Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography A, 1187 (2008) 111–118
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Analysis of carbohydrates in *Lupinus albus* stems on imposition of water deficit, using porous graphitic carbon liquid chromatography-electrospray ionization mass spectrometry

Carla Antonio [a], Carla Pinheiro [b], Maria Manuela Chaves [b,c], Cândido Pinto Ricardo [b], Maria Fernanda Ortuño [b,d], Jane Thomas-Oates [a,*]

[a] *Department of Chemistry, University of York, Heslington, York YO10 5DD, UK*
[b] *Instituto de Tecnologia Química e Biológica (ITQB), Av. Da República, Apartado 127, 2781-901 Oeiras, Portugal*
[c] *Instituto Superior de Agronomia (ISA), Universidade Técnica de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal*
[d] *Centro de Edafología y Biología Aplicada del Segura, Campus Universitario de Espinardo, Apartado de Correos 164, Murcia E-30100, Spain*

Received 7 December 2007; received in revised form 25 January 2008; accepted 5 February 2008
Available online 8 February 2008

Abstract

This work reports the development and application of a negative ion mode online LC-ESI-MS method for studying the effect of water deficit on the carbohydrate content of *Lupinus albus* stems, using a porous graphitic carbon (PGC) stationary phase and an ion trap mass spectrometer. Using this method, separation and detection of several water soluble carbohydrates, ranging from mono-, di-, and oligosaccharides (raffinose, stachyose, and verbascose) to sugar alcohols was achieved in approximately 10 min. This on-line PGC-LC-ESI-MS method shows good linearity with correlation coefficients $R^2 > 0.99$, selectivity, short analysis time, and limits of detection (LOD) ranging from 0.4 to 9 pmol for sugars and 4–20 pmol for sugar alcohols. This PGC-LC-ESI-MS method is sensitive and allowed us to detect even small alterations in carbohydrate levels in *L. albus* stems that resulted from a mild/early water deficit (nmol g^{-1} DW). This paper describes details of our method and its application to the quantitative analysis of water soluble underivatised carbohydrates extracted from *L. albus* stem tissues that have been subjected to early and severe water deficit conditions, followed by a rewatering period.

Keywords: Carbohydrate; Water deficit; Early stress; Severe stress; Porous graphitic carbon; Mass spectrometry; *Lupinus albus*; Stem

1. Introduction

Lupinus albus L. is an important grain legume crop widely cultivated in intensive farming systems. Fully developed lupin grains are of great nutritional value and are widely used in diets of ruminants, primarily due to their unique carbohydrate properties, characterised by negligible levels of starch, and high levels of proteins [1]. Lupins are also cultivated for human consumption (high nutritional protein and oil contents) and as an environmentally friendly fertilizer; they increase the percentage of nitrogen, phosphorus, and organic matter in the soil, and therefore contribute to the improvement of water retention, and other soil characteristics [2].

L. albus is able to withstand water deficit (WD). It has been previously reported that it can survive severe drought conditions during both vegetative and reproductive growth [3,4]. When occurring during vegetative growth, WD affects several organs in different ways. Despite the loss of leaves, *L. albus* plants recover upon rewatering (RW), the stems being the fundamental structures that allow the plant to survive [3]. Furthermore, the metabolism of the two stem components (cortex and stele) was differently affected by WD, suggesting that these two components are important systems for studying the biochemical alterations that allow the plant to survive this stress.

It has been generally proposed that the alterations in the carbohydrate content of a plant under WD act as a metabolic signal to the plant's stress response system; carbohydrates (mainly soluble sugars and sugar alcohols) are also known to

* Corresponding author. Tel.: +44 1904 43 4459; fax: +44 1904 43 2516.
E-mail addresses: cipa500@york.ac.uk (C. Antonio), pinheiro@itqb.unl.pt (C. Pinheiro), mchaves@itqb.unl.pt (M.M. Chaves), ricardo@itqb.unl.pt (C.P. Ricardo), agr004@cebas.csic.es (M.F. Ortuño), jeto1@york.ac.uk (J. Thomas-Oates).

0021-9673/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2008.02.010

act as osmolytes during dehydration [5]. Although they are thus important analytes for understanding the biochemical processes underlying drought resistance, carbohydrates are difficult compounds to analyse due to their high polarity, poor UV absorbance, and the structural variety of isomeric structures. Highly polar compounds are poorly or completely unretained on reversed-phase columns and generally elute close to the void volume without chromatographic separation.

High performance anion-exchange liquid chromatography (HPAEC) with pulsed amperometric detection (PAD) has been used very often for the detection of carbohydrates, and commercial HPAEC-PAD systems are available from Dionex [6–8]. However, the mobile phases used with commercial HPAEC systems contain high concentrations of sodium hydroxide and sodium acetate, and therefore are not compatible with electrospray ionization mass spectrometry (ESI-MS). The high salt content in the samples suppresses ionization and causes capillary blockages and ion source contamination, thus compromising sensitivity [9,10]. To overcome this problem, post-column membrane suppressors were developed by Dionex that were designed to remove the salt from the mobile phase following separation [11]. Although the ion suppressor does desalt, it is not effective enough to make on-line coupling with ESI-MS routinely applicable.

Porous graphitic carbon (PGC) is an LC stationary phase developed by Knox and co-workers as an alternative to the commonly used reversed-phase (RP-) silica packings, and became commercially available in 1988 under the trade name Hypercarb™ [12,13]. The retention mechanism of PGC has been defined as the Polar Retention Effect on Graphite (PREG) [14] determined by a balance of two factors: (i) hydrophobic eluent–analyte repulsions, which occur between a hydrophilic eluent and any nonpolar segments of the analyte, (ii) the interaction of polarizable or polarized functional groups in the analyte with the graphite. PGC stationary phases have advantages over classical RP- and HPAEC columns of stability over the entire pH range, and the use of MS compatible mobile phases without the need for ion-pairing reagents, hence allowing efficient coupling with electrospray ionization. The use of PGC columns has been reported for the separation of mono-, di- and oligosaccharides [15,16], underivatised water soluble oligosaccharides extracted from *Triticum aestivum* stems [17], and more recently for the separation of water soluble sugars and sugar phosphates extracted from *Arabidopsis thaliana* leaves [18]. Here we report the application of a robust on-line LC-ESI-MS method using a PGC stationary phase and an ion trap instrument to follow the alterations in carbohydrate content of *L. albus* stems subjected to a gradual imposition of WD from the early phases to the most severe stress, followed by a rewatering (RW) period.

2. Experimental

2.1. Chemicals

Water and solvents used for chromatography were of HPLC grade and purchased from Fisher (Fisher Scientific, Loughborough, UK). All standards were purchased from Sigma (Poole, UK). Standard stock solutions were prepared in water at a concentration of 1.0 mg mL^{-1} and stored at −20 °C prior to use. Further dilutions were prepared in water.

2.2. Plant material

L. albus L. plants (cv. Rio Maior) were cultivated on a sterilised soil, peat, sand mixture (1:1:1, v/v) in controlled-environment growth chambers: photon flux density 290–320 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), photoperiod (12 h), temperature (19/25 °C, night/day) and relative humidity (65–70%). Twenty-three days after sowing, WD was induced by withholding watering (this caused a natural and slow induction of WD). Plants were collected 4 and 13 days after withholding water (DAW), and 6 and 26 h after rewatering (RW). Control plants were watered throughout the whole period. Sample collection took place 3–5 h after the beginning of the light period. The stem was cut at both extremities (cotyledon level and the shoot/root junction) to separate the vascular (stele) and cortical (cortex) tissues. A small vertical incision produced at the cotyledon extremity allowed the cortex to be peeled off the stele. As the separation occurred at the cambium level, the stele included both the parenchymatous pith tissue and the xylem while the cortex included parenchymatous tissue and the phloem. Stele and cortex tissues were immediately frozen in liquid nitrogen, lyophilised, and stored at −80 °C prior to extraction and LC-mass spectrometric analysis.

2.3. Characterisation of soil and plant water status

Soil water content (Ψ_{soil}) was measured with a ThetaProbe soil moisture sensor (ML2x ThetaProbe coupled with ThetaMeter type HH2 from Delta-T Devices). Leaf water potential was measured with a Sholander pressure chamber (PMS instrument Co, Corvallis, Oregon, USA) at predawn ($\Psi_{leaf\ pd}$). The relative water content (RWC) for leaflets, stems (cortex and stele) was determined according to Rodrigues et al. [19].

2.4. Extraction of water soluble carbohydrates from L. albus stems

Water soluble carbohydrates were extracted from *L. albus* stem tissues (cortex and stele) in chloroform/methanol [18]. Lyophilised stem material was finely ground in liquid nitrogen using a pre-cooled mortar and pestle. Approximately 10–12 mg plant material were transferred to a polypropylene microfuge tube (2.0 mL) containing 250 μL ice-cold chloroform:methanol (3:7, v/v), vortex-mixed, and the frozen mixture was incubated at −20 °C for 2 h to stop metabolism and extract water soluble metabolites. After incubation, 200 μL ice-cold water were added to the tube, and the tube was kept at 4 °C with continuous shaking. The samples were then centrifuged at 17,900 × *g*, at 4 °C, for 10 min. The upper phase was transferred to a new polypropylene microfuge tube (1.5 mL) and kept at 4 °C. The lower chloroform phase was re-extracted with 200 μL ice-cold water, centrifuged as described above, and the second upper phase was added to the first. The combined extract was evaporated to dryness using

a centrifugal concentrator (Savant SpeedVac system, Thermo Electron Corporation, Runcorn, UK). Samples were reconstituted in 100 μL water and centrifuged at 6800 × *g* at 20 °C for 30 min followed by liquid chromatography ion trap mass spectrometric analysis.

2.5. Liquid chromatography-ion trap mass spectrometry conditions

LC-MS analyses were performed on a Thermo Finnigan Surveyor HPLC system coupled to an ion trap mass spectrometer (LCQ DECA XP Plus, Thermo Electron, San Jose, CA, USA), equipped with a Thermo Finnigan orthogonal electrospray interface. Neutral carbohydrates were detected in the negative ion mode using the following MS parameters: ion source voltage −3.0 kV, capillary voltage −20 V, tube lens offset −60 V, capillary temperature 300 °C, sheath gas 40 (arbitrary units) and auxiliary gas 30 (arbitrary units). Mass spectra were acquired over the scan range *m/z* 50–1000, and data were processed using Xcalibur 1.3 software (Thermo Finnigan, San Jose, CA, USA). Precursor ions were selected with an isolation width of 2 Th and activated for 30 ms. Collision induced dissociation (CID) experiments used helium as the collision gas and normalised collision energy settings were in the range 20–30%, depending on the analyte. Chromatographic separation was carried out using a PGC Hypercarb™ column (5 μm, 100 mm × 4.6 mm; Thermo Electron, Runcorn, Cheshire, UK) at a flow rate of 600 μL min^{-1}. The sample injection volume was 20 μL and the PGC column was used at ambient temperature (25 °C). Two binary mobile phases were used during method development. Mobile phase 1 was composed of (A) water and (B) acetonitrile. Mobile phase 2 was composed of (A) water modified with 0.1% (v/v) of formic acid (FA) and (B) acetonitrile modified with 0.1% (v/v) of FA. The gradient elution profile was as follows: 0–5 min, 96% A + 4% B to 92% A + 8% B; 5–7 min, 92% A + 8% B to 75% A + 25% B, and maintained for 3 min, followed by column re-equilibration: 10–12 min, 75% A + 25% B to 50% A + 50% B, and maintained for 4 min; 16–18 min, 50% A + 50% B to 96% A + 4% B and maintained for 10 min.

3. Results and discussion

3.1. PGC-LC-ESI-MS method development

Separation of mono-, di-, and oligosaccharides on PGC columns have been reported by several different groups to be successful using mobile phases composed mainly of acetonitrile and water [15–18]. We therefore started by optimising our LC separation using mobile phases composed of different ratios of water:acetonitrile (mobile phase 1, details in Section 2) and making use of a solution containing a mixture of authentic standard neutral soluble sugars and sugar alcohols that are known to act as osmolytes in plants during dehydration.

The standard mixture was constituted in order to contain representatives of the main compound types: neutral monosaccharide glucose (Glc), neutral disaccharide sucrose (Suc), neutral trisaccharide (raffinose), neutral tetrasaccharide (stachyose), neutral pentasaccharide (verbascose), and the sugar alcohols mannitol (monosaccharide alcohol), and maltitol (disaccharide alcohol). This mixture was separated on a PGC column without derivatisation and all standard compounds were detected in the negative ion mode as formylated molecules

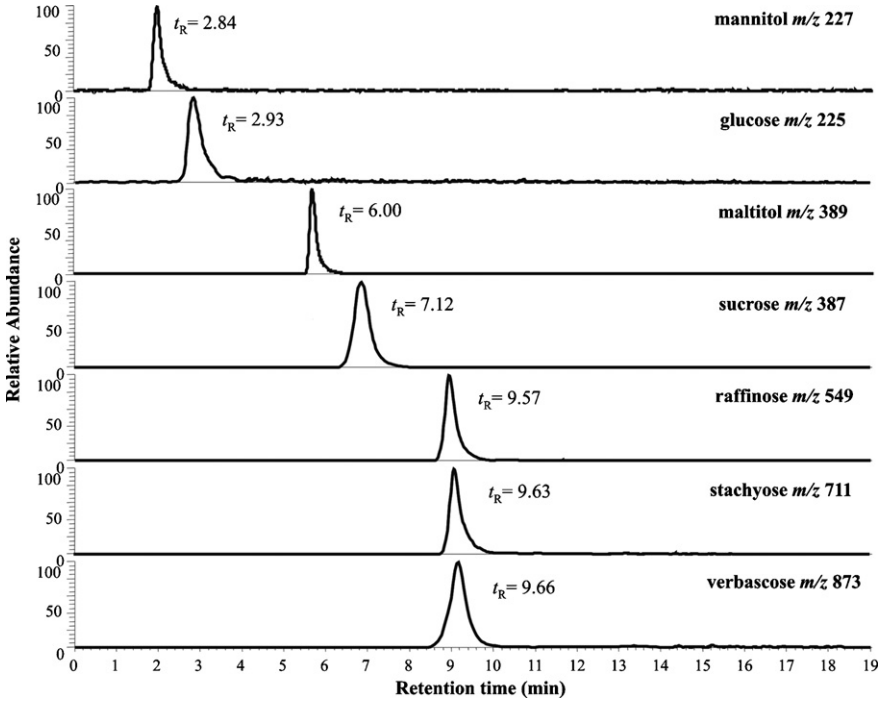

Fig. 1. Extracted ion chromatograms obtained using mobile phase 1 and porous graphitic carbon (PGC) negative ion mode ESI-MS for the separation of a standard mixture of neutral sugars and sugar alcohols (50 μg mL^{-1}). All compounds were detected as formylated molecules $[M + HCOO]^-$.

Table 1
Intra-day and inter-day repeatability of retention times, limits of detection (LOD), limits of quantification (LOQ), and linearity of calibration curves obtained for standard compounds using negative ion PGC-LC-ESI-MS

Standard compounds	Diagnostic ion (*m/z*)	t_R (min)	t_R (intra RSD)[a] (%, $n=3$)	t_R (inter RSD)[b] (%, $n=6$)	LOD[c] (μM)	LOQ[d] (μM)	Amount LOD[e] (pmol)	R^{2f}
Inositol	225 $[M+HCOO]^-$	2.44	0.47	1.48	0.60	2.00	12	0.9981
Mannitol	227 $[M+HCOO]^-$	2.84	0.54	0.96	1.00	3.33	20	0.9985
Fru	225 $[M+HCOO]^-$	2.86	0.68	0.77	2.00	6.70	40	0.9958
Glc	225 $[M+HCOO]^-$	2.93	0.20	1.15	0.45	1.60	9	0.9956
Sorbitol	227 $[M+HCOO]^-$	2.95	0.78	1.47	1.00	3.33	20	0.9989
Galactinol	387 $[M+HCOO]^-$	4.31	0.48	0.50	0.08	0.25	2	0.9958
Maltitol	389 $[M+HCOO]^-$	6.00	0.35	1.11	0.08	0.25	2	0.9991
Suc	387 $[M+HCOO]^-$	7.12	0.41	0.59	0.08	0.25	2	0.9995
Maltose	387 $[M+HCOO]^-$	8.34	0.37	0.71	0.08	0.25	2	0.9931
Raffinose	549 $[M+HCOO]^-$	9.57	0.12	0.50	0.10	0.33	2	0.9966
Stachyose	711 $[M+HCOO]^-$	9.63	0.21	0.41	0.06	0.20	1	0.9970
Verbascose	873 $[M+HCOO]^-$	9.66	0.26	0.42	0.02	0.07	0.4	0.9997

HPLC conditions: Mobile phase 1, Hypercarb PGC column (4.6 mm × 100 mm), 20 μL injection, flow rate 600 μL/min.
[a] Intra-day relative standard deviation (RSD) of retention times ($n=3$ independent measurements).
[b] Inter-day relative standard deviation (RSD) of retention times ($n=6$ independent measurements).
[c] Concentration limit of detection (LOD) calculated at a signal-to-noise ratio (S/N) of 3.
[d] Limit of quantification (LOQ) calculated at a signal-to-noise ratio (S/N) of 10.
[e] LOD of the amount loaded onto column calculated at a signal-to-noise ratio (S/N) of 3.
[f] Correlation coefficients for the standard curves (five points) of relative peak areas against concentration (0–100 μM). Each point on the standard curve is the mean value of 3 independent measurements.

$[M+HCOO]^-$ using electrospray ion trap mass spectrometry (Fig. 1).

Neutral carbohydrates were also observed to ionise as formylated molecules on direct infusion negative ion mode ESI analyses of standard solutions in methanol:water (50:50, v/v) (data not shown), thus showing their affinity for low levels of background formate anions present in the instrument.

In order to ensure that our data would be reproducible in other laboratories using other LC-systems (where formate anions may not be present in the background) we have tested the effect of addition of small amounts (0.1%) of formate to the mobile phase (mobile phase 2, details in Section 2). In this experiment, solvent (A) was water modified with 0.1% of formic acid and (B) acetonitrile modified with 0.1% of formic acid, to ensure that formate anions are present. The same standard mixture as in Fig. 1 was separated on the PGC column using mobile phase 2. We verified that the same elution profile was obtained as before (when no formic acid was present in the mobile phase), as well as that the retention times remained unchanged (all were the same within the standard deviation). We also determined the LODs for all the standard compounds listed in Table 1, and again verified that they were the same (within the standard deviation) as when using mobile phase 1.

Since in our hands good PGC-LC separation and identification of all standard compounds was achieved in approximately 10 min using a binary gradient composed of water and acetonitrile (without the need for addition of formate), we carried out the remainder of the experiments using mobile phase 1. Although raffinose ($t_R=9.57$ min), stachyose ($t_R=9.63$ min), and verbascose ($t_R=9.66$ min) elute very close together, since these compounds have different masses it was decided not to attempt to separate them further, as this would have required extending the gradient and thus the run time. Instead, we chose to make use of the conveniently short run time, by exploiting the ability to differentiate these compounds mass spectrometrically.

Following the optimisation of the LC separation, we have further developed this PGC-method by performing LC-tandem mass spectrometry experiments (MS^n) on the individual authentic standard compounds to record their retention times (t_R), mass-to-charge ratios (*m/z*) (Table 1) and their characteristic ion trap mass spectrometric fragmentation behaviour. Specific ions (formylated molecules $[M+HCOO]^-$, Table 1) were selected as precursors for fragmentation in order to collect MS^2 and MS^3 data. Galactinol ($t_R=4.31$ min), Suc ($t_R=7.12$ min) and maltose ($t_R=8.34$ min) are isomeric compounds detected at *m/z* 387 (formylated molecules $[M+HCOO]^-$, Table 1) and it is clear from their retention times that these are resolved using PGC.

To illustrate typical MS^n data obtainable using this PGC-based method, we describe experimental data for the non-reducing trisaccharide raffinose ($[M+HCOO]^-$ at *m/z* 549) (Fig. 2). Raffinose is a member of the raffinose family oligosaccharides (RFOs), which are non-reducing carbohydrates, consisting of galactose units linked to Suc via α-1,6 glycosidic linkages.

Fig. 2a illustrates the extracted ion chromatogram for raffinose at *m/z* 549 from the full scan negative ion ESI-MS, the extracted ion chromatogram for *m/z* 503 from the full scan product ion spectrum of *m/z* 549, and the extracted ion chromatogram for *m/z* 179 from the full scan product ion spectrum of *m/z* 503 (Fig. 2a). The collision induced dissociation (CID) MS^2 product ion spectrum of raffinose produced an intense ion at *m/z* 503 ($[M-H]^-$) corresponding to the loss of formic acid [HCOOH] (Fig. 2b). Subsequent CID MS^3 analysis, readily achieved using ion trap MS, of the $[M-H]^-$ ion at *m/z* 503 produced intense ions at *m/z* 341 and at *m/z* 179, which are formed by cleavage of the glycoside bond with the loss of one hexose $C_6H_{10}O_5$

(−162 Da), and the loss of two hexoses $C_6H_{10}O_5$ (−324 Da), respectively, and produced cross-ring fragment ions at *m/z* 221, 251, 281, and 311 which are formed by multiple neutral losses of CH_2O (−30 Da) (Fig. 2c).

3.2. Linearity and limits of detection (LOD) of the PGC-LC-ESI-MS method

The potential of the method for quantitative analysis of water soluble carbohydrates from stem tissues of *L. albus* was evaluated by testing intra and interday repeatability of retention times, and the linearity of response of the PGC-LC-ESI-MS method. Intraday repeatability was measured by injecting the same standard solution three times in a single day. Interday repeatability was measured by analysing the same standard solution over 6 different days. Intraday and interday repeatability of retention times using our method gave relative standard deviations (RSD) of less than 2% (Table 1). The linearity of the PGC-LC-ESI-MS response was measured for several analytes ranging from mono- to pentasaccharides and sugar alcohols by recording the responses at different concentrations over the range 0–100 μM. Five-point standard curves were obtained by plotting integrated peak areas versus concentration. Each point on the calibration curve is the mean value of three independent measurements using the PGC-LC-ESI-MS method. The limit of detection (LOD) for each compound was calculated as the min-

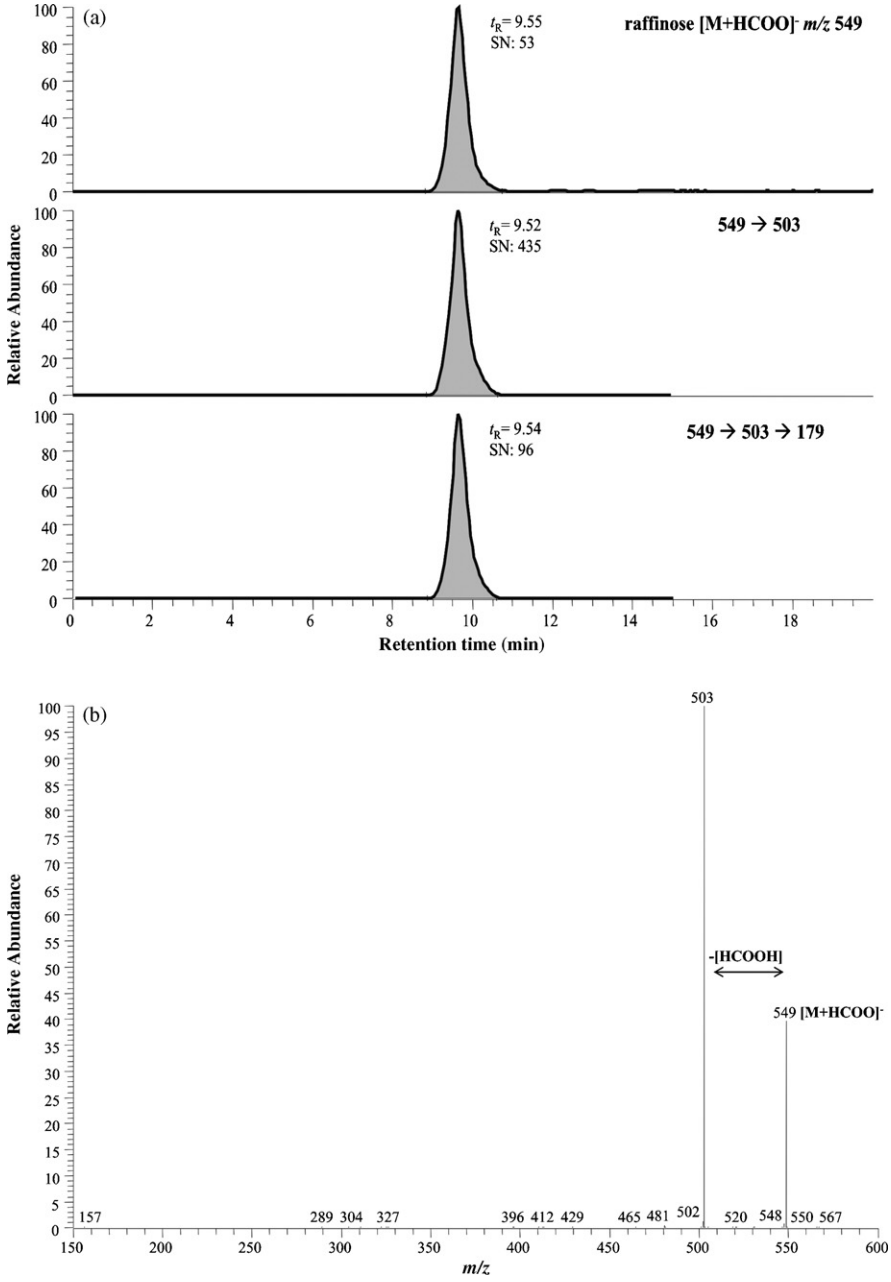

Fig. 2. (a) Extracted ion chromatograms obtained (mobile phase 1) on porous graphitic carbon (PGC) negative ion mode ESI-MS showing the specificity of detection provided by CID MS^n for a standard solution (10 μM) of raffinose, detected at *m/z* 549 ($[M + HCOO]^-$). (b) CID MS^2 spectrum of raffinose (precursor ion $[M + HCOO]^-$ at *m/z* 549). (c) CID MS^3 spectrum of raffinose (precursor ion $[M - H]^-$ at *m/z* 503).

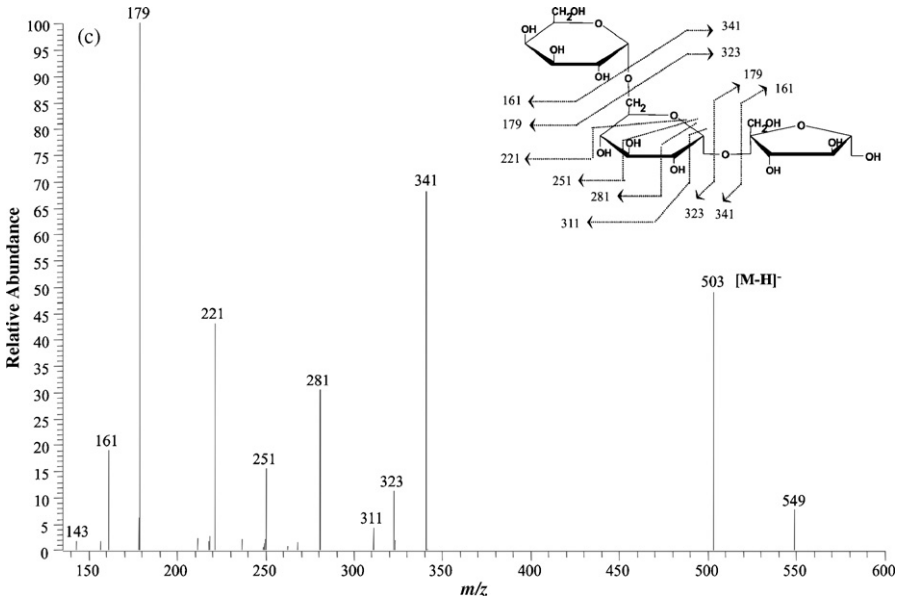

Fig. 2. (*Continued*).

imum amount injected which gave a detector response higher than three times the signal-to-noise ratio (S/N), and the limit of quantification (LOQ) was calculated at a S/N ratio of 10. The on-line PGC-LC-ESI-MS method showed good linearity of response over the concentration range 0–100 μM for all analytes, with correlation coefficients $R^2 > 0.99$ (Table 1).

3.3. Characterisation of the L. albus plant water status during early/mild and severe water deficit (WD), and recovery conditions

The characterisation of the *L. albus* plant water status during development and stress imposition was monitored by means of soil water content (Ψ_{soil}), leaf water potential at predawn (Ψ_{pd}), and relative tissue water content (RWC) (Table 2).

At 4 DAW, the soil water content had decreased by approximately 30–40% when compared to that of well watered (WW) control plants, but the plant water status was barely affected, as the leaf and stem RWCs decreased by only around 5%. Although there was a clear soil water shortage at 4 DAW the stress on the plant can be considered as mild.

At 13 DAW, the soil water content was extremely reduced (by approximately 80%) when compared to WW control plants, and this was significantly reflected in the plant water status: leaf Ψ_{pd} decreased by about 5-fold (down to −1.7 MPa), leaf RWC decreased by approximately 50%, and stem RWC decreased by around 15%. It was clear from these results that at 13 DAW the plant is under severe water deficit. However, the stem components (cortex and stele) were much less affected during the WD period than leaves, as they showed a higher capacity to retain water (only 15% reduction). It was also clear that *L. albus* can withstand severe WD, since upon rewatering (RW) the plant water status was rapidly restored (Table 2). In fact, 6 h after water reintroduction the RWC of leaves and stems was already restored and after 26 h of rewatering the plant water status was similar to that of the control plants.

3.4. PGC-LC-ESI-MS analysis of water soluble carbohydrates from L. albus stem extracts

The results described above (Section 3.3) indicate that two very distinct water deficit phases can be studied in *L. albus* plants corresponding to early stress (4 DAW), and severe stress (13 DAW), and it was shown [3,4] that the stems are fundamental systems that allow *L. albus* plants to survive such WD. We have, thus, applied our on-line PGC-LC-ESI-MS system as a sensitive method to study the effect of water deficit on the water soluble carbohydrate components of *L. albus* stem tissues (cortex and stele) at 4 DAW and 13 DAW, and 6 and 26 h after rewatering.

Table 2
Soil water potential (Ψ_{soil}), leaf water potential at pre-dawn (Ψ_{pd}) and leaf, stem stele and stem cortex relative water content (RWC) during the period of water deficit (WD) imposition and rewatering (RW). WW, well watered plants (control)

DAW	Ψ_{soil} (MPa)		$\Psi_{leaf\ pd}$ (MPa)		Leaf RWC (%)		Stem stele RWC (%)		Stem cortex RWC (%)	
	WW	WD	WW	WD	WW	WD	WW	WD	WW	WD
4 d	64 ± 1.1	35 ± 1.7	−0.23 ± 0.01	−0.28 ± 0.01	90 ± 1.7	87 ± 0.8	91 ± 1.0	92 ± 0.1	68 ± 4.6	64 ± 0.5
13 d	60 ± 8.0	12 ± 0.2	−0.33 ± 0.02	−1.74 ± 0.05	86 ± 2.7	46 ± 4.7	91 ± 0.5	77 ± 0.7	66 ± 3.7	55 ± 2.9
6 h RW	57 ± 3.1	48 ± 4.3	–	–	–	84 ± 2.1	–	91 ± 1.0	–	67 ± 0.3
26 h RW	49 ± 0.5	42 ± 3.2	−0.31 ± 0.03	−0.35 ± 0.04	88 ± 4.2	86 ± 1.7	94 ± 5.3	90 ± 3.0	67 ± 1.7	71 ± 0.3

The application of our on-line PGC-LC-ESI-MS method allowed the separation and identification in a single run of three water deficit responsive metabolites from *L. albus* stem extracts: the water soluble carbohydrates Glc, Suc, and raffinose. The presence of Glc, Suc, and raffinose in stem extracts was demonstrated based on the retention times, masses, and characteristic fragmentation patterns (MS^2 and MS^3) of peaks obtained on analysis of the stem extracts, and their comparison with those obtained for authentic standard compounds (Table 1). Glc, Suc, and raffinose were quantified by PGC-LC-ESI-MS using the calibration curves (Table 1), and the ions at *m/z* 225 for Glc ($[M+HCOO]^-$), *m/z* 387 for Suc ($[M+HCOO]^-$), and *m/z* 549 for raffinose ($[M+HCOO]^-$).

Fig. 3 shows the quantitative results obtained using PGC-LC-ESI-MS for *L. albus* stems (cortex and stele) at 4 DAW (early/mild stress), 13 DAW (severe stress), and subsequent rewatering (RW) for 6 and 26 h for Glc (Fig. 3A), Suc (Fig. 3B), and raffinose (Fig. 3C). Quantitative values for the control plants (well watered) at 4 and 13 days are also shown.

Using this PGC-LC-ESI-MS method, at 4 DAW (early stress) we determined levels of $2.58 \pm 0.32\ \mu mol\ g^{-1}$ of dry weight (DW) for Glc, $1.35 \pm 0.05\ \mu mol\ g^{-1}$ DW for Suc, and much lower levels of $9.34 \pm 1.00\ nmol\ g^{-1}$ DW for raffinose in stem cortex extracts of *L. albus*. In comparison with the early stress, a decrease in sugar amounts was observed at 13 DAW (severe stress) in stem cortex extracts of *L. albus*, with levels determined to be $1.14 \pm 0.08\ \mu mol\ g^{-1}$ DW for Glc, $1.12 \pm 0.03\ \mu mol\ g^{-1}$ DW for Suc, and $2.18 \pm 0.50\ nmol\ g^{-1}$ DW for raffinose. RW caused a subsequent decrease in the levels of Glc and Suc, but not raffinose.

In the stem stele extracts of *L. albus*, the levels of these water soluble carbohydrates were found to be much lower than those in the stem cortex; 4 DAW stele levels of $0.34 \pm 0.06\ \mu mol\ g^{-1}$ DW for Glc, and $0.16 \pm 0.05\ \mu mol\ g^{-1}$ DW for Suc were obtained (raffinose was not detected). In contrast to what is observed in stem cortex tissues, an increase in sugar amounts was observed at 13 DAW (severe stress) in stem stele extracts when compared to early stress, with levels of $0.49 \pm 0.05\ \mu mol\ g^{-1}$ DW for Glc, and $0.38 \pm 0.01\ \mu mol\ g^{-1}$ DW for Suc determined. RW caused the steady decrease of glucose and sucrose.

It was observed that *L. albus* stems accumulate water soluble sugars (Glc, Suc, and raffinose) during WD, and despite the small variations of the plant water status, or the small variations of the stem RWC (Table 2), WD causes large alterations in the carbohydrate content of *L. albus* stems both 4 DAW and 13 DAW. In addition, by using PGC coupled to ESI-MS, we could separate, identify, and detect very small amounts of raffinose ($nmol\ g^{-1}$ DW) in stem cortex tissues, which was found to be highly responsive to WD, reacting rapidly to small changes in the plant water status. Interestingly, its response is higher in early stress (when the plant water status is still largely unaffected) and on RW (when the plant water status is re-established).

Raffinose is a member of the raffinose family oligosaccharides (RFOs). It has been proposed that the primary roles of RFOs in seeds and vegetative tissues are to store and transport carbohydrates, and to function as osmoprotection molecules against abiotic stresses, such as drought, salinity or cold [20,21]. The accumulation of raffinose during seed maturation has also been associated with the development of desiccation tolerance in mature seeds [20,22].

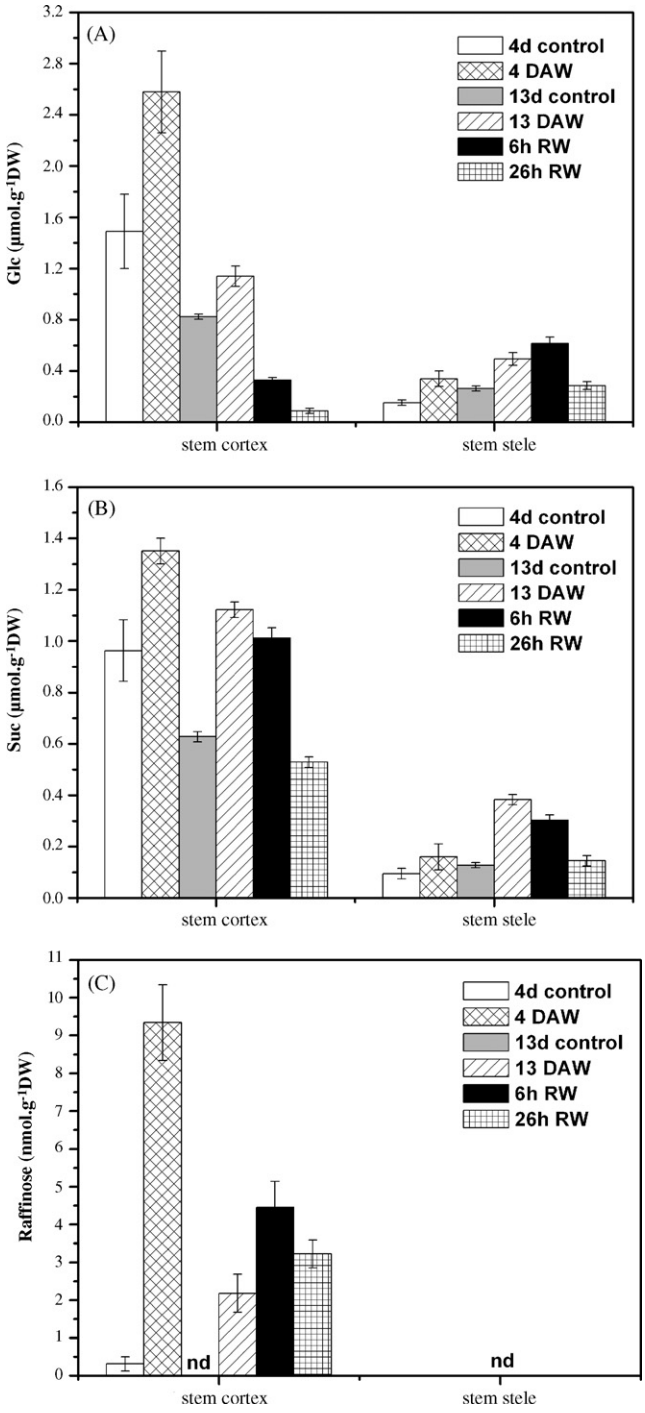

Fig. 3. Water soluble carbohydrate levels in *Lupinus albus* stems obtained by PGC-LC-ESI-MS at 4 days after withholding water (DAW; early water stress), 13 days after withholding water (severe water stress), 6 and 26 h after rewatering (RW). (A) Glucose, (B) sucrose, and (C) raffinose. Data are mean ± S.D. of $n = 2$–3 biological replicates. Each biological replicate is the mean value of $n = 2$–3 independent LC-MS measurements. DW, dry weight. Control, well watered plants.

The presence of raffinose has been previously detected in vegetative tissues of different plant systems exposed to WD (mainly using HPLC with refractive index detection or HPAEC-PAD). In coleus leaves, raffinose levels remained unchanged in response to WD, and were found to vary between 0.1 and 0.2 μmol g^{-1} FW [23]. In perennial ryegrass leaves, raffinose levels decreased in response to WD, leaf bases being less affected (from 0.7 to 0.2 μmol g^{-1} DW) than leaf blades (from 3.1 to 0.7 μmol g^{-1} DW) [24]. On the other hand, in *Arabidopsis thaliana* plants, raffinose was found to be highly responsive to progressive WD, accumulating up to 0.4 μmol g^{-1} FW [20], and a similar response was found recently in the leaves of the angiosperm resurrection plant *Xerophyta viscosa*, where raffinose accumulated up to 35 μmol g^{-1} DW [25].

In *L. albus* stem cortex tissues, not only did we determine much lower levels of raffinose (nmol g^{-1} DW) than those cited above for the leaves of other plants, but we also showed that raffinose does not increase steadily under progressive WD (i.e. a transient accumulation is observed between early and severe stress) which brings into question the role of raffinose in desiccation tolerance in stems. Taking into account that the stem cortex included parenchymatous tissue and the phloem, our results are in agreement with the hypothesis that raffinose is more effectively retained in the phloem leading to higher transport efficiency [26]. However, to our knowledge, transient accumulation of raffinose at such early stages in WD has never been reported. A further study of the effects of progressive WD in other lupin organs (leaves and roots) is underway in order to better understand the physiological role of raffinose in *L. albus* plants.

4. Conclusions

The aim of this study was to develop a robust LC-MS system using a porous graphitic carbon column, suitable for on-line coupling with ESI ion trap tandem MS, for the sensitive analysis of water soluble carbohydrates extracted from the stem tissues of *L. albus* plants subjected to early and severe water deficit conditions, followed by a rewatering period. We have demonstrated that this PGC-LC-based method is capable of separating, detecting and measuring in a single run, water soluble sugars (Glc, Suc and raffinose) in the concentration range 2.18 nmol g^{-1} DW to 2.58 μmol g^{-1} DW from *L. albus* stem tissue extracts.

Such an early stress study (4 d) requires a sensitive technique that can measure small amounts of water deficit responsive metabolites as well as tissue specific responses in *L. albus* stems, induced by a mild drought. This PGC-LC-ESI-MS method provides good linearity, selectivity, short analysis times (approximately 10 min), and LODs in the picomole range (0.4–20 pmol) for a wide variety of sugars and sugar alcohols. It combines the separation power of the LC system with ion trap MS that can perform multistage tandem MS, thus, providing useful structural identification of metabolites from a single sample analysis.

Acknowledgements

C.A. gratefully acknowledges the CHEMCELL Marie Curie Early Stage Research Training Fellowship of the European Community's Sixth Framework Programme under contract number MEST-CT-2004-504345 for financial support. J.T.-O. gratefully acknowledges Thermo Finnigan's support and funding from the Analytical Chemistry Trust Fund, the RSC Analytical Division, and EPSRC. C.P. acknowledges a grant SFRH/BPD/14535/2003 from Fundação para a Ciência e Tecnologia, Portugal.

References

[1] D.S. Petterson, in: J.S. Gladstones, C.A. Atkins, J. Hamblin (Eds.), Lupin as Crop Plants: Biology, Production and Utilization, CAB International, 1998, p. 353.
[2] C. Huyghe, Field Crops Res. 53 (1997) 147.
[3] C. Pinheiro, M.M. Chaves, C.P. Ricardo, J. Exp. Bot. 52 (2001) 1063.
[4] C. Pinheiro, J.A. Passarinho, C.P. Ricardo, J. Plant Physiol. 161 (2004) 1203.
[5] M.M. Chaves, J.P. Maroco, J.S. Pereira, Funct. Plant Biol. 30 (2003) 239.
[6] Y.C. Lee, Anal. Biochem. 189 (1990) 151.
[7] Y.C. Lee, J. Chromatogr. A 720 (1996) 137.
[8] R.R. Townsend, M.R. Hardy, Y.C. Lee, Methods Enzymol. 179 (1989) 65.
[9] J.-P. Antignac, K. de Wasch, F. Monteau, H. De Brabander, F. Andre, B. Le Bizec, Anal. Chim. Acta 529 (2005) 129.
[10] L.L. Jessome, D.A. Volmer, LCGC North America 24 (2006) 498.
[11] W.M.A. Niessen, R.A.M. van der Hoeven, J. van der Greef, H.A. Schols, A.G.J. Voragen, J. Chromatogr. 647 (1993) 319.
[12] M.T. Gilbert, J.H. Knox, B. Kaur, Chromatographia 16 (1982) 138.
[13] J.H. Knox, B. Kaur, G.R. Millward, J. Chromatogr. 352 (1986) 3.
[14] J.H. Knox, P. Ross, Adv. Chromatogr. 37 (1997) 73.
[15] K. Koizumi, Y. Okada, M. Fukuda, Carbohydr. Res. 215 (1991) 67.
[16] K. Koizumi, J. Chromatogr. A 720 (1996) 119.
[17] S. Robinson, E. Bergström, M. Seymour, J. Thomas-Oates, Anal. Chem. 79 (2007) 2437.
[18] C. Antonio, T. Larson, A. Gilday, I. Graham, E. Bergström, J. Thomas-Oates, J. Chromatogr. A 1172 (2007) 170.
[19] M.L. Rodrigues, C.M.A. Pacheco, M.M. Chaves, J. Exp. Bot. 46 (1995) 947.
[20] T. Taji, C. Ohsumi, S. Luchi, M. Seki, M. Kasuga, M. Kobayashi, K. Yamaguchi-Shinozaki, K. Shinozaki, Plant J. 29 (2002) 417.
[21] E. Zuther, K. Büchel, M. Hundertmark, M. Stitt, D.K. Hincha, A.G. Heyer, FEBS Lett. 576 (2004) 169.
[22] K.L. Koster, A.C. Leopold, Plant Physiol. 88 (1988) 829.
[23] W. Pattanagul, M.A. Madore, Plant Physiol. 121 (1999) 987.
[24] V. Amiard, A. Morvan-Bertrand, J.-P. Billard, C. Huault, F. Keller, M.-P. Prud'homme, Plant Physiol. 132 (2003) 2218.
[25] S. Peters, S.G. Mundree, J.A. Thomson, J.M. Farrant, F. Keller, J. Exp. Bot. 58 (2007) 1947.
[26] M.A. Hannah, E. Zuther, K. Buchel, A.G. Heyer, J. Exp. Bot. 57 (2006) 3801.