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An Efficient Method for the Quantification of Hydroxamic Acids From Wheat by Thin Layer Chromatography–Densitometry

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A new method is described for the quantification of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), the main hydroxamic acids in wheat and rye extracts, respectively, in cereal extracts based on densitometry of scanned thin layer chromatographic plates. The method allows the simultaneous quantification of up to five samples, and is linear between 0.5–7 μg and 10–30 μg for DIMBOA and between 0.5–3.0 μg and 10–30 μg for DIBOA. Quantification of DIMBOA by this method generates a linear correlation with results obtained following analysis by high performance liquid chromatography. The possibility of applying this methodology to mixtures of DIBOA and DIMBOA is discussed. © **1998 John Wiley & Sons, Ltd.**

Keywords: hydroxamic acids; benzoxazinones; DIMBOA; DIBOA; thin layer chromatography–densitometry.

INTRODUCTION

Hydroxamic acids present in cereal extracts confer resistance to the plant towards insects such as the European corn borer and several species of aphids (Niemeyer and Pérez, 1995). Hydroxamic acids are present in the plant as 2-β-*O*-D-glucopyranosides which are hydrolysed to the corresponding aglucones when the tissue is damaged (Hofman and Hofmanova, 1971).

Three groups of methods have been described in order to quantify hydroxamic acids in cereal extracts. In the first, the tissue is ground and then either frozen, thawed and heated or simply heated to promote hydrolysis of the glucosides to agluconic benzoxazinones and their further decomposition to benzoxazolinones. These latter derivatives are then separated chromatographically and quantified by UV-VIS spectroscopy (Beck *et al.*, 1957; Molot and Anglade, 1968), by spectrofluorimetry (Bowman *et al.*, 1968) or by infrared spectroscopy (Scism *et al.*, 1974), or are quantified directly by isotopic dilution (Klun and Brindley, 1966), by gas chromatography (GC) (Pessi and Scalorbi, 1979; Malan *et al.*, 1986), or by visual determination after thin layer chromatographic (TLC) separation (Robinson *et al.*, 1982). These methods assume that decomposition of hydroxamic acid aglucones to benzoxazolinones is quantitative, or at least that a single correction factor may be applied to all samples. These asumptions have not been validated by experiments. The decomposition reaction is not quantitative, the yield of benzoxazolinones being not only a function of pH (Niemeyer *et al.*, 1982) and temperature (Woodward *et al.*, 1978a; Bravo and Niemeyer, 1986a), but also of the composition of the reaction medium (Woodward *et al.*, 1978b; Argandoña *et al.*, 1981; Bravo and Niemeyer, 1986b).

A second group of methods is based on the quantification of hydroxamic acids as Fe (III) complexes (Hamilton, 1964; Long *et al.*, 1974; Sullivan *et al.*, 1974). These methods suffer from uncertainties owing to the non-specific nature of the colourimetric reaction of Fe(III) and also to their inability to quantify different hydroxamic acids that may be present in the samples. However, the method has been applied successfully to series of related cereals (Woodward *et al.*, 1979a).

The third group of methods allows for the formation of aglucones and the quantification of these compounds directly by GC (Woodward *et al.*, 1979b) or by high performance liquid chromatography (HPLC) on silica columns (Gutiérrez *et al.*, 1982) or on reverse-phase columns (Lyons *et al.*, 1988; Niemeyer *et al.*, 1989; Xie *et al.*, 1990; Mayoral *et al.*, 1994). This has become the method of choice for hydroxamic acid analysis.

Direct TLC–densitometric quantitation has been applied to a number of natural products in extracts with high accuracy and repeatability, e.g. cuticular waxes (Davyt *et al.*, 1995), carbohydrates (Cesio *et al.*, 1996), steroidal alkaloids (Ferreira *et al.*, 1993), and cineole from the essential oil of Eucalyptus (Rossini *et al.*, 1995). Moreover, the UV spectra taken directly from a TLC plate was useful in identifying a series of flavonoids (García *et al.*, 1993). In this paper, we describe a new method to quantify 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), the main hydroxamic acid in wheat extracts, and/or 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), the main hydroxamic acid in rye extracts, based on the TLC separation from plant extracts of these

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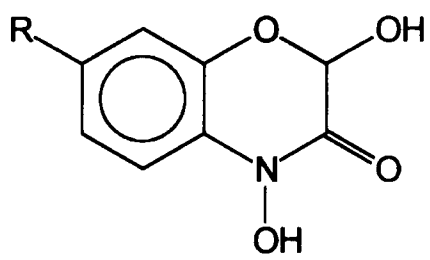

DIBOA: R= H
DIMBOA: R= CH_3O

aglucones, and their quantification by densitometry after scanning the chromatographic layer.

EXPERIMENTAL

Chemicals and instruments. All solvents were of reagent quality or better. Silica gel and polyamide TLC plates were from Machery and Nagel (Düren, Germany). The HPLC system was a Shimadzu (Tokyo, Japan) model LC8A, and the TLC Scanner system was a Shimadzu type CS9000 operating in the zig-zag mode.

Preparation of extracts. Certified seeds of wheat (*Triticum aestivum* L. cv. Estanzuela Tarariras (La Estanzuela, Colonia, Uruguay)) were germinated on dishes with 1 mL water per seed. Shoots of seedlings of different ages (*ca.* 50 mg fresh tissue) were ground in an Agate mortar with three aliquots (0.33 mL each) of water, and left for 15 min at room temperature thus allowing for complete glucoside hydrolysis. The pH was then adjusted to 3.4 using 0.03M phosphoric acid. The suspension was centrifuged for 20 min at 2000 rpm. The supernatant was extracted with diethyl ether (3 × 1 mL) and the organic phases dried over sodium sulphate and evaporated at reduced pressure. The vacuum was released under nitrogen and the residue dissolved in 100 µL of acetonitrile. Aliquots of this solution were spotted (3 × 10 µL) on to TLC layers or injected into the HPLC (20 µL) for quantification.

Thin layer chromatography. Standards of DIMBOA and DIBOA were separated using chloroform: butanol: acetic acid (30:15:1) as mobile phase (system 1; R_f values: DIMBOA = 0.65; DIBOA = 0.52) A different mobile phase was used with wheat extracts since they were shown to contain only DIMBOA: silica gel layers (0.1 mm thickness) were developed using toluene: diethyl ether (1:4; R_f value: DIMBOA = 0.6), and polyamide layers (0.1 mm thickness) with butanol: acetic acid (20:1; system 2; R_f value: DIMBOA = 0.7). Response and standard curves were determined using various exact amounts of DIMBOA and DIBOA solutions (1.00 mg/mL) in acetonitrile spotted with an HPLC syringe on to a 20 × 5 cm precoated polyamide layer. The plate was placed in a saturated (system 2) developing chamber and solvent was allowed to ascend to the top of the plate (5cm). After air drying for 15 min, measurements were performed at $\lambda = 280$ nm, in the zig-zag mode, reading the plate transversally at $R_f = 0.7$.

High performance liquid chromatography. The column (15 × 1 cm id.) was packed with R 18 (Machery-Nagel). The solvent system was a 25:75 mixture of methanol and 0.05‰ phosphoric acid aqueous solution pumped at a flow of 1 mL/min. Acidic solvent was used in order to minimise the decomposition of aglucones (Niemeyer *et al.*, 1982) and peak tailing. UV detection was at $\lambda = 280$ nm; retention times were 3.9 min for DIBOA and 5.4 min for DIMBOA.

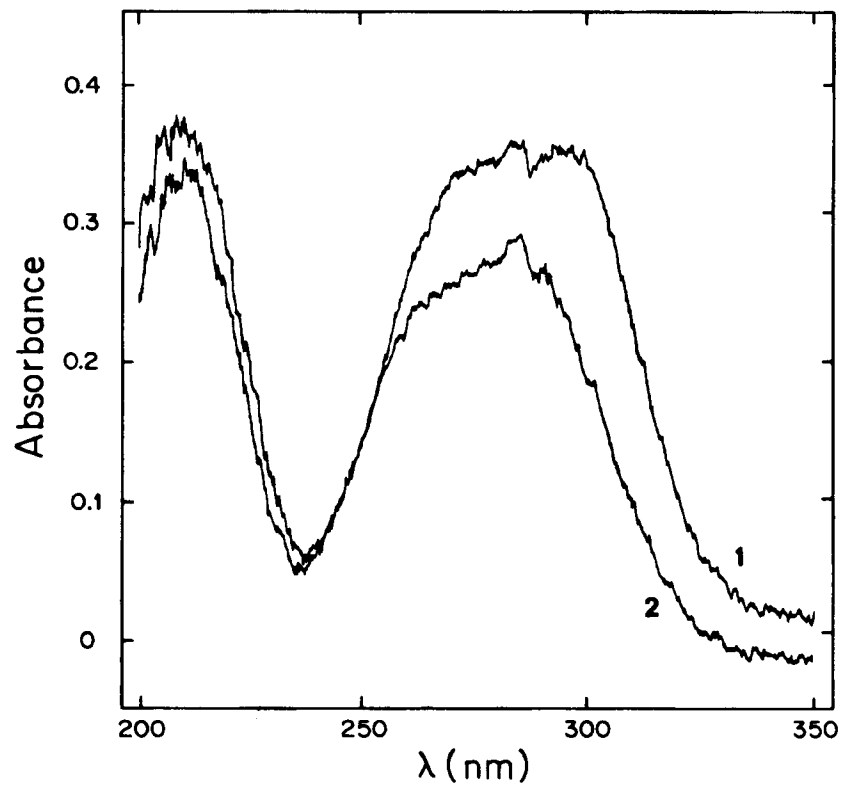

Figure 1. UV spectra of DIMBOA (trace 1) and DIBOA (trace 2) measured over a polyamide thin layer chromatographic layer.

RESULTS AND DISCUSSION

Selection of the TLC conditions and quantitation

Silica gel and polyamide were tested as stationary phases. DIMBOA and DIBOA were found to decompose on the silica gel plate, giving rather stable spots after 20 min exposure to air but lacking a definite absorption maximum in the UV-VIS, therefore, silica gel was discarded as unsuitable for quantification. Good stability of the spots corresponding to hydroxamic acid aglucones was found on polyamide plates and DIMBOA and DIBOA could be separated using solvent system 1.

Selection of the detection reagent

Some reported visualization agents for hydroxamic acids such as ferric chloride, Fast Blue B and Draggendorff reagent did not produce linear responses or showed low sensitivity. UV light was the most effective visualizing agent for DIMBOA and DIBOA, concentrations being measured at $\lambda = 285$ nm. The most suitable wavelength was chosen after taking the spectra of hydroxamic acid spots over the polyamide TLC plate (Fig. 1) with the TLC scanner. Two ranges of linear response were observed for both hydroxamic acids in the case of DIMBOA between 0.5 and 7 µg ($r = 0.995$) and between 10 and 30 µg ($r = 0.998$) (Fig. 2), and in the case of DIBOA between 0.5 and 4 µg and 10 and 30 µg ($r = 0.999$) (Fig. 3). The

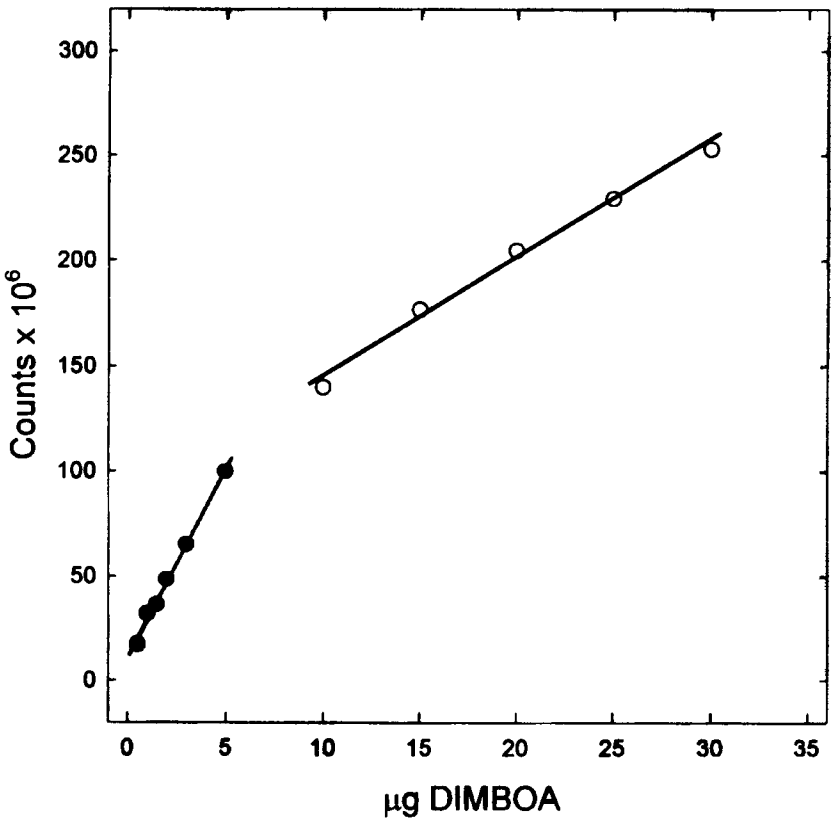

Figure 2. Response curve of the UV detector of the thin layer chromatographic plate scanner to increasing concentrations of DIMBOA.

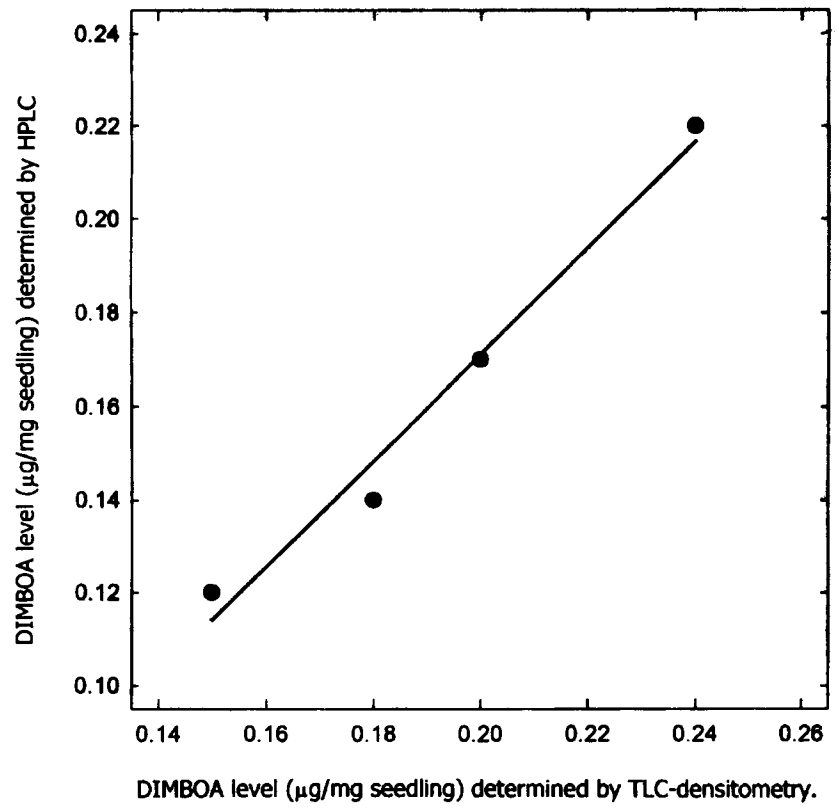

Figure 4. The correlation between independent determinations by thin layer chromatography–densitometry and high performance liquid chromatography of the levels of DIMBOA in four samples of 15-day-old wheat seedlings.

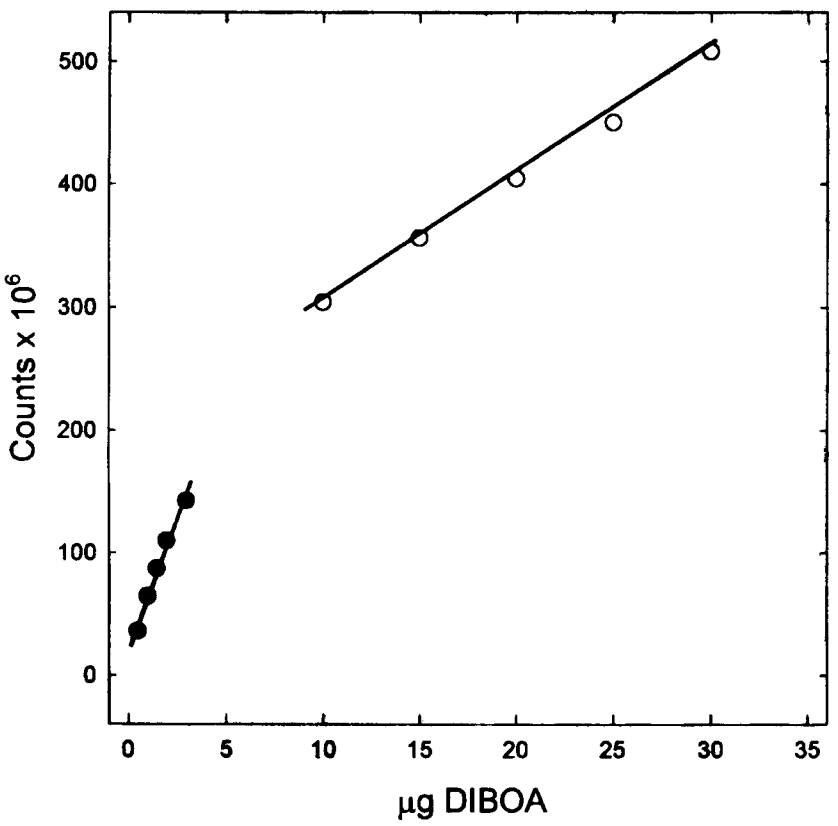

Figure 3. Response curve of the UV detector of the thin layer chromatographic plate scanner to increasing concentrations of DIBOA.

shape of the response curve and, therefore, the linearity ranges were similar for both hydroxamic acids, not surprisingly since the UV chromophores are nearly the same. The lack of linearity of the response may be attributed to the chromatographic quantification since the absorbance of solutions of DIMBOA and DIBOA at comparable dilutions to those used in the chromatographic response curves correlated linearly with concentration (results not shown). Deviations from linearity in quantifications by TLC have been described and theoretical treatments developed (Goldman and Goodall, 1968). However, the preferred method for routine analytical work involves the use of calibration curves within the range of linear response of the detector (Ferreira *et al.*, 1993; Davyt *et al.*, 1995; Rossini *et al.* 1995; Braithwaite and Smith, 1996; Cesio *et al.*, 1996). Moreover, the UV spectra taken directly from a TLC plate was useful to identify a series of flavonoids (García *et al.*, 1993). The same spot of DIMBOA was read 20 times in order to check the repeatability of the measurements: standard deviations of the absorbance readings were always smaller than 4.1%.

Method development

For each plate a calibration experiment was performed. Correlation coefficients for calibration lines were always larger than 0.997. The precision of the method was determined by duplicate spotting and quantification of the same concentration of DIMBOA on five different plates. The coefficient of variation of the absorbance readings was, in all cases, less than 2.3%. Each sample was analysed in duplicate and the plate was scanned transversally: this allowed the simultaneous quantitation of up to five different samples per plate. The total time required for the determination was about 30 min, considering the time employed to spot the samples, chromatographic development, scanning of the plate and treatment of data.

Comparison with high performance liquid chromatography

HPLC has been up to now the method of choice to quantify hydroxamic acids. Nevertheless, each run takes aproximatively 10–15 min, and only one sample can be analysed at one time. Given that hydroxamic acid levels vary with plant age (Argandoña *et al.*, 1981), the concentration of DIMBOA in comparable samples of

seedlings of ages ranging from 5 to 15 days were determined by TLC and by HPLC. The results from both methods correlated linearly ($r = 0.998$, Fig. 4). The main advantage of the TLC determination is that the time of analysis per sample is reduced as more than one sample can be investigated per run, with minimal amounts of extract. Solvent system 1 separated DIMBOA and DIBOA and could be used to quantify simultaneously both hydroxamic acid aglucones in wheat seedlings. In the case of presently analysed extracts of wheat seedlings, only DIMBOA could be detected. However, if both DIMBOA and DIBOA had been observed, the number of samples that could be simultaneously analysed would be reduced to three since the reference curve for DIBOA should be generated over the same plate. Simultaneous quantification of glucosides and aglucones has been described (Lyons *et al.*, 1988). The present methodology involves transformation of naturally-present glucosides to aglucones since it aims at the fast and reliable screening of hydroxamic acids levels as indicators of resistance potential in the plant. Seedlings may be analysed containing hydroxamic acids in the mmol/kg fresh weight range found for a set of wordwide wheats (Nicol *et al.*, 1992). Although our study was limited to wheat, the method should be applicable to other cereals and grasses where hydroxamic acids occur and is particulary suitable when many samples must be quantified fast, accurately and with high repeatability.

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