QUALITY ASSURANCE OF ANALYTICAL METHODS FOR WHEAT ALLELOCHEMICALS

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INTRODUCTION

During the 80s and 90s, several procedures for the separation and quantification of benzoxazinone derivatives in plant extracts were developed (1). 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 (2). Recently, a new method for the quantification of benzoxazinone using LC-tandem mass spectrometry (MS-MS) was developed (3). 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 is to provide accurate methods for the determination of benzoxazinone derivatives in plant material. One way to achieve this purpose was to organize an inter-laboratory study involving the laboratories which are carrying out benzoxazinone analyses in the project. There are six laboratories from four different countries: Denmark, Lithuania, Poland and Spain.

STABILITY STUDIES

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 and further degradation products was carried out. To determine the stability, acidified spiked solutions were stored at room temperature, 4ºC and -20ºC. After 1, 2, 3 and 7 days the solutions were analysed. The experimental design involved three replicate LC-MS analyses of each sample. Results clearly demonstrate that significant losses occurred when solution was stored at room temperature or 4ºC. At -20ºC, 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. As regards aminophenoxazinones and malonamic acids, results showed that significant losses occurred, not only when solution was stored at room temperature but also at 4ºC and -20ºC. APO and AMPO were the most unstable compounds, with approximately 75-100 % of degradation. This degree of degradation was observed after three days of storage. Thus, the instability of APO and AMPO was an important fact to consider for standard solution preparation. Concerning to

the rest of compounds, better stability was observed at -20ºC. At this temperature, AAPO remained stable, whereas HPAA and APH suffered an approximately 20% of degradation. In view of these results and to prevent degradation, storage in acidic conditions at -20ºC was recommended.

INTERLABORATORY STUDIES

The interlaboratory evaluation was divided into two different phases. The first one was focused on the evaluation of different detection systems for allelochemical determination in plant material, whereas the second one was centred on the evaluation of different sample preparation steps, such as extraction and purification. Seven allelochemicals were selected for these studies: DIMBOA-glc, HBOA, DIBOA, HMBOA, DIMBOA, BOA and MBOA.

Evaluation of detection systems

Three different detection systems were evaluated: DAD, MS and MS-MS. The laboratories were asked to determine the concentrations of selected analytes in five different samples, three standard solutions, one purified extract of root material and one purified extract of foliage material.

Results of the exercise showed that in general, repeatability is at a satisfactory level (below 15%) for standard solution analysis. However, large between-laboratory variability was observed when a purified root extract was determined (between 19 and 47%). Worse results were obtained for the purified foliage extract, due to the high complexity of this matrix. Comparison of the three techniques leads to the conclusion that MS approaches (LC-MS and LC-MS-MS) are the most accurate and precise techniques for the determination of benzoxazinone derivatives at nanogram per microlitter level in plant material. Several co-elutions were detected when root or foliage material was analysed by LC-DAD (Figure 1), showing the poor selectivity of this technique. Moreover, their sensitivity is lower than that afforded by MS or MS-MS (Table 1), and some minor benzoxazinone derivatives (HBOA and DIMBOA) could be only detected by MS approaches.

Table 1. Instrumental detection limits, expressed in ng/µL, obtained for the analysisof standard solutions by DAD, MS and MS-MS.

	DAD	MS	MS-MS
HBOA	0.039	0.086	0.003
DIBOA	0.060	0.049	0.038
HMBOA	0.045	0.017	0.003
DIMBOA	0.091	0.075	0.009
BOA	0.048	0.008	0.016
MBOA	0.045	0.006	0.001

Evaluation of sample preparation steps

The two main sample preparation steps, extraction and purification, were evaluated. The laboratories were asked to determine the concentrations of selected analytes in one root extract (for purification evaluation) and one homogenized root (for extract evaluation).

Results of the exercise showed that in general, purification step worked satisfactorily. Intra-laboratory variability values were low, below 12%, and variability between laboratories were similar than those observed for the purified root material in the previous phase. In contrast, the analysis of

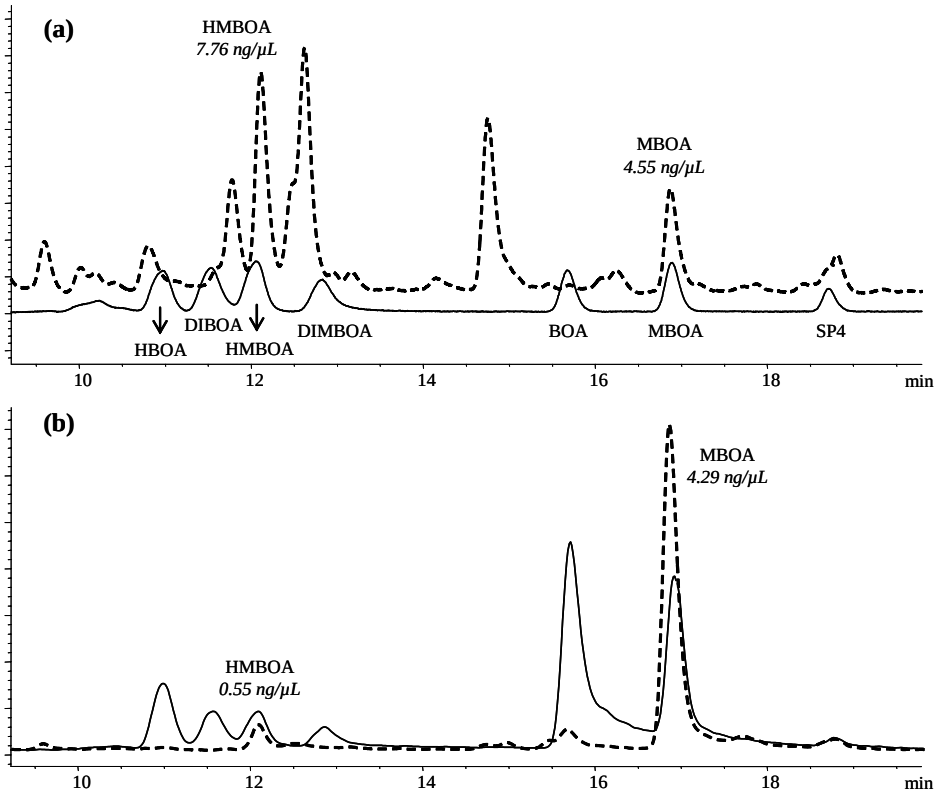

Figure 1. (a) LC-DAD (280 nm) and (b) LC-MS (TIC) chromatograms obtained for a standard solution (2 ng/µL) (—) and for a purified root extract (----).

Table 2. Intra-laboratory variability, expressed in %, for the determination of MBOA in different standards and real samples using different instrumental approaches.

		MS + DAD	DAD	MS
Detection systems	Standard 1	10	7	12
	Standard 2	6	9	3
	Standard 3	9	10	8
	Purified Root Extract	20	25	14
	Purified Foliage Extract	43	38	16
Purification step	Root Extract	28	29	19
Extraction + Purification steps	Homogenized Root	25	31	22

homogenized root material showed higher between-laboratory variability. Moreover, large intra-laboratory variability was also observed (up to 48%).

Table 2 summarizes the intra-laboratory variability obtained for the determination of MBOA in each selected sample, and using different instrumental approaches. It should be pointed that MBOA is the main benzoxazinone derivative found in the analysed wheat samples.

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