# SAMPLE HANDLING AND ANALYSIS OF BENZOXAZINONES AND FURTHER DEGRADATION PRODUCTS IN PLANTS AND SOILS

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## INTRODUCTION

The family of 1, 4-benzoxazin-3-ones have been reported to be involved in the defence of the plant against a wide variety of organisms, and it's occurring in Poaceous plants such as wheat, maize and rye (1). The major degradation products of benzoxazinones in soil are aminophenoxazinones and corresponding malonamic acids. In recent years there has been an increasing focus on the prospects of exploiting allelopathy as an alternative strategy for controlling in particular weeds but also insects and diseases. During the 1980s and 1990s, several procedures were developed for the separation and quantification of benzoxazinones in plant extracts using liquid chromatography (LC) with ultraviolet (UV) detection (2). To overcome the LC-UV limitations, the use of liquid chromatography-electrospray-mass spectrometric method (LC-MS) and tandem mass spectrometry method (LC-MS-MS) have clear advantages. As regards the analysis of aminophenoxazinones, only Zikmundova et al. (3) developed an analytical method using LC-MS. For sample preparation, the most commonly technique used for the extraction of allelochemicals was sonication, using MeOH as solvent without clean up step.

The aim of our study was to develop a rapid and simple analytical method for the determination of the allelochemicals in wheat and soil samples. The analytical method consist on pressurized liquid extraction (PLE) followed by solid phase extraction (SPE) purification. In order to obtain a more sensitive and selective method, the instrumental determination was performed by LC-MS and LC-MS-MS (4).

## MATERIALS AND METHODS

### Target compounds

The main allelochemicals studied are shown in Figure 1, including 8 benzoxazinone derivatives (DIMBOA-glc, DIBOA-glc, DIMBOA, DIBOA, HMBOA, HBOA, MBOA and BOA) and 7 aminophenoxazinones derivatives (HPAA, HPMA, HMPMA, APH, APO, AMPO and AAPO).

### Chromatographic conditions

Analyses were performed on HP 1100. Due to the necessity to work in acidified media we tested some properties of different commercial columns (Lichrocart $C_{18}$, Nucleosil $C_{18}$ and Ultracarb $C_{18}$, Synergi $C_{12}$) such as: durability, stability and also the good separation and peak intensities of all compounds.

### Mass spectrometry conditions

The LC-MSD HP 1100 mass selective detector equipped with an atmospheric pressure ionization source was used. The optimized parameters were: ionization mode, drying gas flow, nebulizer gas pressure, drying gas temperature, capillary voltage, and fragmentor voltage. The analysis of benzoxazinone derivatives was also optimized in LC online with MS-MS detection method. The parameters optimized were: cone voltage and collision energy for each compound.

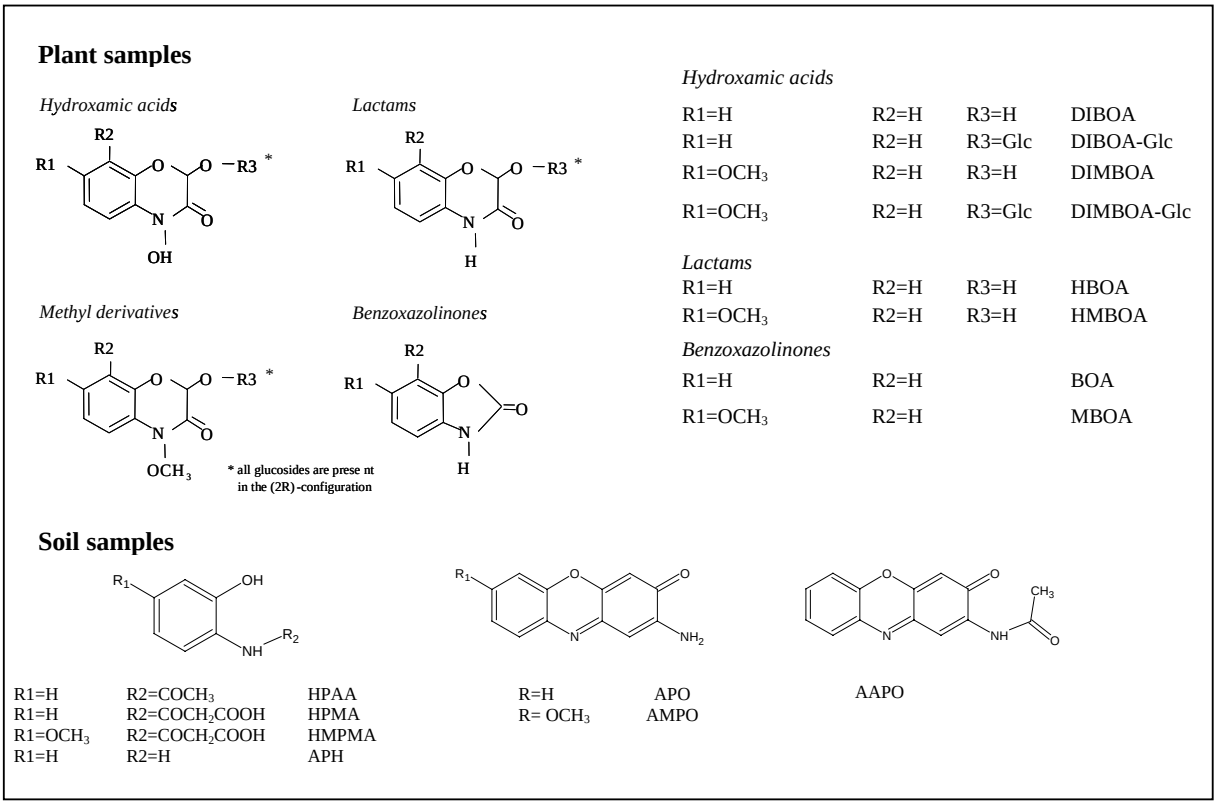


Figure 1. Molecular structures and nomenclature of allelochemicals studied in the present work, including 8 benzoxazinones and 7 aminophenoxazinones derivatives

**Sample preparation**

Excess water was removed from the frozen wheat plants and soil by lyophilisation for 24 hours. The analytes were isolated from foliage, roots and soil using PLE. The optimized parameters of the PLE system were: solvent composition, temperature and % flush volume. In the raw extracts of plants the broad variety of substances (salts, lipids, glycosides, phosphates, peptides, macromolecules and chlorophyll) can influence the quantifications. Thus, purification of the plant sample prior to instrumental analysis is recommended. Aqueous extracts were directly applied to the activated and preconditioned cartridges (LiChrolut® RP $C_{18}$). Two-step elution procedure was used: first, with acidified $H_2O$ (1% HOAc); and second with acidified MeOH/$H_2O$ (1% HOAc). Different proportions of MeOH/$H_2O$ (1% HOAc) (0:100, 20:80, 40:60, 50:50, 60:40, 80:20, 100:0) were studied.

## RESULTS AND DISCUSSION

**Chromatographic conditions**

Among different columns tested, the Synergi MAX-RP 80A LC C12 (Phenomenex) column showed the best results and it was used for all the analysis with a solvent flow-rate of 1mL/min. The sample volume was set at 50 µl. Acidified $H_2O$ (0.05% HOAc) and MeOH (0.05% HOAc) was used as the elution solvents A and B, respectively. Total run time was 35 and 30 minutes for benzoxazinone derivatives and aminophenoxazinone derivatives, respectively.

**Mass spectrometry conditions**

The LC-MSD HP 1100 mass selective detector was used in electrospray interface (ESI). The ESI-MS was selected in negative ion mode for benzoxazinone derivatives and positive for aminophenoxazinone derivatives. The MS analysis were carried out in select ion monitoring mode (SIM), and were confirmed by on line UV detection using a Waters Model 996 photo diode array detector over the range 250-440 nm. 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. The MS-MS analysis was carried out in multiple reaction monitoring mode (MRM). Two transitions were selected. Primary transitions were used for quantification and the secondary transition for confirmation. Comparison between MS and MS-MS instrumental LODs showed that better values were obtained using MS-MS technique, i.e. for HBOA instrumental LODs were 0.09 and 0.003 ng/µL using MS and MS-MS, respectively.

**Sample preparation**

The procedure adopted for the sample preparation involved lyophilistaion, extraction, concentration, filtration and SPE purification (Figure 2). After study of the different PLE parameters, the optimal extraction conditions were as follow: MeOH in acidified media for benzoxazinone derivatives and without acid for aminophenoxazinone derivatives; temperature, 150ºC; flush volume, 60%. The extract was then concentrated, redissolved in $H_2O$, filtrated and applied to the preconditioned cartridges (LiChrolut® RP $C_{18}$). First elution was obtained with 6 mL of $H_2O$ and the second one with 5 mL of MeOH/$H_2O$ (60:40) (1% HOAc). Finally, the extract of fraction 2 was diluted before the

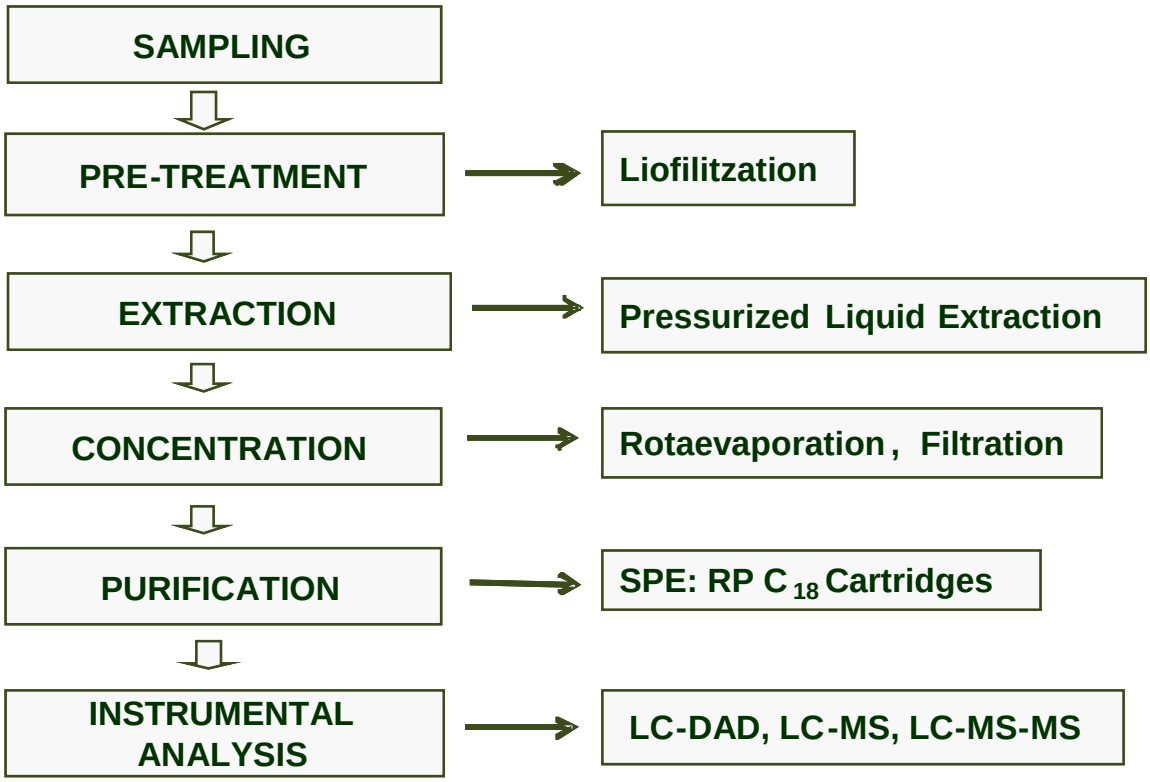


Figure 2. Sample preparation steps for the determination of benzoxazinones and further degradation products in plants and soils

analysis (Foliage: dilution 1:10; and root: dilution 1:5) in order to avoid the ion suppression effect in LC-ESI-MS analysis. The less polar compounds (HMBOA, DIMBOA, BOA, MBOA) were analysed in the second fraction, whereas for the others (Glucosides, HBOA, DIBOA) is necessary to analyse both fractions. In soil samples clean up step was not required. The method detection limits ranged from 2 to 57 µg/g dry weight.

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