ELSEVIER

Journal of Chromatography A, 864 (1999) 315–321

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Simultaneous determination of phenolic acids and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one in wheat (*Triticum aestivum* L.) by gas chromatography–tandem mass spectrometry

Hanwen Wu[a,*], Terry Haig[a], Jim Pratley[a], Deirdre Lemerle[b], Min An[c]

[a]*Farrer Centre for Conservation Farming, Charles Sturt University, P.O. Box 588, Wagga Wagga, NSW 2678, Australia*

[b]*NSW Agriculture, Wagga Agricultural Institute, Wagga Wagga, NSW 2650, Australia*

[c]*Environmental and Analytical Laboratories, Charles Sturt University, P.O. Box 588, Wagga Wagga, NSW 2678, Australia*

Received 23 June 1999; received in revised form 24 September 1999; accepted 27 September 1999

Abstract

A procedure using gas chromatography and tandem mass spectrometry (GC–MS–MS) has been developed for the identification and quantification of some allelochemicals in wheat (*Triticum aestivum* L.). The quantities of allelochemicals in wheat shoots ranged from 2.9 to 110 mg per kilogram of dry shoot residues. Compared with gas chromatography–mass spectrometry (GC–MS), the GC–MS–MS technique significantly increased instrument selectivity and sensitivity, thereby providing more reliable quantitation results in the determination of the phytotoxic compounds examined during this allelopathy research.

Keywords: Wheat; Allelochemicals; *Triticum aestivum*; Phenolic acids; Dihydroxymethoxybenzoxazinone

1. Introduction

Wheat (*Triticum aestivum* L.) has been found to possess allelopathic potential [1,2] and studies have been conducted to apply wheat allelopathy for biological weed control [3,4]. Wheat cultivars differed in their allelopathic ability to suppress the growth of annual ryegrass (*Lolium rigidum* G.) and the degree of allelopathic inhibition was highly associated with the total phenolic contents contained in each wheat extract [4]. *p*-Hydroxybenzoic, vanillic, *p*-coumaric, syringic and ferulic acids have been regarded as some of the major phenolic acids predominantly identified in wheat stubbles and in the soil [1,5]. In addition to the phenolic acids, one of the hydroxamic acids, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), has also been reported as an active allelochemical in wheat [6]. The phytotoxicity of these compounds has been clearly demonstrated [7,8].

Allelopathic compounds are often found as complex mixtures and their determination presents an analytical challenge. Many traditional separation techniques such as paper, thin-layer, and column chromatography have been used for the separation and identification of phenolic compounds, but these methods are limited in separation power [9]. High-performance liquid chromatography (HPLC) gives

*Corresponding author. Tel.: +61-2-6933-2749; fax: +61-2-6933-2812.

E-mail address: 11134425@belgarian.riv.csu.edu.au (H. Wu)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)01034-1

better results and has been widely used for the analysis of phenolics and DIMBOA in plants [5,7,10]. The best separation power has been achieved by capillary gas chromatography (GC) in the determination of phenolic mixtures [9]. Gas chromatography coupled with mass spectrometry (GC–MS) has been regarded as a powerful analytical tool for the characterisation of complex organic mixtures and has successfully been employed in the identification and quantitation of active allelopathic compounds in vulpia (*Vulpia myuros* L. Gmel) [11]. Recent advances in instrumentation have further boosted the analytical power for the determination of allelopathic compounds. Ion trap detectors have been improved to perform tandem mass spectrometry (MS–MS), which enables the ion trap to isolate an ion of interest and then produce characteristic progeny ions by collision-induced dissociation (CID). Compared to single-stage MS, MS–MS technology enhances instrumental selectivity and sensitivity and has become a favoured tool for the quantitative analysis of the complex matrixes encountered in biological and environmental applications [12]. However, this powerful technique has not yet been much employed in allelopathy studies.

Although both phenolic acids and cyclic hydroxamic acids have been reported in relation to wheat allelopathy, no attempt has been made to simultaneously determine these two distinct groups of allelopathic compounds. This study was designed to apply gas chromatography–tandem mass spectrometry (GC–MS–MS) to selectively identify and quantify some phenolic acids and DIMBOA in a very complex matrix of wheat shoot extract.

2. Experimental

2.1. Chemicals and reagents

p-Hydroxybenzoic acid (PHB), vanillic acid (VAN), syringic acid (SYR), *p*-coumaric acid (COU), ferulic acid (FER) and the internal standard (*p*-chlorobenzoic acid) were obtained from Sigma–Aldrich. DIMBOA was kindly provided by Dr. William S. Chilton of the Department of Botany, North Carolina State University, Raleigh, NC, USA. HPLC-grade methanol was obtained from EM Science and the GC-grade diethyl ether was purchased from BDH. The derivatising reagent, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), was obtained from Alltech Australia.

2.2. Analytical procedure

2.2.1. Preparation of calibration standards

Seven standard solutions each containing all six target compounds, i.e., *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid and DIMBOA, were first prepared in methanol at concentrations of 0.05, 0.1, 0.5, 1, 5, 10, 20 μg/ml, respectively. In separate analyses, 1 ml from each of the seven methanolic standard solutions was pipetted into a 2-ml minivial and dried by nitrogen blow-down. A 1-ml volume of internal standard *p*-chlorobenzoic acid at the concentration of 5 μg/ml in methanol was then pipetted into the dry minivial and dried again with nitrogen prior to the derivatisation step.

2.2.2. Preparation of wheat samples

Surface-sterilised and pre-germinated wheat seeds (*Triticum aestivum*, cv. Triller) were grown in a nutrient-free agar medium under aseptic conditions in a controlled growth cabinet with a daily light/dark cycle of 13 h/11 h and a temperature cycle at 25°C/13°C. The fluorescent light intensity in the cabinet was $3.56 \pm 0.16 \cdot 10^3$ lux. Shoots of 17-day-old wheat seedlings were harvested and immediately freeze-dried (Christ Alpha 1-4 freeze dryer, B. Braun Biotech International). An amount of 0.100 g of freeze-dried wheat shoots was cut into 2-mm lengths, ground into powder with a mortar and pestle after the addition of liquid nitrogen and macerated with 3 ml of 0.001 *M* HCl. The entire macerate was transferred into a labelled glass scintillation vial and sonicated at 5°C for 15 min (Unisonics, Australia). The resulting mixture was centrifuged at 20 000 rpm at 10°C for 15 min to remove the debris (Avanti J-30I Centrifuge, Beckman, USA). The supernatant was then collected and extracted three times with 10-ml portions of diethyl ether. The ether layers were combined and evaporated on a rotary evaporator under reduced pressure at 35°C until the volume of residual solution was approximately 2 ml. The 2 ml ether solution was then transferred to a 2-ml minivial

and dried with nitrogen blow-down. A 1-ml volume of internal standard *p*-chlorobenzoic acid at the concentration of 5 μg/ml in methanol was then pipetted into the dry minivial and dried again with nitrogen prior to the derivatisation step.

2.2.3. Derivatisation

The silylation of calibration standards and the wheat shoot sample was accomplished by the addition of 1.00 ml of MSTFA at 60°C for 30 min. The silylated samples were analysed by GC–MS–MS or GC–MS. The large excess of MSTFA ensured that the derivatization was complete. Care was taken to ensure anhydrous conditions during the preparation and derivatization process because of the high sensitivity of trimethylsilyl (TMS) derivatives towards moisture.

2.2.4. GC–MS–MS instrumentation and conditions

GC–MS–MS analysis was carried out on a Varian 3400 CX gas chromatograph coupled with a Varian Saturn 2000 ion trap mass spectrometer. Samples were introduced via a DB-5 MSITD (Ion Trap Tested) fused-silica capillary column of 30 m×0.25 mm I.D., with a stationary phase thickness of 0.25 μm (J&W Scientific, Alltech, Australia). The gas chromatographic conditions for the analysis of wheat allelochemicals were slightly modified from An [7]. The column temperature was initially held at 80°C for 1 min, then programmed to 160°C at a rate of 10°C/min, from 160 to 235°C at a rate of 5°C/min and from 235 to 280°C at a rate of 50°C/min, with a final hold time of 5 min (total run time, 29.9 min). Helium was used as the carrier gas with purity of 99.9999% and its linear velocity was 34 cm/s. Injector temperature was maintained at 280°C, and the injection volume was 1 μl with the splitless mode.

The electron impact ionization (EI) mode with automatic gain control (AGC) was used for MS. The electron multiplier voltage for MS–MS was 1450 V, AGC target was 10 000 counts and filament emission current was 60 μA with the axial modulation amplitude at 4.0 V. The ion trap was held at 200°C and the transfer line at 250°C. Manifold temperature was set at 60°C. Mass spectral scan time from m/z 50 to 450 was 1.0 s (using 3 microscans). Nonresonant CID was used for MS–MS. The associated parameters for the MS–MS method were optimised for each individual compound (Table 1). The method was divided into 10 acquisition segments so that different ion preparation files could be used to optimise the conditions for the TMS derivatives of the chemically distinct internal standard, phenolic acids and DIMBOA. Standard samples of both *p*-coumaric acid and ferulic acid consisted of *trans* and *cis* isomers so that four segments were required to characterise the *p*-coumaric and ferulic acids. The first segment was a 9-min solvent delay, which was necessary for the protection of the electron multiplier from the large solvent peak signal.

2.2.5. Identification and quantitation

Two user libraries (MS and MS–MS library) were generated with the injection of TMS-derivatised authentic reference compounds by GC–MS or GC–MS–MS analysis. The MS library recorded the retention times and the mass spectra of TMS deriva-

Table 1
Ion trap MS–MS method parameters[a]

	Acquisition segment[b]								
	2	3	4	5	6	7	8	9	10
Compound (silylated)	*p*-Chlorobenzoic acid	PHB	VAN	*cis*-COU	SYR	*cis*-FER	*trans*-COU	DIMBOA	*trans*-FER
Segment time (min)	4.10	0.40	2.60	2.00	0.30	0.50	1.10	1.80	8.10
Retention time (min)	9.78	13.30	15.74	16.23	18.23	18.63	19.06	20.16	21.91
Chosen precursor ion (m/z)	213	267	297	293	297	323	293	340	323
Excitation amplitude (V)	54	64	43.6	45	43.6	41	45	46.5	41.2
Excitation storage level (m/z)	60	80	65	65	65	65	65	75	65
Quantifying product ion (m/z)	169	223	267	249	253	293	249	194	293

[a] Mass isolation window (m/z) was set at 3 and excitation time (ms) at 20.
[b] Segment one was the solvent delay.

tives of authentic standards under the chosen chromatographic conditions. Similarly, the MS–MS library recorded the retention times and the daughter mass spectra derived from the specific precursor ions of TMS derivatives of each authentic standard after CID with helium gas. The allelopathic substances were then identified by comparing retention times and mass spectral data with those in either user library, depending upon the type of analysis. All quantitation was performed by the method of internal standardisation using *p*-chlorobenzoic acid at a concentration of 5 μg/ml as the internal standard. The quantitation of these compounds was based on the peak area of the selected daughter ions listed in Table 1 and is reported in units of milligrams per kilogram of dry matter. All calibration standards and the wheat samples were run in triplicate.

3. Results and discussion

Underivatised phenolic acids and DIMBOA have relatively low volatility and are not suitable for direct capillary GC analysis. MSTFA was chosen as the derivatisation reagent to convert analytes into volatile compounds. The GC–MS analysis of silylated wheat shoot extract was repeated three times (Fig. 1). Analysis by GC–MS was able to identify all the target allelopathic compounds, i.e., *p*-chlorobenzoic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid and DIMBOA by comparing retention times and the mass spectra with those of TMS derivatives of their authentic compounds under identical conditions. However, the *p*-hydroxybenzoic acid, *cis*-*p*-coumaric acid, *trans*-*p*-coumaric acid, syringic acid, *cis*-ferulic acid and DIMBOA were strongly coeluted with background substances (Fig. 2), although *p*-chlorobenzoic acid, vanillic acid and *trans*-ferulic acid were well separated. Owing to the complexity of the sample matrix, for each of the six co-eluted analytes shown in Fig. 2, there could not be found unique characteristic GC–MS ions of sufficient intensity to serve as suitable quantifying ions. In order to help illustrate this difficult background matrix problem, the more characteristic analyte ion chromatograms and total ion chromatograms of Fig. 2 have been supplemented with indicative background ion chromatograms from coelutants. Coelution with these analytes is also further evident in the asymmetry of the more characteristic analyte ion profiles. The simple GC–MS results were therefore not considered adequate for accurate quantitation.

The tandem mass spectrometry technique was introduced in order to filter out unwanted chemical backgrounds. After preliminary experimentation, the MS–MS conditions shown in Table 1 were used to analyse the phenolic acids and DIMBOA in wheat

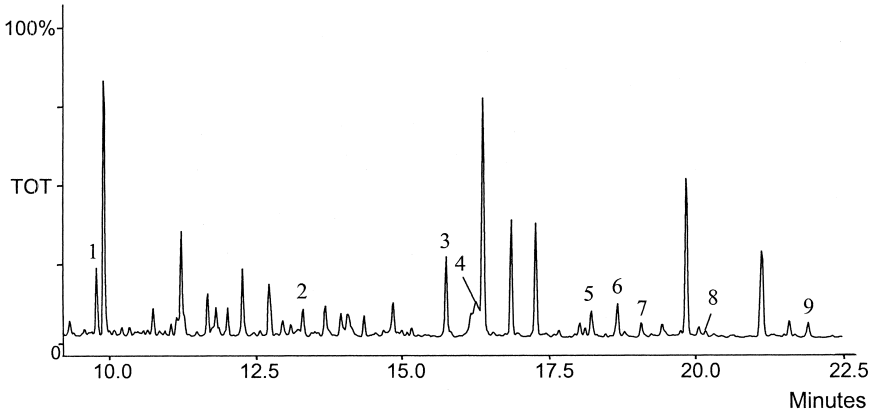

Fig. 1. Reconstructed total ion current chromatogram (RTIC) of derivatized wheat shoot extract obtained from GC–MS. Peaks: 1=*p*-chlorobenzoic acid, 2=*p*-hydroxybenzoic acid, 3=vanillic acid, 4=*cis*-*p*-coumaric acid, 5=syringic acid, 6=*cis*-ferulic acid, 7=*trans*-*p*-coumaric acid, 8=2,4,-dihydroxy-7-methoxy-1,4,-benzoxazin-3-one, 9=*trans*-ferulic acid.

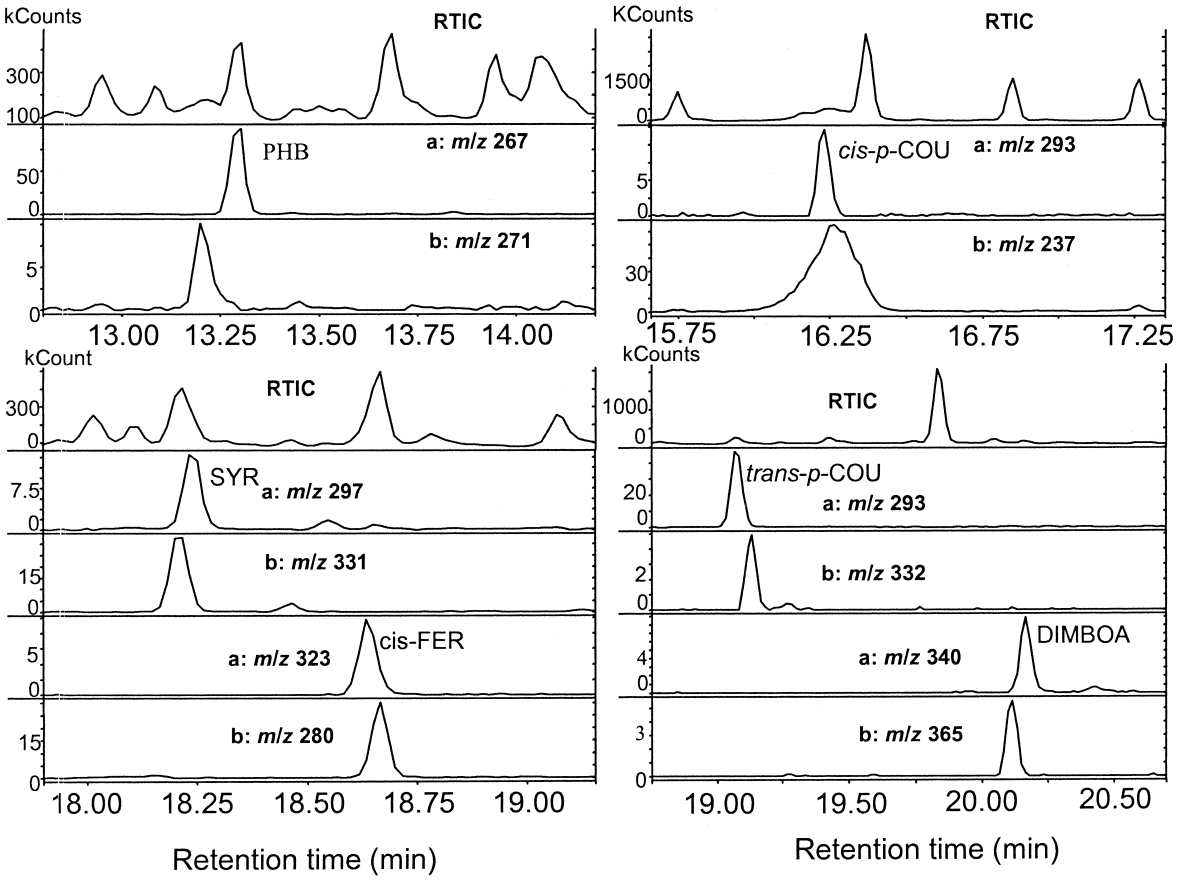

Fig. 2. GC–MS run showing the coelution of analytes with background signals (*: analytes were identified by comparing retention times and mass spectral data with those in the user MS library; a: precursor ion of the analyte; b: background ion).

shoots. The GC–MS–MS analysis of the shoot extract was repeated three times. A highly-resolved chromatogram was successfully obtained (Fig. 3). The MS–MS technique successfully removed most of the unwanted chemical signals and provided a clear background for the quantification of the chosen allelopathic compounds. The precursor ion of the particular compound was isolated and then CID with helium carrier gas gave rise to a characteristic daughter mass spectrum for each analyte (Table 2). The preferential isolation of the particular ions by the MS–MS technique yielded much stronger analyte signals than those of single stage MS analysis, thereby significantly reducing the effect of matrix noise. The signal/noise ratios for all the allelopathic compounds were much lower in GC–MS than in GC–MS–MS (Table 3). The signal/noise ratios for *p*-chlorobenzoic acid, *p*-hydroxybenzoic acid and vanillic acid in the shoot sample were 51, 25 and 29 times higher under MS–MS conditions than under MS conditions. This technique was then employed to quantify the allelochemicals in wheat (Table 2). Wheat shoots contained a higher amount of DIMBOA than of phenolic acids. The allelochemical contents in wheat shoots were 110 mg/kg dry mass for DIMBOA and 2.9 mg/kg dry mass for *cis*-*p*-coumaric acid. The difficulties in the separation and identification of *cis* and *trans* mixtures have been reported [13]. However, the *cis* and *trans* isomers of *p*-coumaric and ferulic acids were successfully sepa-

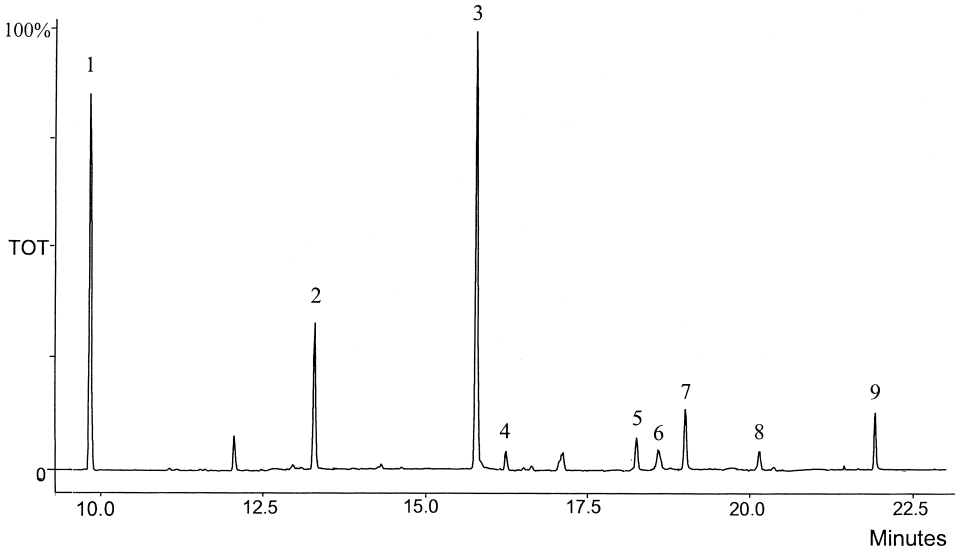

Fig. 3. Reconstructed total ion current chromatogram of derivatized wheat shoot extract obtained from GC–MS–MS. Peaks as in Fig. 1.

Table 2
Important ions present in the daughter mass spectra of silylated compounds in wheat extract by GC–MS–MS and their concentrations

Peak No.	Compound[a]	*m/z*	Quantity±SD (mg/kg dry mass)
1	*p*-Chlorobenzoic acid	213[b], 169 (100%), 141, 138, 133, 131, 105, 91, 75	Internal standard
2	PHB	267, 251, 225, 223 (100%), 207, 193, 179, 147, 103	22±2.3
3	VAN	297, 267 (100%), 253, 237, 225, 224, 223, 181, 147	56±4.3
4	*cis*-*p*-COU	293, 251, 249 (100%), 247, 233, 231, 223, 219, 203	2.9±0.1
5	SYR	297, 271, 255, 253 (100%), 225, 223, 193, 179, 133	11±0.7
6	*cis*-FER	323, 294, 293 (100%), 279, 264, 249, 233, 179, 175	3.9±0.2
7	*trans*-*p*-COU	293, 251, 249 (100%), 247, 233, 231, 223, 219, 203	37±1.5
8	DIMBOA	340, 296, 268, 250, 221, 220, 194 (100%), 192, 147	110±4.8
9	*trans*-FER	323, 294, 293 (100%), 279, 264, 249, 233, 179, 175	89±2.0

[a] Identified as trimethylsilyl (TMS) derivative.
[b] The first ion in each compound list is the precursor ion; all other ions are greater than 5% of the base peak.

Table 3
Signal/noise ratio of GC–MS and GC–MS–MS

Compound	Signal/noise ratio±SD	
	GC–MS	GC–MS–MS
p-Chlorobenzoic acid	34±9.6	1700±52.8
PHB	11±2.2	270±49.1
VAN	88±7.7	2500±38.0
cis-*p*-COU	24±3.5	26±2.1
SYR	13±5.7	68±5.0
cis-FER	17±7.3	42±4.1
trans-*p*-COU	17±9.3	180±14.1
DIMBOA	3.3±2.3	17±3.0
trans-FER	35±11.0	250±17.2

rated under the present chromatographic conditions despite their similar daughter mass spectra (Table 2). Results showed that *trans*-ferulic and *trans*-*p*-coumaric acid were present in higher quantities than their *cis* isomers in wheat shoots.

4. Conclusions

Application of state-of-the-art analytical tools can generate unambiguous results in the determination of responsible allelopathic compounds in a complex mixture of plant origin. In the present study, a

GC–MS–MS technique has been successfully used to obtain a highly resolved chromatogram to determine several key phenolic compounds and DIMBOA. The enhanced sensitivity and selectivity of GC–MS–MS have provided reliable quantitative results for the biologically active compounds in complex mixtures encountered in this allelopathy research and can be employed to screen cultivars for their differential production of allelopathic compounds in a large number of wheat accessions. This technique coupled with DNA technology will facilitate the identification of genetic markers conferring the biosynthesis of allelopathic compounds. The possibilities for genetic manipulation of crop allelopathic potential have been reviewed [14]. The genetic manipulation of crop cultivars might provide crop plants with strong allelopathic potential to compete with weeds, thereby reducing the input of herbicides into agroecosystems.

Acknowledgements

The authors are thankful for financial support from the Australian Cooperative Research Centre for Weed Management Systems and acknowledge the provision of GC–MS–MS instrumentation from the Environmental and Analytical Laboratories at Charles Sturt University.

References

[1] W.D. Guenzi, T.M. McCalla, Agron. J. 58 (1966) 303.
[2] R.W.L. Kimber, Aust. J. Agric. Res. 18 (1967) 361.
[3] J.A. Spruell, Dissertation Abstr. Int., B Sci. Eng. 45 (1984) 1102B.
[4] H. Wu, J. Pratley, D. Lemerle, T. Haig, B. Verbeek, in: Proc. 9th Aust. Agron. Conf., Wagga Wagga, 1998, p. 567.
[5] U. Blum, T.R. Wentworth, K. Klein, A.D. Worsham, L.D. King, T.M. Gerig, S.W. Lyu, J. Chem. Ecol. 17 (1991) 1045.
[6] H.M. Niemeyer, Phytochemistry 27 (1988) 3349.
[7] M. An, Ph.D. Thesis, Charles Sturt University (1995).
[8] F.J. Perez, Phytochemistry 29 (1990) 773.
[9] C.S. Creaser, M.R. Koupai-Abyazani, G.R. Stephenson, J. Chromatogr. 478 (1989) 415.
[10] F.J. Perez, J. Ormeno-Nunez, J. Chem. Ecol. 17 (1991) 1037.
[11] M. An, J.E. Pratley, T. Haig, Rapid Commun. Mass Spectrom. 10 (1996) 104.
[12] C.K. Van Pelt, H. Haggarty, J.T. Brenna, Anal. Chem. 70 (1998) 4369.
[13] E.M. Gaspar, H.C. Neves, Allelopathy J. 2 (1995) 79.
[14] H. Wu, J. Pratley, D. Lemerle, T. Haig, Weed Res. 39 (1999) 171.