PERGAMON

Phytochemistry 53 (2000) 223–229

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Variation of DIMBOA and related compounds content in relation to the age and plant organ in maize

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Received 2 December 1998; received in revised form 6 August 1999; accepted 14 September 1999

Abstract

We report the variation of all 1,4-benzoxazin-3-one derivatives content detectable in maize with plant age in roots and aerial parts. Our results show that the concentration of hydroxamic acids, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (DIMBOA-Glc) and its 8-methoxylated analogue (DIM_2BOA-Glc) is high after seed germination and then decreases with plant age. Nevertheless, these compounds continue to be biosynthesised during 6–10 days after germination. Variation in concentration of N–O-methylated DIMBOA-Glc (HDMBOA-Glc) is similar to the one of hydroxamic acids in aerial parts. On the contrary, in roots, its concentration remains relatively stable with plant age. After 10 days, HDMBOA-Glc becomes the main compound in roots. This compound is also present in higher concentration than hydroxamic acids in the oldest leaf of 20-day-old maize. The presence of four other DIMBOA related compounds in maize plants depends on variety, age and tissue. The role of these compounds in plant resistance to aphids is discussed.

Keywords: Poaceae; Maize; Hydroxamic acids; DIMBOA; 1,4-benzoxazin-3-one; Plant resistance

1. Introduction

Poaceae such as wheat, rye and maize contain hydroxamic acids in high concentrations, up to 44 mmol/kg fresh weight (Copaja, Barria & Niemeyer, 1991) depending on varieties and conditions. These compounds belong to the family of 1,4-benzoxazin-3-ones and are naturally present in the plants as 2-O-β-D-glucosides (Wahlroos & Virtanen, 1959; Hofman & Hofmanova, 1969, 1971). The main hydroxamic acid in maize and wheat is 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-Glc) with lesser amount of 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc) (Tipton, Klun, Husted & Pierson, 1967). The 2-β-D-glucopyranosyloxy-4-hydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one (DIM_2BOA-Glc) is also found in maize (Hofman & Hofmanova, 1971) (Fig. 1). In injured plants, glucosides are hydrolized by β-glucosidase yielding the respective aglycones (Wahlroos & Virtanen, 1959; Mace, 1972). The aglycones are unstable and rapidly converted into the benzoxazolin-2-ones (Woodward, Corcuera, Helgeson & Upper, 1978).

Biosynthesis of hydroxamic acids in maize has several steps in common with tryptophan biosynthesis (Tipton, Wang, Tsao, Lin Tu & Husted, 1973). Recently, Frey et al. (1997) showed that five genes are required for 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) biosynthesis from indole-3-glycerol phosphate. The functions of these genes were demonstrated in vitro. According to Leighton, Niemeyer and Jonsson (1994), glucosylation of hydroxamic acids would be the last step of the pathway.

Hydroxamic acids, and mainly the 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), could play several roles in the poaceae (Niemeyer, 1988). A possible role is the defence against aphids. DIMBOA exerts both toxic and antifeedant effects on aphids

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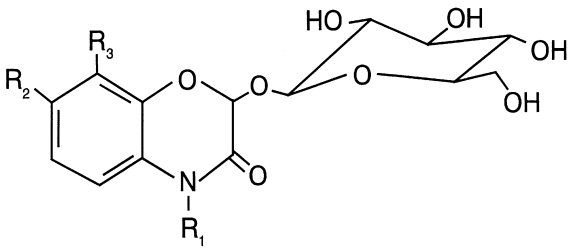
0031-9422/00/$ - see front matter
PII: S0031-9422(99)00498-7



	R_1	R_2	R_3
Hydroxamic acids			
DIBOA-Glc	OH	H	H
DIMBOA-Glc	OH	CH_3O	H
DIM_2BOA-Glc	OH	CH_3O	CH_3O
Lactams			
HMBOA-Glc	H	CH_3O	H
HM_2BOA-Glc	H	CH_3O	CH_3O
N-O-methyl derivatives			
HDMBOA-Glc	CH_3O	CH_3O	H
HDM_2BOA-Glc	CH_3O	CH_3O	CH_3O

Fig. 1. Benzoxazinone derivatives present in maize plants (Magda and Sesnord varieties).

(Niemeyer, Pesel, Franke & Francke, 1989; Givovich & Niemeyer, 1995). Negative relationships were found between DIMBOA concentration in several poaceae (wheat mainly) and aphids performance (Bohidar, Wratten & Niemeyer, 1986; Thackray, Wratten, Edwards & Niemeyer, 1990; Nicol, Copaja, Wratten & Niemeyer, 1992).

DIMBOA-Glc measured as DIMBOA is not detected in cereal seeds (Argandona, Niemeyer & Corcuera, 1981). According to Argandona et al. (1981), a few days after germination, concentration in the plant increases abruptly reaching a maximum and then decreases progressively with plant age. Nevertheless, concentration in the younger leaves at different plant age is always higher (Argandona et al., 1981; Thackray et al., 1990). A substantial variation was also found between the concentrations of hydroxamic acid of different plants (Argandona et al., 1981; Nicol et al., 1992; Xie, Arnason, Philogène, Olechowski & Hamilton, 1992) and plant organs (Argandona et al., 1981; Argandona & Corcuera, 1985).

Other 1,4-benzoxazin-3-one derivatives belonging to two other classes, lactams and N–O-methylated hydroxamic acids, are also found in poaceae (Fig. 1) (Cambier, Hance & de Hoffman, 1999). These compounds are only present as glucosides in noninjured plants (Cambier et al., 1999). Their roles in the plant are not well known, and no data is available in the literature on their distribution and variation in concentration with plant age.

Our objective is to examine variation with age and plant organ of the compounds genuinely present in the plant, the glucosides. This study will give information on the period of biosynthesis of these compounds in the plant. The results obtained will help to understand their roles in maize resistance to aphids.

2. Results

No 1,4-benzoxazin-3-one derivatives were detected in dry seeds. In noninjured maize seedlings, seven 1,4-benzoxazin-3-one glucosides were found. They belong to three classes: hydroxamates (DIBOA-Glc, DIMBOA-Glc and DIM_2BOA-Glc), lactams (HMBOA-Glc and HM_2BOA- Glc) and N–O-methylated derivatives (HDMBOA-Glc and HDM_2BOA-Glc) (Fig. 1). Only three compounds, DIMBOA-Glc, DIM_2BOA-Glc and HDMBOA-Glc, were present both in roots and aerial parts during all 20 days. The concentrations and total amounts per plant were followed for a period of 20 days after germination.

2.1. Variation with plant age

In aerial parts, DIMBOA-Glc concentration was high two days after germination for Magda (11.7 mmol/kg fresh weight) and Sesnord (12.9 mmol/kg fresh weight) varieties. Then, it decreased rapidly with age (Fig. 2a). After 20 days, the concentration was respectively 23 and 43 times lower than after 2 days. DIM_2BOA-Glc (Fig. 2b) and HDMBOA-Glc (Fig. 2c) concentration also decreased rapidly with plant age. DIMBOA-Glc remained the major 1,4-benzoxazin-3-one derivative during the 20 days following germination.

Although concentrations decreased with plant age, total amounts of each compound increased during the first 10 days after germination (Fig. 2f, g and h). This suggests that these compounds continue to be biosynthesised by the plant. The decrease of DIMBOA-Glc and DIM_2BOA-Glc content, observed after 10 days suggests that these compounds are transformed, released and/or degraded by the plant.

In roots, changes of DIMBOA-Glc (Fig. 2d) and DIM_2BOA-Glc (data not shown) concentration were similar to those in aerial parts. Nevertheless, concentrations were always lower. These hydroxamic acids are probably synthesised during the first days and degraded, released and/or transformed by the plant after 10 days (Fig. 2i). Changes of HDMBOA-Glc in roots were very different than those of hydroxamic acids (Fig. 2e). Indeed, the concentration did not decrease with plant age but remained quite stable. Fig. 2j suggests that HDMBOA-Glc is biosynthesised by the plant during the first 15 days. The first six days after germination, the DIMBOA-Glc concentration

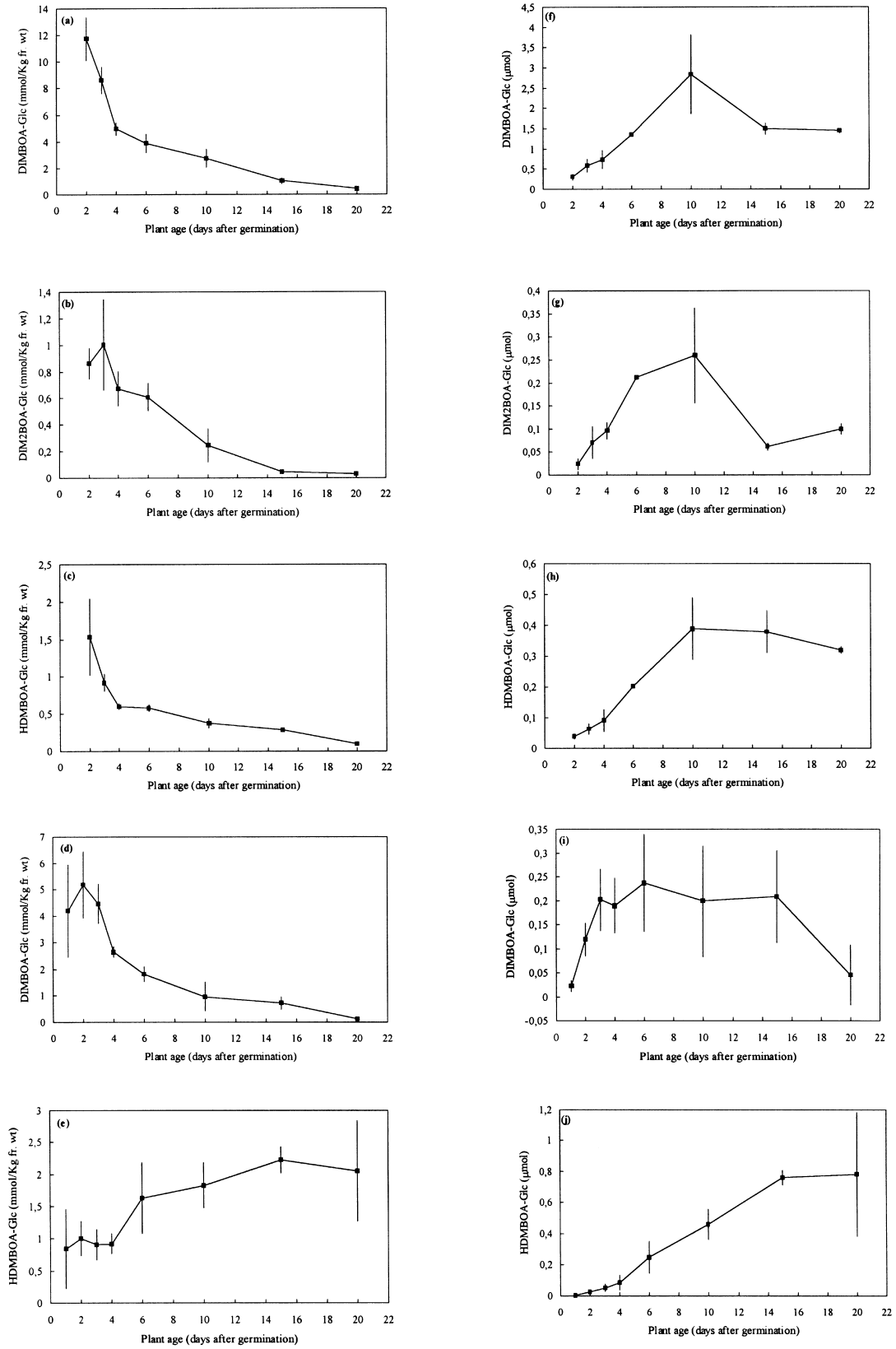


Fig. 2. Variation of benzoxazinone derivatives with age in aerial parts and roots of maize seedlings (Magda variety). (a)–(e): Variation in concentration. (a): DIMBOA-Glc in aerial parts. (b): DIM_2BOA-Glc in aerial parts. (c): HDMBOA-Glc in aerial parts. (d): DIMBOA-Glc in roots. (e): HDMBOA-Glc in roots. (f)–(j): Variation in total amounts per plant. (f): DIMBOA-Glc in aerial parts. (g): DIM_2BOA-Glc in aerial parts. (h): HDMBOA-Glc in aerial parts. (i): DIMBOA-Glc in roots. (j): HDMBOA-Glc in roots. Each point on the graph represents the mean of three replicates. One plant per replicate was used. Vertical lines at data points show standard deviations.

Table 1
Presence (continuous line) of four 1,4-benzoxazin-3-one derivatives in roots and aerial parts during the first 20 days for two maize varieties (Magda and Sesnord). Absence of continuous line means that the compound was not detected

Compounds	Variety and tissue	Days after germination: 1 2 3 4 6 10 15 20
DIBOA-Glc‡	Magda roots	———
	Sesnord roots	———
	Magda aerial parts	———
	Sesnord aerial parts	———
HMBOA-Glc‡	Magda roots	———
	Sesnord roots	———
	Magda aerial parts	———
	Sesnord aerial parts	———
HM_2BOA-Glc‡	Magda roots	———
	Sesnord roots	———
	Magda aerial parts	———
	Sesnord aerial parts	———
HDM_2BOA-Glc‡	Magda roots	———
	Sesnord roots	———
	Magda aerial parts	———
	Sesnord aerial parts	———

‡ The detection limit under the conditions of the experiment is $1\ 10^{-7}$ mmol/tissue.

was higher than HDMBOA-Glc. After this period, HDMBOA-Glc became the major compound.

For each compound, results found for Sesnord variety were similar to those obtained for Magda variety in aerial parts and roots.

Four other 1,4-benzoxazin-3-one derivatives were detected in maize plants. Their presence depended on variety, plant age and plant tissue (Table 1). Concentrations of these compounds were lower than those of DIMBOA-Glc. HM_2BOA-Glc and HDM_2BOA-Glc varied between 0 and 50 μmol/kg fresh weight whereas, DIBOA-Glc and HMBOA-Glc varied between 0 and 1 mmol/kg fresh weight. Roles and relations of these compounds are not known till now.

2.2. Changes of 1,4-benzoxazin-3-ones derivatives content in aerial parts of 20-day-old plant

Changes of DIMBOA-Glc, DIM_2BOA-Glc and HDMBOA-Glc content in the first leave, the oldest and the fourth leave, the youngest of Magda and

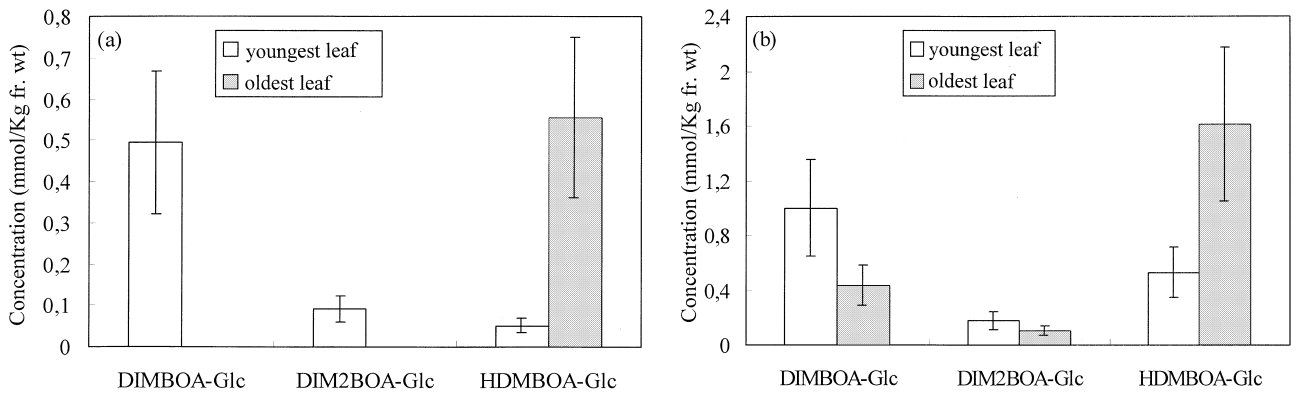


Fig. 3. Concentration of three 1,4-benzoxazin-3-one derivatives present in the oldest and the youngest leaf of 20-day-old maize plants. (a): Magda variety and (b): Sesnord variety. Vertical lines show standard deviations.

Sesnord varieties 20-day-old, fourth stage leave, were also determined. Results (Fig. 3) indicate that the youngest leave contain higher hydroxamic acid levels than the oldest. Argandona et al. (1981) reported similar results after measuring DIMBOA. Concerning the HDMBOA-Glc, we observed the opposite distribution. Indeed, concentration in the first leave is higher.

3. Discussion

Our results show that concentration of DIMBOA-Glc is high two days after germination and then decreases rapidly in roots and aerial parts. Nevertheless, this compound continues to be biosynthesised during about the first 6–10 days following the germination. After this period, the decrease is more rapid than a simple growth dilution effect. This result suggests that hydroxamic acid is released, degraded and/or transformed in the plant. We show for the first time that DIM_2BOA-Glc follows the same pattern. Morse, Wratten, Edwards and Niemeyer (1991) declared that decline of hydroxamic acids in maize leaves was not entirely due to a growth dilution effect. They suggest that abscisic acid is one possible candidate for the factor which control the hydroxamic acid levels of maize leaves.

Variation of N–O-methyl derivative, HDMBOA-Glc, has never been studied according to plant age and plant organ. Our results show that, in aerial parts, concentration of this compound varies with plant age in the same way as that of DIMBOA-Glc. Nevertheless, distribution of this compound is very different. Indeed, its concentration is higher in the oldest leaf than in the youngest. The opposite is observed for DIMBOA-Glc. We show also that variation of HDMBOA-Glc is very different from hydroxamates in the roots. Indeed, it continues to be biosynthesised 15 days after germination and becomes the major 1,4-benzoxazin-3-one derivative in roots. Its level is 12 times more important than DIMBOA-Glc in Magda roots and 3 times in Sesnord roots.

In the literature, HDMBOA-Glc was probably overlooked because its aglycone, HDMBOA is very unstable and is not more detected after plant extraction. Indeed, Grambow, Lückge, Klausener and Müller (1986) have shown that HDMBOA degraded very rapidly into MBOA, the same degradation product of DIMBOA. In a previous work, we confirmed this result (Cambier et al., 1999). Nevertheless, researchers nowadays consider MBOA as resulting exclusively from the DIMBOA degradation and continue to quantify DIMBOA-Glc by the sum of the measured quantities of DIMBOA and MBOA. This method can lead to important errors when concentration of HDMBOA-Glc is high in the plant. In our experiment, this is observed in roots and in the oldest leave of maize plant. These results draw the attention on a possible confusion between role of HDMBOA-Glc and DIMBOA-Glc in the literature. We have avoided these problems with the direct analysis of the glucosylated forms.

In Belgium, aphids, *Metopolophium dirhodum*, are not present on seedlings of maize. In laboratory, breeding of these aphids on maize younger than 15 days is not possible because of their high mortality and low fecundity. Thus, maize seems to have developed a defence mechanism against these aphids for the more sensitive stage. We observed that aphids population begin to develop on 15-day-old maize plants when DIMBOA-Glc and HDMBOA-Glc concentration in aerial parts become very weak. In the literature, several papers have shown that DIMBOA protects poaceae against aphids, *Metopolophium dirhodum* (Argandona, Luza, Niemeyer & Corcuera, 1980; Niemeyer et al., 1989). Nevertheless, DIMBOA is not present in noninjured plants and no research has shown that DIMBOA-Glc converts into DIMBOA during an attack of the plant by aphids. Indeed, Massardo, Zuniga, Perez and Corcuera (1994) showed that DIMBOA-Glc and DIMBOA-β-glucosidase are stored in different compartments (extravacuolar and vacuolar spaces, respectively) and Dreyer & Campbell (1987) stated that aphids can neatly avoid substances compartmentalized inside cell vacuoles by merely bypassing the vacuoles or by probing intercellulary. Thus, it is possible that during the aphids attack, DIMBOA is not present in the plant due to self degradation. In conclusion, it is likely that one or several 1,4-benzoxazin-3-one derivatives play a resistance role to aphids *M. dirhodum* but there is no evidence that this compound is really the DIMBOA.

Our results suggest that it is essential for further studies to focus on the roles and relations of 1,4-benzoxazin-3-one derivatives emphasising the compounds which are really present in the plant and not to restrict the investigation on DIMBOA only.

4. Experimental

4.1. Plant material

Seeds of *Zea mays* L., Magda and Sesnord varieties, were planted in 10 cm (diameter) × 13 cm (height) plastic pots, 1 seed per pot, with vermiculite and watered every two days with 50 ml of a nutrient solution (Cambier et al., 1999). Growth conditions were $20 \pm 2^{\circ}C$, 16 h light/8 h dark photoperiod and a light intensity of 6500 lx. Each seedling was taken out and washed with tap water 1, 2, 3, 4, 6, 10, 15 or 20 days after germination. Roots were measured from one day

after germination, and aerial parts from the time when they become visible, two days after germination. Roots and aerial parts were cut at their junction with the seed and then weighed. The first and the fourth leaf of 20-day-old maize plants, fourth leaf stage, were also cut.

4.2. Extraction method

Roots or aerial parts of each plant were cut in small pieces, put in 60 ml of boiling methanol and heated for 30 min. A standard was then added (1 ml of BOA 0,65 mM per sample). The methanol extract was separated by decantation and evaporated under vacuum. The residue was redissolved in 200 μl of methanol. Preliminary tests showed that over 95% of each 1,4-benzoxazin-3-one derivative were extracted with this method. Each process was repeated at least three times. Samples were stored in a freezer (−20°C) before analysis.

Nongerminated dry maize seeds were macerated in boiling methanol during 30 min. Solid residue was crushed with 10 ml of methanol and a small amount of fine sand. The homogenate was centrifuged (2000 g) for 5 min and the supernatant was combined with the previous extract. The methanol was then evaporated under vacuum. The residue was redissolved in 200 μl of methanol.

4.3. Instrumentation and HPLC–APCI procedures

The method employed for HPLC was similar to the one described by Lyons, Hipskind, Wood and Nicholson (1988) and Cambier et al. (1999). Samples were chromatographed with a Spectra System AS 3000 on a 2,1 mm × 25 cm microbore reversed phase C18 column (5 μm, adsorbosphere). A two-solvent system was used to generate the mobile phase: solvent A was 1% acetic acid in water and solvent B was 100% methanol. Solvents were degassed and filtered permanently. The flow rate was 200 μl/min. The mobile phase at the initiation of each run was a 8:2 ratio (A to B). Before analysis, all samples were passed through a 0.2 μm filter. After injection of 3 μl of this filtrate, a 3 min linear gradient from 8:2 to 5:5 A/B was applied and held for another 17 min. Elution was monitored at 280 nm with a Spectra System UV 1000 detector and mass spectrometry.

The mobile phase flow was connected to a Finnigan MAT (San Jose, CA) TSQ 7000 triple quadrupole mass spectrometer with an atmospheric pressure chemical ionisation source (APCI) interface. Nitrogen was used as both nebulizer (70 psi) and auxiliary (10 psi) gas of the liquid inlet. The APCI source was operated at 7 kV corona discharge. The capillary temperature was 220°C and the vaporiser temperature was 400°C. Spectra were acquired in negative ion mode.

4.4. Semi-quantitative analysis of 1,4-benzoxazin-3-one derivatives

The concentration of DIMBOA-Glc + DIM_2BOA-Glc (two coeluting compounds), and HDMBOA-Glc were estimated using UV detection by comparison of their peak area with the peak area of standard (BOA). The UV extinction coefficients (280 nm) of these compounds were determined in water/methanol (50/50). ε BOA = 2800 ± 200, ε DIMBOA-Glc = $10{,}300 \pm 500$, ε HDMBOA-Glc = 8300 ± 400. ε DIMBOA-Glc and ε DIM_2BOA-Glc were supposed to be identical. Mass spectrometry allowed the semi-quantitative analysis of DIMBOA-Glc and DIM_2BOA-Glc separately. Ratio of peak intensity of DIMBOA-Glc deprotonated molecular ion (372 u) and peak intensity of DIM_2BOA-Glc deprotonated molecular ion (402 u) was used to measure relative concentration of these two compounds supposing the constant response factor. With these methods, the concentrations of these compounds are not exactly known but they give good approximations, maximum experimental error being 25%.

Acknowledgements

V. Cambier has been supported by a Ph.D. fellowship from the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (F.R.I.A.). T. Hance is a Research Associate of the National Fund for Scientific Research (Belgium). The authors are indebted to the Belgium National Fund for Scientific Research (F.N.R.S.).

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