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Journal of Chromatography A, 1047(2004) 69–76
JOURNAL OF CHROMATOGRAPHY A
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First European interlaboratory study of the analysis of benzoxazinone derivatives in plants by liquid chromatography☆

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Received 8 March 2004; accepted 6 July 2004

Abstract

Six laboratories from four different countries participated in the first European interlaboratory comparison exercise within the framework of the "Fate and toxicity of allelochemicals (natural plant toxins) in relation to environment and consumer" (FATEALLCHEM) European Union Project. The study, organized between November 2002 and March 2003, involved the analyses of seven benzoxazinone derivatives in two standard solutions and one purified extract of root material. Results are reported from the first phase of the study that examined the variability associated with different detection methods and different laboratories. The analytical strategies were based on liquid chromatography (LC) with diode array detection, LC coupled to mass spectrometry (MS) and LC coupled to tandem MS. When data from all laboratories were pooled, the relative standard deviation values ranged from 2 to 14% for the determination of target compounds in standard solutions, and between 19 and 47% for the analysis in root material. Comparison of the three detection techniques leads to the conclusion that MS approaches are the most accurate and precise techniques for the determination of benzoxazinone derivatives at ng/μL level in plant material.

Keywords: Interlaboratory studies; Plant material; Benzoxazinones

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 benzoxazinone class in particular are implicated. These compounds are present in the plants as the relatively non-toxic 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].

During the 1980s and 1990s, several procedures for the separation and quantification of benzoxazinone derivatives

☆ Presented at the 3rd Meeting of the Spanish Association of Chromatography and Related Techniques and the European Workshop: 3rd Waste Water Cluster, Aguadulce (Almeria), 19–21 November, 2003.

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doi:10.1016/j.chroma.2004.07.004

in plant extracts were developed [8]. To date liquid chromatography (LC) coupled with ultraviolet (UV) detection has been the most broadly applied technique for the analysis of benzoxazinones. Some LC coupled to mass spectrometry (MS) methods has been developed in order to enhance sensitivity and specificity of LC–UV methods [9]. Recently, a new method for the quantification of benzoxazinone using LC-tandem mass spectrometry (MS–MS) was developed [10]. This 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.

The performance of the laboratories for benzoxazinones had not been tested until now. One of the objectives of the FATEALLCHEM European Union project (fate and toxicity of allelochemicals (natural plant toxins) in relation to environment and consumer) is to provide accurate methods for the determination of benzoxazinone derivatives in plant material. One way to achieve this purpose was to organize an interlaboratory study involving the laboratories which are carrying out benzoxazinone analyses in the project. There are six laboratories from four different countries, and they used different techniques such as LC–diode array detection (DAD), LC–MS and LC–MS–MS.

2. Materials and methods

2.1. Chemicals and materials

The benzoxazinone standards were obtained from commercial and private sources as available. DIMBOA-glc, DIBOA, HBOA, HMBOA and the non-naturally occurring synthetic derivative 2-methoxy-2H-1,4-benzoxazin-3(4H)-one (2′MeO-HBOA, SP4) were purchased Professor Dr. Sicker (University of Leipzig, Germany) (see Fig. 1 for abbreviations). DIMBOA was received from Dr. S. Chilton (University of North Carolina, USA) and MBOA from Dr. F. Macias (University of Cádiz, Spain). BOA was purchased from Sigma–Aldrich.

2.2. Description of the interlaboratory exercise

Seven allelochemicals were selected for the interlaboratory study: DIMBOA-glc, HBOA, DIBOA, HMBOA, DIMBOA, BOA and MBOA (Fig. 1). The laboratories were asked to determine the concentrations of these selected analytes in three different samples, two standard solutions and one purified extract of root material.

Five different standard solutions were prepared and 0.8 mL of each one was distributed to each participant for the preparation of the calibration curves. These solutions contained the seven selected benzoxazinones at concentration levels ranging from 0.01 to 5 ng/μL. Moreover, internal standard (SP4) was added at 1 ng/μL. Two solutions, containing DIMBOA-glc, HBOA, DIBOA, HMBOA, DIMBOA, BOA and MBOA at 0.3 ng/μL (coded *Standard 1*) and at 1.5 ng/μL (coded *Standard 2*) were prepared and ampouled (0.8 mL). Moreover, one purified extract of root material (coded *Root1*) was prepared, using the extraction and purification process proposed by Bonnington et al. [10]. Briefly, lyophilized wheat root was spiked with internal standard (SP4). Sample was extracted by pressurized liquid extraction (PLE) using an ASE 200 (Dionex, Idstein, Germany) apparatus. Extraction conditions for benzoxazinone 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) solid-phase extraction (SPE) cartridges (Merck). The benzoxazinones were eluted using MeOH–acidified water (6:4). Cleaned extract (1.5 mL) was sent to each participant.

An analytical protocol was distributed among the participants describing in detail the LC–DAD, LC–MS and LC–MS–MS methods, etc. However, each participant will decide if they will use the proposed method or own method. The description of the analytical procedures used had to be reported and are included below.

Although the emphasis of the study was on the between-laboratory agreement, five results per determinant per sample obtained on five different days (intra-laboratory variation) were required. Moreover, detection limits (LODs) obtained with each instrumental technique must be calculated and reported. A period of 3 months was given to the participants to complete this work.

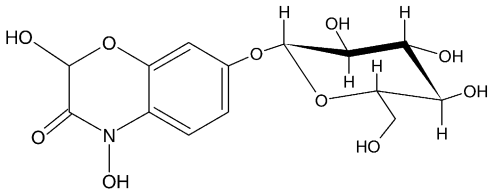
2.3. Methods used by the participants

An overview of the analytical procedures and chromatographic conditions used is given in Table 1. One laboratory was able to perform the analyses by the three different techniques, two other laboratories used the LC–MS method, and the rest of participants used the LC–DAD as instrumental technique. Regarding the chromatographic conditions, two laboratories were using the chromatographic column proposed by the coordinator of the study (Synergi MAX-RP), and two laboratories used the BDS Hypersil C_{18} column. In addition, one laboratory worked with a LiChrospher 100 RP-18, and another laboratory used the Symmetry C_{18} column. Internal calibration (using SP4 as internal standard) was used for quantification by all the participants, with the exception of laboratory 5, which used external calibration.

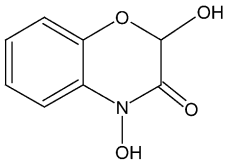
The analytical parameters for LC–MS and LC–MS–MS experiments used by laboratories 1, 2 and 3 are presented in Tables 2 and 3. Different ionization techniques were applied. In general, electrospray ionization (ESI) in mode positive or negative was selected. However, atmospheric pressure chemical ionization (APCI) was also used by laboratory 1.

Hydroxamic acids

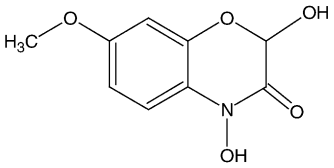
DIMBOA-Glc
2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one glucoside



DIBOA
2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one

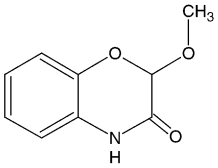


DIMBOA
2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one



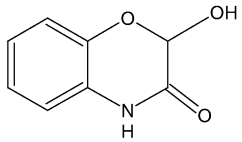
Synthetic derivatives

SP4 (2-MeO-HBOA)
2-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one

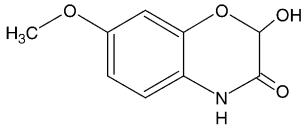


Lactams

HBOA
2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one

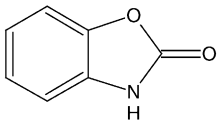


HMBOA
2-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one



Benzoxazolinones

BOA
1,3-benzoxazol-2(3*H*)-one



MBOA
6-methoxy-1,3-benzoxazol-2(3*H*)-one

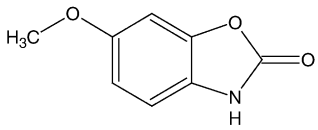


Fig. 1. Structure and nomenclature of selected benzoxazinone derivatives.

3. Results and discussion

3.1. Stability study

The analysis depends upon a good quality assurance of procedure involving reliable sampling and storage. The key issue is the stability of target compounds in solution during transport and storage. Loss of sample integrity for some compounds may limit the reliability of the results obtained.

A preliminary study of the stability of the benzoxazinone derivatives was carried out by the coordinator of the inter-laboratory exercise. The stability of DIMBOA-glc, HBOA, DIBOA, HMBOA, DIMBOA, BOA, MBOA and SP4 in acidified solution was studied. To determine the stability, spiked

Table 1
Analytical procedures and chromatographic conditions used during the interlaboratory exercise by each participant

Laboratory no.	Analytical procedure	Chromatographic conditions				
		Mobile phase A*	Mobile phase B*	Gradient		Column
				t (min)	% B	
1	LC–DAD	Water	MeOH	0	0	Synergi MAX-RP 80A (250 mm × 4.6 mm, 4 μm)
	LC–APCI-MS	Water	MeOH	2	30	
	LC–ESI-MS–MS	Water	MeOH	19	60	
				21	95	
2	LC–ESI-MS	Water–MeOH (9:1)	MeOH	0	10	BDS Hypersil C_{18} (250 mm × 2.1 mm, 5 μm)
				1	10	
				8	70	
				15	70	
3	LC–ESI-MS	Water–MeOH (9:1)	MeOH	1	10	BDS Hypersil C_{18} (250 mm × 2.1 mm, 5 μm)
				8	70	
				15	70	
4	LC–DAD	Water	Water–acetonitrite (6:4)	0	0	Synergi MAX-RP 80A (250 mm × 4.6 mm, 4 μm)
				60	60	
				70	100	
5	LC–DAD	Water	MeOH	0	30	LiChrospher 100 RP-18 (250 mm × 4.0 mm, 5 μm)
				2	30	
				19	60	
				21	100	
6	LC–DAD	Water	MeOH	0	10	Symmetry C_{18} (150 mm × 3.9 mm, 5 μm)
				14	90	

* All the solvents were acidified with acetic acid, with the exception of laboratory 4 where solvents were acidified with H_3PO_4, and laboratory 6 where the acidification was done with formic acid.

solutions were stored at room temperature, 4 and −20 °C. After 1, 2, 3 and 7 days the solutions were analyzed. The experimental design involved three replicate LC–MS analyses of each sample.

Fig. 2 shows the results obtained from the study of the stability of benzoxazinones stored during 7 days at the three different temperatures. Results clearly demonstrate that significant losses occurred when solution was stored at room temperature or 4 °C. At −20 °C, DIMBOA-glc, BOA and MBOA remained stable, HBOA and DIBOA suffered an approximately 10% of degradation, and HMBOA and DIMBOA were the most unstable compounds, with an approximately 20% of degradation. The compound selected as internal standard, SP4, was also checked in this stability test, showing an acceptable stability at −20 °C of storage. Thus, in terms of stability, SP4 is a good selection as internal standard. In view of these results and to prevent degradation, samples were sent to all participants in acidic

Table 2
LC–MS systems and analytical parameters used by participating laboratories 1, 2 and 3

Parameter	Laboratory 1		Laboratory 2	Laboratory 3
	HP1100, MS mode, APCI	Quattro, Micromass, MS–MS mode, ESI	HP1100, MS mode, ESI	API 2000, Applied Biosystems, MS mode, ESI
Vaporizer temperature (°C)	450	–	–	–
Source temperature (°C)	–	150	–	–
Desolvation temperature (°C)	–	350	–	–
Drying gas temperature (°C)	350	–	350	450
Cone voltage (V)	70	15–35	15–60	20
Collision energy (eV)	–	8–20	–	–
Corona voltage (kV)	4	–	–	–
Corona current (μA)	5	–	–	–
Capillary voltage (kV)	–	2.8	4	–
Extractor (V)	–	7	–	–
Lens voltage (kV)	–	0.6	–	–
Drying gas flow rate (L/min)	6	–	10	–
Desolvation gas flow rate (L/h)	–	600	–	–
Nebulizing gas pressure (psi; 1 p.s.i. = 6894.76 Pa)	50	–	20	55
Solvent flow rate (mL/min)	1	0.2	0.2	0.2

Table 3
m/z ions and MS–MS transitions used for quantification (the first one) and confirmation (the second one) of target analytes with the different interfaces used in the interlaboratory exercise

Compound	Laboratory 1		Laboratory 2	Laboratory 3
	MS mode APCI (−)	MS–MS mode ESI (−)	MS mode, ESI (+)	MS mode, ESI (+)
DIMBOA-glc	149, 164	164 > 149, 164 > 121	NA	374
HBOA	164, 108	164 > 136, 164 > 108	166, 148	166, 148
DIBOA	134, 108	134 > 78, 134 > 91	182, 164	182, 164
HMBOA	194	194 > 138, 194 > 179	196, 178	196, 178
DIMBOA	164, 149	164 > 149, 164 > 121	212, 166	212, 166
BOA	134	134 > 78, 134 > 91	136	136
MBOA	164, 149	164 > 149, 164 > 121	166	166
SP4	178, 134	178 > 118, 118 > 90	180, 148	180, 148

NA: not analyzed.

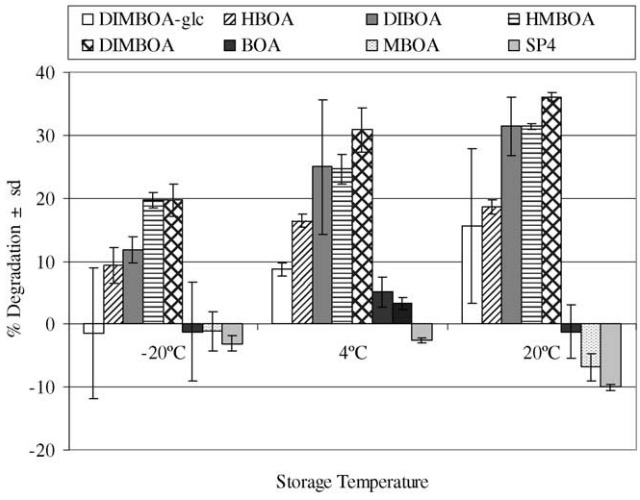


Fig. 2. Degradation (%) of target compounds in a standard solution kept during 7 days under different storage temperatures.

conditions at −20 °C, and storage in these conditions was recommended.

3.2. Interlaboratory study

3.2.1. Standard solutions

Results of six laboratories from four different countries were received. The results obtained for the analysis of different benzoxazinone derivatives in *Standard 1* and *Standard 2* by different instrumental technique are listed in Table 4. Moreover, the mean of means values, as well as the relative standard deviation among laboratories (R.S.D.), are included. Our approach calculated means and R.S.D.s after discarding outlying observations (values out of the range of spiked value ±20%). Only two participants, laboratories 1 and 3, reported results for the seven allelochemicals, including DIMBOA-glc. The rest of participants analyzed the selected analytes, with the exception of DIMBOA-glc.

Results for benzoxazinone derivatives in the more concentrated standard solution (*Standard 2*) are satisfactory with a range of R.S.D. of 2.0–6.5%. Moreover, mean values ranged between 1.48 and 1.52 ng/μL, matching reasonably well with the spiked value (1.5 ng/μL). For this standard solution no outlier laboratories were found. In general terms, the variation from laboratory to laboratory (interlaboratory) was similar than that attributed to the analytical error displayed within laboratories (intra-laboratory). Intra-laboratory R.S.D.s ranged between 0.7 and 9.7% for laboratory 1, 2.3 and 8.0% for laboratory 2, 1.8 and 6.6% for laboratory 3, 2.1 and 5.8% for laboratory 4, and 6.3 and 8.5% for laboratory 5. Only for laboratory 6, higher intra-laboratory R.S.D. values were found, ranging between 0.7 and 19%.

Results obtained for the more diluted standard solution (*Standard 1*) also showed satisfactory mean values, ranging between 0.28 and 0.32 ng/μL (spiked value = 0.30 ng/μL). The R.S.D. values were higher than those obtained for *Standard 2*, but always below 15%. For this standard solution the number of outliers was limited to one, and laboratory 5 presented values below LODs.

As regard the comparison of the different instrumental techniques (LC–DAD, LC–MS and LC–MS–MS) used for the determination of benzoxazinones in standard solutions, we can conclude the following. Results reported by laboratories using MS techniques were not different from those obtained by LC–DAD methods. In some cases the MS laboratories reported lower values, and in some cases these values were somewhat higher. However, MS laboratories may be able to obtain lower LODs. Table 5 shows the detection limits obtained for each compound by the different instrumental approaches. As expected, LODs obtained by MS approaches were lower than those obtained using DAD. Comparison between MS and MS–MS LODs showed that better values were obtained using MS–MS technique, especially for HBOA (0.04–0.09 and 0.003 ng/μL using MS and MS–MS, respectively). Regarding the ionization mode, APCI resulted in higher LODs as compared to ESI values, especially for DIMBOA-glc. Glucoside compounds were not well ionized using APCI mode, whereas using ESI mode the sensitivity of glucosides was similar to the rest of benzoxazinone derivatives.

3.2.2. Root material

The results obtained for the analysis of purified root extract (*Root1*) by the different laboratories are listed in Table 6.

Table 4
Results (expressed in ng/μL) obtained for the analysis of *Standard 1* and *Standard 2*

Benzoxazinone	Spiked level	Laboratory 1			Laboratory 2	Laboratory 3	Laboratory 4	Laboratory 5	Laboratory 6	Mean*	R.S.D. (%)
		DAD	MS	MS–MS	MS	MS	DAD	DAD	DAD		
Standard 1											
DIMBOA-glc	0.3	0.32	ND	0.24	NR	0.29	NR	NR	NR	0.28	14.3
HBOA	0.3	0.30	0.34	0.34	*0.40*	0.34	*0.52*	ND	0.29	0.32	7.7
DIBOA	0.3	0.31	0.30	0.34	*0.41*	0.35	0.31	ND	0.33	0.32	6.1
HMBOA	0.3	0.30	0.29	0.36	*0.38*	0.33	0.29	ND	0.33	0.32	8.9
DIMBOA	0.3	0.30	0.37	0.29	*0.37*	0.30	0.29	ND	0.30	0.31	9.9
BOA	0.3	0.29	0.29	0.29	*0.38*	0.34	*0.23*	ND	0.26	0.29	9.8
MBOA	0.3	0.29	0.29	0.28	*0.37*	0.35	0.30	ND	0.26	0.30	10.2
Standard 2											
DIMBOA-glc	1.5	1.55	1.52	1.46	NR	1.53	NR	NR	NR	1.52	2.6
HBOA	1.5	1.54	1.50	1.56	1.53	1.46	1.55	*0.85*	1.45	1.51	2.9
DIBOA	1.5	1.54	1.48	1.49	1.58	1.49	1.63	1.34	1.56	1.51	5.8
HMBOA	1.5	1.49	1.48	1.55	1.53	1.50	1.55	*2.02*	1.55	1.52	2.0
DIMBOA	1.5	1.48	1.47	1.61	1.51	1.54	1.53	1.32	1.36	1.48	6.5
BOA	1.5	1.54	1.47	1.58	1.55	1.54	1.53	1.30	1.55	1.51	5.9
MBOA	1.5	1.56	1.46	1.47	1.52	1.55	1.55	1.28	1.57	1.50	6.4

Values of each laboratory corresponded to the mean of five replicates. ND: not detected; NR: not reported. Outlier values in italics.
* Mean of means, excluding outlier values (values out of the range of spiked value ±20%).

Table 5
Detection limits (expressed in ng/μL) obtained for the analysis of standard solution

	Laboratory 1			Laboratory 2	Laboratory 3	Laboratory 4	Laboratory 5	Laboratory 6
	DAD	MS	MS–MS	MS	MS	DAD	DAD	DAD
DIMBOA-glc	0.252	1.429	0.071	NR	0.004	NR	NR	NR
HBOA	0.039	0.086	0.003	0.050	0.038	NR	0.07	0.096
DIBOA	0.060	0.049	0.038	0.025	0.022	NR	0.08	0.167
HMBOA	0.045	0.017	0.003	0.025	0.005	NR	0.06	0.186
DIMBOA	0.091	0.075	0.009	0.015	0.006	NR	0.02	0.162
BOA	0.048	0.008	0.016	0.005	0.008	NR	0.03	0.085
MBOA	0.045	0.006	0.001	0.003	0.011	NR	0.03	0.033

NR: not reported.

Moreover, the mean of means values as well as the R.S.D.s among laboratories are included. Our approach calculated means and R.S.D.s after discarding outlying observations. Obvious extreme values, two-fold above the mean value or two-fold below the mean value were not included in the calculations. When data from all laboratories (excepting outliers) were pooled, R.S.D.s ranged from 19 to 47% for the determination of target analytes. It should be pointed that the higher R.S.D. value was obtained for HBOA, which was present in the sample at concentration level very close to LOD. As expected, results for *Root1* are not as good as for standard solutions.

As regard the comparison of the different instrumental approaches (LC–DAD, LC–MS and LC–MS–MS) used for the determination of benzoxazinones in root material, we can conclude the following. MS laboratories may be able to obtain lower LODs. For this reason, some analytes with low concentration level, as HBOA or DIM-

Table 6
Results (expressed in ng/μL) obtained for the analysis of *Root1*

Benzoxazinone	Laboratory 1			Laboratory 2	Laboratory 3	Laboratory 4	Laboratory 5	Laboratory 6	Mean*	R.S.D. (%)
	DAD	MS	MS–MS	MS	MS	DAD	DAD	DAD		
DIMBOA-glc	ND	ND	1.00	NR	1.35	NR	NR	NR	1.18	21.1
HBOA	ND	ND	0.01	0.02	*0.23*	ND	*1.92*	*1.28*	0.02	47.1
DIBOA	ND	ND	ND	ND	ND	ND	ND	*0.86*	ND	–
HMBOA	*7.76*	0.55	0.68	0.57	0.87	0.75	*1.78*	*6.66*	0.68	19.2
DIMBOA	ND	ND	0.20	0.11	0.19	ND	ND	*1.59*	0.17	29.8
BOA	0.21	0.25	0.24	0.41	0.48	0.36	ND	*2.54*	0.33	33.3
MBOA	4.55	4.29	4.80	5.63	5.82	5.72	7.68	4.88	5.42	19.9

ND: not detected; NR: not reported. Outlier values in italics.
* Mean of means, excluding outlier values (two-fold above or two-fold below the mean value).

BOA, could only be detected using MS techniques. For these compounds, results reported by DAD laboratories were below LOD. For the rest of the compounds, results reported by laboratories using MS techniques were generally lower than those obtained by LC–DAD methods due to co-elution peaks in DAD chromatograms. Fig. 3 shows the chromatograms obtained by laboratory 1 using LC–DAD as well as LC–MS. As can be seen, some analytes were well resolved by both techniques leading to similar concentration levels (MBOA). However, some benzoxazinones co-eluted with interferent peaks in the DAD system. These interferences corresponded to other compounds present in root extracts, as flavonoids or other benzoxazinone derivatives. This situation was very clear for HMBOA: the calculated concentration with DAD was 7.8 ng/μL, whereas level of 0.6 ng/μL was estimated using LC–MS. Similar high values were reported by the other DAD laboratories, with the exception of laboratory 4 who reported concentration level 0.8 ng/μL, matching reasonably well with MS data. It should be pointed that chromatographic conditions used by laboratory 4 were different from those used by the rest of participants. Using the chromatographic conditions proposed by the coordinator of the interlaboratory exercise, benzoxazinone derivatives eluted in 20 min approximately. However, 80 min were required for the complete elution of selected analytes using experimental conditions of laboratory 4.

Fig. 3 also shows another important co-elution observed in DAD chromatograms. As can be seen, SP4 co-eluted with another chromatographic peak when LC–DAD was used for *Root1* analysis. This situation could lead to erroneous quantification using internal standard method.

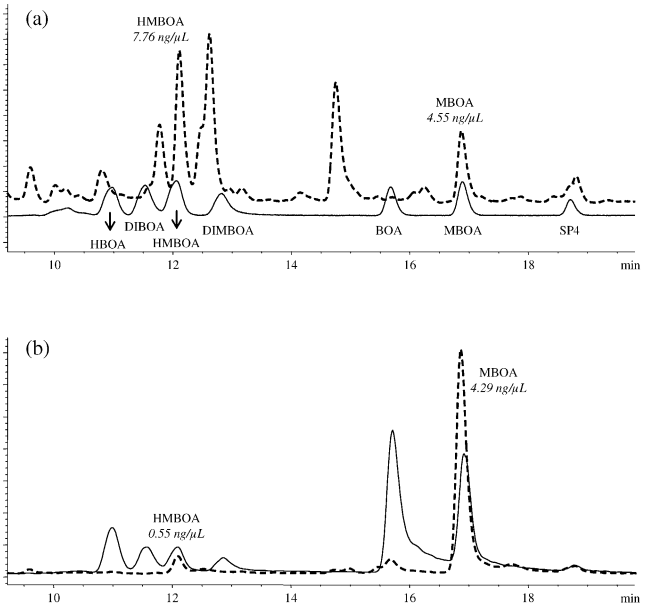


Fig. 3. (a) LC–DAD (280 nm) and (b) LC–MS (TIC) chromatograms obtained for a standard solution (2 ng/μL) (----) and for *Root1* (—) by laboratory 1.

The only compound determined by all the laboratories without outlier values was MBOA. This analyte presented the higher concentration level. Mean of means value was 5.42 ng/μL and the R.S.D. among laboratories 19.9%. These data were calculated with values obtained using LC–DAD, LC–MS and LC–MS–MS approaches. When R.S.D. was re-calculated with values obtained using only MS techniques, an improvement was observed with R.S.D. value of 14.0%. In contrast, re-calculation with data obtained using DAD technique, leads to an increase of R.S.D. value up to 24.6% (Fig. 4). It should be pointed that mean of means value obtained from MS results (5.1 ng/μL) was lower than that obtained from DAD calculations (5.7 ng/μL). More MS–MS data are required in order to compare the capabilities between MS and MS–MS techniques.

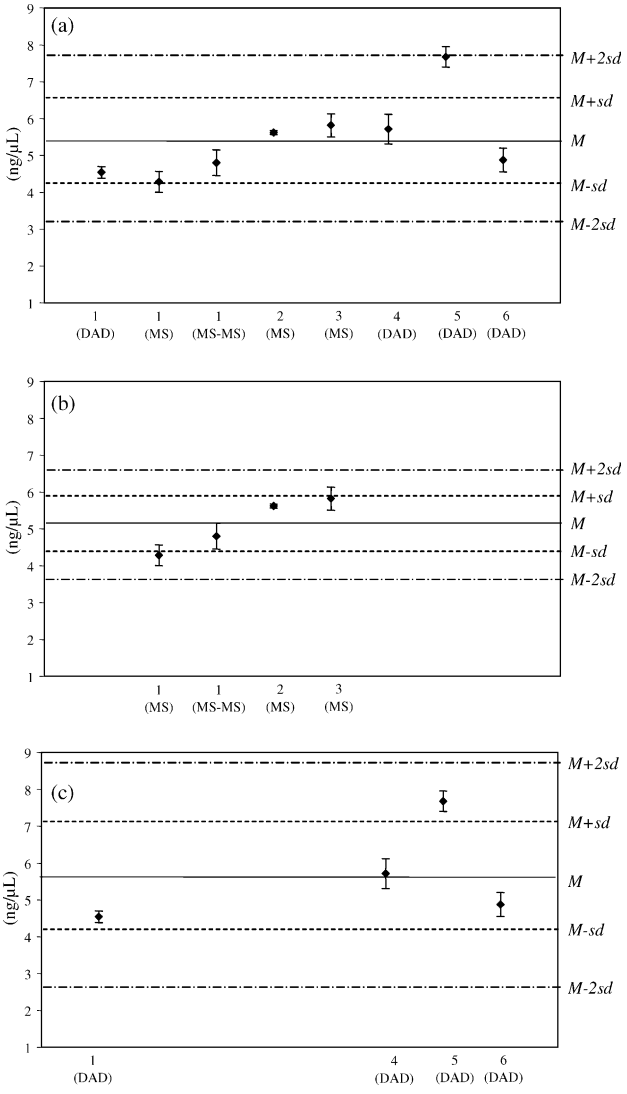


Fig. 4. Interlaboratory MBOA results in *Root1*. Mean of means value ±2 standard deviation obtained when: (a) data from all laboratories are pooled; (b) MS data are pooled; and (c) DAD data are pooled. Values reported by each participant are mean values obtained from five individual determinations, and error bars record the associated standard deviation.

4. Conclusions

Six laboratories representing four countries and responsibles for allelochemical analyses in the FATEALLCHEM European Union project took part in the interlaboratory study. Phase I of this study was designed to check the instrumental approaches used for benzoxazinone determinations: LC–DAD, LC–MS and LC–MS–MS. These techniques were compared in terms of selectivity and sensitivity. Results of the exercise showed that in general, repeatability (as reflected by within-laboratory R.S.D.) is at a satisfactory level (below 15%) for standard solution analysis. However, large between-laboratory variability was observed when a real sample (purified root extract) was determined (between 19 and 47%). Comparison of the three techniques leads to the conclusion that MS approaches (LC–MS and LC–MS–MS) are the most precise techniques for the determination of benzoxazinone derivatives at nanogram per microlitter level in plant material. Several co-elutions were detected when root material was analyzed by LC–DAD, showing the poor selectivity of this technique. Moreover, their sensitivity is lower than that afforded by MS or MS–MS, and some minor benzoxazinone derivatives (HBOA and DIMBOA) could be only detected by MS approaches. It should be pointed that LC–MS–MS also offered improvements to the sensitivity and selectivity, as compared with LC–MS methods.

The next phase (phase II) of the interlaboratory study will incorporate both a standard and a purified foliage extract. It is expected that more difficulties will be found in analyzing foliage material, compared with root material. Moreover, the next phase will incorporate the evaluation of different steps of sample preparation, like extraction and purification.

Acknowledgments

This work was financially supported by the programme “Quality of life and management of living resources (1998 to 2002)” of the European Union, FATEALLCHEM Contract No. QLRT-2000-01967, and by the Spanish Ministerio de Ciencia y Tecnología (AGL2001-4952-E). We would like to thank Professor D. Sicker (University of Leipzig) and Professor S. Chilton (University of North Carolina) for kindly providing standards.

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