Capillary electrophoresis for the determination of major amino acids and sugars in foliage: application to the nitrogen nutrition of sclerophyllous species

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Abstract

Amino acids and sugars are probably the most commonly measured solutes in plant fluids and tissue extracts. Chromatographic techniques used for the measurement of such solutes require complex derivatization procedures, analysis times are long and separate analyses are required for sugars and amino acids. Two methods were developed for the analysis of underivatized sugars and amino acids by capillary electrophoresis (CE). Separation of a range of sugars and amino acids was achieved in under 30 min, with good reproducibility and linearity. In general, there was close agreement between amino acid analyses by CE and HPLC with post-column derivatization. An alternative, more rapid method was optimized for the common neutral sugars. Separation of a mixture of fructose, glucose, sucrose, and fucose (internal standard) was achieved in less than 5 min. How the source of N applied (nitrate or ammonium) and its concentration (8.0 or 0.5 mM) affects the amino acid and sugar composition of leaves from Banksia grandis Willd. and Hakea prostrata R. Br. was investigated. The amino acid pool of Banksia and Hakea were dominated by seven amino acids (aspartic acid, glutamic acid, asparagine, glutamine, serine, proline, and arginine). Of these, asparagaine and glutamine dominated at low N-supply, whereas at high N-supply the concentration of arginine increased and dominated amino-N. Plants grown with nitrate had a greater concentration of proline relative to plants with ammonium. In Banksia the concentration of amides was greatest and arginine least with a nitrate N-source, whereas in Hakea amides were least and arginine greatest with nitrate N-source. The concentration of sugars was greater in Banksia than Hakea and in both species at greater N-supply.

Key words: Capillary electrophoresis, sugars, amino acids, nitrate, ammonium.

Introduction

A number of techniques have been used to quantify sugars and amino acids. For free amino acids, chromatographic methods including gas chromatography (GC) or gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) are most common. These methods frequently rely on a variety of derivatization procedures (Fan et al., 1993; Oh et al., 1994; Ortiz et al., 1994; Gzik, 1996; Chen et al., 1998). Sugars are commonly analysed using HPLC or GC after pre-column derivatization (Honda et al., 1989; Suzuki et al., 1992; Anumula, 1994). These methods offer high sensitivity and resolution, but are expensive of time, reagents and equipment. Equally, derivatization-based HPLC or GC analysis may suffer from interference from excess reagent and/or reaction media, and several derivatives may be produced from the one solute (Heftmann, 1992; Lee and Lin, 1996). High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) offers sensitive analysis of sugars, but amino acids and peptides can cause significant interference (Hardy et al., 1988). Naidu recently described the simultaneous analysis of underivatized sugars, polyols, proline analogues, and betaines in plant extracts by HPLC although the method suffered from interference by amino acids (Naidu, 1998). In summary, the primary drawbacks of existing methods are: (1) cost (time, reagents, equipment), (2) safety and (3) interference.

Capillary electrophoresis (CE) is a relatively new technique (Jorgensen and Lukacs, 1981) that is increasingly being applied to solve problems of separation and quantification.
fication of components of the complex mixtures that are common in biological research. Compared to alternative chromatographic techniques, CE offers advantages of simplicity, speed, and efficiency for the analysis of physiological fluids or tissue extracts. The minimum sample size in CE is often an order of magnitude smaller than that required for GC or HPLC. CE analysis of plant secondary metabolites such as flavonoids, alkaloids, and phenolic acids is now common (reviewed by Tomás-Barberán, 1995). CE methods for analysis of amino acids and sugars have also been described (Lee and Lin, 1996; Bazzanella et al., 1997; Bazzanella and Bächmann, 1998; Soga and Heiger, 1998; Chen et al., 2000). However, most of these methods still require derivatization or complexation of the analytes of interest (Bazzanella et al., 1997; Bazzanella and Bächmann, 1998) owing to the lack of chromophores in most sugars and amino acids. The analysis of undervatized amino acids in plant extracts at pH 11.1 with indirect UV detection using benzoate and the cationic surfactant myristyltrimethylammonium bromide (MTAB) as the background electrolyte has been described previously (Chen et al., 2000). Some 17 amino acids were well resolved although arginine, often a major form of amino-N in plants, was only weakly charged at this pH and could not be resolved from the large water peak (C Warren, unpublished data).

Uptake of either of the two inorganic N sources (cationic ammonium versus the anionic nitrate) will differentially modify the uptake and accumulation of other inorganic cations and anions (Lang and Kaiser, 1994). In general, growth on ammonium reduces the foliar content of cations and increases that of anions relative to nitrate-grown plants (Boxman et al., 1991; Pearson and Stewart, 1993). Plants grown with ammonium have greater concentrations of free amino acids in foliage than nitrate-fed plants (Geiger et al., 1999) and the composition of the total pool of free amino acids may vary with N-source (Barneix et al., 1984; Lavoie et al., 1992; Atilio and Causin, 1996).

The interrelationships of the pools of soluble N and C in foliage are complex. Assimilation of ammonium depends on the supply of C skeletons from the TCA cycle, which may lead to a reduced concentration of soluble carbohydrates (Raab and Terry, 1995). Nitrate assimilation involves a similar anapleurotic synthesis of carboxylates, in addition to the synthesis of carboxylates that is required to maintain the cation–anion balance and intracellular pH, placing similar or greater demands on C supply (Salsac et al., 1987), and concentrations of carboxylates may be equivalent or lower in nitrate- than ammonium-fed plants (Chaillou et al., 1991).

Here two CE methods for the analysis of a wide range of sugars and amino acids in plant extracts and physiological fluids are described, one for the rapid analysis of neutral sugars and a second for simultaneous analysis of sugars and amino acids. Analysis of amino acids by CE is compared with results obtained by a widely used HPLC techniques based on post-column derivatization with ninhydrin. Finally, the CE method was used in an investigation of the response of foliar pools of amino acids and sugars to N-source and supply in B. grandis and H. prostrata, two sclerophyllous species native to southwestern Western Australia. The authors sought, in particular, to test the hypothesis that arginine is a ‘N-sink’ in woody species adapted to low nutrient soils (Nåsholm and Ericsson, 1990; Adams et al., 1995).

Materials and methods

Materials

All reagents were of analytical grade and were dissolved in double glass-distilled water without further purification. Stock solutions of amino acids and sugars (Sigma, St Louis, MO, USA) were prepared at 25 mM, with the exception of aspartic acid (5 mM), cysteine (5 mM) and cystine (15 mM) and stored at −20 °C. The background electrolyte (BGE) was prepared fresh daily as 10 or 20 mM sodium benzoate with 0.5 mM myristyltrimethylammonium bromide (MTAB). BGE was filtered through a Millipore 0.45 μm membrane filter and degassed in a benchtop microfuge prior to use. Electrolyte pH was adjusted to 12.0 with 1.0 and 0.1 M NaOH.

Plant material

Three-month-old seedlings of B. grandis and H. prostrata were obtained from the ALCOA nursery at Marrinup (WA) and transferred to the glasshouse where they were grown in sand culture for 3 months with one-fortieth-strength Hoagland’s solution (with N as 0.25 mM nitrate) applied weekly. When seedlings were 6-months-old they were subjected to one of two N concentrations (0.5 mM or 8 mM N), as nitrate or ammonium. All macro-elements, with the exception of P, were applied in proportion to N as in a full-strength Hoagland solution. P was applied at a constant concentration (0.2 mM), which was estimated to be sufficient for plant growth at the greatest N concentration. Micronutrients were supplied as in a full-strength Hoagland solution. Pots were watered with these nutrient solutions three times per week and with water on the remaining days. Three seedlings of each species were grown for each N-rate by N-form combination. After treatments had been applied for 2 months, leaves were harvested and stored at −20 °C.

Sample extraction

To examine the utility of CE for analysis of plant solutes, several kinds of samples and extraction techniques were examined: extracts from seeds of Vigna unguiculata; extracts from needles of Pinus pinaster; phloem sap from Eucalyptus globulus. Seed and needle tissues were extracted with 20 ml g−1 (fresh weight) of cold (0–4 °C) 50% aqueous ethanol. Phloem sap was collected from E. globulus as previously described (Pate et al., 1998). Routinely, over 50 μl of phloem sap was collected, however, only a few microlitres were required for analysis.

Soluble sugars and amino acids were extracted from B. grandis and H. prostrata with hot water. Leaf material was ground in hot (80 °C) water (10 ml g−1 fresh weight), transferred to a water bath (95 °C) and incubated with periodic agitation for 30 min. Hot-water extracts were centrifuged for
5 min in a microfuge and the supernatant was used as a source of sugars and amino acids with no further pretreatment.

**Instrumentation and electrophoretic procedures**

All analyses using CE were performed with a Bio-Rad 3000 Capillary Electrophoresis system (Bio-Rad, Hercules, CA, USA) controlled by a computer equipped with CE-3000 software (Bio-Rad). Fused-silica capillaries (Bio-Rad) with an inner diameter of 50 µm were used in all analyses. A BGE of benzoate/MTAB was used for all separations owing to its proven suitability for separating amino acids (Chen et al., 2000) and since benzoate and MTAB are widely available and inexpensive. Neutral sugars were separated in a 36 cm capillary (effective length 31.4 cm) with a BGE of 10 mM benzoate, 0.5 mM MTAB (pH 12.0) with a constant separation voltage of −20 kV. For the simultaneous analysis of sugars and amino acids, greater resolution was achieved using a longer 100 cm capillary (effective length 95.4 cm), an increased separation voltage of −25 kV and an increased concentration of benzoate of 20 mM. The concentration of MTAB and pH were unchanged.

Prior to use, new capillaries were pretreated by flushing with 0.1 M NaOH for 10 min, deionized water for 10 min, and BGE for 10 min. Before injection, the capillary was preconditioned with 1.0 M NaOH for 90 s, 0.1 M NaOH for 3 min followed by BGE for 5 min. Prior to analysis, fucose (at a final concentration of 1 mM) was added to samples as an internal standard. Samples were injected by pressure (5 psi s⁻¹) into a capillary at 22°C. The UV detector was set at 225 nm for all experiments.

**Peak identification and quantification**

Peaks were first identified on the basis of migration time (MT), with subsequent confirmation by spiking samples with amino acid and sugar standards. In CE the speed at which different analytes pass the detector varies systematically, as opposed to HPLC where all solutes migrate at the same speed. Peak area in CE analysis is thus a function of MT: slower moving peaks spend longer in the detection window and have larger areas than similar, but faster moving peaks. Hence, analytic concentrations were calculated from normalized peak areas (area/MT) relative to that of the internal standard (fucose).

**Fractionation of samples for comparing CE and HPLC analyses of amino acids**

The identification and quantification of amino acids in *B. grunins* was compared with results obtained by ion-exchange HPLC with lithium-based eluent gradients and post-column derivatization with ninhydrin, as described by Grunau and Swiader (Grunau and Swiader, 1992). As is customary for HPLC analysis, samples must be pretreated to remove interfering analytes and prevent damage to expensive HPLC columns. Hence the cationic fraction (including amino acids) was separated from neutral and anionic fractions (sugars and anions) using strong cation exchange, essentially as described by Blackburn (Blackburn, 1983). In brief, subsamples of extract (400 µl) were applied to disposable SPE columns (500 mg, Strong Cation Exchange, Alltech Assoc, Deerfield, IL, USA). Anions and unchanged solutes were eluted with 2 ml of deionized H₂O. Amino acids were subsequently eluted with 2 ml of 2 M NH₄OH, 500 µl aliquots of the ammonia eluate were dried and redissolved in H₂O. For CE, only a small volume of concentrated sample was required and eluates were redissolved in 100 µl of H₂O (i.e. a similar concentration to the original extract). For HPLC, eluates were redissolved in 2 ml of H₂O (i.e. a 5-fold dilution relative to the original extract).

**HPLC analysis of amino acids**

50 µl of norleucine (250 µM) was added as an internal standard to a 50 µl sample along with 100 µl of precipitant (trichloroacetic acid, Seraprep Plasma Precipitant, Pickering Laboratories, Mountain View, CA, USA). Samples were centrifuged and a 100 µl aliquot was adjusted to pH 2.2–2.4 with lithium hydroxide (0.6 M). Amino acids were separated in a Pickering Laboratories 150 × 3 mm ID, 5 µm column, combined with an HP1101 liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA), an Altex/Hitachi 100–10 spectrophotometer (Beckman Instruments, Fullerton, CA, USA), a Shimadzu CR3A integrator/recorder (Columbia, MD, USA). Column eluents and detection reagents were supplied by Pickering: Lithium eluents (Li 280, Li 750), regenerant (RG003), and ninhydrin reagent (T120). Chromatographic conditions were essentially as described by Grunau and Swiader (Grunau and Swiader, 1992). Proline and hydroxy-proline were detected at 456 nM, all other compounds were detected at 570 nM. Peaks were identified by comparing retention times with a standard solution containing 43 amino acids and related compounds (Sigma, Physiological Standard supplemented with glutamine, phosphoethanolamine, phosphoserine, and asparagine).

**Total N**

Three leaves from each treatment were dried at 80°C for 48 h and ground to a fine powder in a ball mill. Three leaves from each treatment or species were analysed for total N, by Dumas combustion. Finely ground samples (5.5–6.5 mg dry weight) were combusted to CO₂ and N₂ (Roboprep-CN, Europa Scientific, Crewe, UK) before passing into a mass spectrometer (Tracemass, Europa).

**Results**

**Fast separation of neutral sugars**

A mixture of fructose, glucose, sucrose, and fucose (internal standard) were separated in 5 min (Fig. 1a). Sucrose and raffinose could not be separated. Detection limits were: fructose, 0.026 mM; glucose 0.039 mM; sucrose 0.088 mM at a signal-to-noise ratio of three. Calibration curves for fructose, glucose and sucrose were linear over the range 0.1–25 mM (Table 1). Calibration curves for fructose, glucose and sucrose were linear over the range 0.1–5.0 mM with correlation coefficients (r²) between 0.9993 and 0.9997. Reproducibility of quantification was determined by repeated injection (n = 4) of an extract from needles of *P. pinaster*. Migration times (1.4–2.2% RSD) and peak areas (4.1–4.8% RSD) were acceptably reproducible. The recovery of added fructose, glucose and sucrose (0.5 mM of each sugar added prior to extraction) was tested. Recovery at the end of the extraction and analysis procedure was 87% for fructose, compared to 91% for glucose and 77% for sucrose. If added immediately prior to CE analysis, the recovery of all sugars was excellent, approaching 100% in all cases.

The above method was applied to the analysis of neutral sugars in phloem sap from *E. globulus*. Samples were centrifuged prior to analysis, but no other pretreatment was employed. In undiluted sap, the three major sugars were detected, along with ribose and rhamnose plus one unidentified sugar. Quantification of each sugar
Acids (e.g. Asp and Glu) migrated more quickly than neutral or basic amino acids. Arginine (pI = 10.8) migrated slowly, after the neutral sugars, and could not be resolved from the sugar alcohols, mannitol and sorbitol (see inset Fig. 2). Fractionation of samples to separate sugars and alcohols from amino acids (e.g. using cation exchange resins, Blackburn, 1983) can be used if arginine and polyols are present.

Detection limits for amino acids and sugars ranged between 0.02 mM and 0.1 mM (Table 2) and calibration curves for all analytes were excellent. Reproducibility of migration times and peak areas were determined by repeated injection (n = 4) of a seed extract from *V. unguiculata*. The reproducibility of migration time decreased with increasing migration time from 0.5%RSD for Asp (c. 9 min MT) to 2.7%RSD for Suc (c. 23 min MT). Reproducibility of peak area ranged between 2.2–4.1%RSD and generally was better for solutes present in greater concentrations (e.g. Gln, Glc, Suc) compared to those at lesser concentrations (e.g. Ala, Ser, Fru). Recovery through the extraction and analysis procedure was determined by extracting seeds of *V. unguiculata* into 50% EtOH containing 0.25 mM of various solutes. Recovery of added solutes varied between 72% and 117% (Table 2). Recovery of added solutes in hot-water extracts was similar—routine analysis in this laboratory shows recoveries to be consistently greater than 80%.

The above method was applied to the analysis of amino acids in phloem sap of *E. globulus* (Fig. 3). Samples were centrifuged and diluted 50× and reanalysed (Fig. 1b).

Simultaneous separation of amino acids and sugars by CE

A mixture of amino acids and sugars were separated in less than 30 min (Fig. 2). Solutes migrated according to their mass-to-charge ratio or pI. Hence, acidic amino acids (e.g. Asp and Glu) migrated more quickly than neutral or basic amino acids. Arginine (pI = 10.8) migrated slowly, after the neutral sugars, and could not be resolved from the sugar alcohols, mannitol and sorbitol (see inset Fig. 2). Fractionation of samples to separate sugars and alcohols from amino acids (e.g. using cation exchange resins, Blackburn, 1983) can be used if arginine and polyols are present.

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The above method was applied to the analysis of amino acids in phloem sap of *E. globulus* (Fig. 3). Samples were centrifuged and diluted 15-fold with DI water. Phloem sap of *E. globulus* was dominated by glutamine at a native concentration of 5.5 mM. Amino acids present at much lesser concentrations were aspartic acid (0.43 mM) and glutamic acid (0.72 mM). As mentioned above, sucrose/raffinose were by far the most abundant sugars and their concentration was well above the linear range. In undiluted samples, ribose, fructose, rhamnose, glucose, and one unidentified sugar were detected at low concentrations (data not shown).

**Comparison of CE and HPLC analyses of amino acids**

Amino acids present in *B. grandis* were analysed by CE after fractionation with strong cation exchange resin. A

**Table 1. Linearity, sensitivity and reproducibility of the method for rapid analysis of neutral sugars**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Linearity correlation</th>
<th>Detection limit (mM)</th>
<th>Migration time (RSD%)</th>
<th>Peak area (RSD%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fru</td>
<td>0.9996</td>
<td>0.026</td>
<td>1.42</td>
<td>4.64</td>
<td>87</td>
</tr>
<tr>
<td>Glc</td>
<td>0.9997</td>
<td>0.039</td>
<td>1.55</td>
<td>4.06</td>
<td>91</td>
</tr>
<tr>
<td>Suc</td>
<td>0.9993</td>
<td>0.088</td>
<td>2.15</td>
<td>4.77</td>
<td>77</td>
</tr>
</tbody>
</table>
Amino acids and sugars by CE

Fig. 2. Electropherogram of a mixture of amino acids and sugars. Note the break in the X-axis and change in scale. CE conditions: capillary, 100 cm (effective length 95.4 cm) × 50 μm i.d.; BGE, 20 mM benzoate, 0.5 mM MTAB, pH 12.0; applied voltage, −25 kV; temperature 22°C; detection, indirect UV 225 nm. All amino acids and sugars are 0.9 mM. Peaks: 1, aspartic acid; 2, glutamic acid; 3, cysteine; 4, glycine; 5, alanine; 6, serine; 7, asparagine; 8, threonine; 9, gamma-aminobutyric acid; 10, glutamine; 11, proline; 12, ornithine; 13, citrulline; 14, lysine; 15, tryptophan; 16, ribose; 17, mannose; 18, fructose; 19, rhamnose; 20, glucose; 21, galactose; 22, fucose (internal standard); 23, sucrose; 24, arginine; 25, mannitol; 26, sorbitol; 27, pinitol; 28, inositol.

Table 2. Linearity, sensitivity and reproducibility of the method for simultaneous analysis of sugars and amino acids

<table>
<thead>
<tr>
<th>Solute</th>
<th>Linearity correlation ($r^2$)</th>
<th>Detection limit (mM)</th>
<th>Migration time (RSD%)</th>
<th>Peak area (RSD%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>0.9980</td>
<td>0.019</td>
<td>0.46</td>
<td>3.39</td>
<td>77.6</td>
</tr>
<tr>
<td>Glu</td>
<td>0.9962</td>
<td>0.018</td>
<td>0.51</td>
<td>3.81</td>
<td>104.3</td>
</tr>
<tr>
<td>Cys</td>
<td>0.9868</td>
<td>0.099</td>
<td>ND</td>
<td>ND</td>
<td>111.4</td>
</tr>
<tr>
<td>CyS-CyS</td>
<td>0.9953</td>
<td>0.028</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gly</td>
<td>0.9924</td>
<td>0.041</td>
<td>0.79</td>
<td>5.51</td>
<td>72.4</td>
</tr>
<tr>
<td>Ala</td>
<td>0.9857</td>
<td>0.046</td>
<td>0.79</td>
<td>ND</td>
<td>117.9</td>
</tr>
<tr>
<td>Ser</td>
<td>0.9681</td>
<td>0.043</td>
<td>0.82</td>
<td>4.06</td>
<td>ND</td>
</tr>
<tr>
<td>Asn</td>
<td>0.9831</td>
<td>0.043</td>
<td>0.92</td>
<td>2.19</td>
<td>84.7</td>
</tr>
<tr>
<td>GABA</td>
<td>0.9843</td>
<td>0.040</td>
<td>ND</td>
<td>ND</td>
<td>103.1</td>
</tr>
<tr>
<td>Gla</td>
<td>0.9966</td>
<td>0.048</td>
<td>1.02</td>
<td>3.51</td>
<td>82.5</td>
</tr>
<tr>
<td>Phe</td>
<td>0.9810</td>
<td>0.055</td>
<td>ND</td>
<td>ND</td>
<td>79.7</td>
</tr>
<tr>
<td>Orn</td>
<td>0.9983</td>
<td>0.048</td>
<td>ND</td>
<td>ND</td>
<td>79.3</td>
</tr>
<tr>
<td>Cit</td>
<td>0.9862</td>
<td>0.042</td>
<td>1.11</td>
<td>4.01</td>
<td>ND</td>
</tr>
<tr>
<td>Lys</td>
<td>0.9906</td>
<td>0.041</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Arg</td>
<td>0.9892</td>
<td>0.104</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fru</td>
<td>0.9970</td>
<td>0.096</td>
<td>2.29</td>
<td>3.84</td>
<td>106.8</td>
</tr>
<tr>
<td>Glc</td>
<td>0.9973</td>
<td>0.099</td>
<td>2.37</td>
<td>2.83</td>
<td>101.8</td>
</tr>
<tr>
<td>Suc</td>
<td>0.9994</td>
<td>0.075</td>
<td>2.50</td>
<td>2.18</td>
<td>84.0</td>
</tr>
</tbody>
</table>

Fig. 3. Electropherogram of phloem sap from *E. globulus* diluted 15-fold with water. CE conditions and peak identification as in Fig. 2.
total of 15 amino acids were detected and positively identified along with several unidentified compounds (Fig. 4a). The most abundant amino acids were Asp, Glu, Ala, Ser, Asn, GABA, Gln, Orn, Cit, Lys, and Arg (Table 3). Amino acids present at lesser concentrations, or in a minority of samples, were Gly, Thr, Phe, and Pro.

Amino acids present in fractionated extracts of foliage of *B. grandis* were also analysed by HPLC (Table 3). Twenty-six ninhydrin-positive compounds were eluted in 120 min (Fig. 4b; Table 3). Thr and Ser co-eluted about 1 min before Asn, Glu and Gln, which were also poorly resolved, especially Glu and Asn. Aspartic acid was only readily identifiable in one sample, though it was probably present in all. Unknowns were eluted about 0.3 min after Val and between His and Arg (~103 min). Based on retention time, the latter may well be S-methylmethionine (Grunau and Swiader, 1991). Quantification of Arg was difficult owing to instability of the baseline in this region of chromatograms. Identification of phosphoserine was tentative owing to variability in retention times of such weakly bound and readily eluted compounds, especially in complex ionic matrices (Grunau and Swiader, 1991). The most abundant ninhydrin-positive compounds were Thr + Ser, Asn + Glu, Gln, Ala, GABA, Pro, ethanolamine, Orn, Lys, and Arg (Table 3). Other compounds identified but present at lesser concentrations and/or in single samples were Gly, Cit, α-Aminobutyric acid, Val, Met, Allo-isoleucine, Ile, Leu, Tyr, Phe, and His. Neither ethanolamine nor ammonia were detectable by CE.

Amino acids were analysed by CE or HPLC in extracts of *B. grandis* that had been fractionated with strong cation exchange resin (see methods). Separation of Glu from Asn and Thr from Ser was not achieved by HPLC, thus data are presented as the Glu + Asn and Ser + Thr. The concentration of amino acids and amides in the initial extract was calculated (in μmol l⁻¹), taking into account dilution during the fractionation step. Data are means of three independent extractions, one standard error is given in parentheses.

### Table 3. Comparison of CE and HPLC analyses of amino acids in *B. grandis*

<table>
<thead>
<tr>
<th>Amino acid/s</th>
<th>8 mM nitrate</th>
<th>8 mM ammonium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Analytical method</td>
<td>Analytical method</td>
</tr>
<tr>
<td>Asp</td>
<td>88 (10) CE</td>
<td>0 (0) HPLC</td>
</tr>
<tr>
<td>Glu + Asn</td>
<td>129 (9) CE</td>
<td>116 (13) HPLC</td>
</tr>
<tr>
<td>Gly</td>
<td>29 (13) CE</td>
<td>29 (16) HPLC</td>
</tr>
<tr>
<td>Ala</td>
<td>165 (40) CE</td>
<td>153 (8) HPLC</td>
</tr>
<tr>
<td>Ser + Thr</td>
<td>163 (17) CE</td>
<td>200 (19) HPLC</td>
</tr>
<tr>
<td>GABA</td>
<td>102 (33) CE</td>
<td>147 (18) HPLC</td>
</tr>
<tr>
<td>Gln</td>
<td>73 (4) CE</td>
<td>161 (22) HPLC</td>
</tr>
<tr>
<td>Pro</td>
<td>155 (52) CE</td>
<td>63 (7) HPLC</td>
</tr>
<tr>
<td>Orn</td>
<td>22 (11) CE</td>
<td>27 (8) HPLC</td>
</tr>
<tr>
<td>Cit</td>
<td>8 (4) CE</td>
<td>44 (23) HPLC</td>
</tr>
<tr>
<td>Lys</td>
<td>11 (1) CE</td>
<td>27 (9) HPLC</td>
</tr>
<tr>
<td>Arg</td>
<td>67 (67) CE</td>
<td>660 (400) HPLC</td>
</tr>
<tr>
<td>Other aa</td>
<td>3 (3) CE</td>
<td>2 (2) HPLC</td>
</tr>
<tr>
<td>Total aa</td>
<td>1015 (151) CE</td>
<td>1862 (510) HPLC</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>– 357 (41) CE</td>
<td>636 (40) HPLC</td>
</tr>
</tbody>
</table>

Generally there was close agreement between CE and HPLC analyses with the predictable exceptions of Asp and Arg (Table 3). All samples analysed by CE contained Asp, whereas HPLC detected Asp in only one of the
same samples. The concentration of Arg measured by CE was consistently less than that measured by HPLC.

**The effect of N-source and supply on amino acids and sugars in foliage of B. grandis and H. prostrata**

Increased supply of N (8 mM versus 0.5 mM) resulted in increased concentrations of total N in foliage (Table 4). In Banksia, concentrations of total-N were greater in plants supplied with ammonium, whereas in Hakea total-N was greater when nitrate was the N-source. Total concentrations of amino acids and amino-N were also greater when N was supplied at 8 mM instead of 0.5 mM (Fig. 5a). Concentrations of amino acids but not amino-N (owing to differences in proportional distribution of N among amino acids) were greater in Banksia than Hakea. The proportion of total-N present as amino-N was not significantly different between treatments (8.1–13.3%). Increased total concentrations of amino acids in plants supplied with 8 mM instead of 0.5 mM nitrogen were nearly all due to increased concentrations of arginine and other N-rich amino acids such as citrulline and ornithine (Fig. 5a).

Amino acid profiles varied between species and between the rates and forms of supplied N (Fig. 5b). When the concentration of N in the watering solution was 8 mM, arginine provided about half of total amino-N in foliage of ammonium-supplied Banksia and nitrate-supplied Hakea, about 30% in ammonium-supplied Hakea and less than 5% in nitrate-supplied Banksia. When the concentration of N was 0.5 mM, arginine was not detected in extracts of either species, except ammonium-supplied Banksia where it accounted for about 10% of total amino-N. The amides, asparagine and glutamine, contributed between 21% and 54% of amino-N in both species and with either rate or form of supplied N. The response of amides and Arg to N-source differed significantly between species (P<0.05). In Banksia concentrations of amides were greatest and arginine least with a nitrate N-source, whereas in Hakea amides were least and arginine greatest with a nitrate N-source. The proportion of amino-N present as proline was significantly greater in either species grown with a nitrate N-source (6.4–14.6% of amino-N) compared to ammonium N-source (4.4–8.5% of amino-N).

Fructose and glucose dominated the sugar profile of leaves, sucrose was present at lower concentrations. The contribution of each sugar to the total was similar in all

![Graph](attachment:image.png)

**Fig. 5.** Major amino acids in leaves of *B. grandis* and *H. prostrata* grown with ammonium- or nitrate-N at a concentration of 0.5 or 8.0 mM. The concentration of amino acids is expressed (a) per unit dry mass, and (b) as a proportion of total amino-N.

<table>
<thead>
<tr>
<th>Species</th>
<th>N-form</th>
<th>N-rate (mM)</th>
<th>Total N (mg g⁻¹)</th>
<th>Amino acids (µM g⁻¹)</th>
<th>Amino-N (µM g⁻¹)</th>
<th>Amino-N (% of total N)</th>
<th>Soluble sugars (µM g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banksia</td>
<td>Ammonium</td>
<td>0.5</td>
<td>6.1 (0.4)</td>
<td>27.4 (1.8)</td>
<td>40.8 (7.1)</td>
<td>9.4 (1.6)</td>
<td>50.0 (2.7)</td>
</tr>
<tr>
<td>Banksia</td>
<td>Ammonium</td>
<td>8.0</td>
<td>16.9 (1.2)</td>
<td>63.1 (6.6)</td>
<td>143.1 (31.0)</td>
<td>11.9 (2.6)</td>
<td>143.4 (21.1)</td>
</tr>
<tr>
<td>Banksia</td>
<td>Nitrate</td>
<td>0.5</td>
<td>4.6 (0.2)</td>
<td>32.6 (1.6)</td>
<td>43.6 (2.8)</td>
<td>13.3 (0.9)</td>
<td>59.5 (2.4)</td>
</tr>
<tr>
<td>Banksia</td>
<td>Nitrate</td>
<td>8.0</td>
<td>9.6 (1.5)</td>
<td>39.3 (5.1)</td>
<td>55.1 (9.6)</td>
<td>8.1 (1.4)</td>
<td>79.2 (12.0)</td>
</tr>
<tr>
<td>Hakea</td>
<td>Ammonium</td>
<td>0.5</td>
<td>3.6 (0.5)</td>
<td>18.4 (3.7)</td>
<td>24.9 (4.2)</td>
<td>9.7 (1.7)</td>
<td>41.3 (14.6)</td>
</tr>
<tr>
<td>Hakea</td>
<td>Ammonium</td>
<td>8.0</td>
<td>10.5 (0.9)</td>
<td>36.1 (6.4)</td>
<td>71.0 (20.9)</td>
<td>9.6 (2.8)</td>
<td>68.4 (17.6)</td>
</tr>
<tr>
<td>Hakea</td>
<td>Nitrate</td>
<td>0.5</td>
<td>5.0 (0.2)</td>
<td>21.7 (4.5)</td>
<td>29.7 (6.2)</td>
<td>8.5 (1.7)</td>
<td>31.7 (7.8)</td>
</tr>
<tr>
<td>Hakea</td>
<td>Nitrate</td>
<td>8.0</td>
<td>13.2 (0.2)</td>
<td>42.7 (4.7)</td>
<td>84.8 (19.1)</td>
<td>9.1 (2.0)</td>
<td>76.8 (9.9)</td>
</tr>
</tbody>
</table>

**Table 4. Summary of amino acid and sugar composition of B. grandis and H. prostrata**

Plants were grown with either nitrate or ammonium as N-source at a concentration of either 0.5 mM or 8.0 mM. Amino acids and sugars were extracted with hot water and analysed by CE. Data are means of three replicates, standard error is indicated in parentheses.
treatments, thus results are presented here as the sum of Fru, Glc and Suc (Table 4). Concentrations of sugars were significantly greater in Banksia than Hakea and at greater rather than lesser N supply.

Discussion

Analytical methodology

The ability to quantify a range of amino acids and sugars in a single electrophoretic analysis (Figs 2–4) without derivatization or other pretreatment of samples, is a useful adjunct to existing methods in plant analysis. Total analysis time, including capillary conditioning, was in the order of 35 min. GC and HPLC analysis of sugars and amino acids generally requires derivatization, and analysis of both classes of solutes in the one run is seldom feasible. Moreover, combined analysis times are typically much greater than those reported here (Chen et al., 1998; Naidu, 1998; Noctor and Foyer, 1998).

Using CE, sugars are generally analysed in the pH range 11.8–12.2 (Soga and Heiger, 1998) and amino acids at pH 11.0–11.2 (Chen et al., 2000), owing to both the intrinsic properties of CE and of the compounds of interest. Accurate analysis of Arg by CE will probably require use of cation exchange resins, or other standard techniques, to separate amino acids from alcohols and poly-alcohols (Redgwell, 1980; Blackburn, 1983; Stabler et al., 1987). Samples were routinely fractionated using such methods and then amino acids and sugars were analysed separately. The abbreviated method for analysis of neutral sugars (Fig. 1) decreases analysis time to less than 5 min and could conceivably be reduced to less than 2 min with slight modifications to the separation system (Zemann et al., 1997).

As well as advantages, the CE methods described have some drawbacks. Amino acids and carbohydrates are weak absorbers of UV radiation (with the obvious exception of tryptophan). Hence, the indirect detection of underderivatized samples was employed which results in low sensitivity compared to derivatization-based chromatographic methods. Sensitivity may also be reduced by the short pathlength (50 μm) of CE capillaries and by the adverse effects of high pH on indirect UV detection. For example, large concentrations of hydroxide reduce the transfer ratio (Ma and Zhang, 1992), and sensitivity is less than that reported for analysis at lesser pH using a similar separation system (cf. Chen et al., 2000). None the less, limits of detection of the described method are better than those achieved for analysis of amino acids as cations by direct detection (Klampfl et al., 1998) and the simultaneous detection of sugars with amino acids can save considerable analytical time when the concentrations of solutes of interest are greater than the detection limits reported here. HPLC analysis suggested that nine amino acids not detected by CE were present at low concentrations and their combined contribution to total amino-N was well under 5%. With further work and without major advances in technology, it is expected that for most solutes CE detection limits will fall appreciably, at least by an order of magnitude. Sensitivity can be readily increased by derivatizing with a UV-absorbing or fluorescent species and separation by micellar electrokinetic capillary chromatography (MEKC) (Bazzanella et al., 1997). Alternatively, preconcentrating samples prior to analysis (e.g. by evaporation or solid-phase extraction), is a cheap means of improving detection ‘limits’ while retaining the CE advantages of time and cost.

Run-to-run variability is probably greater in CE than in comparable chromatographic techniques. Changing conditions in the capillary wall, particularly charge, affect migration times, peak shapes and, in extreme cases, the migration order of analytes (Jimidar et al., 1993). Routine washing of the capillary between consecutive runs with 1.0 M NaOH (90 s), 0.1 M NaOH (3 min) and the background electrolyte (5 min) improve reproducibility, even when analysing complex matrices such as plant samples. Slowly migrating species may only partially dissociate and small changes in electrolyte pH during a run can greatly affect their net charge and electrophoretic mobility. Some of this variation may be overcome by use of internal standards and calculation of effective migration time or effective mobility (Beckers et al., 1991). Fucose (1 mM) was routinely used as an internal standard although, especially for those species with greater pI, benzyl alcohol (0.05%, v/v) is possibly a better internal standard since it migrates with the electro-osmotic flow (EOF), and is more indicative of the ‘state’ of the capillary (Beckers et al., 1991).

Ethanolamine was detected at high concentrations only by HPLC (since it does not possess anionic moieties at the pH used in our CE analysis). Many studies suggest amino acids are the main forms of soluble N in foliage (Pate, 1983; Millard, 1988) although others have reported large concentrations of ethanolamine and other amines (Kawaguchi et al., 1991; Tertuliano and Le Ru, 1992). CE analyses similar to that described here will underestimate the pool of soluble N in cases where ethanolamine and other amines (and ammonia?) are abundant.

Accurate quantification of arginine is problematic, irrespective of which common chromatographic/electrophoretic method is employed. Baseline instability in HPLC analysis introduces considerable uncertainty as does only partial dissociation of arginine in CE analysis.

Does variation in N-source and N-concentration affect amino acids and sugars?

The free amino acid pool in foliage of Banksia and Hakea was dominated by seven amino acids that comprised over...
75% of amino-N (Fig. 5b). This finding is consistent with previous studies showing that relatively few of the large number of free amino acids found in foliage dominate both the total pool and the response to N-form and availability (Sagiska, 1987; Näsholm and Ericsson, 1990; Näsholm et al., 1994; Adams et al., 1995; Ohlson et al., 1995). At low N supply the amides asparagine and glutamine comprised the greatest fraction of amino-N; consistent with the importance of glutamine in the assimilation of inorganic N through the GS/GOGAT pathway and with the dominance of Gln in the transport of N in the Proteaceae (Stewart et al., 1993; Jeschke and Pate, 1995). At high N-supply, concentrations of arginine increased and dominated amino-N. Previous studies have suggested that arginine is frequently accumulated in species adapted to nutrient-poor habitats if rates of N supply are greater than normal (e.g. Pinus sylvestris, Näsholm and Ericsson, 1990; Eucalyptus spp., Adams et al., 1995).

The form of N supplied did not affect total concentrations of amino acids, the fraction of total N present as amino-N, or the concentrations of sugars (Table 4). More generally, ammonium-grown plants have a greater concentrations of free amino acids than nitrate-fed plants (Causin and Barneix, 1993; Ohlson et al., 1995; Geiger et al., 1999), probably as a consequence of differences in sites, patterns and rates of N assimilation. For example, Kpodar et al. reported that ammonium-fed soybean had four times more serine and five times more glutamine and asparagine than nitrate-fed plants, and aspartate concentrations were 24 times greater in nitrate than ammonium-fed plants (Kpodar et al., 1993). It is difficult, however, to generalize about species native to Australia since much of the literature is based on northern hemisphere or crop species. For example, the characteristically nutrient poor soils on which the Australian flora have evolved are frequently strongly ammonifying, producing nitrate only in short bursts after rain (Pate, 1994) or for longer periods after fire (Stewart et al., 1993; Attiwill et al., 1996). Many Australian native species are thus strongly adapted to ammonium nutrition (Adams, 1996; Turnbull et al., 1995) whereas their northern hemisphere or crop counterparts are well adapted to nitrate nutrition. The general case for adaptation of species to the nitrogen supply characteristics of their environment is similarly well developed (Stewart et al., 1989).

Banksia and Hakea grow in fire-prone woodlands in south-western Western Australia where the strongly Mediterranean climate produces an equally strong seasonal pattern of nitrogen availability (Grierson and Adams, 2000). Nitrate is sparingly available (mainly in spring and autumn) and ammonium is the dominant form of available N for most of the year. The increase in proportion of amino-N present as proline when both species were grown with nitrate (6.4–14.6% of amino-N) is probably related to accumulation of nitrate in the vacuole requiring a counteracting, osmotically compatible solute in the cytoplasm (Salsac et al., 1987). The general observation that arginine and related compounds (ornithine and citrulline) are, like proline, of the glutamate ‘family’ of amino acids (Ireland, 1997) and were responsible for the majority of the differences between treatments (Fig. 5a), raises further interesting questions about the interactive nature of the response of native species to nitrogen and water supply. For example, it seems logical that species with well-developed gross characteristics (i.e. the described characteristics of sclerophyll), have equally well-developed metabolic abilities to assimilate and store N when available and then use N effectively to cope with drought. The differing effects of ammonium and nitrate on total N and proportional distribution of N among the amino acids and amides require further experimentation before generalizations are made about the response of such characteristically sclerophyllous species from Mediterranean environments to nitrate and ammonium nutrition.

Conclusions

The simultaneous analysis of amino acids and sugars in a single electrophoretic run has been demonstrated. Analysis times are less than with most chromatographic methods, there is no need for derivatization with toxic species, sample volumes are small, consumption of reagents is low, and the capillary columns used are inexpensive. Detection by indirect UV offers significant advantages, but some disadvantages via interference from compounds with a similar electrophoretic mobility. The significance of such interference varies. For example, sugar phosphates are present in many plant samples, migrate along the acidic and neutral amino acids, and produce an indirect UV response, yet do not interfere with quantification of sugars or amino acids. The described methods may be readily adapted to other plant species and tissues and made specific for either amino acids or sugars or sugar phosphates through inclusion of simple pre-analysis treatment of samples.

Seven amino acids comprised over 75% of amino-N in extracts from leaves of Banksia and Hakea. The amides contained the bulk of amino-N when N was supplied at 0.5 mM. At 8 mM N, arginine accumulated and dominated amino-N, consistent with previous studies identifying arginine and related compounds as clear indices of N-status. Nitrate-grown plants had a greater concentration of proline than those grown with ammonium, which may be related to an accumulation of nitrate in the vacuole of nitrate-grown plants.

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References


