908
% SD	5.14	15.60	1.74	17.35	7.11	5.95

Table 5. Quantification of Allelochemicals and Metabolites in Roots and Foliages of Different Wheat Varieties Using PLE-SPE-LC-MS-MS[a,b]

	HBOA	DIBOA	HMBOA	DIMBOA	BOA	MBOA
foliage						
1	<LOD	<LOD	4.29	<LOD	<LOD	27.92
2	<LOD	<LOD	2.81	<LOQ	<LOD	29.09
3	<LOD	<LOD	12.08	<LOD	<LOD	37.19
4	<LOD	<LOD	12.54	1.84	<LOD	38.05
5	<LOQ	<LOD	63.00	78.89	<LOD	173.85
6	<LOD	<LOD	19.15	18.90	<LOD	86.18
roots						
1	<LOD	<LOD	3.08	4.03	<LOD	89.67
2	<LOD	<LOD	3.77	0.58	<LOD	80.39
3	<LOQ	<LOD	12.87	2.06	4.74	139.08
4	<LOD	<LOD	12.15	0.46	0.70	117.15
5	<LOQ	<LOD	32.80	15.19	<LOD	206.75
6	<LOD	<LOD	16.82	3.76	<LOD	127.59

[a]Values expressed in μg/g of dry weight. [b] <LOD, below limit of detection; <LOQ, below limit of quantification.

required to ensure stability of the glucoside derivatives (immediate workup) as described in the literature[8,9] was not possible. Deglucolysation occurs rapidly and can only be avoided by immediate deactivation of the enzymes responsible. As such, considerable degradation of the glucosides and aglucones in the samples obtained here could be expected.

Method precision was checked by analyzing one foliage sample in triplicate. A sample with detectable amounts of all six naturally occurring benzoxazinone derivatives was selected. The mean values obtained, as well as the associated standard deviations, are presented in Table 4. As can be seen, satisfactory results were obtained for HBOA, HMBOA, BOA, and MBOA, with relative standard deviation below 10%. For DIBOA and DIMBOA, higher standard deviations were found, with values of 16 and 17%, respectively.

The quantified results obtained for the foliage and root samples for the six different wheat cultivars are presented in Table 5. The major metabolite detected in the wheat foliage and root extracts was MBOA in all cases. The detected levels of MBOA were in the range of 27−207 μg/g of dry weight. The content in roots exceeded that found in foliage for all samples analyzed. Further presentation and discussion of the sample preparation and quantification results will be addressed elsewhere as these experiments are continuing.[21]

CONCLUSIONS

In this work, a new methodology for the chromatographic separation, characterization, and quantification of a broad range of benzoxazinone derivatives is described. A novel C_{12}-modified, TMS end-capped reversed-phase LC column was used in the on-line chromatographic separation, which offered improvements to component resolution, analyte stability and peak shape, and column integrity. The applicability of ESI-MS-MS methods to the characterization of these allelochemicals has been demonstrated, with generic trends determined thereby providing a diagnostic tool for unknowns in real samples. The advanced analytical method developed could thus be applied to the simultaneous quantification and screening of the allelochemicals in wheat plant extracts. The broad range of benzoxazinones that are produced by plants and the further potential transformation of these to a variety of metabolites in plant and soil environments results in a complex analyte mixture in extracts. An analytical method with the potential to detect a range of these compounds is thus a highly useful tool. The method offers significant improvements to detection limits and unequivocal identification and quantification and eliminates the adverse effects from matrix interference associated with the more conventionally applied UV detection method.

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