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Analysis of plant analytes using capillary electrophoresis and high performance liquid chromatography

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Analysis of Plant Analytes Using Capillary Electrophoresis and High Performance Liquid Chromatography

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The declaration page
is not included in this version of the thesis

Abstract

Plants contain an enormous array of organic and inorganic components, the analysis for which may involve a wide range of methods. The focus of this study was to develop high performance liquid chromatography and capillary electrophoresis methods for the analysis of three classes of analytes: osmoregulants, minerals and amino acids.

Firstly, this study explored the potential of capillary electrophoresis for the analysis of three very common osmoregulants (proline, glycine betaine and mannitol). A diverse array of methods has been reported for determining each of these analytes, however, the literature on osmoregulants and their analysis is quite disjointed and traverses both biological and chemistry fields. Therefore, a comprehensive review of this literature has been completed (Chapter 2). Considerably fewer methods are available for the simultaneous determination of these osmoregulants, compared to individual analysis. In chapter 3, a method is described for the simultaneous analysis of proline and betaine by capillary electrophoresis at low pH and specifically various cationic probes for the indirect detection of proline and betaine were explored. Sulfanilamide was identified as a suitable probe and was employed to quantify proline and betaine in spinach and beetroot. However, this method could not detect mannitol as it is not charged at low pH.

In Chapter 4, a high performance liquid chromatography method for the simultaneous determination of all three osmoregulants is described. For separation, a NH_2 column with formic acid and acetonitrile as the mobile phase were used. The high performance liquid chromatography evaporative light scattering detection method was applied to determine osmoregulants in *Stylosanthes guianensis*, *Atriplex cinerea* and *Rhagodia baccata* plant extracts. A complementary method, using a C_{18} column with heptafluorobutyric acid added to acetonitrile was used for verification of the analytes.

Secondly, the potential for using capillary electrophoresis was investigated to simplify and shorten the complex sample preparation procedure. Chapter 5 describes a capillary electrophoresis method that allows direct injection from plant tissues. The experiments highlighted that uncontrolled hydrodynamic injection of sample on piercing of food sample resulted in non-reproducibility. The

addition of hydroxypropylmethlycellulose to the background reduced the uncontrolled hydrodynamic injection up to 95% for all of the analytes. The sample was injected electrokinetically and an imidazole buffer consisting of hydroxypropylmethlycellulose was used for separation. The issue of reducing the reliance on prior separation is also relevant to minerals, thus the developed capillary zone electrophoresis-UV method was applied for the direct injection of inorganic cations from apple, mushroom, zucchini, green bean and strawberries. The applicability of the method across fruit varieties was determined by analysing four apple varieties including red delicious, fuji, pink lady and royal gala.

Thirdly, the potential of the direct injection method was explored for the analysis of amino acids in zucchini. As amino acids are present at low concentrations and lack a chromophore, a more sensitive detector, capacitively coupled contactless conductivity, and pre-concentration of amino acids using isotachophoresis (leading electrolyte = HCl, terminating electrolyte = hydroxyproline) was performed. The separation of amino acids was carried using acetic acid. For minimising uncontrolled hydrodynamic injection poly(ethylene oxide) was used. Using this method sensitive detection of amino acids was possible (Chapter 6). In short, the developed methods allow for quick, inexpensive, sensitive and efficient analysis of plant components.

Acknowledgements

Many individuals have contributed to this research and I would like to extend my thanks and appreciation to the following people.

I would like to express my special gratitude to my principal supervisor, Assoc. Prof. Mary Boyce, for continuous help and support during the course of my PhD. I would like to thank her for excellent guidance and professional advice on my research, and patiently correcting my writing. Mary, thank you for sharing your knowledge and experience on capillary electrophoresis and practical issues beyond textbooks. I could not have wished for a better or friendlier supervisor and mentor.

My sincere thanks must go to my co-supervisor, Prof. Michael Breadmore (UTAS), for immense knowledge and expert advice on capillary electrophoresis. Michael, I have learnt so much from you and I am thankful to you for always being available through emails or personally, for all the insightful discussions, brilliant comments and suggestions. I would also like to thank my co-supervisor (for the first 2 years of my PhD), Dr. Ian Bennett, for all the help, encouragement and support. I am also grateful to my collaborator, Dr. Rossane Guijt, for all the thoughtful comments and discussions about my project.

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I thank all of my friends and fellows at ECU and UTAS for making my PhD journey enjoyable.

Last but not the least; I am grateful for my family for all the emotional and moral support which was essential during my PhD journey.

List of Abbreviations

CE	Capillary electrophoresis
HPLC	High performance liquid chromatography
EOF	Electroosmotic flow
CZE	Capillary zone electrophoresis
MEKC	Micellar electrokinetic chromatography
CMC	Critical micelle concentration
SDS	Sodium dodedyl sulphate
MS	Mass spectrometer
PA	Pulsed amperometric
EC	Electro chemical
LIF	Laser induced fluorescence
C^4D	Capacitively coupled contactless conductivity detector
FASS	Field amplified sample stacking
LVSS	Large volume sample stacking
ITP	Isotachophoresis
LE	Leading electrolyte
TE	Terminating electrolyte

NP-HPLC	Normal phase high performance liquid chromatography
RP-HPLC	Reversed-phase high performance liquid chromatography
RI	Refractive index
ELSD	Evaporative light scattering detector
TFA	Trifluoroacetic acid
ICP-MS	Inductively coupled plasma-mass spectrometry
FMOC	9-fluorenylmethyl chloroformate
DBS	Dabsyl chloride
NDA	Naphthalene-2,3-dicarboxyhydrate
OPA	*o*-phthalaldehyde
PIC	Phenylisocyanate
DNS	Dansyl chloride
AQC	Ammoniumquinolyl-N-hydroxysuccinimidylcarbamate
GC	Gas chromatography
MTBSTFA	N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide
C_{18}	Octadecyl carbon chain
UPLC	Ultra performance liquid chromatography
NMR	Nuclear magnetic resonance
FABMS	Fast atomic bombardment mass spectrometry

FID	Flame ionization detector
TBDMS	Tert-butyldimethylsilyl
TSIM	N-(trimethylsilyl)imidazole
MALDI	Matrix-assisted laser desorption ionization
DI	Direct injection
LOD	Limit of detection

Publications and Abstracts

Following is the list of publications and abstracts that have been completed during the course of candidature rising from this thesis.

Papers in Refereed Journals

Kalsoom, U.; Boyce M. C. *J. Agric. Food Chem*. 2015, (Manuscript submitted) **(Chapter 2)**

Kalsoom, U.; Breadmore, M. C.; Guijt, R. M.; Boyce, M. C. *Electrophoresis* 2014, *35,* 3379-3386. **(Chapter 3)**

Kalsoom, U.; Boyce, M. C.; Bennett, I. J.; Veraplakorn, V. *Chromatographia* 2013, *76*, 1125-1130 **(Chapter 4)**

Kalsoom, U.; Guijt, R. M.; Boyce, M. C.; Townsend, A. T.; Haselberg, R.; Breadmore, M. C. *Anal. Chem*. 2015 (Manuscript submitted) **(Chapter 5)**

Peer-Reviewed Conference Abstracts

Kalsoom, U.; Boyce, M. C.; Bennett, I. J.; Veraplakorn, V. 2013. "Simultaneous Determination of Key Osmoregulants in Halophytes Using HPLC-ELSD." 40th International Symposium on High Performance Liquid Phase Separations and Related Techniques. Hobart, Australia. 17-21st November 2013.

Invited Oral Presentations– Keynote Speaker

Kalsoom, U.; Guijt, R. M.; Boyce, M. C.; Townsend, A. T.; Haselberg, R.; Breadmore, M. C. Electrokinetic Injection of Minerals from Whole Fruits and Vegetables. 20th 21st International Symposium on Electro- and Liquid Phase- Separations Techniques. Natal, Brazil. 4–8th October 2014.

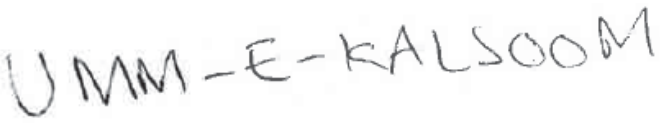
Statement of Contribution

This thesis consists of published, submitted papers and unpublished work. The bibliographical details of the work are provided for each chapter. All data collection, experimental work and data analysis was carried by Umme Kalsoom (the candidate).

The thesis outline was planned and prepared by the candidate in consultation with the primary supervisor, Mary C. Boyce. The experimental design was planned by the candidate in consultation with Mary C. Boyce and Michael C. Breadmore (co-supervisor).

The original thesis was prepared by candidate and Mary C. Boyce provided feedback and comments on each draft until it was ready for submission. Michael C. Breadmore also provided feedback on the final draft of the thesis before submission.

Student Signature:



Primary Supervisor Signature

Date: 5. 03. 2015

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Chapter 1 Introduction and Literature Review

1.1. Introduction

Plants are composed of a broad range of chemical components with incredibly diverse structures. These chemical components are required for performing a variety of functions and are not only beneficial for plants but also for humans [1]. Some of the chemical components present in plants include primary metabolites (e.g. carbohydrates, lipids and amino acids), secondary metabolites (e.g. flavonoids and terpenoids), antioxidants (e.g. vitamins, polyphenols and ascorbic acid), minerals (e.g. sodium, potassium and calcium), and osmoregulants (e.g. amino acids, sugars and quaternary ammonium compounds) [1, 2]. However, this research focused on three classes of plant analytes including osmoregulants, minerals and amino acids.

There are numerous methods available for analysis of each set of analytes. For example, for the three most common osmoregulants i.e. proline, betaine and mannitol, a plethora of methods varying from simple colorimetric [3] to more sophisticated approaches such as high performance liquid chromatography (HPLC) [4] and capillary electrophoresis (CE) [5] have all been used for the determination of each analyte. In most studies when two or more osmoregulants are investigated, each are analysed separately [4, 5]. When the analytes are determined simultaneously, the methods tend to suffer from poor sensitivity and long run times. Therefore, there is a need to develop more sensitive and efficient HPLC and CE methods for simultaneous analysis of commonly studied osmoregulants to minimise time, sample and solvent waste. The determination of three analytes using a single technique is challenging as each of them possess significantly different properties. For example the challenge with CE analysis is that it is not possible to make all three analytes (i.e. proline, betaine and mannitol) charged at any given pH. Proline and betaine are positively charged

at low pH and mannitol is neutral, and at high pH mannitol and proline are negatively charged whereas betaine carries no charge.

Sample preparation was also addressed in this research. For most studies, sample pre-treatment prior to analysis is essential. For example, analysis of inorganic mineral cations in food samples usually requires sample preparation involving drying, powdering, digestion, filtration, etc [6, 7]. Genccelep *et al*. (2009) digested dried mushroom samples using concentrated acid for the analysis of inorganic cations by atomic absorption spectrophotometric (AAS) method. The acid digest were diluted (to make the acid concentration suitable for the instrument) and filtered before analysis [7]. Similarly the analysis of amino acids also generally requires pretreatment including; freeze drying, pulverisation, extraction with a solvent, centrifugation and filteration [8]. These procedures are usually extensive and complex. Moreover, there are many drawbacks to sample preparation such as; sample and solvent loss, contamination (addition of new and distinct species) and degradation of sample [6, 7] thus affecting the analysis and interpretation of results. Therefore, efficient methods that require minimum sample preparation are in demand. The focus of the research presented here was to explore the potential of CE and HPLC for development of sensitive, efficient and rapid methods for concurrent determination of key osmoregulants (i.e. proline, betaine and mannitol) and to exploit the ability of CE to minimise the steps involved in sample preparation of inorganic cations and amino acids.

1.2. Capillary Electrophoresis (CE)

1.2.1. Background

CE has been used extensively for determination of plant analytes [5, 9-11]. The advantages of CE over other analytical techniques include fast analysis; ability to separate a mixture of samples varying from charged to neutral analytes; a wide range of background electrolyte (BGE) compositions available and ability to easily change the separation mechanism.

Additionally, minimum sample and solvent consumption, makes it a simple, rapid, low cost and an environment friendly approach.

1.2.2. Basic Mode of Operation

In CE, separation occurs in a fused silica capillary with both ends immersed in a buffer. A voltage is applied across the two ends of the capillary with the anode typically at the capillary inlet and the cathode at the detector end (capillary outlet) (Fig 1). Movement of the electrolyte in CE, generally known as the running buffer, occurs when a potential difference (up to ± 30 kV) is applied across the capillary. Under the influence of applied voltage, the buffer moves in bulk toward the detector. This bulk movement of buffer is called electroosmotic flow (EOF) [12].

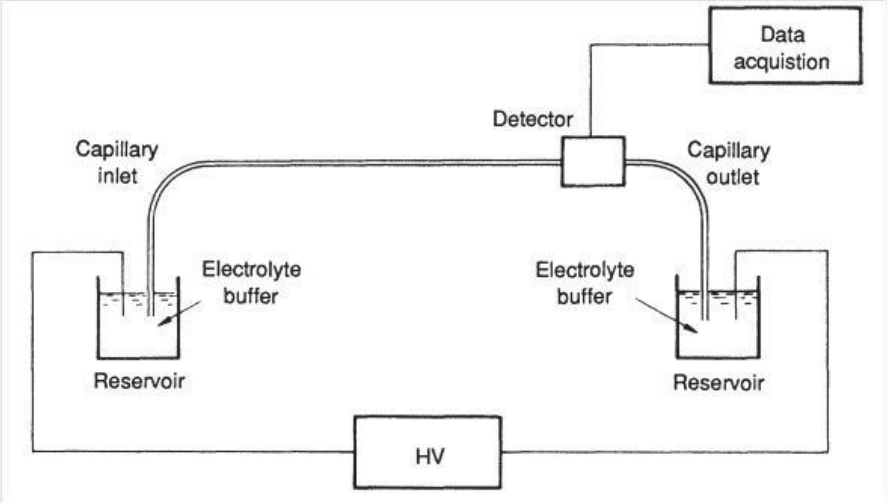

Fig 1. A typical capillary electrophoresis instrument with capillary and two electrodes dipped in the buffer reservoirs and a detector [13]

This EOF is due to the formation of the electric double layer at the buffer/fused silica capillary interface. The pH of buffer plays an important role in generating the EOF. At low pH the silanol groups on the capillary surface are protonated and the surface is not charged and

therefore there is no EOF. At high pH the capillary walls become charged on contact with the buffer due to the formation of surface silanol groups. This charged surface attracts the opposite charges resulting in the formation of inner tightly bound and outer diffused layers (Fig 2). Under the influence of an applied electric field the loosely bound outer layer moves in bulk carrying the solute particles with it [14].

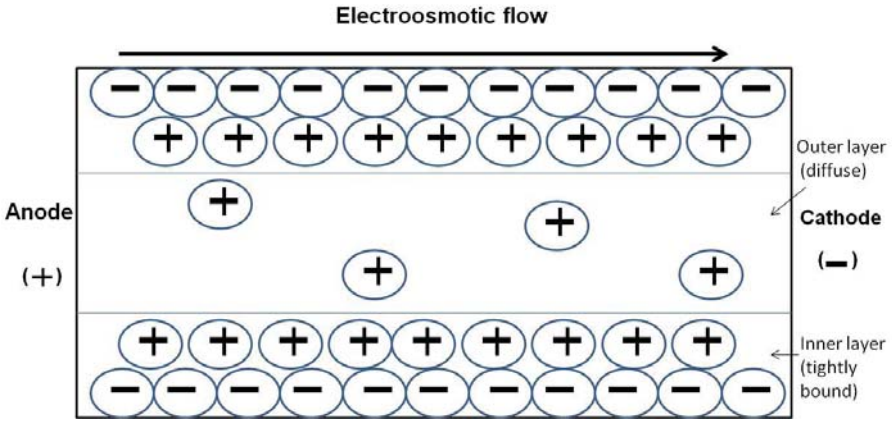

Fig 2. EOF in a fused silica capillary

1.2.3. Modes of Separation

Two common separation modes of CE are capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC). CZE is suitable for charged species [15] while MEKC was developed to also allow for separation of neutral species [16].

1.2.3.1. Capillary Zone Electrophoresis (CZE)

In CZE, separation is based on differences in mobility of the charged species under the influence of an applied electric field. The mobility of an analyte depends on the charge to mass ratio i.e. smaller highly charged species are more mobile compared to larger, minimally charged ions. In a typical CE set-up, a buffer at high pH generates an EOF toward the detector (or cathode end), and when a sample is injected, the cations migrate toward the cathode; the total mobility is the sum of the EOF and the inherent mobility of the

cations. As the movement of anions is in the opposite direction to the EOF they will reach the detector end only if the EOF is greater than the innate mobility of the anion. Neutral species reach the detector with the running buffer and are not resolved [12, 17].

1.2.3.2. Micellar Electrokinetic Chromatography (MEKC)

In MEKC, separation is based on the distribution of solute between the pseudo-stationary phase and the running buffer. Micelles form the pseudo-stationary phase and are generated when a surfactant is dissolved in a buffer above its critical micelle concentration (CMC). Sodium dodecyl sulphate (SDS) is the most commonly used anionic surfactant and at high pH, migrates towards the anode. Neutral analytes migrating with the EOF can interact with SDS and experience a decrease in velocity (Fig 3). Generally, the more hydrophobic the analyte the more it interacts with the SDS phase and the later it moves [18], therefore, the polar/ ionic species migrates sooner than the less polar analytes.

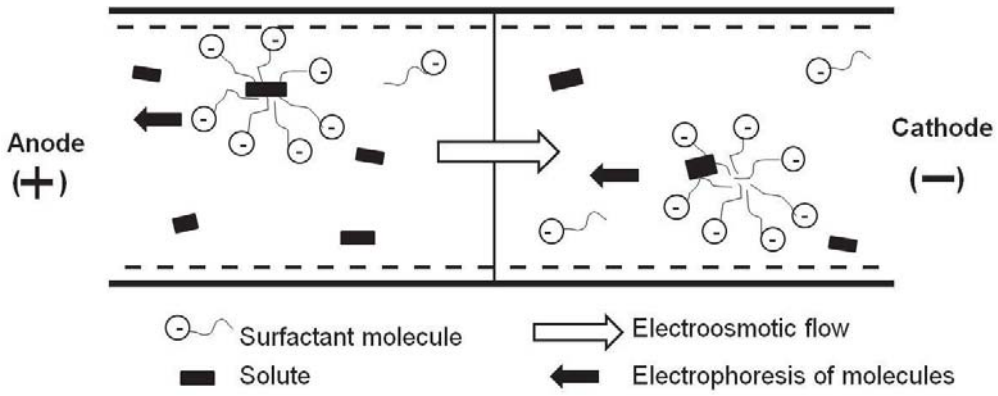

Fig 3. Separation principle in MEKC

1.2.4. Detection

A variety of detectors are commercially available to be used with CE including mass spectrometer (MS) [9, 19, 20], pulsed amperometric (PA) [21, 22], electro chemical (EC) [23,

24], laser induced fluorescence (LIF) [25, 26], capacitively-coupled contactless conductivity detector [21, 27-32] and UV/Vis [10, 33-44]. The latter is the most commonly used detector because its cost and operational complexity is low. An essential pre-requisite of UV/Vis detection is the presence of a chromophore in the analyte. When the analyte lacks a UV absorbing group, detection is usually carried out by derivatization or indirect detection.

Derivatization is mostly used to improve detection by incorporation of a UV absorbing group to the analyte [45], however, it can also be employed to change the hydrophobic properties or charge to mass ratio of an analyte to enhance separation [46]. Derivatization is classified as pre- [41], post-, and/or on-capillary depending on the place of reaction in the CE set-up [45]. Selection of a suitable method for derivatization depends on the physiochemical properties of the analyte and the reagent, purpose of derivatization (i.e. whether derivatization is required for separation or detection), and simplicity of the reaction [47]. However, formation of side products, incomplete reaction, heat/light and pH sensitive derivatives, and in some cases requirement for special equipment limits the usefulness of this approach [47]. Derivatization for UV detection can be avoided by using an alternative approach, indirect detection.

1.2.4.1. Indirect Detection

In indirect detection, a strongly absorbing electrolyte, generally referred to as a probe, is added to the BGE. The displacement of the probe, by the UV transparent analyte of the same charge, results in a significant decrease in absorbance and a negative peak is detected. These negative signals can be easily inverted into positive peaks [48, 49]. The limit of detection and the shape of a peak are related to the concentration and mobility of the probe. A highly absorbing probe in low concentration is the best way to improve the limits of detection [50]. The peak shape is also affected by mobility of the probe. The combination, of a highly mobile probe with the analytes of low mobility, results in tailed peaks while the fast moving analytes with a slow probe give rise to fronted peaks. To obtain acceptable peak shape and improve the detection limits, the mobility of the probe and the analyte should

closely match [50]. However, availability of a limited choice of probes and selecting a suitable probe for a particular set of analytes is challenging.

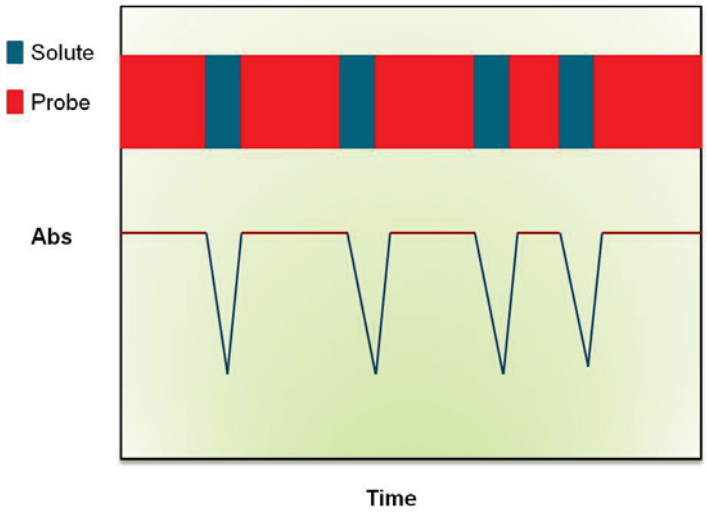

Fig 4. Displacement of UV absorbing probe by UV transparent analytes

An alternative to UV detection, capacitively-coupled contactless conductivity detection, has been used considerably in recent years [21, 27-32, 51-56] due to its ability to detect charged analytes without the requirement of a complex derivatization procedure.

1.2.4.2. Capacitively-Coupled Contactless Conductivity Detection (C^4D)

C^4D is a specific mode of conductivity detector in which it is not necessary for the electrodes to be in direct contact with the solution [27]. Detection can be performed in the capillary by placing the electrode outside the capillary wall. In C^4D, the detection is based on differences in the distribution of electromagnetic field between two electrodes. The electromagnetic field depends mainly on the conductivity of the solution. Therefore, when sample containing different ionic species compared to BGE pass through the detector, a change in conductivity is observed. This change in conductivity is measured by the electrode and a signal is recorded in the form of a peak [30]. Although C^4D provides improved detection, the

determination of trace level of analytes in real sample may still require further improvement in sensitivity. Therefore, the pre-concentration of analytes before analysis is often required.

1.2.5. Sample Pre-concentration Techniques

The application of CE to real samples where analytes are present in trace amounts is hampered by poor concentration sensitivity. Another approach to improve detection sensitivity is concentration of analytes before analysis. This is achieved by focusing the analytes into a narrow zone in the capillary during the sampling phase, prior to separation [57-60]. The most common approaches are: field amplified sample stacking (FASS), large volume sample stacking (LVSS), sweeping, on-column isotachophoresis, pH-mediated sample stacking, and electro-stacking [38] have been used. However, only isotachophoresis will be discussed here in detail.

Isotachophoresis is a pre-concentration technique, used for enhancing the sensitivity and selectivity of ionic species. In a typical isotachophoresis system, the sample is sandwiched between a leading electrolyte (LE) and a terminating electrolyte (TE). LE is marked as a high mobility (low electric field) zone and TE is a slow mobility (high electric field) zone. Therefore, sample ions experience high mobility in TE and are slowed down when they enter into LE. As a result of this, the sample ions are focused at LE/TE interface. On the application of electric potential, all ions migrate with the same mobility between LE and TE forming an ion train (continuous zones) of analytes depending on their mobility range. Once IPT has established, the analytes cannot move out of their zone, therefore ITP not only causes concentration enhancement but also zone compression/sharpening [61] resulting in improved sensitivity and peak shapes respectively. After focusing the ions are separated by electrophoresis.

Another more traditional analytical approach, high performance liquid chromatography, has also been used for analysis of a variety of plant samples.

1.3. High Performance Liquid Chromatography (HPLC)

1.3.1 Background

HPLC is a separation technique that has been around since 1970s [62]. This technique has been broadly used for the separation of a diverse range of samples varying from highly polar to non-polar in nature. The application of HPLC for the analysis of a variety of plant analytes [63-71] has been well explored due to its advantages over other analytical techniques; such as versatility, ease of use, ability to determine analytes of varying polarity, high sensitivity, and availability of a wide range of well developed robust methods.

1.3.2. Basic Principle

In HPLC, the mobile phase is pumped through a stainless steel column at high pressure. The column is packed with an inert material (usually fused silica), which is coated with the stationary phase. The sample dissolved in mobile phase is injected and the analytes are resolved as they move through the column at varying rates depending on their interaction with the stationary phase. The interation of solute with two phases can be manipulated by selecting various mobile and stationary phases [72].

There are several modes of separation but reversed-phase HPLC (RP-HPLC) is the most common. Typically in RP-HPLC, silica particles are coated with a non polar stationary phase such as a long chain hydrocarbon (e.g. C_{18}). The mobile phase usually consists of a polar solvent mixture such as methanol water. In this case, non-polar analytes are “squeezed out” of the mobile phase and interact with the more non-polar stationary phase. Each analyte in the sample mixture interacts slightly differently with the stationary phase resulting in a different retention time, which distinguishes them from each other. Polar analytes in contrast are more soluble in the mobile phase compared to the non polar stationary phase and are less retained in the column [72]. The retention of polar analytes in the RP column can be increased by adding an ion pairing reagent (IPR) to the mobile phase. IPR has both an ionic group and a non-polar tail (or alkyl group). For separation of positively charged analytes an IPR with negative ionic group is added to the mobile phase and for anions a

positively charged IPR is used [73, 74]. The IPR forms an ion pair with the analyte making it less polar and more hydrophobic. The analyte in this form interacts more strongly with the stationary phase and is retained. Trifluoro acetic acid (TFA) [75], sodium perchlorate [75], and pentadecafluorooctanoic acid [76] are some examples of ion pairing agents.

1.3.3. Detection

A variety of detectors including, refractive index (RI) [77, 78], MS [79, 80] and UV/ visible [65, 81, 82] have been used in combination with HPLC. As with CE, UV detection is the most abundantly used mode for HPLC analysis due to a number of advantages over other detectors. As previously mentioned for CE (see section 1.2.4.), the poor sensitivity associated with UV detection can be improved by derivatization. The derivatised UV absorbing product is usually less polar when compared to the native analyte and separation on a reversed phase column (e.g. C_{18}) is enhanced [83, 84]. When derivatization is not preferred due to a number of limitations (as mentioned previously is section 1.2.4) an alternative detector such as evaporative light scattering detector, can be used to improve sensitivity of some analytes.

1.3.3.1. Evaporating Light Scattering Detection (ELSD)

ELSD is a relatively new technique that has been developed, in part, to allow sensitive analysis of amino acids and sugars [76, 85, 86]. In ELSD the response is related to the mass of solute [87] and any species less volatile than the mobile phase can be detected. In this detector, the effluent from the column is transported to the nebulisation chamber where it is transformed to a mist with the help of a high pressure inert gas (usually nitrogen). These small droplets are then evaporated in the drift tube (evaporation tube) and the remaining solid particles are allowed to enter the optical cell. A beam of light strikes the analyte and intensity of scattered light is measured by a photomultiplier (Fig 5). As derivatization is not required the polar analytes are retained on the non-polar C_{18} column by adding an ion pairing reagent to the mobile phase (see Section 1.3.3).

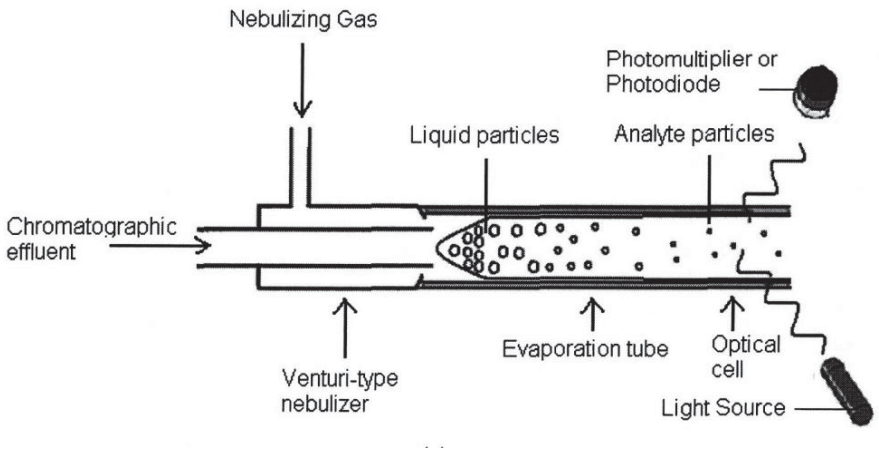

Fig 5. Principle of evaporative light scattering detection [88]; the chromatographic effluent is transformed to a mist and the solid particles detected after leaving the evaporation tube.

1.4. Plant Analysis

An enormous range of components in plants have been studied using numerous analytical approaches. However, the discussion here will be limited to the HPLC and CE methods reported for the analysis of three set of analytes including; osmoregulants, minerals and amino acids.

1.4.1. Osmoregulants

Plants produce low molecular mass metabolites known as osmoregulants in response to environmental stresses such as drought, salinity and water logging [89]. These osmoregulants perform a variety of functions in plants such as maintenance of osmotic balance to minimise water loss [90] increases in tolerance to dehydration [91], scavenging of free radicals [92, 93] maintenance of sufficient cell turgor to improve the growth [94], stabilization of the sub-cellular structures [95] and regulation of co-enzymes. Osmoregulants include sugars, sugar alcohols, amino acids and quaternary ammonium compounds. The three most commonly studied osmoregulants are proline, mannitol and betaine [96].

Proline is the most commonly explored α-amino acid and is polar in nature. It has a carboxylic acid (-CH_3COOH, pKa_1= 1.95) functional group which makes it negatively charged under highly alkaline conditions and the amino group (-NH_2, pKa_2= 10.64) [75] which makes it positively charged under acidic conditions. (Fig 6)

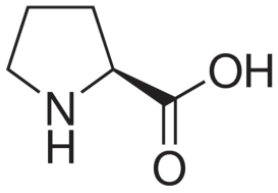

Fig 6. Structure of Proline

Betaine is the most commonly studied quaternary ammonium compound. Betaine is a zwitterionic compound and carries a positive charge at the quaternary ammonium functional group and a negative charge at carboxylate group (Fig 7). The pK_a of the carboxylic group of betaine is 4.00 [97], therefore under acidic conditions the carboxylic group becomes neutral as a result of protonation resulting in an overall positive charge on betaine from the nitrogen of the amino group.

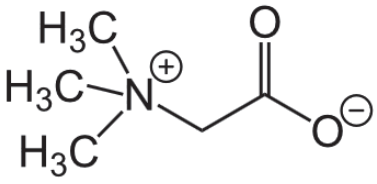

Fig 7. Structure of Betaine

Mannitol is the most commonly examined sugar alcohol [98] and is polar in nature (Fig 8). The pK_a value of mannitol is 13.5 and is therefore negatively charged at high pH [99].

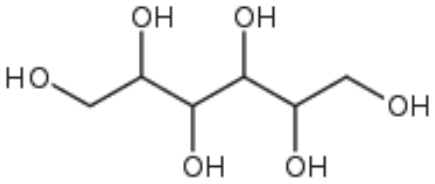

Fig 8. Structure of Mannitol

1.4.1.1. Analysis by CE and HPLC

There are a variety of methods available for the determination of each osmoregulant. These methods include; colorimetry, HPLC, gas chromatography (GC), CE and nuclear magnetic resonance (NMR) spectroscopy. Photometry and HPLC are by far the most common. These methods are discussed more extensively in a review article presented in Chapter 2. CE and HPLC methods used for the analysis of three most commonly explored osmoregulants are discussed below.

Proline (as an osmoregulant) has been analysed using a variety of methods in a wide range of samples. The reported HPLC methods vary both in terms of separation mechanism and detection mode. Separation of proline is usually achieved using an ion exchange column when no derivatization is required [4] and for less polar proline derivatives a RP [100] column such as C_{18} is used [101, 102]. For detection, UV, LIF, RI, and MS have all been reported [103]. However, UV/Vis is the most commonly used mode of detection and derivatization is carried out to improve sensitivity of the UV transparent proline molecule. A variety of derivatising agents have been reported including, ninhydrin [104], 9-fluorenyl-methylchloroformate (FMOC) [105], phenylthiocarbamyl [106] and *o*-phthalaldehyde (OPA) [107].

CE analysis of proline, as an osmoregulant is limited; there is only one report by Nishimura *et al*, 2001, in which separation of proline and betaine using CZE in combination with UV

detection (at low wavelength) [5] has been achieved. However, they experienced poor sensitivity due to employing direct UV detection.

A wide range of methods have been reported for analysis of betaine. As with proline, HPLC methods demonstrated for betaine differ in terms of separation mechanism and mode of detection. As betaine can be charged at low pH, ion exchange columns [107-109] are commonly used for separation of betaine. However the use of a RP column [110] has also been reported and retention is increased by derivatization or addition of an ion pairing agent to the mobile phase [111]. For detection, UV/Vis is the most commonly used detection mode. Using UV, detection at low wavelength [107-109] and after derivatization with 2-naphthycyl trifluoromethane sulfonate [112], 4-bromo-phenacyl triflate [113] and 4-isophenyl trifoliate [114] have all been reported.

CE has also been used for analysis of betaine. For example, analysis of betaine using CZE in combination with UV detection at low wavelength (195 nm) has been reported [5]. However, this method lacked sensitivity due to non-UV absorbing properties of betaine. The poor sensitivity can be improved by derivitisation of betaine to form *p*-bromophenacyl esters for UV detection and separation can be achieved using both CZE [115] and MEKC [5]. However, these methods are complicated and the derivatives are sensitive to pH and thermal changes.

Mannitol has been explored widely and a number of HPLC and CE methods have been reported. HPLC analysis usually involves separation in alkaline conditions using an anion exchange chromatography in combination with pulsed PA detection [92, 116]. Sensitivity can be improved by using fluorescence or UV detection. As for proline and betaine detection with a fluorescence or UV detector is often achieved after derivatization and separation of the less polar mannitol derivatives is carried using a RP column [117]. 1-isopropyl-3-(3-dimethylaminopropyl) carbodiimide perchlorate (IDC) and benzoic acid are among the most common derivatising agents used for the fluorescent detection of mannitol [117].

It can be noticed that the above described HPLC and CE methods are for the determination of a single osmoregulant. In a study, where two or more osmoregulants are of interest, each analyte is often determined by a separate method [105, 107, 118-125]. For example, Canamas *et al*. (2007) used a separate method for the determination of proline (RP-HPLC with fluorescence detection) and betaine (HPLC using RI detector) from the extracts of the same plants [105]. Similarly, Hassine *et al*. (2008) used RP-HPLC with UV detection for betaine and a colorimetric method for proline determination from the extracts of the same plant [123]. These methods are time consuming and labour-intensive and a method that allows simultaneous determination of three commonly explored osmoregulants is desirable.

1.4.1.1.1. Simultaneous Determination of Osmoregulants

There are few reports in which attempts to analyse osmoregulants simultaneously have been made [5, 4]. Naidu (1998) determined sugars, sugar alcohols, proline, its analogues and betaines simultaneously using HPLC coupled to a UV detector [4]. As the detection was achieved at low wavelength the sensitivity of the UV transparent osmoregulants was low. As mentioned previously in this chapter that ELSD can be used to achieve better sensitivity for amino acids and sugars in particular. However, prior to the current study, ELSD had not been used for the simultaneous determination of all three common osmoregulants.

CE has also been applied for simultaneous analysis of osmoregulants. Nishimura *et al*. (2001) reported a method for concurrent determination of proline and betaine using UV detection at low wavelength (190 nm) and at low pH [5]. However, as they were using UV detection the sensitivity was low (100 μM) for both analytes i. e. proline and betaine. The poor sensitivity can be improved by using indirect UV detection; however, prior to the present study, it has not been used for analysis of proline and betaine.

The analysis of all three osmoregulants simultaneously using both CZE and MEKC is challenging. In CZE mode, at any given pH it is not possible to develop a charge at all three osmoregulants. For instance, at low pH proline and betaine carry a positive charge and can be separated by CZE but mannitol remains neutral and hence cannot be resolved from other neutral analytes in the sample matrix. Similarly, at high pH, proline and mannitol can be

separated as anions but betaine remains neutral and migrates with the EOF. The challenge with employing MEKC is that the osmoregulants are less hydrophobic and do not interact effectively with hydrophobic pseudostationary phase and hence elute unresolved.

1.4.2. Plant Minerals

Minerals are divided in to macronutrients (e.g. potassium, sodium, calcium, and magnesium) and micronutrients (e.g. zinc, copper and iron) depending on the quantity of minerals required or present in the human body [26]. However, only macronutrients including potassium, sodium, calcium, and magnesium will be discussed here.

Macronutrients are important for a healthy functioning body [126, 127]. The significance of calcium for healthy bones and teeth is well established and potassium is known to play an important role in balancing the body fluids and muscle contraction. This awareness has resulted in an increase interest in consumption of a nutrient rich diet. A major portion of these nutrients is obtained from eating vegetables and fruits [60]. However, the concentration of these nutrients can vary significantly in different type of fruits and vegetables [128]. Furthermore, the nutrient composition of different food is of interest to a health conscious public [129, 130]. Therefore, as each new variety of fruit or vegetable comes on the market, the nutritional composition is comprehensively determined.

The composition of nutrients in fruit and vegetables is also important in determining the food quality. For example, an imbalance in calcium leads to development of dark spots, and internal breakdown in apples [131, 132]. Monitoring the macronutrients in fruits and vegetables can inform growers when application of nutrients to the soil for uptake by plants might be beneficial [133, 97].

1.4.2.1. Mineral Analysis

A large number of methods including; atomic absorption spectroscopy [134], inductively coupled plasma-mass spectrometry (ICP-MS) [135], ion chromatography [136] and CE [137] have all been reported for mineral analysis, however, only CE methods will be discussed in detail.

CE has been used widely for mineral analysis. UV [33, 34, 39] is one of the most commonly employed modes of detection. For UV, direct detection after complexation with a UV absorbing compound such as 2,6- pyridine dicarboxylic acid [134] and indirect detection both have been reported [33, 34, 39, 139]. However, CZE with indirect UV detection has been most frequently used for mineral analysis [33, 34, 39]. Generally, imidazole is used as a visualising agent (probe) for indirect UV analysis of mineral cations [34, 38, 40, 42, 48, 49]. The mineral cations have very similar electrophoretic mobilities which results in poor selectivity and co-migration of two or more than two ions. To improve selectivity, a complexing agent is usually added to the BGE to form complexes with metals [139]. For example Francois *et al*. (1995) improved the selectivity by adding 18-crown-6-ether to the BGE [140]. Similarly, Lee & Yin (1994) showed the importance of complexing agents in enhancing the selectivity of ions and suggested glycolic acid, α-hydroxyisobutyric acid or succinic acid as useful complexing agents for separation of the metal cations [141].

Independent of which method is chosen for mineral analysis, the sample pre-treatment before analysis is essential in order to make the minerals available for analysis when studying the real samples.

1.4.2.2. Sample Pre-treatment before Analysis

The sample preparation for minerals usually involves drying, grinding or pulverizing the dried sample, extraction or digestion of the sample usually with concentrated nitric acid to remove matrix interferences, filtration and dilution of the acid digest [142, 143]. This multistep sample pre-treatment procedure is tedious and time consuming. The drying process alone can take more than 24 hours [144]. In addition, digestion requires the use of concentrated nitric acid (purity = 99%) which is an expensive and hazardous solvent. Furthermore, sample pre-treatment provides many opportunities for sample contamination and can also result in sample decomposition. Not surprisingly there is increasing interest in reducing and minimising sample preparation steps.

1.4.2.3. Minimising Sample Pre-treatment

In light of above described issues, several approaches have been taken to simplify and speed up the conventional sample preparation procedures. The commonly implemented alternatives include focused microwave induced combustion (FMIC), use of ultrasound radiation, and extraction using a suitable solvent. FMIC provides excellent destruction of organic matrix with minimum use of time and energy. In FMIC digestion is carried out in large open vessels which provide opportunity to process a large amount of sample (almost 10 g) as the gases produced as a result of digestion do not result in pressure build up. However, consumption of large volume of concentrated acid is the major drawback of this process [145]. An alternative to acid digestion procedure is the extraction of the minerals from the sample matrix with the help of ultrasound radiation at ambient temperature and pressure [146]. For example, WieteskaIn *et al*. (1996) extracted mineral cations from vegetables using the equivalent concentration of HCl and HNO_3 to provide a quick, low cost and less hazardous procedure for mineral extraction [147]. The advantages of ultrasound extraction include; low cost, less time and solvent consumption [148]. However, degradation and changes in sample composition are the major limitations of this approach [149]. In some studies, solvent extraction has been used as an alternative to acid digestion to provide less hazardous and quick sample preparation. For example Fukushi *et al*. (1997), extracted Ca^{2+} from vegetables using boiling water. Although it provides a simplified sample preparation procedure, however, weighing, crushing, boiling of vegetable (15-20 min), cooling, filtration and dilution before analysis of metals [151] are still complex and may not result in extraction of all analytes.

Another technique, direct injection, has also been used in several studies to completely avoid the sample preparation step. For instance, direct sampling from rat's brain using CE has been reported [151]. In this method, the capillary was injected directly into the rat's brain. This approach allowed injection of both intra- and extra- cellular taurine whereas the traditional technique, dialysis, only allowed the determination of extracellular taurine. However, the direct injection method only provided qualitative information of the analyte. Quantitation was not achieved as it was difficult to control the amount of sample injected

into the capillary. Therefore, a technique that allows qualitative and quantitative analysis without any sample preparation would be highly advantageous. Such a method will not only overcome the issue of sample contamination during preparation step but will also provide inexpensive and quick analysis and may pave the way for rapid on-site analysis.

1.4.3. Amino Acid Analysis

Amino acids are organic compounds of biological significance consisting of an amine ($-NH_2$) and carboxylic acid (-COOH) functional groups [152]. Amino acids are used for synthesis of proteins and are precursors of other molecules such as tryptophan which is the precursor for synthesis of serotonin [153], similarly tyrosine and phenylalanine are precursors for catecholamine neurotransmitters dopamine, epinephrine and norepinephrine [154] and arginine is a precursor of nitric oxide which is vital for a variety of biological processes [155]. The human body cannot synthesise all the amino acids required for essential biological processes and these amino acids are obtained through the consumption of a plant-based diet [156].

There are a plethora of methods available for analysis of amino acids; however, CE methods only will be introduced here. Separation of amino acids has been achieved both by CZE [26, 36, 157] and MEKC [158-160] in combination with a variety of detectors such as UV [161], LIF [26, 120], C^4D [26, 28], amperometric [22] and MS [162]. However, as mentioned previously UV detection is the mode of choice and sensitivity of UV transparent amino acids [163] is usually improved by derivatizing agents including; FMOC [87, 164], dabsyl chloride [165], naphthalene-2,3-dicarboxyaldehyde (NDA), o-phthaldehyde (OPA) [166], phenylisocyanate (PIC) [167] and fluorescamine, 2,4-dinitrophenyl(DNP), dansyl chloride (DNS), and 6-ammoquinolyl-N-hydroxysuccinimidylcarbamate (AQC) [45]. However, each derivatizing agent has pros and cons; for example, PIC forms highly stable derivatives with amino acids having detection limit at nanogram levels, however, PIC is not generally recommended as it reacts with almost every compound having an active hydrogen, causing the formation of many side products and resulting in complicated spectra [167]. Similarly,

while the reaction with OPA is quick, the formation of side products and light sensitive derivatives are major limitations of this process.

An alternative detector, C^4D, has become well recognised for simple and sensitive analysis without the need for derivatization [28, 29, 31, 40, 53]. There are a variety of methods reported for C^4D detection of amino acids [32,51, 52, 54, 55, 168, 169]. However, analysis of amino acids at low pH using acetic acid as the BGE is the most commonly reported method [52, 168] with C^4D detection. As with minerals, amino acids also require an inevitable sample preparation procedure before analysis.

1.4.3.1. Sample Pre-treatment before Analysis

The extraction of amino acids prior to analysis is essential when investigating real samples. It usually involves freezing with liquid nitrogen [170] or drying, grinding or crushing, extraction with a solvent, centrifugation and filtration of the extracted analytes [171]. This sample preparation step is complex and time consuming. In addition, it results in contamination and loss of sample and solvent. Alternatives such as ultrasound driven extraction [172] of amino acids from vegetables has been reported. For example, extraction of amino acids from grapes using ultrasound radiations has been reported. Although this method speeds up the extraction step, it still requires grinding, centrifugation, and filtration. Therefore, a simple and quick method for direct analysis of amino acids with minimum or no sample pre-treatment is highly desirable.

1.5. Project Aims

This project aims to;

1. To explore the potential of CE for development of a sensitive, robust and rapid method for simultaneous determination of key osmoregulants i.e. proline, betaine and mannitol.
2. To explore the ability of HPLC for sensitive and quick determination of key osmoregulants i.e. proline, betaine and mannitol simultaneously.

3. Explore the potential of CE for direct electrokinetic injection of inorganic cations and and amino acids from whole fruits and vegetables in order to minimise the cumbersome sample pre-treatment procedures.

1.7 Research Outline, Methods and Techniques

In the following section the outline of the research framework, developed methods and techniques used this project are described. This section provides a link between different chapters of this thesis, and a detailed explanation of each experiment is provided in the consecutive chapters. The discussion here is presented in the same order as the chapters in this thesis.

1.7.1. Determination of Key Osmoregulants in Plants by CE and HPLC

In Chapter 3, a CE method has been developed for simultaneous analysis of two commonly explored osmoregulants proline and betaine. Their separation was achieved at low pH using CZE and detection was carried using indirect UV detection. Probes were evaluated for their ability to identify and quantify proline and betaine. The suitability of these probes was tested on the basis of molar absorbtivity, electrophoretic mobilities and pK_a values. Based on these parameters sulfanilamide, was identified to be the appropriate probe for both analytes. Therefore, a BGE containing sulfanilamide (pH adjusted using H_2SO_4) was used for separation and analysis of proline and betaine. Separation parameters such as pH and probe concentration were studied in order to obtain maximum peak efficiency and sensitivity. For validation of the method, inter-day and intra-day reproducibility and the linearity of the detector response to varying concentration of two analytes was determined. The robustness of the developed method was determined by separation and quantification of proline and betaine in spinach and beetroot. The identity of two analytes in real samples was confirmed based on migration time. Beetroot and spinach extracts were spiked with proline and betaine to further confirm the identity of these analytes. Using this method, the recovery for proline and betaine in the real samples was also determined.

Chapter 4 presents a method for simultaneous determination of three key osmoregulants including proline, betaine and manitol using HPLC in combination with ELSD. For development of this method, an amino column was used for separation due to requirement of volatile mobile phase with ELS detection. The retention time of proline and betaine was increased by adding an IPR to the mobile phase. For validation of the method, the linearity of detector response with various concentrations of three analytes i.e proline, betaine and mannitol and repeatability in retention time for three analytes were investigated. A second method developed on a C_{18} column with a completely different mechanism of separation provided an alternative to validate the identity of peaks and quantities of analytes measured using NH_2 method. The robustness of the method was investigated by determination of key osmoregulants in halophytes (*Stylosanthes guianensis*, *Atriplex cinerea* and *Rhagodia baccata*) and the results obtained using the developed amino column method were validated with the alternative C_{18} method.

1.7.2. Direct Injection of Fruits and Vegetables for CE Analysis

In Chapter 5, a CE method for direct injection of inorganic cations from whole fruits and vegetables is presented. In this work, CZE was used for separation in combination with indirect UV detection for identification of inorganic mineral cations. The BGE consisted of imidazole (pH adjusted using acetic acid). The viscosity of BGE was increased by adding a polymer, hydroxypropylmethyl cellulose, to allow precise and repeatable injection of inorganic mineral cations in to the capillary. The robustness of method was tested by applying the method to a variety of fruits and vegetables including zucchini, apple, mushroom, tomato, green bean, and strawberry. Zucchini, apple and mushroom were chosen for determination of inorganic mineral cations. For quantititation, external standards prepared for each food sample including apple, mushroom and zucchini were used. The results obtained using the developed CZE method was validated by an ICP-MS method. The applicability of the method across different varieties of a food sample was determined by analysing four varieties of apple including red delicious, fuji, pink lady and royal gala. The external standards prepared from red delicious were used to quantify mineral in fuji, pink

lady and royal gala and so on. The variations in results were calculated to compare the results obtained from using different apple matrixes.

Chapter 6 extends the applicability of direct injection method developed in Chapter 5. The direct injection method for minerals was successfully applied for the analysis of amino acids in plant tissues. A CZE method using acetic acid as a separation buffer was developed for the determination of amino acids in plant tissues. As amino acids are in trace quantities in plants and possess non-UV absorbing properties, C^4D detector was used instead of UV detection to achieve better sensitivity. In addition, pre-concentration of analytes before analysis using isotachophoresis (ITP) was employed to enhance sensitivity and obtain sharp peaks. The viscosity of buffer was increased by adding poly(ethylene oxide) to the BGE. Using the developed method, direct injection, ITP and identification of amino acids in zucchini was carried.

1.8. References

[1]. Springob, K.; Kutchan, T. M. Introduction to the Different Classes of Natural Products. *Plant-derived Natural Products: Synthesis, Function, and Application*. Eds, Osbourn, A. E., Lanzotti, V. New York, USA: Springer, 2009, pp 104.

[2]. Weil, A. *Rodale's 21st-Century Herbal: A Practical Guide for Healthy Living Using Nature's Most Powerful Plants.* Eds, Balick, M. New York, USA: Rodale, 2014, pp 62.

[3] Bates, L. S. *Short Commun.* Plant and Soil, **1973**, 39, 205-207.

[4] Naidu, B. P. *Aust. J. Plant Physiol*. **1998**, 25, 793–800.

[5]. Nishimura N.; Zhang, J.; Abo M.; Okubo, A.; Yamazaki, S. *Anal. Sci*. **2001**, 17, 929–932.

[6] Genccelep, H.; Uzun, Y.; Tuncturk, Y.; Demirel, K. *Food Chem*. **2009**, 113, 1033−1036.

[7] Tucker, K. L.; Hannan, M. T.; Chen, H. L.; Cupples, L. A.; Wilson, P. W. F.; Kiel, D. P. *Am. J. Clin. Nutr*. **1999**, 69, 727−736.

[8] Fish, W. W. *Food Nutr. Sci.* **2012**, 47, 863–871.

[9]. Bai, Y.; Chang, C. L.; Du, F. Y.; Tan, Z. J.; Bai, Y.; Liu, H. W. *Electrophoresis* **2014**, 35, 1984–1988.

[10]. Boiteux, J.; Vargas, C. S.; Pizzuolo, P.; Lucero, G.; Silva, M. F. *Electrophoresis* **2014**, 35, 1709–1718.

[11]. Tomasbarberan, F. A. *Phytochem. Anal.* **1995**, 6, 177–192.

[12]. Boyce, M. *J. Chem. Edu*. **1999**, 76, 815–819.

[13]. Gordon, M. J.; Huang, X.; Pentoney, S. L.; Zare, R. N. *Science* **1988**, 241, 224–228.

[14]. Copper, C. L. *J.Chem. Edu*. **1998**, 75, 343–347.

[15] Xuan, X.; Li, D. *Electrophoresis* **2005**, 26, 166–175.

[16] Zdunczyk, P.; Ciska, E.; Amarowicz, R. *Pol. J. Food Nutr. Sci.* **2004**, 13, 369–372.

[17] Boyce, M. C. *J. Chrom. A* **1999**, 847, 369–375.

[18] Otsuka, K.; Terabe, S. *Bull. Chem. Soc. Jpn*. **1998**, 71, 2465–2481.

[19] Barroso, A.; Gimenez, E.; Benavente, F.; Barbosa, J.; Sanz-Nebot, V. *Anal. Chim. Acta* **2015**, 854, 169–177.

[20] Gonzalez-Dominguez, R.; Garcia, A.; Garcia-Barrera, T.; Barbas, C.; Gomez-Ariza, J. L. *Electrophoresis* **2014**, 35, 3321–3330.

[21] Gimenes, D. T.; Marra, M. C.; Abarza Munoz, R. A.; Angnes, L.; Richter, E. M. *Anal. Method*. **2014**, 6, 3261–3267.

[22] Guo, Y.; Colon, L. A.; Dadoo, R.; Zare, R. N. *Electrophoresis* **1995**, 16, 493–497.

[23] Chen, G.; Zhang, L. Y.; Wu, X. L.; Ye, J. N. *Anal. Chim. Acta* **2005**, 530, 15–21.

[24] Martin, A.; Angel Lopez, M.; Cristina Gonzalez, M.; Escarpa, A. *Electrophoresis* **2015**, 36, 179–194.

[25] Arlt, K.; Brandt, S.; Kehr, J. *J. Chrom. A* **2001**, 926, 319–325.

[26] Cheng, Y. F.; Dovichi, N. J. *Laser Techniques in Luminescence Spectroscopy*. Eds, Vodinh, T.; Eastwood, D.L. American Scoiety for Testing and Materials: Chelsea, London, 1990, Vol. 1066, pp 151–159.

[27] Brito-Neto, J. G. A.; da Silva, J. A. F.; Blanes, L.; do Lago, C. L. *Electroanalysis* **2005**, 17, 1198–1206.

[28] Coufal, P.; Zuska, J.; van de Goor, T.; Smith, V.; Gas, B. *Electrophoresis* **2003**, 24, 671–677.

[29] Elbashir, A. A.; Aboul-Enein, H. Y. *Biomed. Chrom*. **2012**, 26, 990–1000.

[30] Guijt, R. M.; Evenhuis, C. J.; Macka, M.; Haddad, P. R. *Electrophoresis* **2004**, 25, 4032–4057.

[31] Mori, M.; Kaseda, M.; Yamamoto, T.; Yamada, S.; Itabashi, H. *Anal. Bioanal. Chem*. **2012**, 402, 2425–2430.

[32] Pormsila, W.; Morand, R.; Kraehenbuehl, S.; Hauser, P. C. *J. Chrom. B-Anal. Technol. Biomed*. *Life Sci.* **2011**, 879, 921–926.

[33] Aupiais, J. *Chromatographia* **1997**, 44, 303–312.

[34] Beck, W.; Engelhardt, H. *Chromatographia* **1992**, 33, 313–316.

[35] Chen, H.; Xu, Y.; Ip, M. P. C. *J. Liq. Chrom. Rel. Technol*. **1997**, 20, 2475–2493.

[36] Chen, Z. L.; Warren, C. R.; Adams, M. A. *Chromatographia* **2000**, 51, 180–186.

[37] Cortacero-Ramirez, S.; Segura-Carretero, A.; Cruces-Blanco, C.; de Castro, M. H. B.; Fernandez-Gutierrez, A. *Food Chem*. **2004**, 87, 471–476.

[38] Gao, J.; Fan, H.; Yang, W.; Sun, X.; Li, C.; Mao, X.; Wang, J.; Wang, R.; Jia, Z. *Cent. Eur. J. Chem*. **2008**, 6, 617–621.

[39] Haumann, I.; Boden, J.; Mainka, A.; Jegle, U. *J. Chrom. A* **2000**, 895, 269–277.

[40] Johns, C.; Yang, W. C.; Macka, M.; Haddad, P. R. *J. Chrom. A* **2004**, 1050, 217–222.

[41] Kaale, E.; Leonard, S.; Van Schepdael, A.; Roets, E.; Hoogmartens, J. *J. Chrom. A* **2000**, 895, 67–79.

[42] Shi, M.; Gao, Q.; Feng, J.; Lu, Y. *J. Chrom. Sci*. **2012**, 50, 547–552.

[43] Takeda, S.; Fukushi, K.; Chayama, K.; Nakayama, Y.; Tanaka, Y.; Wakida, S.; *J. Chrom. A* **2004**, 1051, 297–301.

[44] Vitali, L.; Della Betta, F.; Costa, A. C. O.; Vaz, F. A. S.; Oliveira, M. A. L.; Vistuba, J. P.; Favere, V. T.; Micke, G. A. *Talanta* **2014**, 123, 45–53.

[45] Underberg, W. J. M.; Waterval, J. C. M. *Electrophoresis* **2002**, 23, 3922–3933.

[46] Waterval, J. C. M.; Lingeman, H.; Bult, A.; Underberg, W. J. M. *Electrophoresis* **2000**, 81, 4029–4045.

[47] Padarauskas, A. *Electrophoresis* **2003**, 24, 2054–2063.

[48] Boyce, M. C.; Breadmore, M.; Macka, M.; Doble, P.; Haddad, P. R. *Electrophoresis* **2000**, 21, 3073–3080.

[49] Doble, P.; Macka, M.; Haddad, P. R. *Trend. Anal. Chem*. **2000**, 19, 10–17.

[50] Macka, M.; Johns, C.; Doble, P.; Haddad, P. R. *Liq. Chrom. Gas Chrom*. **2001**, 19, 178–188.

[51] Schuchert-Shi, A.; Hauser, P. C. *Chirality* **2010**, 22, 331–335.

[52] Strieglerova, L.; Kuban, P.; Bocek, P. *J. Chrom. A* **2011**, 1218, 6248–6255.

[53] Doan, T. K.; Kubáň, P.; Kubáň, P.; Kiplagat, I. K.; Boček P. *Electrophoresis* **2011**, 32, 464–471.

[54] Tuma, P.; Samcova, E.; Andelova, K. *J. Chrom. B-Anal. Technol. Biomed. Life Sci*. **2006**, 839, 12–18.

[55] Tuma, P.; Samcova, E.; Opekar, F.; Jurka, V.; Stulik, K. *Electrophoresis* **2007**, 28, 2174–2180.

[56] Zhang, C.-L.; Tian, J.; Qin, W.-D.; Chin. *J. Anal. Chem*. **2010**, 38, 1497–1500.

[57] Hanai, T. Basic Concenpts of High Performance Liquid Chromatography. *HPLC, A Practical Guide. RSC Chromatography Monographs*. Eds, Smith, R.M. Cambridge, UK: The Royal Society of Chemistry. 1999, pp 1–9.

[58] Roosens, N. H.; Willem, R.; Li, Y.; Verbruggen, I.; Biesemans, M.; Jacobs, M.; *Plant Physiol.* **1999**, 121, 1281–1290.

[59] Tezara, W.; Mitchell, V. J.; Driscoll, S. D.; Lawlor, D. W. *Nature* **1999**, 401, 914–917.

[60] Tucker, K. L.; Hannan, M. T.; Chen, H. L.; Cupples, L. A.; Wilson, P. W. F.; Kiel, D. P. *Am. J. Clin. Nutr*. **1999**, 69, 727–736.

[61] Garcia-Schwarz, G.; Rogacs, A.; Bahga, S. S.; Santiago, J. G. *J. Vis. Exp.* **2012**, 61, e3890.

[62] Pauli, G. F.; Chen, S.-N.; Friesen, J. B.; Mc Alpine, J. B.; Jaki, B. U. *J. Nat. Prod*. **2012**, 75, 1243–1255.

[63] Abdullah, N. F.; Hussain, R. M. *J. Liq. Chrom. Rel. Technol*. **2015**, 38, 289–293.

[64] Foerster, N.; Ulrichs, C.; Schreiner, M.; Mueller, C. T.; Mewis, I. *Food Chem*. **2015**, 166, 456–464.

[65] Haroon Khalid, S.; Liew, K.; Loh, O.; Peh, K. *Food Chem*. **2015**, 170, 321–326.

[66] He, X.; Li, J.; Zhao, W.; Liu, R.; Zhang, L.; Kong, X. *Food Chem.* **2015**, 171, 405–411.

[67] Li, G.; Liu, S.; Sun, Z.; Xia, L.; Chen, G.; You, J. *Food Chem.* **2015**, 170, 123–130.

[68] Marti, R.; Valcarcel, M.; Herrero-Martinez, M. J., Cebolla-Cornejo, J., Rosello, S. *Food Chem.* **2015**, 169, 169–179.

[69] Megias, C.; Cortes-Giraldo, I.; Giron-Calle, J.; Vioque, J.; Alaiz, M. *Talanta* **2015**, 131, 95–98.

[70] Nam, T.-G.; Lee, S. M.; Park, J. H.; Kim, D. O.; Baek, N.; Eom, S. H. *Food Chem.* **2015**, 170, 97–101.

[71] Pyrzynska, K.; Sentkowska, A. *Crit. Rev. Anal. Chem*. **2015**, 45, 41–51.

[72] McMastr, C, M. HPLC, Advantages and Disadvantages of HPLC. *A Practical User's Guide*. Seconed Edition. United States of America: John Wiley & Sons, Inc., Hoboken, New Jersey. 2007, pp 3–15.

[73] Horvath, C.; Melander, W.; Molnar, I.; Molnar, P. *Anal. Chem*. **1977**, 49, 2295–2305.

[74] Varvara, A.; Monciu, C-M.; Arama, C.; Popescu, C.; *Farmacia* **2009**, 57, 442–451.

[75] Shibue, M.; Mant, C. T.; Hodges, R. S. *J. Chrom. A* **2005**, 1080, 58–67.

[76] Chaimbault P, Petritis K.; Elfakir C.; Dreux M.; *J. Chrom. A* **1999**, 855, 191–202.

[77] Kupina, S.; Roman, M. J. *Aoac Int*. **2014**, 97, 498–505.

[78] Zhang, Y.; Li, H. F.; Ma, Y.; Jin, Y.; Kong, G.; Lin, J. M. *Talanta* **2014**, 129, 404–410.

[79] Nebot, C.; Guarddon, M.; Seco, F.; Iglesias, A.; Miranda, J. M.; Franco, C. M.; Cepeda, A. *Food Control* **2014**, 46, 495–501.

[80] Naira, S. N.; Mhatre, M.; Menon, S.; Shailajan, S. *J. Pharm. Biomed. Anal*. **2014**, 100, 190–198.

[81] Lv, Y.-K.; Zhang, J.-Q.; Guo, Z.-Y.; Zhang, W.; Sun, H.-W. *J. Liq. Chrom. Rel. Technol.* **2015**, 38, 1–7.

[82] Syedl, H. K.; Liew, K. B.; Loh, G. O. K.; Peh, K. K. *Food Chem*. **2015**, 170, 321–326.

[83] Ko, J. H.; Huang, H. Z.; Kang, G. W.; Cheong, W. J. *Bull. Kor. Chem. Soc*. **2005**, 26, 1533–1538.

[84] Hami, Z.; Amini, M.; Kiani, A.; Ghazi-Khansari, M. *Iran. J. Pharm. Res*. **2013**, 12, 911–916.

[85] Fernandez-Hernandez, A.; Mateos, R.; Garcia-Mesa, J. A.; Beltran, G.; Fernandez-Escobar, R. *Span. J. Agri. Res*. **2010**, 8, 1183–1190.

[86] Liu, J. X.; Zhang, Y. W.; Yuan, F.; Chen, H. X.; Zhang, X. X. *Electrophoresis* **2014**, 35, 3258–3263.

[87] Han, Y.; Chen, Y. *Electrophoresis* **2007**, 28, 2765–2770.

[88] Megoulas, N. C.; Koupparis, M. A. Crit. *Rev. Anal. Chem*. **2005**, 35, 301–315.

[89] Servillo, L.; Giovane, A.; Balestrieri, M. L.; Cautela, D.; Castaldo, D. *J. Agric. Food Chem.* **2011**, 59, 274–281.

[90] Sassi, S.; Aydi, S.; Hessini, K.; Gonzalez, E. M.; Arrese-Igor, C.; Abdelly, C. *Afr. J. Biotechnol*. **2010**, 9, 6061–6069.

[91] Khedr, A. H. A.; Abbas, M. A.; Wahid, A. A. A.; Quick, W. P.; Abogadallah, G. M. *J. Exp. Bot*. **2003**, 54, 2553–2562.

[92] Shen, B.; Hohmann, S.; Jensen, R. G.; Bohnert, H. J. *Plant Physiol*. **1999**, 121, 45–52.

[93] Zhu, J.-K. *Trend. Plant Sci*. **2001**, 6, 66–71.

[94] Trinchant, J. C.; Boscari, A.; Spermato, G.; Van de Sype, G.; Le Rudulier, D. *Plant Physiol*. **2004**, 135, 1583–1594.

[95] Ashraf, M.; Foolad, M. R. *Environ. Exp. Bot*. **2006**, 59, 206–216.

[96] Takagi, H. *Appl. Microbiol. Biotechnol*. **2008**, 81, 211–223.

[97] Bramlage, W.; Drake, M.; Lord, W. J. The Influence of Mineral Nutrition on the Quality and Storage Performance of Pome Fruits Grown in North America. *Mineral Nutrition of Fruit Trees: Studies in the Agricultural and Food Sciences*. Atkinson, D.; Jackson, J. E.; Sharples, R.O. eds. Butterworths, London, pp. 29–39.

[98] Paroni, R.; Fermo, I.; Molteni, L.; Folini, L.; Pastore, M. R.; Mosca, A.; Bosi, E. *J. Chrom. B-Anal. Technol. Biomed. Life Sci.* **2006**, 834, 183–187.

[99] Rovio, S.; Yli-Kauhaluoma, J.; Siren, H. *Electrophoresis* **2007**, 28, 3129–3135.

[100] Bhaskaran, S.; Smith, R. H.; Newton, R. J. *Plant Physiol*. **1985**, 79, 266–269.

[101] Fermin, B. C.; Radinsky, J. A.; Kratochvil, R. J.; Hall, J. E.; Lo, Y. M. *J. Food Sci*. **2003**. 68, 2667–2671.

[102] Saito, K.; Kohama, J.; Sakamoto, Y.; Iwasaki, Y.; Ito, R.; Horie, M.; Nakazawa, H. *J. Aoac Int*. **2011**, 94, 482–486.

[103] Oufir, M.; Schulz, N.; Vallikhan, P. S. S.; Wilhelm, E.; Burg, K.; Hausman, J. F.; Hoffmann, L.; Guignard, C. *J. Chrom. A* **2009**, 1216, 1094–1099.

[104] Schwacke, R.; Grallath, S.; Breitkreuz, K. E.; Stransky, E.; Stransky, H.; Frommer, W. B.; Rentsch, D. *Plant Cell* **1999**, 11, 377–391.

[105] Can˜ama´s, T. P.; Vin˜as, I.; Usall, J.; Magan, N.; Morello´, J. R.; Teixido, N. *Lett. Appl. Microbiol*. **2007**, 45, 6–12.

[106] Fougere, F.; Lerudulier, D.; Streeter, J. G. *Plant Physiol*. **1991**, 96, 1228–1236.

[107] Martino, D. C.; Delfine, S.; Pizzuto, R.; Loreto, F.; Fuggi, A. *New Phytol*. **2003**, 158, 455–463.

[108] Bessieres, M. A.; Gibon, Y.; Lefeuvre, J. C.; Larher, F. *J. Agric. Food Chem.* **1999**, 47, 3718–3722.

[109] Chendrimada, T. P.; Neto, M. G.; Pesti, G. M.; Davis, A. J.; Bakalli, R. I. *J. Sci. Food Agric*. **2002**, 82, 1556–1563.

[110] Teixido, N.; Canamas, T. P.; Usall, J.; Torres, R.; Magan, N.; Vinas, I. *Lett. Appl. Microbiol*. **2005**, 41, 248–252.

[111] Kalsoom, U.; Boyce, M. C.; Bennett, I. J.; Veraplakorn, V. *Chromatographia* **2013**, 76, 1125–1130.

[112] Zwart, F. J.; Slow, S.; Payne, R. J.; Lever, M.; George, P. M.; Gerrard, J. A.; Chambers, S. T. *Food Chem*. **2003**, 83, 197–204.

[113] Mar, M. H. H.; Ridky, T. W.; Garner, S. C.; Zeisel, S. H. *Nutr. Biochem*. **1995**, 6, 392–398.

[114] Misra, N.; Gupta, A. K. *Plant Sci.* **2005**, 169, 331–339.

[115] Zhang, J.; Nishimura, N.; Okubo, A.; Yamazaki, S. *Phytochem. Anal*. **2002**, 13, 189–194.

[116] Tarczynski, M. C.; Jensen, R. G.; Bohnert, H. J. *Proc. Nat. Aca. Sci.* **1992**, 89, 2600–2604.

[117] Katayama, M.; Matsuda, Y.; Kobayashi, K.; Kaneko, S.; Ishikawa, H. *Biomed. Chrom*. **2005**, 20, 440–445.

[118] Rudulier, D. L.; Bouillard, L. *Appl. Environ. Microbiol.* **1983**, 46, 152–159

[119] Rhodes, D. Rich, P. J. *Plant Physiol*. **1988**, 88, 102–108.

[120] Graham, J. E.; Wilkinson, B. J. *J. Bacteriol*. **1992**, 174, 2711–2716.

[121] Bussis, D.; Heineke, D. *J. Exp. Bot*. **1998**, 49, 1361–1370.

[122] Huq, S. M. I.; Larher, F. *New Phytol*. **1983**, 93, 209–216.

[123] Hassine, A. B.; Ghanem, M. E.; Bouzid, S.; Lutts, S. *J. Exp. Bot*. **2008**, 59, 1315–1326.

[124] Kumar, S. G.; Reddy, A. M.; Sudhakar, C. *Plant Sci.* **2003**, 165, 1245–1251.

[125] Briens, M.; Larher, F. *Plant Cell Environ.* **1982**, 5, 287–292.

[126] Genccelep, H.; Uzun, Y.; Tuncturk, Y.; Demirel, K. *Food Chem*. **2009**, 113, 1033–1036.

[127] Tůma, J.; Skalický, M.; Tůmová, L.; Bláhová, P.; Rosůlková, M. *Plant Soil Environ*. **2004**, 50, 18–26.

[128] Jodral-Segado, A. M.; Navarro-Alarcon, M.; de la Serrana, H. L. G.; Lopez-Martinez, M. C. *Sci. Total Environ.* **2003**, 312, 47–58.

[129] Greer, F. R.; Krebs, N. F.; Comm, N. *Pediatrics* **2006**, 117, 578–585.

[130] Song, Y. Q.; Manson, J. E.; Buring, J. E.; Liu, S. M. *Diabetes Care* **2004**, 27, 59–65.

[131] Perring, M. A. *J. Sci. Food Agri*. **1986**, 37, 591–606.

[132] Fallahi, E.; Conway, W. S.; Hickey, K. D.; Sams, C. E. *Hortscience*. **1997**, 32, 831–835.

[133] Cooper, T.; Bangerth, F. *Sci. Hort*. **1976**, 5, 49–57.

[134] Lavedrine, F.; Ravel, A.; Villet, A.; Ducros, V.; Alary, J. *Food Chem*. **2000**, 68, 347–351.

[135] Nour, V.; Trandafir, I.; Ionica, M. E. *Not. Bot. Horti. Agrobot. Cluj Napoca*. **2010**, 38, 228–233.

[136] Fredrikson, M.; Carlsson, N. G.; Almgren, A.; Sandberg, A. S. *J. Agri. Food Chem*. **2002**, 50, 59–65.

[137] Kvasnicka, F. *J. Sep. Sci*. **2005**. 28, 813–825.

[138] Tomoyoshi, S.; Ross, G. A. *J. Chrom. A* **1999**, 834, 65–71.

[139] Lin, T. I.; Lee, Y. H.; Chen, Y. C. *J. Chrom. A* **1993**, 654, 167–176.

[140] Francois, C.; Morin, P.; Dreux, M. *J. Chrom. A.* **1995**, 706, 535–553.

[141] Lee, Y. H.; Lin, T. I. *J. Chrom. A* **1994**, 675, 227–236.

[142] Carducci, C. N.; Dabas, P. C.; Muse, J. O. *J. Aoac Int*. **2000**, 83, 1167–1173.

[143] Yang, Q.; Hartmann, C.; Smeyersverbeke, J.; Massart, D. L. *J. Chrom. A* **1995**, 717, 415–425.

[144] Falandysz, J.; Szymczyk, K.; Ichihashi, H.; Bielawski, L.; Gucia, M.; Frankowska, A.; Yamasaki, S. I. *Food Addit. Contam*. **2001**, 18, 503–513.

[145] Matusiewicz, H. Systems for Microwave Assisted Wet Digestion: *Microwave-Assisted Sample Preparation for Trace Element Determination*. Eds, Flores, E. M. M. Elsevier: Poland. 2014, pp 77–96.

[146] Sanz, E.; Munoz-Olivas, R.; Camara, C. *J. Chrom. A* **2005**, 1097, 1–8.

[147] Wieteska, E.; Zioek, A.; Drzewinska, A. *Anal. Chim. Acta* **1996**, 330, 251–257.

[148] Bendicho, C.; De La Calle, I.; Pena, F.; Costas, M.; Cabaleiro, N.; Lavilla, I. *Trac-Trend. Anal. Chem*. **2012**, 31, 50–60.

[149] Ebringerova, A.; Hromadkova, Z. *Cent. Eur. J. Chem*. **2010**, 8, 243-257.

[150] Oguri, S.; Nomura, M.; Fujita, Y. *J. Chrom. B-Anal. Technol. Biomed. Life Sci.* **2006**, 843, 194–201.

[151] Fukushi, K.; Takeda, S.; Wakida, S.; Higashi, K.; Hirro, K. *J. Chrom. A* **1997**, 759, 211–216.

[152] Wagner, I.; Musso, H. *Angew. Chem. Int. Ed. Engl.* **1983**, 22, 816–828.

[153] Savelieva, K. V.; Zhao, S.; Pogorelov, V. M.; Rajan, I.; Yang, Q.; Cullinan, E.; Lanthorn, T. H. *Plos One* **2008**, 3, e3301.

[154] Ritter, H.; Schulz, G. E. *Plant Cell* **2004**, 16, 3426–3436.

[155] Tejero, J. S.; Biswas, A.; Wang, Z. Q.; Page, R. C.; Haque, M. M.; Hemann, C.; Zweier, J. L.; Misra, S.; Stuehr, D. J. *J. Bio. Chem*. **2008**, 283, 33498–33507.

[156] Kirma, M.; Araujo, W. L.; Fernie, A. R.; Galili, G. *J. Exp. Bot*. **2012**, 63, 4995–5001.

[157] Vcelakova, K.; Zuskova, I.; Kenndler, E.; Gas, B. *Electrophoresis* **2004**, 25, 309–317.

[158] Carlavilla, D.; Moreno-Arribas, M. V.; Fanali, S.; Cifuentes, A. *Electrophoresis* **2006**, 27, 2551–2557.

[159] Viglio, S.; Fumagalli, M.; Ferrari, F.; Iadarola, P. *Electrophoresis* **2010**, 31, 93–104.

[160] Jaworska, M.; Szulinska, Z.; Wilk, M.; Anuszewska, E. *Talanta* **2010**, 83, 513–520.

[161] Soga, T.; Ross, G. A. *J. Chrom. A* **1999**, 837, 231–239.

[162] Olivares, J. A.; Nguyen, N. T.; Yonker, C. R.; Smith, R. D. *Anal. Chem*. **1987**, 59, 1230–1232.

[163] Zunic, G.; Jelic-Ivanovic, Z.; Colic, M.; Spasic, S. *J. Chrom. B* **2002**, 772, 19–33.

[164] Ptolemy, A. S.; Britz-McKibbin, P. *Analyst* **2005**, 130, 1263–1270.

[165] Sato, Y.; Bounoshita, M.; Iwaya, K.; Miyaji, T.; Saito, M. *Bunseki Kagaku* **2010**, 59, 823–838.

[166] Ptolemy, A. S.; Tran, L.; Britz-McKibbin, P. *Anal. Biochem*. **2006**, 354, 192–204.

[167] Montero, C. M.; Dodero, M. C. R.; Sánchez, D. A. G.; Barroso, C. G. *Chromatographia*, **2004**, 59, 15–30.

[168] Kuban, P.; Bocek, P. *J. Chrom. A* **2012**, 1234, 2–8.

[169] Anouti, S.; Vandenabeele-Trambouze, O.; Cottet, H. *Electrophoresis* **2010**, 31, 1029–1035.

[170] Carpenaruiz, R.; Sopena, A.; Ramon, A. M. *Plant Soil* **1989**, 119, 251–254.

[171] Carratu, B.; Boniglia, C.; Giammarioli, S.; Mosca, M.; Sanzini, E. *J. Food Sci*. **2008**, 73, 23–28.

[172] Carrera, C.; Ruiz-Rodriguez, A.; Palma, M.; Barroso, C. G. *Ultrason. Sonochem.* **2015**, 22, 499–505.

Chapter 2 Extraction and Quantitative Determination of Osmoregulants in Plants

This chapter in in the process of submission as a review article. All efforts were made to keep the original features of this article except minor changes e.g. layout, numbering, font size and style were carried in order to maintain a consistent formatting style of this thesis.

2.1. Abstract

Osmoregulants are substances produced by plants exposed to extreme environmental conditions. These osmoregulants protect plants during stress by performing several functions including scavenging of free radicals and maintenance of osmotic balance. They are extracted from the plant of interest and quantified to estimate the level of a plant's tolerance to the stress applied. The three most commonly explored osomoregulants include proline, mannitol and glycine betaine. Several different methods have been reported for their extraction and mostly a different solvent system is used for each osmoregulant being studied. Similarly, there are a variety of methods reported for the quantification of these osmoregulants with many studies using a separate method for determination of each analyte. However, there have been some methods reported for simultaneous determination of these osmoregulants. The purpose of this article is to review the methods reported for extraction and quantification of osmoregulants.

2.2. Introduction

Osmoregulants are low molecular weight metabolites produced by plants in response to stress. They include sugars (e.g. sucrose and trehalose), sugar alcohols (e.g. mannitol), amino acids (e.g. proline and glutamate), quaternary ammonium compounds (e.g. glycine betaine and carnitine) and tetrahydropyrimidines (e.g. ecotine and hydroxyecotine) [1]. The most commonly studied osmoregulants are mannitol, proline and glycine betaine commonly referred to as betaine [2, 3]. These compounds perform a variety of functions in plants to protect them in stressed environments. The functions performed by osmoregulants include: maintaining osmotic balance to prevent water losses resulting an increase in tolerance to dehydration [4]; maintaining sufficient cell turgor to improve growth [5]; stabilization of the sub-cellular structures [6], regulation of co-enzymes [7] and scavenging of free radicals to protect plants from membrane degradation [7, 8].

The positive relationship between accumulation of osmoregulants in plants and increased stress tolerance has seen a number of approaches adopted to enhance their concentration in plants [9-13]. These include exogenous application by adding the osmoregulants to the soil or foliar spraying [9, 10] plant breeding [11] and genetic engineering where the gene responsible for producing osmoregulants is introduced to plants [12, 13]. Whatever the approach, there is a need to extract and monitor osmoregulant concentrations in plants and hence determine stress tolerance. There are a variety of methods reported for both the extraction and quantification of osmoregulants in plants. This review will outline the key methods reported for extraction and analysis of the three most commonly studied osmoregulants: mannitol, proline and betaine. In particular, the review will focus on newer, more efficient methods for the analysis of these key osmoregulants. There is an extensive literature available on the analysis of these compounds as their role extends beyond their osmoregulant capabilities. However, this review will focus on the literature where these analytes are investigated in their role as an osmoregulant.

2.3. Extraction of Osmoregulants

Solvent extraction is an essential first step required for analysis of plant osmoregulants. A wide range of solvents have been reported for the extraction of each class of osmoregulant and in many cases with little justification. For example, a variety of methods have been reported for the extraction of amino acids from plants. Extraction using hot water [14], various concentrations of aqueous sulfosalicylic acid including 3% [15-18], 5% [19], 8% [20] and 10% [21], 70% boiling methanol [22], 95% ethanol [23] and a mixture of methanol: chloroform: water (65: 25: 15) [24] have all been reported. Aqueous sulfosalicylic acid [20, 25-30] and a solvent system consisting of various compositions of methanol: chloroform: water [31-33] have been most commonly used for extraction of proline.

For quaternary ammonium compounds and particularly betaine; 80% ethanol, [34, 35] water [19, 36], methanol: chloroform: water mixtures [37], methanol: acetonitrile (1: 9) [18] and methanol [38, 39] have all been reported. However, different compositions of methanol: chloroform: water [40-46] and water [27, 46-48] are the most commonly used extracting media for betaine.

Similarly, sugars and sugar alcohols have been extracted using boiling 80% ethanol [49], methanol: water: chloroform (1: 1: 0.6) [50], methanol: water (1: 1) [51] and hot water [52, 53]. However, aqueous ethanol is the most common extracting solvent system used for mannitol [49, 54-57].

In many studies different extracting solvents were used to individually extract each osmoregulant from the plant of interest [22, 56, 58, 59]. For example, Martino *et al*. (2003) extracted proline, along with other amino acids, using an ethanol and water mixture (80: 20 v/v), and betaine using distilled water from spinach leaves to study the effect of salt stress on the accumulation of these osmoregulants [58]. Similarly, Jouve *et al*. (2003) used 3% sulfosalicylic to extract proline and then in a separate extraction process used 80% ethanol to extract mannitol along with other sugars from *Populus tremula* plants where these analytes were studied as markers for improvement in stress resistance for breeding programmes [56]. However, there are some examples of concurrent extraction of

osmoregulants. Simultaneous extraction of proline and betaine using aqueous ethanol has been reported [60]. Similarly, a mixture of methanol: chloroform: water (65: 25: 15) has been used for simultaneous extraction of proline and betaine from oak leaves for investigating the effect of environmental stress [31]. Likewise, the combined extraction of amino acids including proline, and betaines has been demonstrated using methanol: water (80: 20) [58, 61]. The report by Naidu (1998) is one of the few examples of where combined extraction of proline, mannitol and betaine was undertaken and a methanol: chloroform: water (65: 25: 15) solvent system was employed [62]. In all these studies, a solvent for extraction is chosen without providing the reason for priority of one solvent over the other for a particular osmoregulant.

There have been some investigations to determine optimal extraction of osmoregulants. For instance, Bessieres *et al*. (1999) investigated the best extracting solvent for betaine by comparing cold water, ethanol: chloroform: water (12: 5: 3) and ethanol: water (9: 1) [63]. They concluded that water was the best solvent for extraction being the least expensive and as efficient as the other extraction systems tested. Similarly, Nishimura *et al*. (2001) compared three solvent systems including hot water, 80% ethanol and a mixture of methanol: chloroform: water (12: 5: 3) for their ability to extract proline and betaine from higher plants grown under elevated salt concentrations [36]. The reported extraction of betaine was independent of solvent; however, extraction in hot water (80 ^{o}C) for 20 minutes was optimal for proline. Therefore, as hot water is optimal for proline and as effective as other extracting solvents for extracting betaine, it can be concluded that hot water is an appropriate solvent for combined extraction of these two analytes. It also has the added advantage of being inexpensive and non-toxic. An investigation of optimal conditions for extraction of mannitol has not been reported but hot water has been used in some studies. For example, extraction of mannitol after sonification with distilled water from the cells of *Rhizobium meliloti* to investigate the effect of osmotic values of the medium on the accumulation of mannitol and other sugars as osmoregulants has been demonstrated [52].

Based on above discussion, it can be concluded that a mixture of methanol: chloroform: water, aqueous ethanol, and water are the extracting systems that has been used most

commonly for the extraction of all three osmoregulants including; proline, betaine and mannitol (See Table 1). Additionally, methanol: chloroform: water has been chosen frequently for simultaneous extraction of three osmoregulants in various studies and it has also been reported for simultaneous extraction of these analytes [62]. However, use of this system for extraction is not recommended as chloroform is a hazardous chemical and is not environment friendly. Moreover, comparison of water with other extracting systems including; methanol: chloroform: water and aqueous ethanol, has shown that it is optimal solvent for extraction of proline and provides comparable results to other solvents for betaine extraction [36]. In addition to this, use of hot water for mannitol extraction has also been reported [53]. Furthermore, given that sugar and sugar alcohols are polar is nature and they should be readily soluble in hot water, a hot water extract for mannitol also seems a sensible choice. Therefore, it can be concluded that the quick and inexpensive simultaneous extraction of three osmoregulants (i.e. proline, betaine and mannitol) can be carried using an environment friendly solvent system such as hot water.

Table 1. A list of solvents reported for extraction of three most common osmoregulants i.e. proline, betaine and mannitol.

Analyte	Extracting solvent	Matrix	Ref
Proline	Hot water	Rice	[14]
	3% sulfosalicylic acid	Aspen (*Populus tremula* L.)	[56]
	3% sulfosalicylic acid	Tomato plants	[15]
	3%s ulfosalicylic acid	Maiz plants	[16]
	3% sulfosalicylic acid	Sugarbeet	[17]
	3% sulfosalicylic acid	Green gram	[18]
	5% sulfosalicylic acid	*Altriplex halimus* L	[19]
	8% sulfosalicylic acid	Xerophytes and mesophytes	[20]
	10% sulfosalicylic acid	Wheat plants	[21]
	70% methanol	Tomato pollens	[22]
	95% ethanol	Tomato plants	[23]
	MCW (65:25:15)	Melaleuca species	[24]
	2% sulfosalicylic acid	Rice leaves	[25]
	3% sulfosalicylic acid	Tomato plants	[26]
	3% sulfosalicylic acid	Mulberry leaves	[27]
	3% sulfosalicylic acid	Sugarbeet	[28]
	3% sulfosalicylic acid	Tomato leaves	[30]
	MCW (15:5:1 v/v/v)	Sugarcane callus culture	[33]
Betaine	80% ethanol	Enterococcus feacalis	[34]
	Water	Higher plants	[36]
	Water	Altriplex halimus L	[19]
	Methanol	Zea mays	[38]
	Methanol	Thai jasmine rice	[39]
	Methanol :anhydrous acetonitrile (1:9)	Green gram	[18]

Analyte	Extracting solvent	Matrix	Ref
	MCW (70:20:10, v/v/v)	Barely plants	[40]
	MCW (12:5:1, v/v/v)	Suaeda maritima shoots	[41]
	MCW (10:5:6, v/v/v)	Zea mays L	[42]
	MCW (12: 5: 1, v/v)	Limonium Species and other halophytes	[48]
	MCW (12: 5: 1, v/v)	Cereals and grasses	[44]
Betaine	MCW (12: 5: 1, v/v)	Tobacco plants	[45]
	MCW, (12: 5: 1, v/v)	Barley leaves	[46]
	Ethanol	Rape leaf	[35]
	Water	Spinach leaves	[58]
	80% ethanol	Corn Kernels	[49]
	MCW (12:5:3)	Ligneous plants	[50]
	hot water	*Ligustrum lucidum* Ait	[53]
	80% ethanol	Muskmelon Fruit	[64]
Mannitol	80% ethanol	Celery Petioles	[54]
	80% ethanol	*Phaselous vulgaris* leaves	[55]
	80% ethanol	Aspen (*Populus tremula* L.)	[56]
	80% ethanol	Celery	[57]
Proline, betaine	70% ethanol	Bacterial strains	[60]
Proline, betaine	MCN (60:25:15, v/v/)v	Oak leaves	[31]
Proline, betaine	Ethanol: water (80:20)	Spinach leaves	[58]
Proline, betaine, mannitol	MCW (65:25:15, v/v/v)	Peanut and cotton	[62]

2.4. Quantification of Osmoregulants

A number of approaches have been reported for quantification of each osmoregulant. The key methods reported for each osmoregulant of interest are described below.

2.4.1. Proline.

Proline is an α-amino acid and is polar in nature. It has a carboxylic acid (-CH_3COOH, pK_{a1}= 1.95) functional group which makes it positively charged under acidic conditions and an amino group (-NH_2, pK_{a2} = 10.64) which makes it negatively charged under alkaline conditions [65].

Proline has been extensively analysed using a variety of methods including; colorimetry [66-68], chromatography [69-72] and capillary electrophoresis (CE) [3, 36]. Colorimetry is one of the most popular techniques employed for proline analysis [66-68]. As proline lacks a colour absorbing functional group it can only be analysed after formation of coloured derivatives. In 1957 Chinard reported proline, at low pH, forms a red product after reaction with ninhydrin in the presence of glacial acetic acid and phosphoric acid, and that this compound could be used to quantify for proline [73]. However, other amino acids interfered with the determination of proline and an additional ion-exchange or paper chromatography step was required to remove these interferences prior to analysis. Improvements were made to the method to make it more selective for proline but they reduced the applicability of the method for routine and rapid sampling. Bates *et al*. (1973) suggested a simplified more effective method where filtered extracts were reacted with ninhydrin and glacial acetic acid at 100 ^{o}C for 1 hour [68]. The derivatized proline product was extracted with toluene. While this method was an improvement as interferences from free amino acids were minimised, interferences from sugars was an issue. Magne & Larher (1992) observed that phosphoric acid in the ninhydrin reagent was responsible for the formation of the green coloured complex with sugars particularly with sucrose [74]. Therefore, they suggested the preparation of ninhydrin reagent without phosphoric acid and the use of dilute acetic acid for the analysis of extracts rich in sucrose. While colorimetric methods suffer from poor

sensitivity and selectivity, they are still routinely used as they are quick and require no specialised instrumentation. To obtain better sensitivity and selectivity, chromatographic approaches such as gas chromatography (GC) and high performance liquid chromatography (HPLC) have been adopted for determination of proline.

GC separates the analytes based on their boiling point and or polarity. The volatilised analytes are transported through the column by an inert gas, typically helium or hydrogen where they are selectively retained by the solid, liquid or polymeric stationary phase which usually coats the inner wall of the separation column [75]. GC's high resolving power makes it ideal for complex samples such as plant extracts. While GC is ideal for the analysis of volatile compounds, non-volatile analytes can be derivatized to make them more volatile and hence suitable [76]. Derivatization of functional groups possessing active hydrogens e.g. -SH, -OH, -NH and -COOH is of primary importance as they are polar thereby reducing volatility [77, 78]. The active hydrogen group is usually replaced with a trimethylsilyl group [79] such as trimethylchlorosilane (TMCS) [70], trimethylsilylimidazole (TMSI), N-methyltrimethylsilyltrifluoroacetamide (MSTFA) [70, 71], and N-methyl-N-t-butyldimethylsilyltrifluoroacetamide (MTBSTFA) [72]. The derivatives are less polar and sufficiently volatile to allow their elution from the separation column at temperatures that do not cause thermal decomposition of analyte.

GC has been employed for the analysis of proline [70-72, 80]. For instance, GC analysis of proline along with 150 other metabolites in potato tubers was achieved after derivatization with a mixture of MSTFA and TMCS. A mass spectrometer (MS) was employed for detection [70]. Similarly, determination of proline in grapes for estimating the water and salt stress was achieved after derivatization with MSTFA [71]. GC-MS using MTBSTFA to derivatize proline was employed to study the performance of alfalfa plants exposed to water stress [72].

In HPLC, analytes generally partition between two liquid phases, the stationary phase and mobile phase. The nature of the stationary phase determines the mechanism of separation. A non-polar stationary phase is ideal for the separation of non-polar analytes while an ion

exchanger as the stationary phase is suitable for the separation of charged analytes including amino acids. Reversed phase (RP) HPLC, using a non-polar stationary phase and a polar mobile phase is the most commonly used HPLC system. While it is best suited for non-polar analytes, retention of polar analytes such as amino acids is possible by adding an ion pairing reagent (IPR) to the mobile phase. The IPR forms an ion pair with the polar analyte reducing its polarity and enhancing its interaction the non-polar stationary phase [81] Trifluoro acetic acid (TFA) [82, 83], sodium perchlorate [83] and pentadecafluorooctanoic acid [84] are some examples of IPR.

HPLC has been used extensively for the analysis of proline in plants [2, 62, 85, 86]. The methods described vary in terms of separation mechanism and detection mode. As proline is a polar analyte, separation is often achieved on an ion exchange column. For example, Naidu (1998) analysed proline in peanut and cotton plants exposed to water stress using a cation exchange column and UV detection at low wavelength [62]. UV detection lacks sensitivity for proline, however, this can be overcome by derivatization to impart strong UV absorbing properties. The derivatized product is usually less polar than proline itself and separation on a RP [85] column such as a octadecyl carbon (C_{18}) or an amino column is more suitable [87]. Analysis of proline along with other amino acids in alfalfa plants exposed to extreme saline conditions was carried after derivatization with phenylthiocarbamyl to achieve sensitive UV detection and separation was achieved on a RP column [86]. Other derivatizing agents suitable for UV detection of proline include; ninhydrin [22], 9-fluorenyl-methylchloroformate (FMOC) [67, 87], and o-phthalaldehyde (OPA) [58]. Derivatization has its drawbacks, it is complicated and time consuming and may lead to formation of side product. It can be avoided by using MS instead of UV detection. For example, sensitive analysis of proline extracted from oak leaves using HPLC in combination with MS detection to investigate the effect of drought stress on the accumulation of osmoregulants [31].

Capillary electrophoresis is both an alternative and complementary technique to HPLC and other chromatographic approaches. The distinctive features of CE include, less sample and solvent volume required, rapid analysis times and its ability to simultaneously analyse samples of widely varying polarity [88]. Capillary zone electrophoresis (CZE) and micellar

electrokinetic chromatography (MEKC) are two of the most commonly used modes of CE. In CZE, separation is based on differences in mobility of the charged species under the influence of an applied electric field. The mobility of an analyte depends on the charge to mass ratio i.e. smaller highly charged species are more mobile when compared to larger, minimally charged ions [89]. In MEKC, separation is based on the distribution of solute between the pseudo-stationary phase (micelles) and the running buffer. Neutral analytes migrating with the EOF can interact with micelles and experience a decrease in velocity. Generally, the more hydrophobic the analyte the more it interacts and the later it elutes [90, 91], therefore, the polar/ ionic species move faster than the less polar analytes.

Although a number of CE methods have been reported for proline analysis [92-95], there are only few publications that analyse proline as an osmoregulant. Nishimura et al. 2001 separated proline and other analytes in a number of plant species using CZE and direct UV detection at low wavelength [36]. They experienced poor analyte sensitivity; however, this has since been remedied by using indirect detection [3]. In indirect detection, background electrolyte (BGE) contains a strongly absorbing electrolyte (also known as probe) carrying the same charge as the analyte. The displacement of UV absorbing probe by a UV transparent analyte results in a significant decrease in absorbance and a negative peak is detected [96]. A sensitive measurement for proline in spinach and beetroot was achieved using a novel probe sulphanilamide [3]. CE in combination with MS has also been used for high mass accuracy and efficient resolution of proline [97]. For example, Urano et al. (2009) used CE-MS for separation and detection of proline and other analytes to compare the metabolic profile of wild type and mutant Arabidopsis in relation to dehydration [98].

2.4.2. Betaine

Betaine is a zwitterionic compound; it possesses a positive charge at the quaternary ammonium functional group and a negative charge at the carboxylate group [99]. The pK_a of carboxylic group of betaine is 4.00 [100] which make it possible to develop a positive charge at low pH.

Similar to proline, colorometric analysis of betaine typically relies on removal of interferences by thin layer chromatography, paper chromatography or ion exchange chromatography followed by visualisation of betaine with dragendorff's reagent [101]. KI-I [102, 103] ammonim reineckates [104, 105] and phosphotungstic acid [105] are the other colorimetric reagents reported for the analysis of betaine. However, all of these methods lack sensitivity and are not specific for a particular quaternary ammonium compound. The other limitation is that these methods provide qualitative or semi-quantitative information only. The later drawback can be overcome by using scanning reflectance densitometry in combination with separation techniques [106]. Using the approach, TLC plates sprayed with dragendorff's reagent are scanned with a spectrophotometer and the reflectance of the background usually yellow or red at a particular wavelength is observed. The quenching of red or yellow spots is measured and is used for quantification of betaines [41]. The limitations of these methods have prompted the development of more specific and quantitative methods for the analysis of betaine.

Pyrolysis-GC has been repeatedly used for the analysis of betaines [107, 108]. It provides a quick and powerful tool for analysing complex and non-volatile samples without the need for derivatization [109]. In pyrolysis, large molecules are thermally broken down into small fragments which are then identified and quantified by GC. For example, accumulation of betaine in [57] species of cereals and grasses after exposing them to water stress has been reported after pyrolysis. The detection was achieved by using flame ionisation detector (FID) [44]. The same method was also used by Ladyman *et al*. (1980) for studying the effect of a water deficit on the distribution and metabolism of betaine in barley plants [107].

HPLC provides selective and quantitative information and a number of methods have been reported for betaine. As betaine is charged at low pH, ion exchange columns [58, 63, 110] are commonly used for its separation. However the use of a RP column [37] has also been reported where retention is increased by derivatization or the addition of an IPR to the mobile phase. For detection, UV [58, 63, 110-113], RI [37] and MS [114] have all been used, however, UV is the most commonly used mode. As betaine lacks a choromophore detection is only possible at low wavelengths using UV [58, 63, 110] and for sensitive detection

derivatization is essential [112, 113]. Betaine and its analogues were determined in vegetables after detivatization with 2-naphthycyl trifluoromethane sulfonate for UV detection and separation was performed using a RP column [113]. 4-bromo-phenacyl triflate [112] and 4-isophenyl trifoliate [18] have also been used for derivatization of betaine. The derivatization procedure can be avoided by using evaporative light scattering detection (ELSD); Shin *et al*. (2012) developed a method for the separation using HILIC column and detection using ELSD for analysis of betaine in *Fructus Lycii* [99]. A limited use of HPLC with MS detection has also been reported for betaine determination; Wood *et al*. (2002) used HPLC-MS/MS for the characterisation of betaines in four different plants [115].

CE, in both MEKC and CZE modes, has been reported for the analysis of betaines. Analysis by CZE in combination with UV detection at low wavelength (195 nm) was used to determine betaine in eighteen different plants (e.g. cotton, wheat, barley and alfalfa) [36]. Derivatization of betaine to form p-bromophenacyl esters for more sensitive UV detection after separation by both CZE [116] and MEKC [36] has also been demonstrated. However, the ester derivatives are sensitive to pH and thermal changes. Recently, Kalsoom *et al*. (2014) developed an indirect detection method as an alternative to derivatization for UV analysis [3].

Another analytical technique, nuclear magnetic resonance (NMR) offers well-resolved, unique and highly predictable spectra for small molecules. In NMR spectroscopy, the magnetic properties of certain atomic nuclei e.g. ^{1}H, ^{13}C, ^{15}N, ^{19}F, and ^{31}P are utilised to determine physical and chemical properties of atoms or molecules. There are a number of reports in which NMR spectroscopy has been used for determination of betaine [10, 117-120]. Accumulation of betaine in wild-type and genetically engineered *Arabidopsis thaliana* was examined using NMR spectroscopy to evaluate the success of the transgenic plant [117]. However, large sample volumes, long run times and poor sensitivity are the major limitations of this technique.

Fast atom bombardment mass spectrometry (FABMS) is an ionisation technique that has been used for the determination of chemical structure. In FABMS, the analyte (dissolved in a

non-volatile organic phase such as glycerol) is bombarded with a high energy beam of ions (xenon or argon) to create ions. As a result, a permanent positive charge is created on the analyte by formation of adduct ion $[M+H]^+$ with H^+, Na^+ or K^+. These ions are then separated on the basis of charge to mass ratio. This technique yields a spectrum that is stable for a significantly long period. In addition, short analysis time [121] and generation of more structural information in comparison to MS/ MS methods are the major advantages of this technique [122]. This technique has been used for analysis of betaines [43] as a permanent positive charge is created on the zwitterionic form of the analyte by the formation of adduct with the negative charge of carboxyl group [106]. Another approach is to derivatize the carboxyl group with an alcohol to form an ester leaving a permanent positive charge on the betaine. This method was used to determine betaine in transgenic tobacco plants [48] and in various species of Limonium species to investigate its osmoregulatory role [45].

2.4.3. Mannitol

Mannitol is a sugar alcohol and is polar in nature [123]. The pK_a value of mannitol is 13.5 and can only be negatively charged at high pH [124].

Mannitol has been explored widely for a variety of reasons and using a broad range of techniques including photometry [125, 126], chromatography [127-129], CE [53, 130] and NMR [131].

Similar to proline and betaine, early analysis of mannitol also involved colorimetric methods. For colorimetric analysis, mannitol is oxidised with periodic acid in the presence of formic acid [132] and the formaldehyde produced is estimated by colorimery after coloration with chromotropic acid [133]. As is typical of other colometric techniques, it is not specific to mannitol and suffers interferences from other sugars.

Another technique, paper chromatography has also been used for the analysis of mannitol [134]. In paper chromatography, mannitol and other sugar alcohols separated on a paper are detected by a colouring agent. A variety of colouring agents including *p*-anisidine,

perchloric acid, and alkaline periodate-permanganate have all been used for the detection of polyols [134]. However, non specificity and semi-quantitative analysis are the major limitations of this approach.

A limited use of GC has also been reported for analysis of mannitol in its role as an osmoregulant [70]. As previously mentioned for proline, Roessner, *et al*. (2000) analysed of 150 analytes in potato tubers which also included mannitol using GC-MS [70].

HPLC has been used for determination of mannitol. As mannitol is a polar molecule and lacks a fluorescent or UV absorbing group, HPLC analysis usually involves separation under alkaline conditions using anion exchange chromatography in combination with pulsed amperometric detection (PAD) [135, 136]. Improved sensitivity can be achieved using MS detection. Sensitive determination of mannitol in poplar leaves grown under drought was achieved by PAD followed by MS detection [137]. Though PDA provided sensitive detection, co-elution with matrix interferences was an issue for plant samples. Combining MS with PDA provided a sensitive and selective determination of mannitol along with other carbohydrate in plant extracts. As mannitol and other carbohydrates are negatively charged under highly alkaline conditions, separation was achieved using an anion exchange column [137].

CE has also been used for the analysis of mannitol, though less frequently. The effect of salt stress in Kandelia candel plants was estimated using CE; mannitol was separated using CZE after complexation with borate and indirect mode was used for detection [138].

For many of the studies presented here, the osmoregulants were for the most part isolated and measured independently [19, 27, 58, 60, 87, 139-143]. For example, Canamas *et al*. (2007) determined proline levels in plant tissues by using RP-HPLC with fluorescence detection [87]. The same authors also analysed betaine extracted from the same plant with an HPLC system fitted with a RI detector. Similarly, Hassine *et al*. (2008) determined betaine by RP-HPLC in combination with UV detection and proline by a colorimetric method and both osmoregulants were extracted from the same plant [19]. The cost and time associated with completing independent experiments for osmoregulants isolated from the same plant

has prompted the development of simultaneous methods for the analysis of the three most commonly explored osmoregulants.

2.5. Simultaneous Determination of Osmoregulants

Some attempts have been taken to quantify osmoregulants simultaneously from plant extracts. Jones *et al*. (1986), estimated betaines and proline in barley leaves grown under water deficit conditions using ^{1}HNMR techniques [119]. While this method was sensitive for betaines, it was not suitable for accurate determination of proline, particularly at low levels [118]. Oufir *et al*. (2009) used HPLC to determine proline, its analogues and betaine in oak leaves with photodiode array (PDA) detection and an anion exchange column [31] for separation. However, the sensitivity achieved with PDA was insufficient and only proline and hydroxyproline were detected. The same researchers successfully separated proline, betaine and its analogues using a size exclusion column for separation and MS for detection [31] and long run time limited the usefulness of this method. GC–MS has also been used for the simultaneous analysis of 150 analytes (including proline and mannitol) in potato tubers [70] and because MS detection was employed full separation of the analytes was not necessary. Table 2. A list of analytical methods reported for the analysis of three most common osmoregulants, proline, betaine and mannitol. Naidu (1998) determined sugars, sugar alcohols, proline, its analogues and betaines simultaneously in peanut and cotton plants using HPLC coupled to a UV detector [62]. As detection was achieved at low wavelength the sensitivity of the UV transparent osmoregulants was poor. The lack of sensitivity was somewhat addressed by Kalsoom et al. (2013) who used ELSD [2]. The requirement for a relatively volatile mobile phase negated the use of an ion-exchange column. A C_{18} non-polar column and the inclusion of an ion pairing reagent in the buffer to enhance the retention of the polar osmoregulants successfully separated the analytes prior to analysis by ELSD. The method was used to investigate proline mannitol and betaine concentrations in halophytes.

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Table 2. A list of analytical methods reported for determination of three most common osmoregulants i.e. proline, betaine and mannitol.

Analyte	Analytical method	Comments	Matrix	Ref
Proline	Colorimetry	Colouring reagent-Acid ninhydrin	*Atriplex halimus* L.	[19]
	Colorimetry	Colouring reagent-isatin	Grape juice and wine	[67]
	Colorimetry	Colouring reagent-Acid ninhydrin	Soybean and sorghum	[68]
	GCMS	Derivatising reagent-MSTFA	Grapes	[144]
	GCMS	Derivatising reagent-MTBSTFA	Alfalfa plants	[72]
	HPLC-UV	dansylated derivatives, C_{18} column	Sorghum bicolour	[85]
	RPHPLC-UV	phenylthiocarbamyl derivatives	Alfalfa	
	HPLC-UV	Ninhydrin derivatives	Tomato pollen	[22]
	HPLC-UV	FMOC	Grape juice and wine	[67]
	HPLC-UV	FMOC	Pantoea agglomerans	[87]
	HPLC-UV	OPA	Spinach leaves	[58]
	HPLC-MS	Ligand exchange chromatograhy, electrospray Ionisation MS	Oak leaves	[31]
	CE-MS		*Arabidopsis thaliana*	[98]
Betaine	Colorimetry	Colouring reagent-Dragendorff's reagent	Halophytes	[101]
	Colorimetry	Colorimetric reagent- KI	Zoysiagrasses	[103]
	Colorimetry	Ammonium reineckates	Sugar beet	[104]
	Thin layer electrophoresis+ scanning reflectance densitometry	Plates sprayed with Dragendorff reagent	Suaeda maritime	[41]
	GC-pyrolysis	FID detection	Cereals and grasses	[44]
	GC-pyrolysis	FID detection	Barley plants	[107]
	HPLC-UV	Ion exchange column, detection 195 nm	Spinach	[58]
	HPLC-UV	Ion exchange column,		[64]
	HPLC-UV	4-isophenyl trifoliate, silica column	Green gram	[18]
	HPLC-UV	Reverse phase column	*Altriplex halimus* L	[19]

Analyte	Analytical method	Comments	Matrix	Ref
	HPLC-ELSD	HILIC column	*Fructus Lycii*	[99]
	HPLC-MS/MS		Four plants	[115]
	CE-UV	CZE mode, 195nm	Eighteen plants	[36]
	MEKC-UV	p-bromophenacyl esters	Eighteen plants	[36]
	CZE-UV	p-bromophenacyl esters	Higher plants	[116]
	NMR Spectroscopy		Arabidopsis thaliana	[117]
Bataine	NMR Spectroscopy		Barely	[118]
	NMR Spectroscopy		Rice plants	[119]
	NMR Spectroscopy		Barely	[120]
	NMR Spectroscopy		Tobacco	[10]
	FABMS		Sugarcane	[43]
	FABMS		Tobacco	[45]
	FABMS		Limonium species	[48]
	FABMS		Higher plants	[106]
	HPLC-RI		Pantoea agglomerans	[87]
	HPLC-PAD	Anion exchange chromaography	Tobacco	[136]
Mannitol	HPLC-PAD	Anion exchange chromaography	Yeast	[135]
	Colorimetry	Colouring reagent-Chromotropic acid	Fungi and green plants	[133]
	HP anion exchange electrospray MS	Anion exchange chromaography	Poplar leaves	[137]
Proline, betaine	HPLC-PAD	Anion exchange column	Oak leaves	[31]
Proline, betaine	HNMR Specroscopy	Barely leaves		[118]
Proline, mannitol	GCMS	Derivatisation with MSTFA and TMCS	Potato tubers	[70]
Proline, betaine, mannitol	HPLC-ELSD	RP column	Halophytes	[2]
Proline, betaine, mannitol	HPLC-UV	Detection at 195nm	Peanut, Melaleuca,cotton	[62]
Proline, betaine	CE-UV	Detection at 195nm	higher plants	[36]
proline+betaine+mannitol	CE-UV	Indirect detection	Spinach, beet root	[3]

CE in combination with UV has also been used for simultaneous analysis of osmoregulants. For example Nishimura *et al*. (2001) determined proline and betaine simultaneously using UV detection at low wavelength (190 nm) and at low pH [36]. However, the sensitivity of this method was poor as direct UV detection was employed. The poor sensitivity was improved by using indirect detection at 214 nm at low pH [3]. The simultaneous analysis of three osmoregulants by CE is challenging. At any given pH it is not possible to develop a charge on all three osmoregulants. For instance, at low pH proline and betaine carry a positive charge and can be separated by CZE but mannitol remains neutral and elutes unresolved from other neutral analytes. Similarly, at high pH, proline and mannitol can be resolved in their anionic forms but mannitol remains neutral and again elutes with other neutral analytes unresolved and hence cannot be identified. However, an alternative detector, mass spectrometry (MS), can be used for further identification of analytes. In MS detection is based on the molecular mass of the analytes and as each analyte has different mass it can be easily identified (ref). Furthermore, using MS detection, it would be possible to identify mannitol from other analytes on the basis of molecular mass even if it remained unresolved, thus making the simultaneous analysis of three osmoregulants possible. Therefore, there is need for development of methods using CE in combination with MS to provide sensitive and selective methods for simultaneous analysis of osmoregulants. Another approach that has potential to simultaneously determine all three omosregulants is a dual-capillary sequential injection-capillary electrophoresis (SI-CE) configuration that has been used for the simultaneous determination of cations and anions [145]. This unit has two capillaries in parallel, one at low pH and other at high pH, allowing the separation of cations and anions simultaneously. There is a possibility that the three osmoregulants can be analysed simultaneously using this simple and novel configuration. These methods for simultaneous analysis of osmoregulants will allow the biologist and plant physiologist studying water logging and salinity to analyse the osmoregulants in minimum time and cost when three of them are studied together.

2.6. Conclusion

The individual extraction and quantification of osmoregulants, when two or more analytes are studied, is time consuming and labour intensive. Simultaneous extraction of all three key osmoregulants (mannitol, proline and betaine) is possible using a number of solvents, one of which is hot water. Similarly, for analysis of osmoregulants, coloromeric methods are still commonly used to determine each of the osmoregulants individually. However, methods for simultaneous determination of osmoregulants using various techniques e.g. NMR spectroscopy, GC-MS, HPLC in combination with both UV and ELSD detection are also available. A variety of methods for simultaneous analysis of osmoregulants available provide a freedom of choice to the user to select a method based on the analytes under study, and sensitivity and selectivity requirements of the analysis. In addition to this, simultaneous extraction and analysis of osmoregulants is fast, simple, requires less solvent for extraction, minimise the waste, less labour-intensive and inexpensive in comparison to individual extraction an analysis.

2.7. References

1. Takagi, H. *Appl. Microbiol. Biotechnol.* **2008**. 81, 211-223.
2. Kalsoom, U.; Boyce, M. C.; Bennett, I. J.; Veraplakorn, V. *Chromatographia* **2013**. 76, 1125-1130.
3. Kalsoom, U.; Breadmore, M. C.; Guijt, R. M.; Boyce, M. C. *Electrophoresis* **2014**.
4. Khedr, A. H. A.; Abbas, M. A.; Wahid, A. A. A.; Quick, W. P.; Abogadallah, G. M. *J. Exp. Bot.* **2003**. 54, 2553-2562.
5. Trinchant, J. C.; Boscari, A.; Spermato, G.; Van de Sype, G.; Le Rudulier, D. *Plant Physiol.* **2004**. 135, 1583-1594.
6. Ashraf, M.; Foolad, M. R. *Environ. Exp. Bot.* **2006**. 59, 206-216.
7. Bhauso, T. D.; Radhakrishnan, T.; Kumar, A.; Mishra, G. P.; Dobaria, J. R.; Patel, K.; Rajam, M. V. *Sci. World J.* **2014**. 2014, 125967-125967.
8. Eung-Jun Park; Jeknic, Z.; Chen, T. H. H. *Plant Cell Physiol.* **2006**. 47, 706–714.
9. Heuer, B. *Plant Sci.* **2003**. 165, 693-699.
10. Holmstrom, K. O.; Somersalo, S.; Mandal, A.; Palva, T. E.; Welin, B. *J. Exp. Bot.* **2000**. 51, 177-185.

11. Morgan, J. M. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **1984**. 35, 299-319.

12. Sakamoto, A.; Murata, N. *Plant Cell Environ.* **2002**. 25, 163-171.

13. Quan, R. D.; Shang, M.; Zhang, H.; Zhao, Y. X.; Zhang, J. R. *Plant Biotechnol. J.* **2004**. 2, 477-486.

14. Lutts, S.; Majerus, V.; Kinet, J. M. *Physiol. Plant.* **1999**. 105, 450-458.

15. Claussen, W. *Plant Sci.* **2005**. 168, 241-248.

16. Sultan Köşkeroğlu; Tuna, A. L. *Acta Physiol. Plant.* **2010**. 32, 541-549.

17. Ghoulam, C.; Foursy, A.; Fares, K. *Environ. Exp. Bot.* **2002**. 47, 39-50.

18. Misra, N.; Gupta, A. K. *Plant Sci.* **2005**. 169, 331-339.

19. Hassine, A. B., Ghanem, M.E., Bouzid, S., and Lutts, S.;. *J. Exp. Bot.* **2008**. 59, 1315-1326.

20. Wang, S. M.; Wan, C. G.; Wang, Y. R.; Chen, H.; Zhou, Z. Y.; Fu, H.; Sosebee, R. E. *J. Arid Environ.* **2004**. 56, 525-539.

21. Sawahel, W. A.; Hassan, A. H. *Biotechnol. Lett.* **2002**. 24, 721-725.

22. Schwacke, R.; Grallath, S.; Breitkreuz, K. E.; Stransky, E.; Stransky, H.; Frommer, W. B.; Rentsch, D. *Plant Cell* **1999**. 11, 377-391.

23. Rivero, R. M.; Ruiz, J. M.; Romero, L. M. *Plant Biol.* **2004**. 6, 702-707.

24. Naidu, B.; Paleg, L.; Jones, G. P. *Aust. J. Bot.* **2000**. 48, 611-620.

25. Hsu, S. Y.; Hsu, Y. T.; Kao, C. H. *Biol. Plant.* **2003**. 46, 73-78.

26. Alian, A.; Altman, A.; Heuer, B. *Plant Sci.* **2000**. 152, 59-65.

27. Kumar, S. G.; Reddy, A. M.; Sudhakar, C. *Plant Sci.* **2003**. 165, 1245-1251.

28. Gzik, A. *Environ. Exp. Bot.* **1996**. 36, 29-38.

29. Summart, J.; Thanonkeo, P.; Panichajakul, S.; Prathepha, P.; McManus, M. T. *Afr. J. Biotechnol.* **2010**. 9, 145-152.

30. Umebese, C. E.; Olatimilehin, T. O.; Ogunsusi, T. A. *Amer. J. Agri. Biol. Sci.* **2009**. 4, 224-229.

31. Oufir, M.; Schulz, N.; Vallikhan, P. S. S.; Wilhelm, E.; Burg, K.; Hausman, J.-F.; Hoffmann, L.; Guignard, C. *J. Chromatogr. A* **2009**. 1216, 1094-1099.

32. Roosens, N. H.; Willem, R.; Li, Y.; Verbruggen, I.; Biesemans, M.; Jacobs, M. *Plant Physiol.* **1999**. 121, 1281-1290.

33. Errabii, T.; Gandonou, C. B.; Essalmani, H.; Abrini, J.; Idaomar, M.; Skali-Senhaji, N. *Afr. J. Biotechnol.* **2006**. 5, 1488-1493.

34. Pichereau, V.; Bourot, S.; Flahaut, S.; Blanco, C.; Auffray, Y.; Bernard, T. *Microbiol.* **1999**. 145, 427-435.

35. Gibon, Y.; Bessieres, M. A.; Larher, F. *Plant Cell Environ.* **1997**. 20, 329-340.

36. Nishimura, N.; Zhang, J.; Abo, M.; Okubo, A.; Yamazaki, S. *Anal. Sci.* **2001**. 17, 929-932.

37. Teixido, N.; Canamas, T. P.; Usall, J.; Torres, R.; Magan, N.; Vinas, I. *Lett. Appl. Microbiol.* **2005**. 41, 248-252.

38. Rhodes, D.; Rich, P. J.; Myers, A. C.; Reuter, C. C.; Jamieson, G. C. *Plant Physiol.* **1987**. 84, 781-788.

39. Cha-um, S.; Slipaibulwatana, K.; Kirdmanee, C. *J. Agron. Crop Sci.* **2006**. 192, 25-36.

40. Muller, H.; Eckert, H. *J. Chromatogr.* **1989**. 479, 452-458.

41. Gorham, J.; Coughlan, S. J.; Storey, R.; Jones, R. G. W. *J Chrom A* **1981**. 210, , 550–554.

42. Chen, W. P.; Li, P. H.; Chen, T. H. H. *Plant Cell and Environment* **2000**. 23, 609-618.

43. Paquet, L.; Rathinasabapathi, B.; Saini, H.; Zamir, L.; Gage, D. A.; Huang, Z. H.; Hanson, A. D. *Aust. J. Plant Physiol.* **1994**. 21, 37-48.

44. Hitz, W. D.; Hanson, A. D. *Phytochemistry* **1980**. 19, 2371-2374.

45. Nuccio, M. L.; Russell, B. L.; Nolte, K. D.; Rathinasabapathi, B.; Gage, D. A.; Hanson, A. D. *Plant J.* **1998**. 16, 487-496.

46. Hanson, A. D.; Nelsen, C. E. *Plant Physiol.* **1978**. 62, 305-312.

47. Manivannan, P.; Jaleel, C. A.; Sankar, B.; Kishorekumar, A.; Somasundaram, R.; Lakshmanan, G. M. A.; Panneerselvam, R. *Colloids Surf. B* **2007**. 59, 141-149.

48. Hanson, A. D.; Rathinasabapathi, B.; Chamberlin, B.; Gage, D. A. *Plant Physiol.* **1991**. 97, 1199-1205.

49. Shaw, J. R.; Dickinson, D. B. *Plant Physiol.* **1984**. 75, 207-211.

50. Gomez, L.; Rubio, E.; Augé, M. *J. Sci. Food Agric.* **2002**. 82, 360-369.

51. Bhandari, P.; Kumar, N.; Singh, B.; Kaul, V. K. *J Chromatogr. A* **2008**. 1194, 257-261.

52. Breedveld, M. W.; Zevenhuizen, L.; Zehnder, A. J. B. *J. General Microbiol.* **1990**. 136, 2511-2519.

53. Chen, G.; Zhang, L. Y.; Wu, X. L.; Ye, J. N. *Analytica Chimica Acta* **2005**. 530, 15-21.

54. Stoop, J. M. H.; Pharr, D. M. *J. Amer. Soc. Hort. Sci.* **1994**. 119, 237-242.

55. Sassi, S.; Aydi, S.; Hessini, K.; Gonzalez, E. M.; Arrese-Igor, C.; Abdelly, C. *Afr. J. Biotechnol.* **2010**. 9, 6061-6069.

56. Jouve, L.; Hoffmann, L.; Hausman, J. F. *Plant Biol.* **2004**. 6, 74-80.

57. Stoop, J.; Pharr, D. M. *Plant Physiol.* **1994**. 106, 503-511.

58. Martino, D. C.; Delfine, S.; Pizzuto, R.; Loreto, F.; Fuggi, A. *New Phytol.* **2003**. 158, 455-463.

59. Sairam, R. K.; Rao, K. V.; Srivastava, G. C. *Plant Sci.* **2002**. 163, 1037-1046.

60. Rudlier, D. L.; Bouillard, L. *Appl. Environ. Microbiol.* **1983**. 46, 152-159

61. Robert, H.; Le, M. C.; Blanco, C.; Jebbar, M. *Appl. Environ. Microbiol.* **2000**. 66, 509-517.

62. Naidu, B. P. *Aust. J. Plant Physiol.* **1998**. 25, 793-800.

63. Bessieres, M.-A.; Gibon, Y.; Lefeuvre, J. C.; Larher, F. *J. Agric. Food Chem.* **1999**. 47, 3718-3722.

64. Hubbard, N. L.; Pharr, D. M. *J. Amer. Soc. Hort. Svi.* **1990**. 115, 798-802.

65. Ludescher, R. D., *Physical and Chemical properties of Amino acids and Proteins. Food proteins propertise and chracterisation*, ed. S. Nakai and H. Modler. 1996, USA: W. Wiley-VCH.

66. Troll, W.; Lindsley, J. *J. biol. chem.* **1955**. 215, 655-60.

67. Long, D.; Wilkinson, K. L.; Poole, K.; Taylor, D. K.; Warren, T.; Astorga, A. M.; Jiranek, V. *Journal Agri. Food Chem.* **2012**. 60, 4259-4264.

68. Bates, L. S.; Waldren, R. P.; Teare, I. D. *Plant and Soil* **1973**. 39, 205-207.

69. Katona, Z. F.; Sass, P.; Molnar-Perl, I. *J. Chromatogr. A* **1999**. 847, 91-102.

70. Roessner, U.; Wagner, C.; Kopka, J.; Trethewey, R. N.; Willmitzer, L. *Plant J.* **2000**. 23, 131-142.

71. Cramer, G. R.; Ergul, A.; Grimplet, J.; Tillett, R. L.; Tattersall, E. A. R.; Bohlman, M. C.; Vincent, D.; Sonderegger, J.; Evans, J.; Osborne, C.; Quilici, D.; Schlauch, K. A.; Schooley, D. A.; Cushman, J. C. *Funct. Integr. Genomic.* **2007**. 7, 111-134.

72. Aranjuelo, I.; Molero, G.; Erice, G.; Christophe Avice, J.; Nogues, S. *J. Exp. Bot.* **2011**. 62, 111-123.

73. Chinard, F. P. *The Journal of biological chemistry* **1952**. 199, 91-5.

74. Magne, C.; Larher, F. *Anal. Biochem.* **1992**. 200, 115-118.

75. Karasek, F. W.; R.E., C., *Basic Gas Chromatography-Mass Spectrometry: Principles and Techniques*. Amsterdam, Netherland: Elsevier Science.

76. Berezkin, V. G.; Viktor, R. A.; Irina, B. N. a., *Gas Chromatography of Polymers*. 2011, Amsterdam, The Netherlands: Elsevier Scientific Publishing Company, Amsterdam.

77. Zaikin, V. G.; Halket, J. M. *Eur. J. Mass Spectrum.* **2003**. 9, 421-434.

78. Sobolevsky, T. G.; I.R., A.; Miller, B.; Oriedo, V.; Chernetsova, E. S.; Revelsky, I. A. *J. Sep. Sci.* **2003**. 26, 1474–1478.

79. Kühnel, E.; Laffan Dr, D. D. P.; Lloyd-Jones, G. C.; Martínez del Campo, T.; Shepperson, I. R.; Slaughter, J. L. *Angew. Chem. Int. Edi.* **2007**. 46, 7075–7078.

80. Shulaev, V.; Cortes, D.; Miller, G.; Mittler, R. *Physiol. Plant.* **2008**. 132, 199-208.

81. Horvath, C.; Melander, W.; Molnar, I.; Molnar, P. *Anal. Chem.* **1977**. 49, 2295-2305.

82. Neves, H. J. C. d. *Chromatography* **1997**. 20, 115-118.

83. Shibue, M.; Mant, C. T.; Hodges, R. S. *J. Chromatogr. A* **2005**. 1080, 58-67.

84. Chaimbault P, P. K., Elfakir C, and M., D.;. *J Chrom A* **1999**. 855, 191-202.

85. Bhaskaran, S.; Smith, R. H.; Newton, R. J. *Plant Physiol.* **1985**. 79, 266-269.

86. Fougere, F.; Lerudulier, D.; Streeter, J. G. *Plant Physiol.* **1991**. 96, 1228-1236.

87. Can˜ama´s, T. P.; Vin˜as, I.; Usall, J.; Magan, N.; Morello´, J. R.; Teixido, N. *Lett. Appl. Microbiol.* **2007**. 45, 6-12.

88. Boyce, M. C. *J. Chromatogr. A* **1999**. 847, 369-375.

89. Boyce, M. *J. Chem. Edu.* **1999**. 76, 815-819.

90. Otsuka, K.; Terabe, S. *Bull. Chem. Soc. Jpn* **1998**. 71, 2465—2481

91. Terabe, S.; Miyashita, Y.; Shibata, O.; Barnhart, E. R.; Alexander, L. R.; Patterson, D. G.; Karger, B. L.; Hosoya, K.; Tanaka, N. *J. Chromatogr.* **1990**. 516, 23-31.

92. Sato, S.; Soga, T.; Nishioka, T.; Tomita, M. *Plant J.* **2004**. 40, 151-163.

93. Arlt, K.; Brandt, S.; Kehr, J. *J. Chromatogr. A* **2001**. 926, 319-325.

94. Smith, J. T. *Electrophoresis* **1997**. 18, 2377-2392.

95. Kawai, M.; Iwamuro, Y.; Iio-Ishimaru, R.; Chinaka, S.; Takayama, N.; Hayakawa, K. *Anal. Sci.* **2011**. 27, 857-860.

96. Boyce, M. C.; Breadmore, M.; Macka, M.; Doble, P.; Haddad, P. R. *Electrophoresis* **2000**. 21, 3073-3080.

97. Obata, T.; Fernie, A. R. *Cell. Mol. Life Sci.* **2012**. 69, 3225-3243.

98. Urano, K.; Maruyama, K.; Ogata, Y.; Morishita, Y.; Takeda, M.; Sakurai, N.; Suzuki, H.; Saito, K.; Shibata, D.; Kobayashi, M.; Yamaguchi-Shinozaki, K.; Shinozaki, K. *Plant J.* **2009**. 57, 1065-1078.

99. Du Shin, H.; Suh, J. H.; Kim, J.; Lee, H.; Eom, H. Y.; Kim, U.; Yang, D.-H.; Han, S. B.; Youm, J.-R. *Bull. Kor. Chem. Soc.* **2012**. 33, 553-558.

100. Farwick, M.; Siewe, R. M.; Kramer, R. *J. Bacteriol.* **1995**. 177, 4690-4695.

101. Stumpf, D. K. *Plant Physiol.* **1984**. 75, 273-274.

102. Grieve, C. M.; Grattan, S. R. *Plant and Soil* **1983**. 70, 303-307.

103. Patton, A. J.; Cunningham, S. M.; Volenec, J. J.; Peicher, Z. J. *Crop Sci.* **2007**. 47, 2170-2181.

104. Focht, R. L.; Schmidt, F. H. *J. Agri. Food Chem.* **1956**. 4.

105. Carruthers, A.; Oldfield, J. F. T.; Teague, H. J. *Analyst* **1960**. 85, 272-275.

106. Rhodes, D.; Hanson, A. D. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **1993**. 44, 357-384.

107. Ladyman, J. A. R.; Hitz, W. D.; Hanson, A. D. *Planta* **1980**. 150, 191-196.

108. Hanson, A. D.; Wyse, R. *Plant Physiol.* **1982**. 70, 1191-1198.

109. Sobeih, K. L.; Baron, M.; Gonzalez-Rodriguez, J. *J. Chromatogr. A* **2008**. 1186, 51-66.

110. Chendrimada, T. P.; Neto, M. G.; Pesti, G. M.; Davis, A. J.; Bakalli, R. I. *J Sci Food Agric* **2002**. 82, 1556-1563.

111. Rienth, M.; Romieu, C.; Gregan, R.; Walsh, C.; Torregrosa, L.; Kelly, M. T. *J. Agri. Food Chem.* **2014**. 62, 3384-3389.

112. Mar, M.-H. H.; Ridky, T. W.; Garner, S. C.; Zeisel, S. H. *Nutr. Biochem.* **1995**. 6, 392-398.

113. Zwart, F. J.; Slow, S.; Payne, R. J.; Lever, M.; George, P. M.; Gerrard, J. A.; Chambers, S. T. *Food Chem.* **2003**. 83, 197-204.

114. MacKinnon, S. L.; Hiltz, D.; Ugarte, R.; Craft, C. A. *J. Appl. Phycol.* **2010**. 22, 489-494.

115. Wood, K. V.; Bonham, C. C.; Miles, D.; Rothwell, A. P.; Peel, G.; Wood, B. C.; Rhodes, D. *Phytochemistry* **2002**. 59, 759-765.

116. Zhang, J.; Nishimura, N.; Okubo, A.; Yamazaki, S. *Phytochem. Anal.* **2002**. 13, 189-194.

117. Hayashi, H.; Alia; Mustardy, L.; Deshnium, P.; Ida, M.; Murata, N. *Plant J.* **1997**. 12, 133-142.

118. Graham P. Jones; Bodapati P. Naidu; Starr, R. K.; Paleg, L. G. *Aust. J. Plant Physiol* **1986**. 13, 649-658

119. Shirasawa, K.; Takabe, T.; Takabe, T.; Kishitani, S. *Ann. Bot.* **2006**. 98, 565-571.

120. Arakawa, K.; Katayama, M.; Takabe, T. *Plant Cell Physiol.* **1990**. 31, 797-803.

121. Benfenati, E.; Debellis, G.; Chen, S.; Bettazzoli, L.; Fanelli, R.; Tacconi, M. T.; Kirschner, G.; Pege, G. *J. Lipid Res.* **1989**. 30, 1983-1986.

122. Ahn, S.; Hong, J. Y.; Hong, M. K.; Jang, Y. P.; Oh, M. S.; Jung, J. H.; Hong, J. *Rapid Comm. Mass Spectrom.* **2009**. 23, 3158-3166.

123. Paroni, R.; Fermo, I.; Molteni, L.; Folini, L.; Pastore, M. R.; Mosca, A.; Bosi, E. *J. Chromatogr. B-Anal. Technol. Biomed. Life Sci.* **2006**. 834, 183-187.

124. Rovio, S.; Yli-Kauhaluoma, J.; Siren, H. *Electrophoresis* **2007**. 28, 3129-3135.

125. Yamamoto, A.; Ohmi, H.; Matsunaga, A.; Ando, K.; Hayakawa, K.; Nishimura, M. *J. Chromatogr. A* **1998**. 804, 305-309.

126. Graefe, H.; Gutschow, B.; Gehring, H.; Dibbelt, L. *Clin. Chem. Lab. Med.* **2003**. 41, 1049-1055.

127. Eades, D. M.; Williamson, J. R.; Sherman, W. R. *J. Chromatogr-Biomed. Appl.* **1989**. 490, 1-8.

128. Miki, K.; Butler, R.; Moore, D.; Davidson, G. *Clin. Chem.* **1996**. 42, 71-75.

129. Marsilio, R.; D'Antiga, L.; Zancan, L.; Dussini, N.; Zacchello, F. *Clin. Chem.* **1998**. 44, 1685-1691.

130. Soga, T.; Heiger, D. N. *Anal. Biochem.* **1998**. 261, 73-78.

131. Makkee, M.; Kieboom, A. P. G.; Vanbekkum, H. *J. Royal Neth. Chem. Soc.* **1985**. 104, 230-235.

132. Halsall, T. G.; Hirst, E. L.; Jones, J. K. N. *J. Chem. Soc.* **1947**. 172, 1427-32.

133. Lewis, D. H.; Smith, D. C. *New Phytol.* **1967**. 66, 185-&.

134. Cerbulis, J. *Anal. Chem.* **1955**. 27, 1400-1401.

135. Shen, B.; Hohmann, S.; Jensen, R. G.; Bohnert, H. J. *Plant Physiol.* **1999**. 121, 45-52.

136. Tarczynski, M. C.; Jensen, R. G.; Bohnert, H. J. *Proc. Nat. Acad. Sci. USA* **1992**. 89, 2600-2604.

137. Guignard, C.; Jouve, L.; Bogeat-Triboulot, M. B.; Dreyer, E.; Hausman, J. F.; Hoffmann, L. *J. Chromatogr. A* **2005**. 1085, 137-142.

138. Zhu, Z.; Pei, Z. M.; Zheng, H. L. *Russ. J. Plant Physiol.* **2011**. 58, 226-232.

139. HUQ, S. M. I.; LARHER, F. *New Phytol.* **1983**. 93.

140. Rhodes, D.; Rich, P. J. *Plant Physiol.* **1988**. 88, 102-108.

141. Graham, J. E.; Wilkinson, B. J. *J. Bacteriol.* **1992**. 174, 2711-2716.

142. Bussis, D.; Heineke, D. *J. Exp. Bot.* **1998**. 49, 1361-1370.

143. Briens, M.; Larher, F. *Plant Cell Environ.* **1982**. 5, 287-292.

144. Carrera, C.; Ruiz-Rodriguez, A.; Palma, M.; Barroso, C. G. *Ultrasonics Sonochemistry* **2015**. 22, 499-505.

Chapter 3 Evaluation of Potential Cationic Probes for the Detection of Proline and Betaine

This chapter has been published as a research article in Electrophoresis, 2014, vol 35, pp 3379–3386. All efforts were made to keep the original features of this article except minor changes e.g. layout, numbering, font size and style were carried in order to maintain a consistent formatting style of this thesis.

3.1. Abstract

Osmoregulants are the substances that help plants to tolerate environmental extremes such as salinity and drought. Proline and betaine are two of the most commonly studied osmoregulants. An indirect UV capillary electrophoresis method has been developed for simultaneous determination of these osmoregulants. A variety of reported probes and compounds were examined as potential probes for indirect detection of proline and betaine. Mobility and UV absorption properties highlighted sulfanilamide as a potential probe for indirect analysis of proline and betaine. Using 5 mM sulfanilamide at pH 2.2 with UV detection at 254 nm, proline and betaine were separated in less than 15 min. The limits of detection for proline and betaine were 11.6 µM and 28.3 µM, respectively. The developed method was successfully applied to quantification of these two osmoregulants in spinach and beetroot samples.

3.2. Introduction

Environmental stresses such as salinity, drought, temperature extremes and water logging effect the growth, productivity and quality of plants [1]. To tolerate these stresses plants produce low molecular weight metabolites such as amino acids and quaternary ammonium compounds which are generally known as osmoregulants [2]. Proline is the most commonly studied amino acid osmoregulant [2] and glycine betaine (betaine) is the most commonly explored quaternary ammonium osmoregulant [3]. These osmoregulants protect plants in stressed environments by performing several functions including suppression of free radicals, regulation of osmotic balance and storage of nitrogen and carbohydrates [4]. This basic understanding of the role of osmoregulants has resulted in an increased interest in the application to plants in order to increase yield and quality [5]. For this purpose, osmoregulants are applied externally [6] or plants rich in osmoregulants are selected for breeding by traditional means or by genetic engineering [7]. Therefore, the concentration of these osmoregulants is often studied to estimate a plant's ability to survive in stressed conditions or to determine the success of the new breeds.

There are a variety of methods reported for analysis of each osmoregulant. Proline can be determined by colorimetry (after derivatization with ninhydrin) [8], reversed phase-high performance liquid chromatography (RP- HPLC) and capillary electrophoresis (CE). Furthermore, HPLC methods described vary in terms of sample preparation (e.g. derivatising agents used) and detection mode [3, 9-12] Similarly, CE analysis of proline has been reported with a variety of detection modes with UV and LIF being the most common ones. The commonly reported labelling agents for UV detection include 1-(9-fluorenyl) ethyl chloroformate, fluorescamine, FMOC, OPA, and PITC and for LIF are fluorescein isothiocyanate, dansyl chloride, and OPA [13].

Similarly, betaines have been analysed both by HPLC and CE, however, the reported HPLC methods vary in the mechanism of separation and the mode of detection [14-19]. CE analysis of *p*-bromophenacyl esters of betaines with UV detection using capillary zone

electrophoresis (CZE) [20] and micellar electrokinetic chromatography (MEKC) [21] separation have been reported.

When a study involves both osmoregulants i.e. proline and betaine each analyte is usually determined by an individual method [22, 23]. However, there are some HPLC methods reported for simultaneous determination of proline and betaine. For example, Naidu reported HPLC-UV analysis of proline and betaine at low wavelength [24]. Similarly, Kalsoom *et al*., analysed proline and betaine simultaneously using HPLC in combination with evaporative light scattering detection [4]. Surprisingly, application of CE to simultaneous analysis of proline and betaine is limited. There is only one method reported by Nishimura *et al*. to analyse proline and betaine simultaneously at low pH using direct UV (195nm) detection [25]. However, poor sensitivity (100 µM) due to the poor absorptivity of proline and betaine and long run time (30 min) are the major drawbacks of this method. In this work, the poor sensitivity is addressed by the development of an indirect UV detection method.

In indirect detection (ID), background electrolyte (BGE) contains a strongly absorbing electrolyte co-ion (also known as probe) or counter-ion [26]. The displacement of a UV absorbing co-ion or counter ion by a UV transparent analyte results in a significant decrease in absorbance and a negative peak is detected [27]. A fundamental requirement for the separation by CZE is that the analytes must be charged. At high pH proline is negatively charged but betaine remains neutral and cannot be detected. It is only at low pH that both betaine and proline are positively charged and ID using a cationic probe can be employed. Imidazole, 4-aminopyridine [28], and creatinine [29] are examples of commonly used cationic probes for the separation of alkali and alkaline earth metals, but their potential for the detection of proline and betaine has yet not been explored. The present work investigates the potential of some of the reported probes and identifies new probes for simultaneous determination of proline and betaine using indirect UV detection.

3.3. Materials and Methods

3.3.1. Chemicals

8-hydroxyquinoline was purchased from Merck Pty LTD, Melbourne, Australia and 1-naphthylamine from Merck KGaA Darmstadt, Germany. All other chemicals including; 3-amino-1,2,4-triazole, imidazole, creatinine, 4-aminopyridine, 2-amino-6-picoline, 4-aminomethyl benzoic acid, *p*-toluidine, 4-amino benzoic acid, 2-phenyl-2-imidazole, 2-ethyl-4-methylimidazole, 1-butylimidazole, 2-amino-4-picoline, 2-isopropylimidazole, sulfanilamide, proline, betaine and cysteine were obtained from Sigma Aldrich Sydney, Australia.

3.3.2. Instrumentation

A Hewlett Packard 3D CE (Waldbron, Germany) instrument equipped with an on column diode array UV absorbing detector and Agilent offline data analysis was used throughout the study.

The separation voltage was set at +25 kV and all separations were achieved with the cassette temperature set at 30 ^{o}C. Untreated fused silica capillary (Polymicro, Phoenix, AZ, USA) with an internal diameter of 50 µm and a total length of 50 cm (41.5 cm to the detector), was used for separation. The sample was injected by pressure at 50 mbar for 5s. These conditions were kept constant throughout the analysis unless otherwise stated.

A Shimadzu (Perth, Australia) UV mini 1240 spectrophotometer was used to obtain spectrophotometric data for all the selected probes. The spectrophotometer consisted of 1 cm quartz cell for both sample and reference.

3.3.3. Standards and Sample Solutions

For probe mobility measurements, a 100 mg/ L standard of 8-hydroxyquinoline, 2-phenyl-2-imidazole, *p*-toulidine, and 1-naphthyamine was prepared in 5% ethanol. A 100 mg/ L solution of all other probes including; 3-amino-1,2,4-triazole, imidazole, 2-ethyl-4-

methylimidazole, 2-isopropylimidazole, 1-butylimidazole, 2-phenyl-2-imidazole, creatinine, 4-aminopyridine, 2-amino-6-picoline, 2-amino- 4-picoline, 4-aminobenzoic acid, 4-aminomethyl benzoic acid, and sulfanilamide was prepared in milli Q water.

For comparison of probe function, BGE containing 2.5 mM probe was prepared and the pH was adjusted to 2.5 with 1 M H_2SO_4.

For optimisation studies BGE containing 1, 2, 3, 4, 5 mM sulfanilamide and adjusted to pH 2.5 with 1 M H_2SO_4 were prepared. Also BGE containing 2.5 mM sulfanilamide at pH 2.2, 2.4, 2.6 and 2.8 were prepared.

For method validation, aqueous standards of proline and betaine in the range of 5-100 mg/L were prepared from a stock solution of 500 mg/L.

For quantitative measurement, aqueous standards of proline and betaine in the range 5-100 mg/L were prepared. The line of best fit for concentration versus peak area was used to determine the concentration of the analyte in plant extracts.

3.3.4. Procedures

The capillary was conditioned daily with 0.1 N NaOH, Milli Q water and BGE for 10 min each. The capillary was purged with 0.1 N NaOH and Milli Q water for two min at the end of the day and stored overnight. The capillary was flushed with BGE for 2 min prior to each run.

The mobility measurements were made using 20 mM NaH_2PO_4 buffer at pH 2.5. A 100 mg/L solution of each selected probe was injected with 0.3% mesityl oxide as an EOF marker.

For separation, 2.5 mM solution of each probe at pH 2.5, adjusted with 1 M H_2SO_4, was used. Detection was carried at the maximum absorption wavelength of each probe (given in Table 2). Peak area of the analytes was used to calculate linearity and reproducibility.

3.3.5. Extraction of Plant Material

Fresh beetroot (*Beta vulgaris*) and spinach (*Spinacia oleracea)* samples were purchased from the supermarket. For extraction, approximately 0.5 g of material was mixed for 1 min with 10 mL of 80% ethanol in a blender. This mixture was then shaken for 20 min and filtered. The filtrate was collected and stored at 6 ^{o}C for further analysis.

3.4. Results and Discussion

Three probes identified from the literature, imidazole, creatinine and 4-aminopyridine were tested as potential probes for the ID of proline and betaine. Separate BGE containing each of the probes (2.5 mM probe adjusted to pH 2.5 with 1 M H_2SO_4) resolved proline and betaine in less than 20 mins (Fig 1). In terms of sensitivity, the imidazole probe performed the poorest, with LODs of 180.3 µM and 208.3 µM for proline and betaine respectively whilst 4-aminopyridine, as the probe, performed the best, with LODs of 89.6 µM and 128.2 µM for proline and betaine respectively (Table 1). It can be clearly seen in Fig 1 that broad and tailed peaks were obtained using all the reported probes which resulted in poor efficiency data for both analytes i.e. proline and betaine (Table 1). The peak tailing is probably because the probes are faster than the analytes as Doble *et al*., 2000 described that a slower probe results in peak fronting and a faster probe causes peak tailing, [30]. The mobility mismatch between analyte and probe increases electrodispersion resulting in poor peak shapes and efficiency [31]. The electromigration dispersion can be minimised by matching the mobility of the analyte and probe, and keeping the concentration of probe as high and analyte as low as possible [32]. Therefore, to improve the peak shapes and efficiency it is really important to match the mobility of probe with the analytes.

Furthermore, it was confirmed experimentally that the mobility of the probes imidazole, creatinine and 4-aminopyridine (μ_{eff} 4.99x10^{-4}cm^2/V.s, 3.69 x10^{-4}cm^2/V.s and 4.40 x10^{-4}cm^2/V.s, respectively) were about 5 times faster than the analytes proline and betaine (8.17x10^{-5}cm^2/V.s and 9.4x10^{-5}cm^2/V.s respectively). Therefore a search for a lower mobility probe was undertaken.

The ideal probe should have a mobility value similar to proline and betaine to improve peak shape and a high molar absorptivity to improve sensitivity. In the search for probes with mobility lower than imidazole, creatinine and 4-aminopyridine, structural derivatives with a higher molecular weight, and hence higher size to charge ratio, were selected including 2-isopropylimidazole, 1-butylimidazole, 2-ethyl-4-methylimidazole, 2-phenyl-2-imidazole, and 4-aminomethyl benzoic acid. The amino group in reported probes (i.e. imidazole, creatinine and 4-aminopyridine) is basic in nature and is responsible for the cationic properties of the probes. Therefore, the priority was given to primary and secondary amines when selecting potential probes. As aromatic compounds generally have higher UV absorbance than non-aromatic compounds, aromatic compounds were also favoured when identifying potential probes.

Table 1. Determination of LOD and theoretical plates for commonly reported and selected potential probes

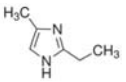
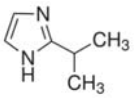
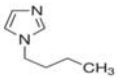
	LOD (μM)		Efficiency (Plates/ meter)	
Probe	Proline	Betaine	Proline	Betaine
Imidazole	180.3± 21.0	208.3± 5.2	53,000± 5,700	55,000± 1,900
Creatinine	113.6± 34.2	149.3± 23.9	35,000± 2,600	31,000± 1,100
4-aminopyridine	89.6± 10.5	128.2± 20.5	46,000± 2,200	39,000± 1,100
4-aminobenzoic acid	87.5± 2.0	115.1± 1.5	53,000± 1,700	54,000± 1,600
Sulfanilamide	19.8± 2.5	45.7± 4.5	98,000± 5,500	54,000± 2,700

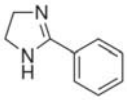
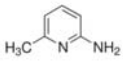
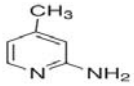
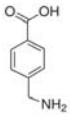
(Mean ± standard error, n=3)

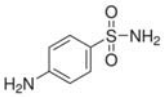
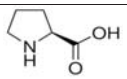
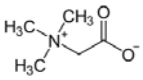
The probes selected based on these criteria are listed along with their molecular weights and chemical structures in Table 2. The molar absorptivity and mobility for each of these probes were determined experimentally and the data are presented in Fig 2. Several of the probes, despite having a molar mass in excess of the reported probes, recorded very small

shifts in mobility. Furthermore, their molar absorptivities were very similar all within the range 4000-10,000 $L.mol^{-1}cm^{-1}$ (Table 2).

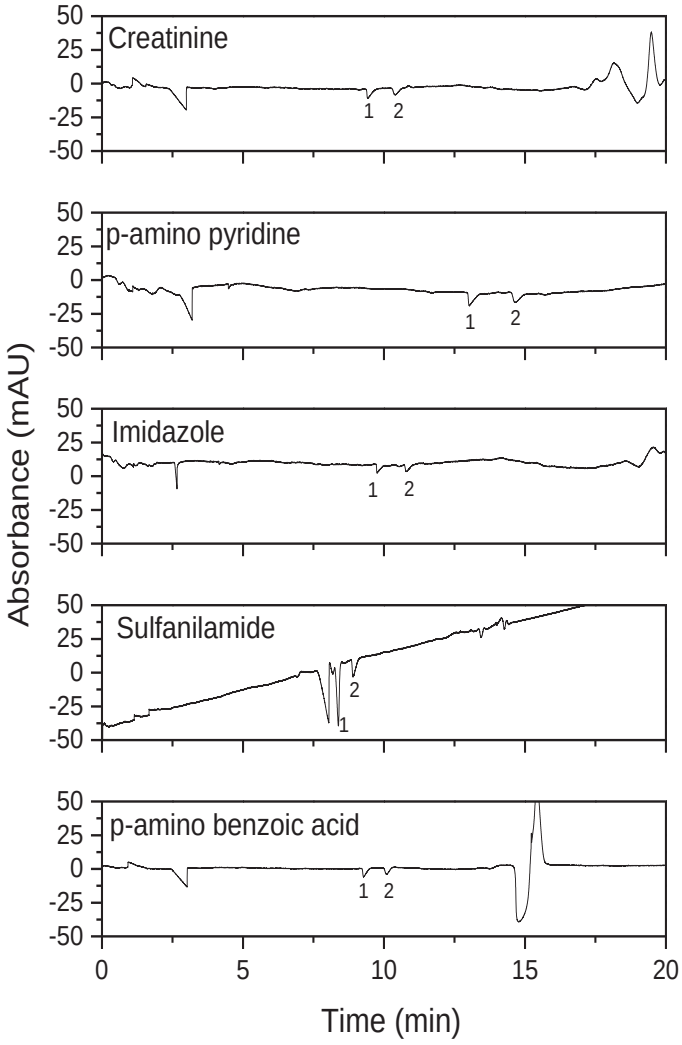
Table 2. Structure and molecular weight, pK_a, spectrophotometric, effective mobility and molar absorbtivity data for the reported and selected potential probes

Probe	Structure	Molecular weight	pK_a	λ_{max} (nm)	μ_{eff} ($cm^2/V.s$)	ε ($L.mol^{-1}cm^{-1}$)
8-hydroxyquinoline		145.16	9.89, 5.13 [37]	214	3.25×10^{-4}	6241
3-amino-1,2,4-triazole		84.08	4.14 [38]	214	4.31×10^{-4}	4035
Imidazole		68.08	6.95 [39]	214	4.99×10^{-4}	4043
2-ethyl-4-methylimidazole		110.16	8.68 [39]	214	3.56×10^{-4}	9319
2-isopropylimidazole		110.16	7.97 [39]	214	3.55×10^{-4}	8129
1-butylimidazole		124.18	7.21 [39]	214	3.60×10^{-4}	4222

2-phenyl-2-imidazole		146.19	-	214	3.11×10^{-4} 2.79×10^{-4}	5906
Creatinine		113.12	2.63, 14.3 [40]	214, 230	3.69×10^{-4}	6040
4-aminopyridine		94.11	9.40 [41]	214	4.4×10^{-4}	4404
2-amino-6-picoline		108.12	-	214	3.78×10^{-4}	3827
2-amino-4-picoline		108.12	7.41 [42]	214	4.02×10^{-4}	4951
4-aminobenzoic acid		137.14	2.50, 4.87 [42]	214, 230	3.37×10^{-4}	17115
4-aminomethyl benzoic acid		151.16	-	214, 254	2.86×10^{-4}	7285
p-toluidine		107.17	5.10 [43]	214, 230	3.41×10^{-4}	4736

1-naphthylamine		143.19	3.92 [44]	214, 230	3.15×10^{-4}	32000
Sulfanilaminde		172.2	10.99, 2.27 [45]	254, 280	4.44×10^{-5}	11,640
Proline		115.13	10.6, 1.99 [46]		9.4×10^{-5}	-
Betaine		117.14	2.17 [47]		8.17×10^{-5}	-

1



2

3 **Fig 1.** Electropherograms of separation of proline and betaine using previously reported and
4 selected detection probes. Peak identification: 1. proline and 2. betaine. Experimental
5 conditions: sample was injected for 5 s at 50 mbar into a 50 cm long, 50 µm i.d. capillary.
6 Separation at +25 kV, UV detection at 214 nm for creatinine, 4-aminopyridine, imidazole, 4-

aminobenzoic acid and 254 nm for sulfanilamide using a probe concentration of 2.5 mM adjusted to pH 2.5 using 1 M H_2SO_4 as BGE.

1-naphthylamine with its high molar absorptivity and mobility more consistent with the analytes appears to be the ideal probe; however, it was discarded because of its toxic (carcinogenic) properties. The slightly slower 4-aminobenzoic acid, compared to imidazole, creatinine and 4-aminopyridine, was tested as a potential probe. Separation efficiencies were comparable with those obtained for imidazole, creatinine and 4-aminopyridine (Fig 1, Table 1). Despite its relatively high molar absorptivity, it did not result in a significant improvement in detection limit (Table 1). The significantly lower mobility of the sulfanilamide probe did result in an improvement in peak shape and height. There was slight peak fronting observed as the probe had a mobility lower than the analytes (Fig 1). Sulfanilamide provided improvements in LOD, 10 fold for proline and 5 fold for betaine (Table 1) compared to when imidazole was used as the probe. Also the separation efficiency for proline improved almost two fold when using sulfanilamide instead of imidazole, but the efficiency for betaine only improved slightly (Table 1). Based on these results, sulfanilamide was selected as the detection probe for further optimisation.

3.4.1. Optimisation of Sulfanilamide BGE

Buffer parameters such as pH and probe concentration were optimised to improve peak shapes and LOD. For pH optimisation, 2.5 mM solution of sulfanilimide (BGE) was used and the pH was varied between 2.2- 2.8. At a pH less than 2.2, the baseline became unstable and the capillary broke after several runs, whilst proline and betaine co-migrated above pH 2.8. Optimal detection limits and highest separation efficiency were obtained at pH 2.2 (Table 3).

The concentration of sulfanilamide was then varied between 1 and 5 mM keeping the pH constant (pH= 2.5); above 5 mM the baseline became unstable possibly due to adsorption of sulfanilamide onto the capillary wall. For both analytes, the optimal efficiency was obtained

when using 5 mM sulfanilamide. Because of the lower sensitivity of the method for betaine, the optimal concentration based on sensitivity was selected for betaine at 5 mM sulfanilamide, resulting in a LOD for proline of 25.5 µM, and 37.7 µM for betaine (Table 3).

Table 3. Optimisation of pH and sulfanilamide concentration for proline and betaine analysis

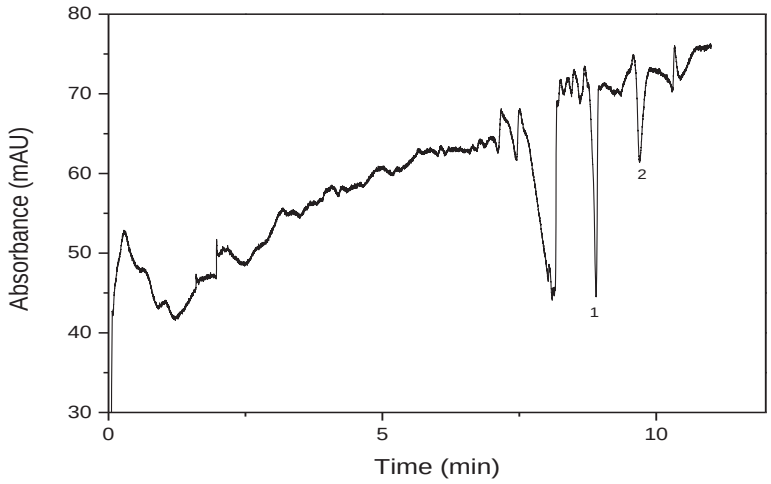
Parameter	LOD (µM)		Efficiency (Plates/ meter)	
	Proline	Betaine	Proline	Betaine
pH 2.2	15.3± 0.7	36.2± 1.7	125,500± 5,000	69,000± 1,000
pH 2.4	19.3± 2.7	43.3± 4.9	98,000± 5,000	54,000± 2,700
pH 2.6	33.4± 9.5	63.3± 18.4	68,500± 2,300	44,700± 3,600
pH 2.8	20.1± 4.92	72.2± 8.9	64,400± 3,500	46,500± 1,700
1 mM	17.9± 0.8	61.3± 3.2	44,000± 1,800	51,000± 1,800
2 mM	21.0± 2.0	49.9± 4.0	82,000± 3,400	42,000± 4,000
3 mM	19.8± 2.6	41.7± 3.7	110,000± 4,500	49,000± 2,000
4 mM	26.6± 1.5	48.3± 5.9	97,000 ± 5,500	44,000± 1,700
5 mM	23.5± 3.1	37.7± 3.8	123,000± 1,500	62,000± 2,700
5 mM, pH= 2.2	11.5± 0.6	28.3± 3.18	112,000± 5,000	70,000± 5,000

(Mean ± standard error, n=3)

Using the optimum conditions of 5 mM sulfanilamide (Fig 2), pH 2.2, the LOD for proline and betaine was improved to 11.6± 0.6 µM and 28.3± 3.2 µM respectively (Table 3). Better peak efficiencies (plates/ meter) for both proline (112,000± 5,000) and betaine (70,000± 3,000) were obtained in comparison to imidazole (Table 3). The optimised method is almost 10 fold more sensitive for proline and 4 fold more sensitive for betaine compared to the direct detection method reported by Nishimura [25]. Though the developed method is less sensitive compared

45 to commonly used esterification method for betaine [20], it has the advantage of avoiding a
46 complicated and time consuming (almost 75 mins) derivatization process.

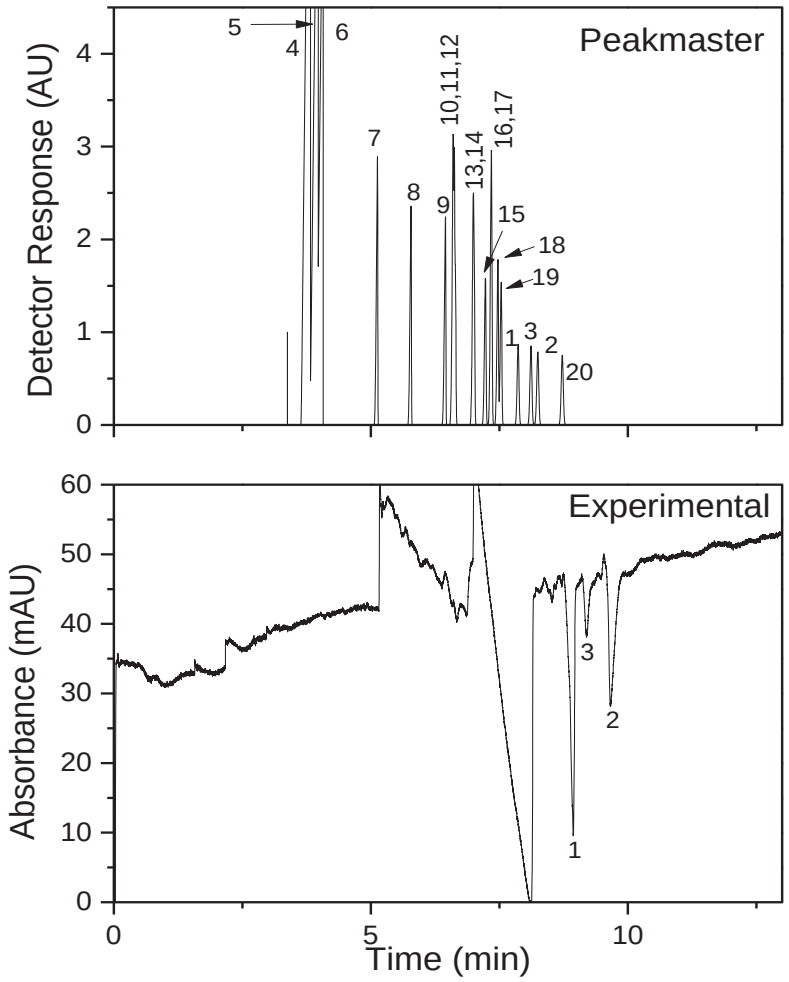
47 When applying any method to real samples it is important to anticipate likely interfering
48 compounds. In the determination of osmoregulants, amino acids are a likely source of
49 interference. Peak Master (http://web.natur.cuni.cz/~gas/) was used to estimate the co
50 migration of amino acids with proline and betaine. Eighteen amino acids available in the Peak
51 Master database were selected to predict their separation and migration under the optimised
52 conditions. The simulated electropherogram (Fig 3) indicates that none of the amino acids co-
53 migrate with proline or betaine, only cysteine has a migration time close to betaine. The
54 robustness of this simulation was tested by spiking a solution containing betaine and proline
55 with cysteine. As predicted by Peak Master, cysteine and betaine were baseline resolved at low
56 concentration (50 mg/ L), with identification and quantification still possible at higher
57 concentrations (Fig 3).



58

59 **Fig 2.** Electropherograms of separation of proline and betaine using the optimised method.
60 BGE, 5 mM sulfanilamide at pH 2.2 adjusted by 1 M H_2SO_4, UV detection at 254 nm.

61



62

63 **Fig 3.** Simulated electropherogram of the separation of proline and betaine in presence of 19
64 amino acids and experimentally obtained electropherogram for proline (100 mg/ L) and betaine
65 (100 mg/ L) in the presence of cysteine (50 mg/ L) using 5mM sulfanilamide at pH 2.2 as BGE.
66 Peak identification; 1. proline, 2. betaine, 3. cysteine, 4. lysine, 5. histidine, 6. arginine, 7.
67 glycine, 8. alanine, 9. isoleucine 10. valine, 11 leucine, 12. serine, 13. asparagine, 14.

thereonine, 15. methionine, 16. glutamine, 17.glutamic acid, 18.tyrosine, 19. phenylalanine, 20. aspartic acid.

3.4.2. Linearity & Reproducibility

The calibration curve indicated a good linear relationship between the concentration and peak area with r^2= 0.998 and r^2= 0.990 for proline and betaine, respectively, over the range of 5-100 mg/ L.

Reproducibility of the developed method was checked by obtaining intra- and inter-day precision. For intra-day reproducibility, a 50 mg/ L standard of proline and betaine was injected three times and variation in the migration time and peak area was calculated. The RSD in peak area were acceptable at 10.72 % and 5.28 % for proline and betaine respectively. The RSD for migration time were very good at 0.21 % for proline and 0.25 % for betaine.

Inter-day reproducibility was carried by injecting 100 mg/ L standard of proline and betaine over three consecutive days. Migration time repeatability for proline and betaine was good at 2.39 % and 2.54 % respectively. Peak area repeatability was good at 5.18 % for proline and 9.51 % for betaine.

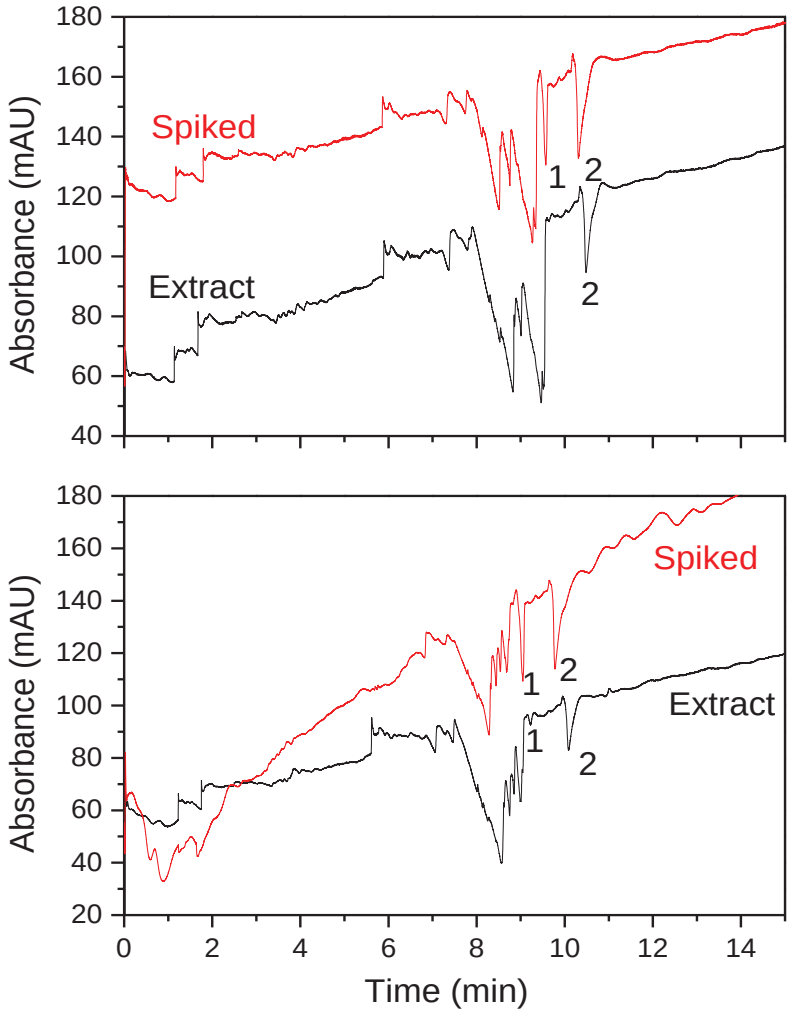
3.4.3. Application to Plant Extracts

Spinach has some proline [33] and high levels of betaine naturally present [34]. Similarly, beetroot has been reported to have naturally high levels of betaine, and has the capacity to accumulate betaine under stress [35]. Spinach and beetroot samples were selected for application of the developed separation method. The ethanolic extracts (2 mL) of plants were dried in air and the residue was redissolved in MilliQ water (1 mL) and filtered. The filtrate was then injected into the capillary for analysis. Spinach extract showed a small peak at around 9 min which was suspected to be proline (Fig 4). The presence of proline was confirmed with spiking. The concentration of proline in spinach was recorded to be 0.9± 0.0 mg/100 g, which is

92 in close agreement with the reported concentration of proline (0.5 mg/100 g) in spinach. Based
93 on migration time, a peak at around 10 mins in both spinach and beetroot samples (Fig 4) was
94 confirmed to be betaine by spiking. The level of betaine was determined at 144.7± 2.8 mg/100
95 g (n= 3) in beetroot and at 104.4 ± 2.7 mg/100 g (n= 3) in spinach. The value obtained for the
96 beetroot sample agrees well with the range reported by Zeisel *et al.* (114–297 mg/100 g) [36].
97 The concentration of betaine determined in spinach is between the values reported by Zeisel *et*
98 *al*. (599 mg/100 g) [36] and by Zhang *et al*. (35 mg/100 g) [20].

99 For recovery test, the plant extract was spiked with 50 mg/L of betaine standard. Recovery of
100 betaine in both spinach and beetroot extracts was found to be 90%.

101



102

103 **Fig 4.** Application of the developed CZE-ID method to spiked and non spiked extracts from a)
104 beetroot b) spinach. Peak identification; 1. proline 2. betaine. Experimental conditions as
105 reported in Fig 3.

3.5. Concluding Remarks

A new method for the indirect UV detection of proline and betaine was developed using a novel indirect absorption probe. Sulfanilamide with slow mobility and good molar absorptivity was selected as a suitable probe for ID of proline and betaine. This quick (10 min), robust and sensitive CZE-ID method is an attractive alternative to derivitistion. The developed method was successfully applied to the identification and quantification of betaine in spinach and beetroot extracts.

3.6. References

[1] Cushman, J. C. *Amer. Zool*. **2001**, 41, 758–769.

[2] Servillo, L.; Giovane, A.; Balestrieri, M. L.; Cautela, D.; Castaldo, D. *J. Agric. Food Chem*. **2011**, 59, 274–281.

[3] Martino, C. D.; Delfine, S.; Pizzuto, R.; Loreto, F.; Fuggi, A. *New Phytol*. **2003**, 158, 455–463.

[4] Kalsoom, U.; Boyce, M. C.; Bennett, I. J.; Veraplakorn, V. *Chromatographia* **2013**, 76, 1125–1130.

[5] Naidu, B. P. Aust. *J. Plant Physiol*. **1998**, 25, 793–800.

[6] Nagao, K.; Takeuchi, M.; Miyazawa, Y.; Sato, H.; Yokota, H. Y.; Kita, K. *Grassl. Sci.* **2005**, 51, 269–270.

[7] Jenks, M. A.; Hasegawa, P. M.; Jain, S. M. *Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops*, Springer, The Netherlands 2007.

[8] Koskeroglu, S.; Tuna, A. L. *Acta Physiol. Plant*. **2010**, 32, 541–549.

[9] Reverter, M.; Lundh, T.; Lindberg, J. E. *J. Chromatogr. B* **1997**, 696, 1–8.

[10] Bank, R. A.; Jansen, E. J.; Beekman, B.; te Koppele, J. M. *Anal. Biochem*. **1996**, 240, 167–176.

[11] Fabiani, A.; Versari, A.; Parpinello, G. P.; Castellari, M.; Galassi, S. *J. Chromatogr. Sci.* **2002**, 40, 14–18.

[12] Minocha, R.; Long, S. *J. Chromatogr. A*, **2004**, 1035, 63–73.

[13] Prata, C.; Bonnafous, P.; Fraysse, N.; Treilhou, M.; Poinsot, V.; Couderc, F. *Electrophoresis* **2001**, 22, 4129–4138.

[14] Martino, C. D.; Delfine, S.; Pizzuto, R.; Loreto, F.; Fuggi, A. *New Phytol*. **2003**, 158, 455–463.

[15] Bessieres, M. A.; Gibon, Y.; Lefeuvre, J. C.; Larher, F. J. *Agric. Food Chem*. **1999**, 47, 3718–3722.

[16] Chendrimada, T. P.; Neto, M. G.; Pesti, G. M.; Davis, A. J.; Bakalli, R. I. *J. Sci. Food Agric.* **2002**, 82, 1556–1563.

[17] Teixido, N.; Canamas, T. P.; Usall, J.; Torres, R.; Magan, N.; Vinas, I. *Lett. Appl. Microbiol*. **2005**, 41, 248–252.

[18] Mar, M. H. H.; Ridky, T. W.; Garner, S. C.; Zeisel, S. H. *J. Nutr. Biochem*. **1995**, 6, 392–398.

[19] Zwart, F. J.; Slow, S.; Payne, R. J.; Lever, M.; George, P. M.; Gerrard, J. A.; Chambers, S. T. *Food Chem*. **2003**, 83, 197–204.

[20] Zhang, J.; Nishimura, N.; Okubo, A.; Yamazaki, S. *Phytochem. Anal*. **2002**, 13, 189–194.

[21] Nishimura, N.; Zhang, J.; Abo, M.; Okubo, A.; Yamazaki, S. *Anal. Sci*. **2001**, 17, 103–106.

[22] Can˜ama´s, T. P.; Vin˜as, I.; Usall, J.; Magan, N.; Morello´, J. R.; Teixido, N. *Lett. Appl. Microbiol.* **2007**, 45, 6–12.

[23] Hassine, A. B.; Ghanem, M.; Bouzid, S.; Lutts, S. *J. Exp. Bot.* **2008**, 59, 1315–1326.

[24] Naidu, B. P. Aust. *J. Plant Physiol.* **1998**, 25, 793–800.

[25] Nishimura, N.; Zhang, J.; Abo, M.; Okuno, A.; Sunao, Y. *Anal. Sci*. **2001**, 17, 1929–1932.

[26] Collet, J.; Gareil, P. *J. Chrom. A* **1995**, 716, 115–122.

[27] Boyce, M. C.; Breadmore M.; Macka M.; Doble P.; Haddad, P. R. *Electrophoresis* **2000**, 21, 3071–3080.

[28] Shakulashvili, N.; Faller, T.; Engelhardt, H. *J. Chromatogr. A* **2000**, 895, 205–212.

[29] Jimidar, M.; Hamoir, T.; Degezelle, W.; Massart, D. L.; Soykenc, S.; Vandewinkel, P. *Anal. Chim. Acta* **1993**, 284, 217–225.

[30] Doble, P.; Macka, M.; Haddad, P. R. *Trends Anal. Chem*. **2000**, 19, 10–17.

[31] Beckers, J. L. *J. Chrom. A* **1999**, 844, 321–331.

[32] Johns, C.; Macka, M.; Haddad, P. R. *Electrophoresis* **2003**, 24, 2150–2167.

[33] Meychik, N. R.; Nikolaeva, Y. I.; Yermakov, I. P. *Amer. J. Plant Sci*. **2013**, 4, 427–435.

[34] Craig, S. A. *Am. J. Clin. Nutr*. **2004**, 80, 539–549.

[35] Neelwarne, B. Red *Beet Biotechnology: Food and Pharmaceutical Applications*, Springer, New **York** 2012.

[36] Zeisel, S. H.; Mar, M. H.; Howe, J. C.; Holden, J. M. *J. Nutr*. **2003**, 133, 1302–1307.

[37] Bardez, E.; Devol, I.; Larrey, B.; Valeur, B. *J. Phys. Chem. B* **1997**, 101, 7786–7793.

[38] Erçağ, A.; Şişmanoğlu, T.; Pura, S. *J. Serb. Chem*. Soc. **2005**, 70, 1057–1066.

[39] Lenarcik, B.; Ojczenasz, P. J. *Heterocyclic Chem*. **2002**, 39, 287–290.

[40] Ambrose, R. T.; Ketchum, D. F.; Smith, J. W. *Clin. Chem*. **1983**, 29, 256–259.

[41] Smith, D. T.; Shi, R.; Borgens, R. B.; McBride, J. M.; Jackson, K.; Byrne, S. R. *Eur. J. Med. Chem.* **2005**, 40, 908–917.

[42] Buckingham, J. *Dictionary of Organic Compounds*, Vol. 8, Chapman and Hall., London 1990.

[43] Lu, G. H.; Wang, C.; Bao, G. Z. *Environ. Toxicol. Chem*. **2003**, 22, 272–275.

[44] Elliot, J. J.; Mason, S. F. *J. Chem. Soc.* **1959**, 2352–2359.

[45] Dewick, P. *Essentials of Organic Chemistry*, Wiley: Chichester, England, 2006.

[46] Dawson, R. M.; Chaplin, R. M. *Data for Biochemical Research*, Clarendon Press, Oxford 1959.

[47] Singh, R.; Haque, I.; Ahmad, F. *J. Biol. Chem*. **2005**, 280, 11035–11042.

Chapter 4 Simultaneous Determination of Key Osmoregulants in Halophytes Using HPLC-ELSD

This chapter has been published as a research article in Chromatographia, 2013, Vol. 76, pp 1125-1130. All efforts were made to keep the original features of this article except minor changes e.g. layout, numbering, font size and style were carried in order to maintain a consistent formatting style of this thesis.

4.1. Abstract

Osmoregulants are the substances produced by plants that assist in tolerating environmental stresses. Three commonly analyzed osomoregulants include mannitol, betaine and proline. A simple, sensitive and rapid HPLC-ELSD method has been developed for the simultaneous analysis of these common osmoregulants in plant extracts. Osmoregulants were extracted using 80% ethanol and separated on an NH_2 column using 0.1% formic acid and acetonitrile as the mobile phase. Retention time repeatability was 0.85%, 1.50%, and 0.93% for mannitol, betaine and proline respectively. The limit of detection (µmole) was 1.43×10^{-4}, 7.81×10^{-5} and 1.08×10^{-4} for mannitol, betaine and proline respectively. The developed method was applied to three different plant extracts, *Stylosanthes guianensis*, *Atriplex cinerea* and Rhagodia baccata. A second method using a C_{18} column with 0.1% heptafluorobutyric acid and acetonitrile as the mobile phase proved to be a useful complementary method for verifying tentative peak identifications.

4.2. Introduction

Environmental stresses such as drought, salinity and temperature extremes adversely affect the growth and development of plants. To cope with these environmental factors, plants produce secondary metabolites including sugars, sugar alcohols, amino acids and quaternary ammonium salts which are collectively referred to as osmoprotectants or osmoregulants [1]. The three most commonly analyzed osmoregulants are proline [1], mannitol [2], and glycine betaine (betaine) [3].

These osmoregulants increase the plant's tolerance to stress by performing various functions, such as, facilitating osmotic adjustments in water-stressed plants [4], scavenging of free radicals [5, 6], stabilization of the sub-cellular structures [7], storage of nitrogen and carbohydrates [3], and regulation of co-enzymes. This understanding has resulted in an interest in application of osmoregulants to commercially important plants and crops to induce stress tolerance and in turn to improve the quality and yield of the product [8]. For this purpose, osmoregulants are introduced by foliar application [9], traditional breeding [10] or genetic engineering [11]. Therefore, osmoregulants are often studied to investigate the performance of the plant's physiological and biochemical mechanisms [12] or to estimate the tolerance of plants during environmental stress [13].

A number of methods have been reported for quantification of each osmoregulant. For example, proline has been detected colorimetrically (after reaction with ninhydrin) [14-17] and by reversed phase high performance liquid chromatography (RP-HPLC). Betaine is usually analysed by HPLC using an ion exchange column and UV-Vis detection at low wavelength [3, 18, 19] or using a RP column with refractive index detection [20]. Derivatization of betaine to impart UV absorbing abilities and to improve retention on RP columns has also been reported [21, 22]. Mannitol is usually detected by colorimetric methods, gas chromatography or HPLC coupled to a UV detector [23]. The latter method requires derivatization of the sample prior to analysis to impart UV absorbing characteristics [23].

In most studies, where more than one osmoregulant is of interest, each species is quantified by a separate technique. Canamus *et al*. (2007) determined proline levels in plant tissues by using RP-HPLC with fluorescence detection. The same authors also analysed betaine extracted from the same plant with an HPLC system fitted with a RI detector [24]. Attempts have been made to simultaneously analyse the three commonly investigated osmoregulants (i.e. proline, betaine and mannitol). For example, Oufir *et al*. (2009) investigated proline, its analogues and betaine simultaneously by HPLC using photodiode array (PDA) detection and an anion exchange column [12]. The sensitivity achieved with PDA was insufficient and only proline and hydroxyproline (with detection limits of 2 μM) were effectively measured. The same researchers successfully separated proline, betaine and its analogues using a size exclusion column for separation and mass spectroscopy for detection. Co-extraction of matrix neutral compounds (carbohydrates, polyols, and pigments), along with osmoregulants, and column degradation due to adsorption of matrix components, limited the usefulness of this method [12]. Naidu's method is one of the few studies in which separation and quantification of proline, betaine and mannitol has been achieved simultaneously. An ion-exchange HPLC column coupled to a UV detector was used for analysis [8]. This approach is attractive as it does not involve a derivatization procedure, however, preliminary purification was necessary to minimise interferences from other plant constituents. The sensitivity was also limited due to using UV/Vis detector [25]. The poor detection of amino acids and sugars by UV/Vis detection should be overcome by using evaporative light scattering detection (ELSD).

To date, no work has been reported for the simultaneous analysis of proline, betaine and mannitol in plants using ELSD. Here we present a rapid, sensitive, robust, and reliable HPLC-ELSD method for the simultaneous determination of these three osmoregulants.

4.3. Materials and Method

4.3.1. Chemicals

The amino acid standards were purchased from different suppliers: methionine, alanine, arginine from BDH Chemicals, Poole, England. Leucine and lysine were obtained from Hopkins and Williams LTD., Chadwell Health Essex, England. Glycine was from Ajax Chemicals, Melbourne, Australia. Proline, betaine, isoleucine, valine, glutamine, threonine, and histidine were from Sigma Aldrich, Sydney, Australia. Glucose and sucrose were obtained from Merck, Melbourne Australia. Trehalose was from Fluka, Buchs, Germany. Fructose from BDH Chemicals, Poole England., and raffinose was obtained from Sigma Aldrich, Sydney, Australia. HPLC grade acetonitrile (ACN) and formic acid and analytical grade trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) were all obtained from Sigma Aldrich, Sydney, Australia.

4.3.2. Samples and Standard Solution

Individual stock solutions (500 ppm) of amino acids and sugars were prepared in milli Q water. A stock solution (500 ppm) containing the three osmoregulants proline, betaine, and mannitol was prepared in milli Q water. From this stock solution, standards in the range of 10-500 ppm were prepared in both water and 0.1% formic acid: ACN (1: 1).

4.3.3. Plant Material and Extraction

Osmoregulants were extracted from three different plants (*Stylosanthes guianensis*, *Atriplex cinerea* and Rhagodia baccata). These plants were grown under elevated salt conditions (500 mM NaCl) in Edith Cowan University, Perth. WA.

For extraction, approximately 0.5 g of fresh plant material (accurately weighed) was ground to a powder aided by liquid nitrogen and using a mortar and a pestle. The finely powdered plant material was extracted with 80% ethanol (5.0 mL) for 10 min (with agitation) at room

temperature. The extract was then centrifuged at approximately 6000 RCF and the supernatant collected. The residue was extracted with a fresh aliquot of 5.0 mL ethanol and the process repeated. The supernatants were combined, filtered and stored at 4 ^{o}C for further analysis.

4.4. Instrumentation and Conditions

Chromatographic analysis was carried out using a HPLC system consisting of a Varian (Melbourne, Australia) 230 gradient pump and a Varian (Melbourne, Australia) 400 auto-sampler. A Prevail (Alltech Associates Australia, Melbourne, Australia) 5 µm C_{18} column (25 cm, 4.60 mm ID) and a Phenomenex (Sydney, Australia) 5 µm NH_2 (25 cm, 4.60 mm ID) column were used.

For the C_{18} column, the mobile phase consisted of 0.1% HFBA and ACN at a flow rate of 1.0 mL min^{-1}. The following gradient was used for separation: Initial conditions 100% B, 0% C; 0-1 min 95% B, 5% C; in 1-15 min 95-70% B, 5-30% C; in 15-20 min 70-60% B, 30-40% C (where "B" is 0.1% HFBA and "C" is ACN). The column was maintained at room temperature throughout separation.

For the NH_2 column, a combination of 0.1% formic acid and ACN was used for the mobile phase and at 1.5 mL min^{-1} flow rate. The gradient conditions were as follows: Initial conditions 1% B, 99% C; 1-8 min 1-10% B, 99-90% C; 8-15 min 15%B, 85% C; 15-20 min 20% B, 80% C and 30% B, 70% C from 20-25 min (where "B" is 0.1% formic acid and "C" is ACN). The column temperature was maintained at 35 ^{o}C throughout the separation.

Detection was carried out using an Alltech ELSD 800, (Melbourne, Australia) detector. The nebulizer used industrial grade N_2 gas at a flow of 3 L min^{-1} for the C_{18} column and 2 L min^{-1} for the NH_2 column. The drift tube temperature was set at 115 ^{o}C and 90 ^{o}C for C_{18} and NH_2 columns respectively.

4.5 Results and Discussion

The present study aimed to develop a method for the simultaneous determination of three commonly produced osmoregulants (mannitol, proline, and betaine) in the plants. For this purpose both a C_{18} and an amino column were trialed for their ability to separate the three key osmoregulants. C_{18} columns have been reported for the separation of a number of amino acids in a variety of samples [26, 27]. Using a C_{18} column and the ion-pairing agent, HFBA, the three analytes were resolved in less than 10 min (see Fig 1a).

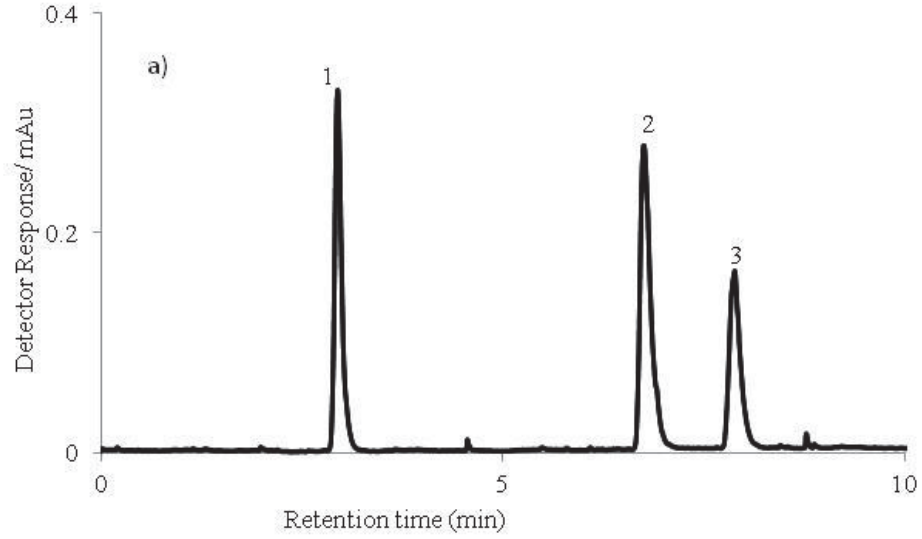
As the method is required for quantification of osmoregulants in plant extracts that are likely to contain a number of other compounds including amino acids and sugars, the potential for these analytes to interfere was studied. Ten amino acids either commonly present in plants or available in our laboratory (glycine, arginine, glutamine, threonine, histidine, valine, lysine, methionine, isoleucine, and leucine) were added to the osmoregulant test mixture. They were all resolved from each other and the analytes of interest (Fig 1b). The sugars commonly present in plant extracts (glucose, fructose, sucrose, raffinose, and trehalose) were also studied to determine the likelihood of interference. The highly polar sugar molecules interacted weakly with the reverse phase C_{18} column and eluted early. Fructose and glucose co-eluted with mannitol while sucrose was fully resolved from all other sugars and amino acids (Fig 1b). Given the likelihood of glucose and fructose being present in plant extracts, an alternative method that allowed the separation of mannitol from these sugars was required.

An NH_2 column provides an alternative mechanism of separation to the C_{18} column. In this instance the NH_2 groups on the column surface act as a weak anion exchanger. NH_2 columns have been used in our laboratory to resolve sugars using a water/ACN mobile phase. Using this mobile phase mannitol eluted early while proline and betaine eluted later but were unresolved. Furthermore, the inclusion of several amino acids in the osmoregulant test mixture resulted in partial or co-elution with proline and betaine. Ion-pairing agents were trialed in an attempt to resolve proline and betaine. The addition of HFBA and TFA to the mobile phase resulted in

broad peaks and with very little improvement in resolution. However, the addition of 0.1% formic acid to the water/CAN mobile phase successfully resolved all three osmoregulants in less than 25 min (Fig 2a).

The ten amino acids available (glycine, arginine, glutamine, threonine, histidine, valine, alanine, methionine, isoleucine and leucine) was then examined as potential interferences. All amino acids, with the exception of glutamine, eluted after mannitol and before proline and betaine (Fig 2b). Glutamine eluted after proline and betaine. While threonine and glycine were not baseline resolved from proline, even when they were present at high concentrations the proline peak was still clearly identifiable. The addition of key sugars (glucose, fructose, sucrose, raffinose, and trehalose) to the osmoregulant test mixture did not cause any interference and they were all resolved from each other and the three osmoregulants (Fig 2b). Therefore, this method also has the advantage of monitoring the key sugars present in plant extracts.

Five standards in the range of 25 ppm to 500 ppm were run on both the C_{18} and NH_2 columns. A polynomial relationship was observed for the three osmoregulants of interest (r^2= 0.995, r^2= 0.994, and r^2= 0.994 on the C_{18} column and r^2= 0.998, r^2= 0.995, and r^2= 0.995 on the NH_2 column for mannitol, betaine and proline respectively). Retention time repeatability over five runs was good at 0.46%, 0.39% and 1.21% on the C_{18} column and 0.85%, 1.50%, and 0.93%, on the NH_2 column for mannitol, betaine and proline.



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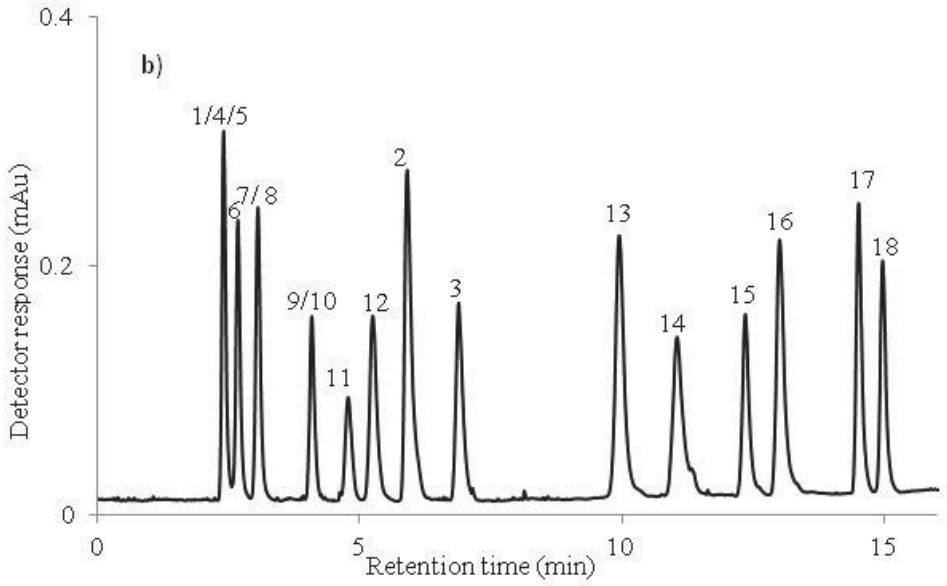

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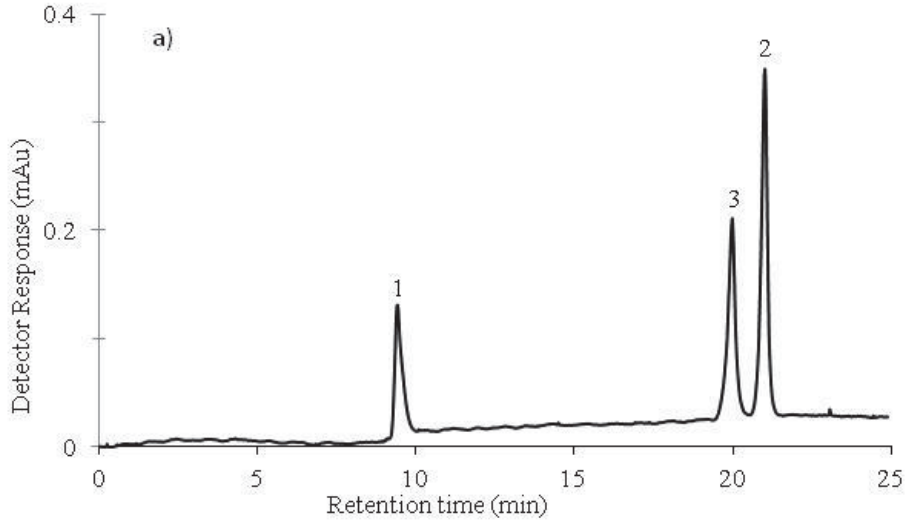
345

346 **Fig 1.** Separation on a C_{18} column using a mobile phase consisting of 0.1% HFBA and ACN in
347 gradient mode (see Text for more details). (a) separation of osmoregulants, peak identification:
348 1. mannitol 2. betaine, 3. proline. (b) separation of a mixture of osmoregulants, amino acids
349 and sugars (100 ppm), peak identification: 1. mannitol, 2. betaine, 3. proline, 4. fructose, 5.

350 glucose, 6. sucrose, 7. trehalose, 8. raffinose, 9. glycine, 10. arginine, 11. glutamine, 12.
351 threonine, 13. histidine, 14. valine, 15. lysine, 16. methionine, 17. isoleucine, 18. leucine.



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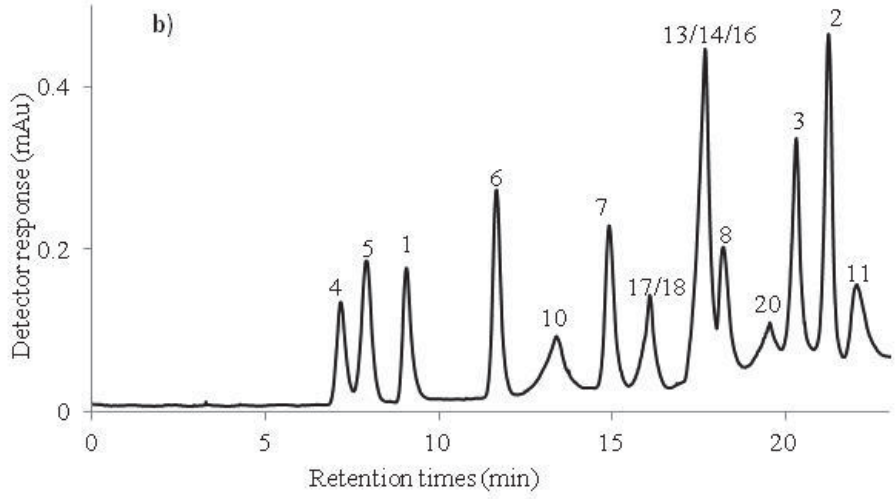

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355 **Fig 2**. Separation on a NH_2 column using a mobile phase consisting of 0.1% formic acid and ACN
356 in gradient mode (see Text for more details) (a) separation of osmoregulants, peak

identification: 1. mannitol, 2. betaine, 3. proline (b) separation of a mixture of osmoregulants, amino acids and sugars (100 ppm), peak identification: 1. mannitol, 2. betaine, 3. proline, 4. fructose, 5. glucose, 6. sucrose, 7. trehalose, 8. raffinose, 10. arginine, 11. glutamine, 13. histidine, 14. valine, 16. methionine, 17. isoleucine, 18. Leucine, 20. alanine.

The limits of detection (LOD) for the method were calculated based on a signal to noise ratio of 3 and are given in Table 1. The detection limits reported for the HPLC-ELSD method described here are almost 10 times more sensitive than the UV/Vis method reported by Naidu *et al*. (See Table 1). [8].

The theoretical plates recorded for the three key osmoregulants are given in Table 1. The theoretical plates are excellent for the three analytes, particularly, for betaine and proline, highlighting the high separation capabilities of the developed methods.

4.6. Application

Three plants extracts were analysed for the key osmoregulants using HPLC and the NH_2 method. A peak at 18.61 min was recorded for the *Stylosanthes guianensis* extract which was tentatively identified as proline based on retention time and spiking (Fig 3a). However, retention time alone is not sufficient for identification so the same extract was separated using the C_{18} method. The chromatogram obtained using the C_{18} column further supported the presence of proline (Fig 3b). Furthermore, both methods recorded similar concentrations of proline, 7.81 µmol and 7.12 µmol for the NH_2 and C_{18} columns respectively. Similarly, *Rhagodia baccata* extracts were also analysed using both the NH_2 and C_{18} methods. The chromatogram obtained using the NH_2 method indicated the presence of betaine (Fig 4a) which was confirmed by using the C_{18} method (Fig 4b). Both columns gave quantitatively similar results, 14.50 µmol and 16.71 µmol for NH_2 and C_{18} columns respectively. Therefore, it can be concluded that the

381 C_{18} method, with its different mechanism of separation clearly provides a useful
382 complementary method in confirmatory studies.

383 The value of a second confirmatory method when using UV/ Vis or ELSD detection was further
384 highlighted for the analysis of the *Atriplex cinerea* extract. The separation of the extract using
385 the NH_2 method (Fig 5a) determined the betaine concentration to be 116.18 µmol g^{-1}. However,
386 using C_{18} method (Fig 5b), the concentration of betaine was recorded at 34.57 µmol g^{-1}. The
387 very different results indicating that at least one analyte co-eluted with betaine on the NH_2.

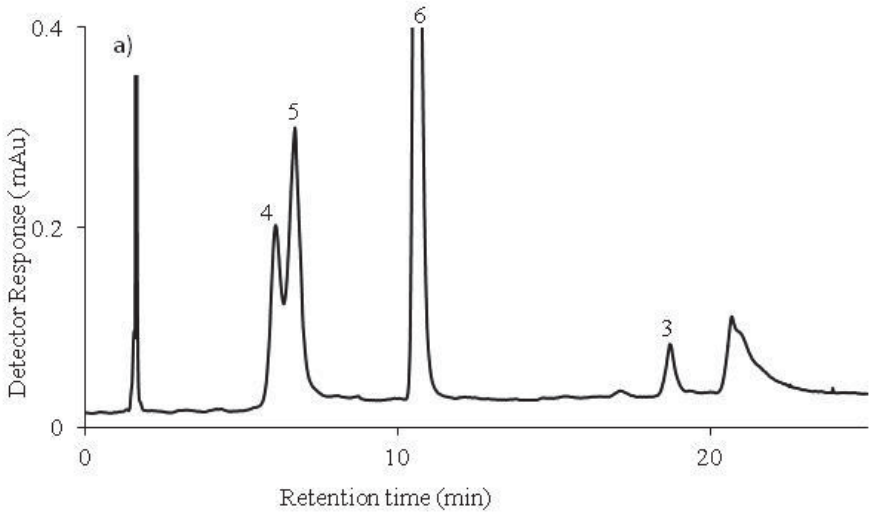
388 and (b) a C_{18} column and a mobile phase consisting of 0.1 % formic acid and ACN in gradient
389 mode (see Text for more details). The extract was diluted 3 fold for the C_{18} separation. Peak
390 identification: 2. betaine, 6. sucrose.

391 The NH_2 method has an added advantage in that it also provides researchers with the
392 opportunity to simultaneously monitor sugar concentrations, particularly the commonly
393 analysed sucrose, glucose and fructose.

394

395 **Table 1.** Limits of detection and efficiency data for osmoregulants separated on both an NH2
396 and a C18 column.

Analyte	**LOD (µmole)**			**Theoretical plates/column**	
	C18	NH_2	Naidu [8]	C_{18}	NH_2
Mannitol	8.00×10^{-5}	1.43×10^{-4}	2.50×10^{-3}	6515	6699
Betaine	1.38×10^{-4}	7.81×10^{-5}	5.00×10^{-4}	12051	51889
Proline	2.28×10^{-4}	1.08×10^{-4}	1.00×10^{-3}	12809	35007



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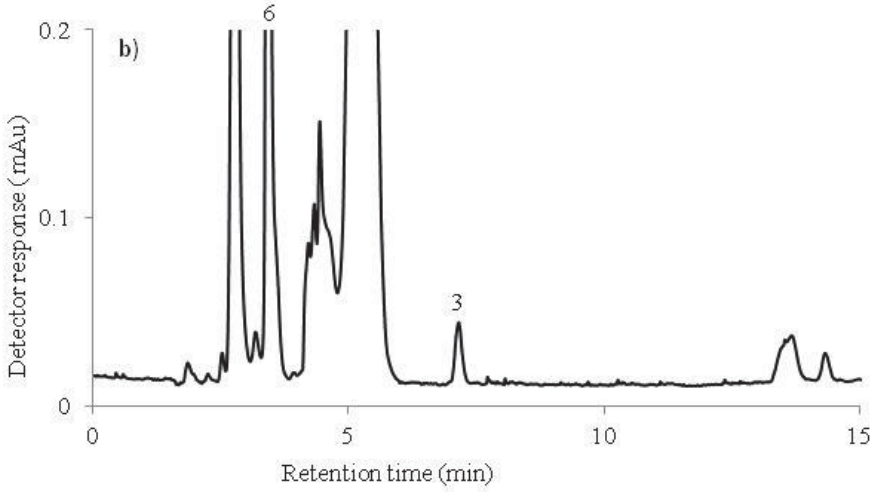

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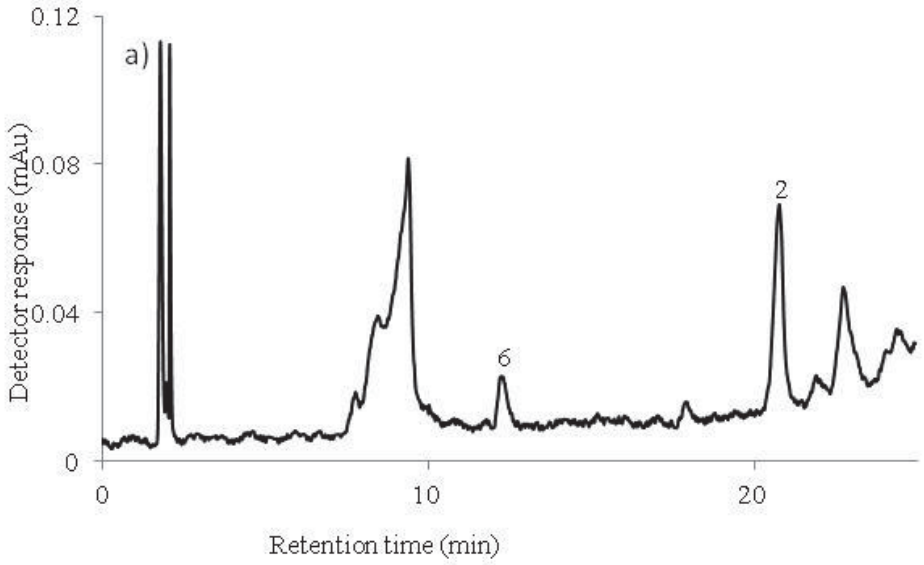
400 **Fig 3.** Chromatograms showing the separation of osmoregulants in *Stylosanthes guianensis*
401 extract on (a) an NH_2 column using a mobile phase consisting of 0.1% formic acid and ACN in
402 gradient mode and (b) a C_{18} column using a mobile phase consisting of 0.1% HFBA and ACN in
403 gradient mode (see Text for more details). Peak identification: 3. proline, 4. fructose, 5. glucose,
404 6. sucrose



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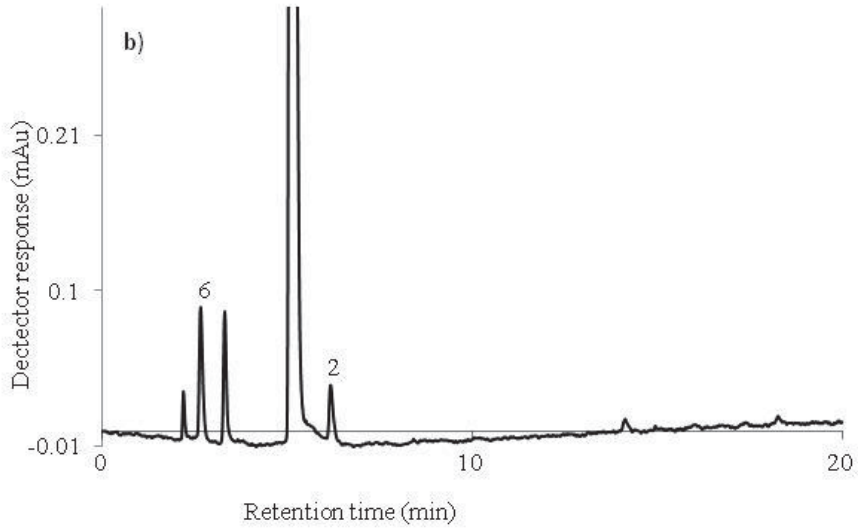

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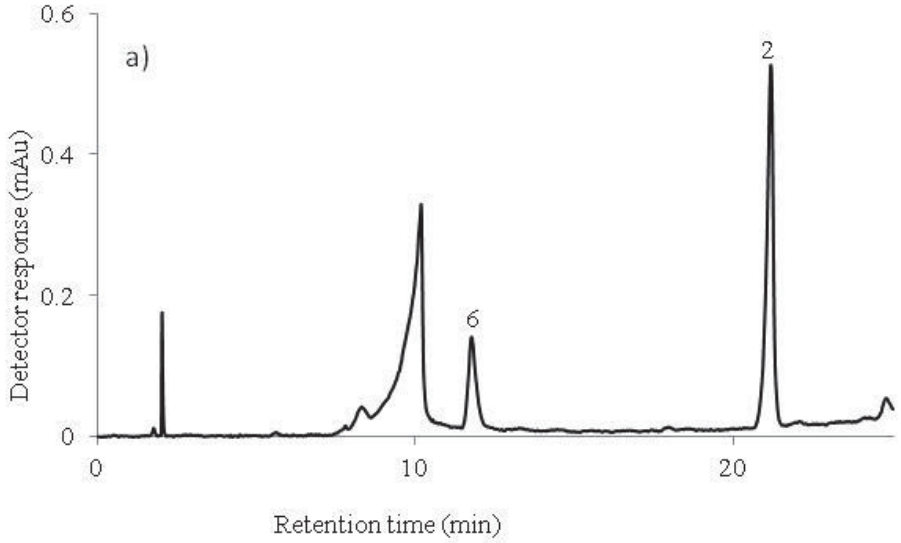
408 **Fig 4**. Chromatograms showing the separation of osmoregulants in stressed *Rhagodia baccata*
409 extract using (a) an NH_2 column and a mobile phase consisting of 0.1 % formic acid and ACN in
410 gradient mode and (b) a C_{18} column and a mobile phase consisting of 0.1 % formic acid and ACN
411 in gradient mode (see Text for more details). Peak identification: 2. betaine, 6. sucrose.



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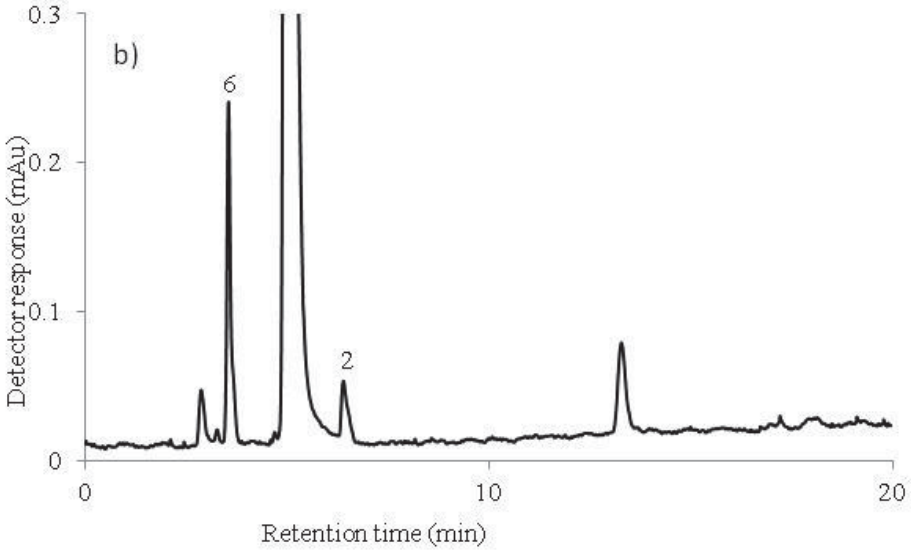

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415 **Fig 5**. Chromatogram showing separation of osmoregulants in Atriplex cinerea extract using (a)
416 a NH2 column and a mobile phase consisting of 0.1 % formic acid and ACN in gradient mode

4.7. Conclusion

This paper presents a robust and a relatively quick HPLC-ELSD method for the simultaneous analysis of the osmoregulants, proline, betaine and mannitol. This method which employs a NH_2 separation column is also useful for the concurrent analysis of sugars. While the C_{18} column was not suitable for the analysis of mannitol due to co-elution with glucose and fructose it is a valuable complementary tool for proline and betaine analysis.

4.8. References

[1] Servillo, L.; Giovane, A.; Balestrieri, M. L.; Cautela, D.; Castaldo, D. *J. Agric. Food Chem.* **2011**, 59, 274–281.

[2] Iwamoto, K.; Shiraiwa, Y. *Mar Biotechnol*. **2005,** 7, 407–415.

[3] Martino, C. D.; Delfine, S.; Pizzuto, R.; Loreto, F.; Fuggi, A. *New Phytol*. **2003,** 158, 455–463.

[4] Sharp, R. E.; Poroyko, V.; Hejlek, L. G.; Spollen, W. G.; Springer, G. K.; Bohnert, H. J.; Nguyen, H. T.; *J. Exp. Bot.* **2004**, 55, 2343–2351.

[5] Shen, B.; Hohmann, S.; Jensen, R. G.; Bohnert, H. J.; *Plant Physiol*. **1999,** 121, 45–52.

[6] Zhu, J. K. *Trend. Plant Sci*. **2001**, 6, 66–71.

[7] Ashraf, M.; Foolad, M. R.; *Environ. Exp. Bot*. **2006** 59, 206–216.

[8] Naidu, B. P. *Aust. J. Plant Physiol.* **1998**, 25, 793–800.

[9] Ali, Q.; Ashraf, M. *J. Agron. Crop Sci*. **2011**, 197, 258–271.

[10] Sahi, C.; Singh, A.; Kumar, K.; Blumwald, E.; Grover, A. *Funct. Integr. Genomic.* **2006**, 6, 263–284.

[11] Sakamoto, A.; Murata, N. *J. Exp. Bot*. **2000**, 51, 81–88.

[12] Oufir, M.; Schulz, N.; Vallikhan, P. S. S.; Wilhelm, E.; Burg, K.; Hausman, J. F.; Hoffmann, L.; Guignard, C.; *J. Chrom. A* **2009**, 1216, 1094–1099.

[13] Verslues, P. E.; Agarwal, M.; Agarwal, S.K.; Zhu, J.; Zhu, J. K. *Plant J*. **2006,** 45, 523–539.

[14] Claussen, W. *Plant Sci*. **2005,** 168, 241–248.

[15] Köşkeroğlu, S.; Tuna, A. L. *Acta Physiol. Plant* **2010,** 32, 541–549.

[16] Naidu, B. P.; Paleg, L. G.; Jones, G. P.; *Aust. J. Bot*. **2000,** 48, 611–620.

[17] Bates, L. S.; Waldren, R. P.; Teare, I. D. *Plant Soil* **1973,** 39, 205–207.

[18] Bessieres, M. A.; Gibon, Y.; Lefeuvre, J. C.; Larher, F. *J. Agric. Food Chem*. **1999,** 47, 3718–3722.

[19] Chendrimada, T.P.; Neto, M. G.; Pesti, G. M.; Davis, A. J.; Bakalli, R. I.; *J. Sci. Food Agric.* **2002,** 82, 1556–1563.

[20] Teixidó, N.; Cañamás, T.P.; Usall, J.; Torres, R.; Magan, N.; Viñas, I. *Lett. Appl. Microbiol.* **2005,** 41, 248–252.

[21] Mar, M. H.H.; Ridky, T. W.; Garner, S. C.; Zeisel, S. H. *J. Nutr. Biochem.* **1995,** 6, 392–398.

[22] Zwart, F. J.; Slow, S.; Payne, R. J.; Lever, M.; George, P. M.; Gerrard, J. A.; Chambers, S. T. *Food Chem.* **2003,** 83, 197–204.

[23] Katayama, M.; Matsuda, Y.; Kobayashi, K.; Kaneko, S.; Ishikawa, H. *Biomed. Chrom.* **2005,** 20, 440–445 .

[24] Cañamás, T. P.; Viñas, I.; Usall, J.; Magan, N.; Morelló, J. R.; Teixidó, N. *Lett. Appl. Microbiol*. **2007,** 45, 6–12.

459 [25] Lucena, R.; Cárdenas, S.; Valcárcel, M.; *Anal. Bioanal. Chem.* **2007,** 388, 1663–1672.

460 [26] Bai, C.; Reilly, C. C.; Wood, B. W. *Plant Physiol*. **2006**, 140, 433–443.

461 [27] Qu, J.; Wang, Y.; Luo, G.; Wu, Z.; Yang, C. *Anal. Chem*. **2002,** 74, 2034–2040.

462

Chapter 5 Direct Electrokinetic Injection of Inorganic Cations from Whole Fruits and Vegetables for Capillary Electrophoresis Analysis

This chapter has been submitted as a technical note in Analytical Chemistry. All efforts were made to keep the original features of this article except minor changes e.g. layout, numbering, font size and style were carried in order to maintain a consistent formatting style of this thesis.

5.1. Abstract

A novel approach for the direct injection from plant tissues without any sample pre-treatment has been developed by simply placing a small piece of the material into a capillary electrophoresis vial followed by application of a voltage for electrokinetic injection. Separations of sodium, potassium, calcium and magnesium were achieved using a BGE comprising 10 mM imidazole and 2.5 mM 18-crown-6-ether at pH 4.5. The addition of 2% (m/v) hydroxypropylmethyl cellulose to the separation buffer allowed for precise and accurate electrokinetic injection of ions from the plant material by halting the movement of tissue fluid into the capillary. This method provides both qualitative and quantitative data of inorganic cations, with quantitation in zucchini, mushroom and apple samples in agreement with sector field inductively coupled plasma mass spectrometric analysis (r^2= 0.97, n=9). This method provides a new way for rapid, quantitative analysis by eliminating sample preparation procedures, and has great potential for a range of applications in plant science and food chemistry.

5.2. Introduction

Minerals and vitamins are essential nutrients required for a healthy functioning body [1, 2] with vegetables and fruits being an important source [3]. The relative abundance of minerals in different foods vary significantly [4] and is of interest to a health conscious public [5, 6]. Furthermore, seasonal variations [7, 8] and growing [9] and storage conditions can impact the nutrient levels and hence quality f a food [10]. Therefore, efficient methods for the analysis of minerals in food are necessary.

Determination of inorganic mineral cations, such as Ca^{2+}, Na^{+}, Mg^{2+} and K^{+} in fruits and vegetables is typically achieved by atomic spectroscopy [7], such as inductively coupled plasma-mass spectrometry (ICP-MS) [11], but alternatives such as ion chromatography [12] and capillary electrophoresis (CE) [13] have also been reported. In all cases sample preparation is required and typically involves drying and pulverizing the sample followed by acid digestion and dilution [2-4, 14, 15]. Fukushi *et al.* reported an electrophoresis method for free calcium in vegetable that was slightly simpler, but still required boiling pulverized vegetable for 15-20 min, cooling, filtering and making to volume prior to analysis [16]. Sample preparation is not only time consuming and labor intensive but also provides opportunity for sample contamination and analyte loss. A simpler method for direct analysis of plant tissue is highly desirable.

Methods for direct analysis of tissues of biological or clinical interest have emerged over the last decade [17-20]. For example, the direct determination of drugs in tissue samples have been achieved using mass spectrometry (MS) in combination with matrix-assisted laser desorption/ionization (MALDI)-MS [14]. MS methods are typically limited to providing qualitative information of the analytes. Quantitative information in direct analysis of a bulk sample was obtained by MS in combination with internal extractive electrospray ionization. The capillary tip was placed inside the sample and a solvent was introduced into the sample matrix to extract the analytes at high voltage (± 4.5 kV) for direct injection into the MS [20]. The signal intensities were highly dependent on the position of the ESI capillary in the sample with slight

changes in capillary position resulting in differences in the injected sample volume, compromising repeatability. The approach also required samples to be precisely cut to ensure a uniform size and shape to achieve reproducible results, which combined with the solvent required for the extraction of analytes from the sample matrix, complicates the method.

Analytical separation techniques offer the possibility of separating target analytes based on their physicochemical properties, avoiding the reliance on the resolving power of the mass spectrometer. CE is known for its ability to perform rapid separations with very small sample volumes, and there are two reports in which analytes have been directly injected from tissue samples. For example, Oguri *et al*. reported the direct sampling from rat's brain using CE in combination with laser induced fluorescence (LIF) for the analysis of taurine [21]. Electrokinetic injection was performed by piercing a rat's brain with the capillary and allowed for the determination of both intra- and extra-cellular taurine, an advantage in comparison with microdialysis only extra-cellular taurine can be sampled. However, this approach only provided qualitative information, as it was not possible to control the amount of sample injected. Also, sampling was achieved only from the surface to minimize the accidental release of taurine from damaged tissues. The use of a tapered capillary was suggested as a way to minimize damage and for sampling deep inside the brain. This approach was subsequently employed by Wang *et al*. who etched the capillary to a sharp point using HF and used this to detect the anticancer drug doxorubicin in human liver tissue [22]. For direct sampling from thin slices of liver tissues, a negative pressure of -7.6 kPa for 2s was applied. However, etching is a hazardous process and the resulting fragile capillary is likely to break when directly sampling from more solid samples such as many plant tissues. This method also required tissues to be cut into very thin slices (5 µm) to prevent large injections, thus making it technically demanding and unsuitable for analysis of intact plant tissues. In addition to this, electrokinetic injection of intracellular content of single cells using CE in combination with laser induced fluorescence (LIF) have also been demonstrated [23, 24] demonstrating the potential of electrophoresis to provide information on biological systems.

In this paper, our aim was to develop a simple and robust method for the direct injection of ions from plant tissue. This would be attractive because it would eliminate the requirement for sample treatment, reducing contamination and improving analytical simplicity. While there work required the tissue to be cut and placed in a CE vial, when implemented in a more portable platform, and extended to other analytes, it may form the basis for rapid on-site analysis of food products to inform agricultural production and nutrition as well as food safety.

5.3. Experimental

5.3.1. Chemicals

Imidazole, 18-crown-6 ether, sodium chloride, potassium chloride, hydoxypropyl methyl cellulose (HPMC) (viscosity 3500-5600 cP, 2% in H_2O, 20 ^{o}C), sodium hydroxide, acetic acid and nitric acid were all purchased from Sigma Aldrich (Sydney, Australia). Calcium chloride dihydrate was from Univar (New South Wales, Australia). Magnesium chloride hexahydrate was from BDH Laboratory Supplies (Poole, England).

5.3.2. Instrumentation

A Hewlett Packard 3D CE (Waldbron, Germany) instrument equipped with a diode array UV absorbance detector and Agilent Chemstation software Rev. A. 08.03 (847) was used. The instrument was connected to the building nitrogen supply to provide up to 6 bar of pressure using the external adaptor provided with the instrument.

The cassette temperature set at 30 ^{o}C. Untreated fused silica capillaries (Polymicro, Phoenix, AZ, USA) with an internal diameter of 50 µm and outer diameter of 350 µm were used for separation unless otherwise stated. Initially, the length of capillary and separation voltage was kept at 100 cm (91.5 cm to the detector) and +20 kV. However, to minimize the blockage of

559 capillary because of the high viscosity buffer, the capillary was shortened to 50 cm (41.5 cm to
560 the detector) for the repeatability experiments and separation was carried at +8 kV. The
561 capillary length was further reduced to 40 cm (31.5 cm to the detector) for the separation of
562 cations in other fruits and vegetables at +5.5 kV).

563 ### 5.3.3. CE Analysis

564 A new capillary was conditioned sequentially with 0.1 N NaOH, deionized water and BGE for
565 15 min each at 5 bar. Once in use, the capillary was flushed daily with deioninzed water and
566 then BGE for 10 min each at 5 bar at the start of the day. At the end of each day, the capillary
567 was flushed with Milli Q at 5 bar for 10 min and stored in MilliQ water. The capillary was flushed
568 with BGE for 2 min prior to each run.

569 For separation the BGE was 10 mM imidazole, 2.5 mM 18-crown-6-ether, 2% (w/ v) HPMC at
570 pH 4.5, adjusted with acetic acid, was used prepared daily from a 10X stock solution. The BGE
571 was replaced after every 5 runs. For detection, the maximum absorption wavelength (214 nm)
572 of imidazole was used.

573 ### 5.3.4. Standards and Sample Solutions

574 Standard solutions of K^+ (350,000 µg/mL), Ca^{2+} (100,000 µg/mL) and Mg^{2+} (100,000 µg/mL)
575 were prepared in water from KCl, $CaCl_2.2\ H_2O$ and $MgCl_2.6\ H_2O$ salts.

576 For preparation of the zucchini gel, 50.0 mL of hot water was blended with 50.0 g of zucchini
577 (2 min or until zucchini formed a paste with water). To this hot mixture 5.0 g of gelatin was
578 added and mixed with a magnetic stirrer for approximately 1 min. The mixture was degassed by
579 sonication (10 min), poured into plastic moulds and allowed to solidify for 1 h in a refrigerator (3
580 ^oC). The gelatin slices were then cut into approximately 5 mm cubes for direct injection and CE
581 analysis.

For quantitation of cations in whole fruit, a series of external calibration standards were prepared using a fruit or vegetable matrix. 10 mL of the paste prepared as above was spiked with the appropriate range of standards. For spiking, a rough estimate of cation concentration in the given fruit and vegetable was considered and standards falling in that range were prepared. Spiked standards of zucchini were prepared as follows; K^+ (0−14, mg/mL), Mg^{2+} and Ca^{2+} (0−0.900 mg/mL). For apple, standards in the range of 0−1.5 mg/mL, 0−0.2 mg/mL, and 0−0.075 mg/mL for K^+, Ca^{2+} and Mg^{2+}, respectively were prepared, for four different varieties of apples: fuji, pink lady, red delicious, and royal gala. For mushroom, the standards were in the range of 0−9, mg/mL, 0−0.04 mg/mL, and 0−0.3 mg/mL for K^+, Ca^{2+} and Mg^{2+}, respectively. The mushroom matrix was prepared by blending 50 g of mushroom with 100 mL of Milli Q water. The spiked standards of each fruit or vegetable were used to construct a calibration curve and from that curve the concentration of analytes in that particular fruit or vegetable was determined.

5.3.5. ICP-MS Analysis

A 10 g sample was cut into small pieces with a knife. Three replicates were placed in a freezer (-20 ^{o}C) for 3 h or until completely frozen. The frozen samples were transferred to a freeze dryer (-37 ^{o}C) and left for 3 days or until completely dry. Dried samples were weighed and crushed to a fine powder using a mortar and pestle. The powdered samples were stored in polypropylene tubes at room temperature prior to further processing.

Approximately 250 mg of dried and powdered sample was transferred to a polypropylene vessel (SCP Science, Quebec, Canada) and 5.0 mL of concentrated nitric acid (Suprapur, Merck, Darmstadt, Germany) added. Sample vials were transferred to a digestion block (DigiPREP-MS, SCP Science, Quebec, Canada) and were allowed to sit for approximately 60 min before heating. Covered vessels were heated at 95 ^{o}C for 4 h. Following digestion samples were diluted to 50 mL using ultra HP water, and further diluted by a factor of 10 before analysis. Digestions were

performed under clean conditions in a dedicated extraction unit (SCP Science, Quebec, Canada) under a flow stream of (High-efficiency particulate arrestance) HEPA filtered air. A Thermo Scientific Element 2 Sector Field ICP-MS (Bremen, Germany) was used for the determination of total elements. This instrument operates using multiple spectral resolutions minimizing overlap from major polyatomic interferences. Mg and Ca were monitored in medium resolution mode (i.e. m/Δm>4000) with K^+ analyzed using high resolution conditions (m/Δm>9500). Quantification was via the method of external calibration using multi-element mixed standards (QCD Analysts, Spring Lake, USA) with indium added to all standards and samples as internal standard. The entire digestion and analysis procedure was validated using the National Bureau of Standards Certified Reference Material Tomato Leaves 1573. Measured and certified values were found to be in agreement to within ±5%, while relative standard deviation between replicates (n=3) was less than 10%. Multiple blank samples (n= 3) were also prepared and were found to be of negligible concentration compared to samples analysed.

5.4. Results and discussion

Sample preparation is often a complex, time consuming, labor intensive and hence expensive step which can be avoided in CE by injecting directly from samples, provided this is practically feasible and can be done in a controlled manner. To evaluate the feasibility of directly injecting from fruit and vegetables for CE analysis, a piece of zucchini was cut into a 5 mm^3 piece and placed directly in a 1.5 mL CE vial and positioned in the instrument (Fig. 1). Electrokinetic injection was performed by applying 5 kV for 5 s followed by separation at +25 kV using an imidazole BGE at pH 4.5 containing 18-crown-6-ether [25]. Four peaks were observed, identified as K^+, Ca^{2+}, Na^+ and Mg^{2+} based on their migration times (Fig 2B). Surprisingly, blockage of the capillary from zucchini residue was not observed, even after multiple runs, and no carryover of residue was observed on the capillary or electrode. Unfortunately, the peak area repeatability was rather poor (RSD ≥100%. n= 10). It was speculated that the poor repeatability was due to the expulsion of fluid from the zucchini as a result of the capillary wall squashing the zucchini,

resulting in hydrodynamic injection into the capillary. To investigate this blank injections (0 kV for 5 s) were performed from individual pieces of zucchini (n=10). Again all analytes were detected, confirming the idea of unwanted hydrodynamic injection. Moreover, this process was not repeatable, with peak area RSD > 100% (Table 1). This issue was previously identified as an issue by Wang *et al*.; their solution was to etch the outer edge of the fused silica capillary and to use a thin 5 µm slice of tissue to reduce the pressure applied [22]. However, this approach reduces applicability and would require consistent and even cutting of the vegetable into very thin (5 µm) slices. Furthermore, the etched capillary is more fragile and may be damaged when sampling fruits and vegetables that are not soft.

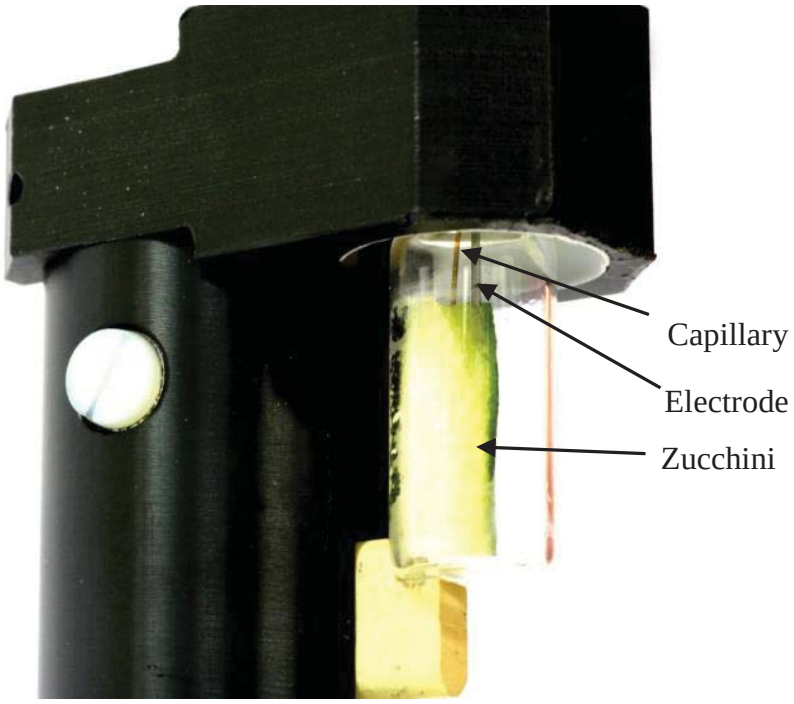
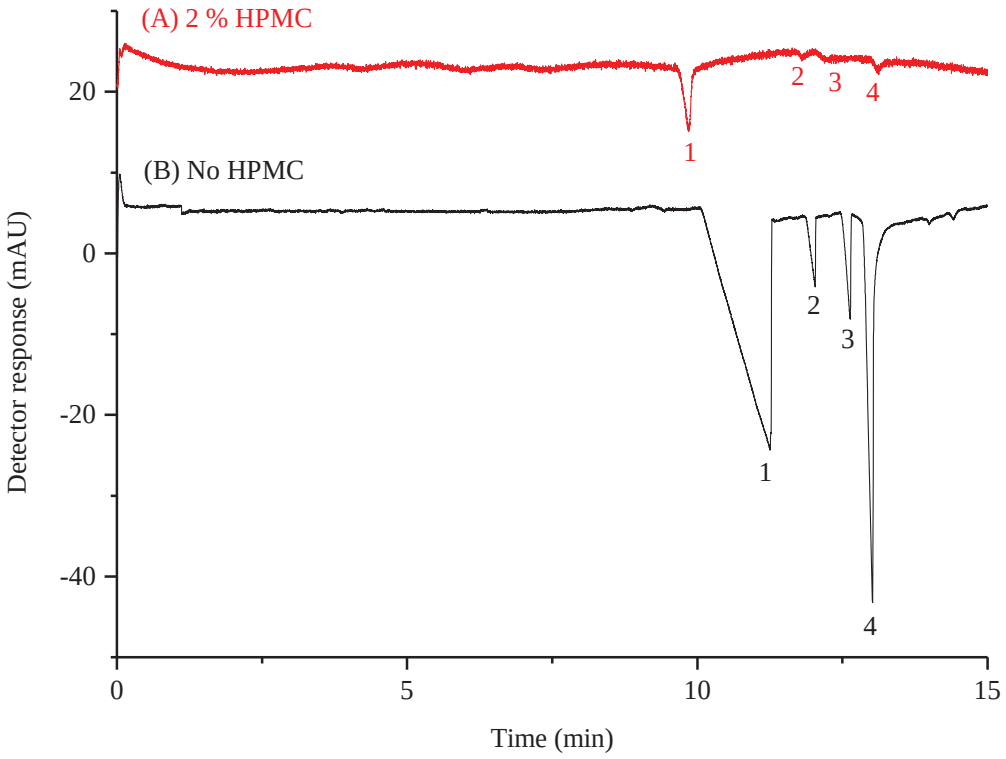


Fig 1. Direct injection of cations from a piece of zucchini in a commercial capillary electrophoresis system.



645

646 **Fig 2.** Electropherograms for separation of cations in zucchini. Peak identification: 1. K^+, 2. Ca^{2+},
647 3. Na^+, and 4. Mg^{2+}. Experimental conditions: sample was injected for 5 s at 0 kV into a 100 cm
648 long, 50 µm I.D. capillary. Separation at +20 kV, UV detection at 214 nm using 10 mM imidazole
649 buffer containing 2.5 mM 18-crown-6-ether adjusted to pH 4.5 using acetic acid as BGE (A)
650 without adding HPMC to the BGE (B) adding 2 % (m/v) HPMC to the BGE.

Table 1. % Variation in peak area for zucchini replicates determined using direct injection CE

	Standard BGE[a] and injection (0 kV, 5s) of zucchini				BGE with 2 % (m/v) HPMC and injection (5 kV, 5 s) of zucchini				BGE with 2 % (m/v) HPMC and injection (5 kV, 5 s) of zucchini gel			
No of Replicates	Peak Area (mAU)				Peak Area (mAU)				Peak Area (mAU)			
	K	Ca	Mg	Na	K	Ca	Mg	Na	K	Ca	Mg	Na
1	9.5	14	11	56	830	86	110	79	350	71	82	320
2	14	11	11	81	990	71	150	35	390	71	72	170
3	78	36	57	99	880	73	120	39	410	72	72	220
4	26	34	36	84	880	74	120	32	450	71	71	160
5	8.2	4.4	7.2	66	920	73	110	70	430	75	75	110
6	3.2	11	11	72	940	61	120	33	410	72	77	290
7	11	5.3	7.5	45	940	62	140	39	410	66	73	130
8	6.1	4.6	5.2	49	970	77	130	37	410	68	78	260
9	160	21	51	73	870	73	110	31	430	85	78	130
10	8.2	21	19	46	920	77	140	35	410	69	75	120
*Ave	33	14	21	66	920	73	130	43	410	72	75	150
**STD	49	11	19	18	49	7.2	13	16	26	5.2	3.4	91
***RSD (%)	140	78	94	28	5.4	9.9	11	39	6.3	7.2	4.5	56
ICP (mg/g)	42	2.2	2.4	0.078								

[a] The BGE consisted of 10 mM imidazole and 2.5 mM 18-crown-6-ether at pH 4.5.

*Average

**Standard Deviation

*** Relative standard deviation

An alternative approach to limit the fluid squeezed into the capillary proposed here is by increasing the viscosity of the BGE. To examine this idea 2% (m/v) HPMC was added to the imidazole BGE (above 2 % (m/v) concentration the HPMC was not soluble in water). The resulting electropherogram confirmed that the HPMC polymer reduced the injection of fluid from the zucchini into the capillary (Fig. 2) with the peaks being reduced in size by 92% for K^+, 88% for Ca^{2+}, 90% for Na^+ and 97% for Mg^{2+}. Using this HPMC system, the repeatability (n=10) increased considerably with peak area RSD ≤ 11% for K^+, Ca^{2+} and Mg^{2+} (Table 1). However, the results for Na^+ were imprecise likely arising from instrument contamination as it was not cleaned to remove Na^+. Therefore, quantitative Na^+ results were considered unreliable and not pursued further. However, this data demonstrates that it is possible to reproducibly inject directly from zucchini without damaging or blocking the capillary, as can be seen in Fig. 3. The capillary length was chosen to be 50 cm to minimize the blockage of capillary from high viscosity buffer or zucchini. To keep the migration time consistent the separation voltage was decreased accordingly. There was no deterioration in peak shape, area or height, and only a slight change in migration time, most likely due to small changes of the EOF. While the addition of HPMC significantly improved the performance of the method, there is still the question of whether the injection variability is due to the heterogeneity of zucchini– the complex and heterogeneous nature of different parts of plants is well documented [26-28] or simply analytical variability. To evaluate this, a homogeneous zucchini gel was prepared by blending the zucchini with gelatin. Electrokinetic injection (5 kV, 5 s) from 10 pieces of gel was performed and less than 5% RSD peak area for three analytes including K^+, Ca^{2+} and Mg^{2+} was obtained (Table 1). This suggests that the direct injection method is reproducible, and that the slightly higher RSD in whole zucchini is related to the heterogeneous nature of zucchini. A blank of gelatine (0.5g/10 mL, m/v) was also performed to establish the ions already present. The electropherogram for gelatine blank demonstrated a peak for K^+ (peak area= 68.42), Ca^{2+} (peak area= 68.42), and Mg^{2+} (peak area= 4.86). As K^+ and Mg^{2+} peaks are very small compared to concentrations of these ions in the zucchini, the peak area of these ions has been approximately halved (Table 1) when mixed 1:1. For Ca^{2+} where the concentrations are similar, the concentration in zucchini

jelly is a sum from gelatine and zucchini and therefore the peak area for this ion (Table 1) remains almost the same even after 1:1 dilution of zucchini.

Zucchini is a soft and moist vegetable which allows easy penetration of the capillary and electrode into the flesh. For direct injection to be a more general approach, it must be applicable to other fruits and vegetables. Apples and green beans were selected as examples of hard tissues; tomato, and strawberry were selected as examples of moist tissues; and mushroom as an example of a dry tissue. Injection was performed again by voltage (5kV for 5 s) with the resulting electropherograms presented in Fig. 4. Peaks were observed for all of the tissue samples and the concentration of cations varied with the sample indicating the general applicability of the method.

While the above data shows the potential for qualitative analysis, it would be significantly more attractive if the approach was quantitative. The standard method for quantitation of cations in vegetables typically involves drying the plant tissue, pulverizing it to a fine powder, acid digestion [15] and analysis by an atomic spectroscopy method [2]. Samples of zucchini, apple and mushroom were prepared in this manner and analyzed by sector field ICP-MS.

The same samples were also analyzed by CE using the direct injection approach. A universal calibration was initially examined but was found to be inaccurate due to the influence of the different matrix of the different fruit. Standard addition is typically used to overcome this; however, this was not possible here due to the inability to spike the tissue sample with known amounts of the ions. Instead an external calibration series using a matched matrix was constructed (see section 2.4. “standards and sample solutions”). To minimize the variation in results due to heterogeneity, the skin was taken off from all samples before measurement.

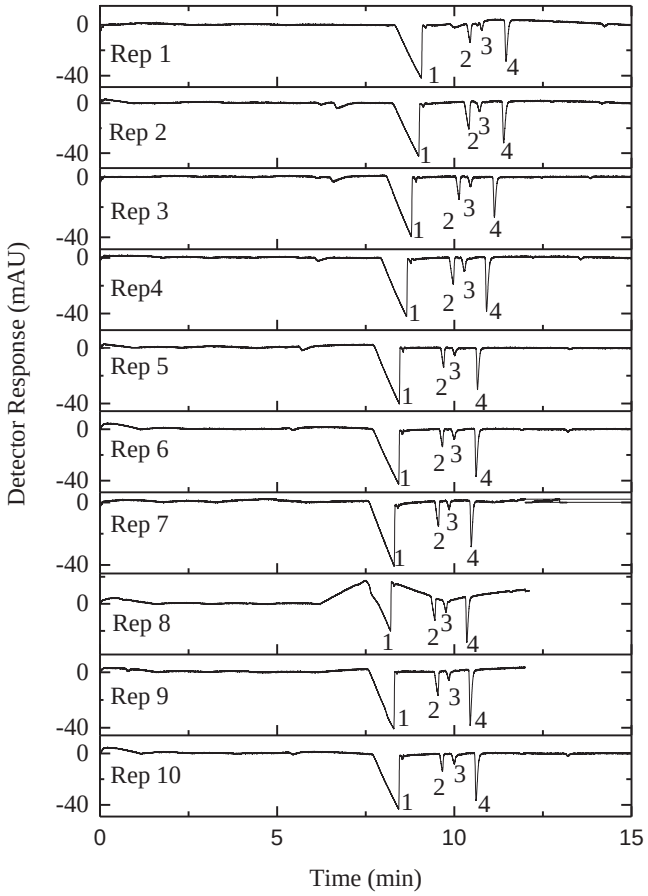


Fig 3. Electropherograms for separation of cations in ten replicates of zucchini. Peak identification: 1. K^+, 2. Ca^{2+}, 3. Na^+, and 4. Mg^{2+}. Experimental conditions: sample was injected for 5 s at +5 kV into a 50 cm long, 50 µm I.D. capillary. Separation at +8 kV, UV detection at 214 nm using 10 mM imidazole buffer containing 2.5 mM 18-crown-6-ether adjusted to pH 4.5 using acetic acid and 2 % (m/v) HPMC added to the BGE.

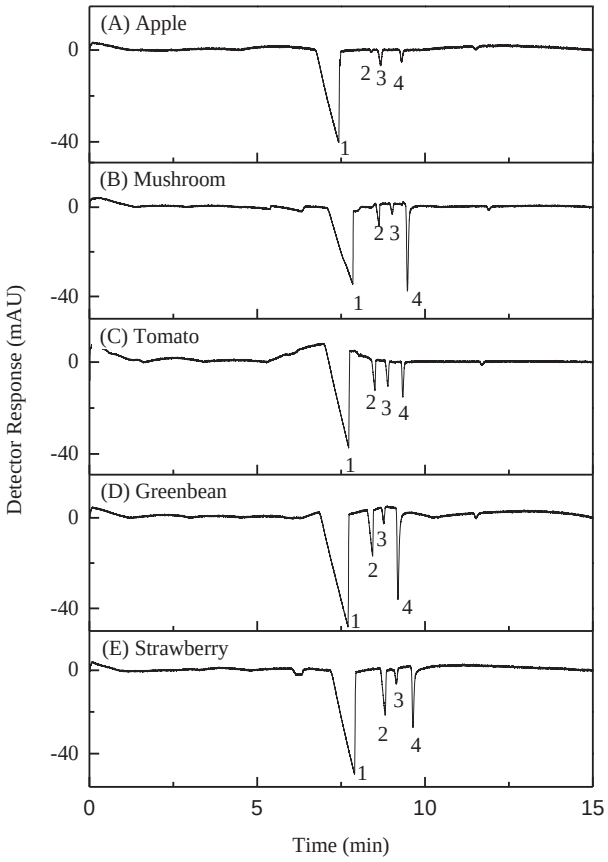


Fig 4. Electropherograms for separation of cations. Peak identification: 1. K^+, 2. Ca^{2+}, 3. Na^+, and 4. Mg^{2+}. Experimental conditions: sample was injected for 5 s at 10 kV into a 40 cm long, 50 µm I.D. capillary. Separation at +5.5 kV, UV detection at 214 nm using 2 % (m/v) HPMC (m/v), 10 mM imidazole buffer containing 2.5 mM 18-crown-6-ether adjusted to pH 4.5 using acetic acid as BGE (A) apple (B) mushroom (C) tomato (D) green bean (E) strawberry.

Excellent agreement was found between the direct injection CE and ICP-MS quantitative data as seen in the co-relation plot (r^2 = 0.97) (Fig. 5). Logarithm of the concentration (µg/g fresh weight) values was taken to clearly illustrate all data points. The maximum deviation of two methods was found to be less than 15 % for all three analytes in all three samples except for Mg (99%) in mushroom. Comparing determined ICP-MS and CE values for Mg in mushroom with example literature estimates provided no clues as to which value may be incorrect. However, the literature indicates that there is a strong correlation between the concentration of Ca and Mg [29-31]. As the concentrations of these two analytes determined by CE are similar in contrast to ICP-MS, this suggests that the ICP results for Mg may be questionable. Furthermore, CE results (mg/g) for K^+ (44), Ca^{2+} (0.11), and Mg^{2+} (0.19) in dry weight (DW) of mushroom (1 g of fresh weight= 0.17 g dry weight) are in close agreement with the range (mg/g) reported by Uzun *et al*. for K^+ (5.9- 29), Ca^{2+} (0.041-5.7), and Mg^{2+} (0.18-1.9) [31]. Similarly for zucchini (1 g fresh weight= 0.13 g dry weight), the concentration (mg/g DW) of inorganic cations i.e. K^+ (38), Ca^{2+} (2.01), and Mg^{2+} (1.9) determined by CE agrees with the range reported by Valdivieso *et al*. for K^+ (14–48), Ca^{2+} (0.80–5.1) and Mg^{2+} (1.3–3.5) [32]. For apple (1 g fresh weight= 0.25 g dry weight), quantities (mg/g DW) of K^+ (4.8), Ca^{2+} (0.16) and $Mg^{2=}$ (0.21) are very close to K^+ (5.1–6.8), Ca^{2+} (0.11–0.22), and Mg^{2+} (0.18–0.22) concentrations reported by Moggia *et al* [33].

The above data show that quantitative results can be obtained when the calibration series (spiked aliquots of fruit/vegetable smoothie) is generated using flesh from the same piece of fruit/vegetable being analysed by direct injection, however, this is highly unpractical. To examine the ability to use a single fruit calibration series for closely related fruit, four different apple varieties (fuji, pink lady, red delicious and royal gala) were analysed. The amount of K^+, Ca^{2+} and Mg^{2+} in each of the four apples using each of the four calibration series generated for each apple is shown in Table 2, along with the % difference from the indicative result. The indicative result was determined from the matched calibration, for example, the values determined from a pink lady apple using the pink lady calibration series. The average difference was 12% (n=33) and the maximum difference was 30%. Given the simplicity of the method and the ability to use a single apple calibration series for multiple apple varieties, this represents a rapid and simple way to quantitate the inorganic cations.

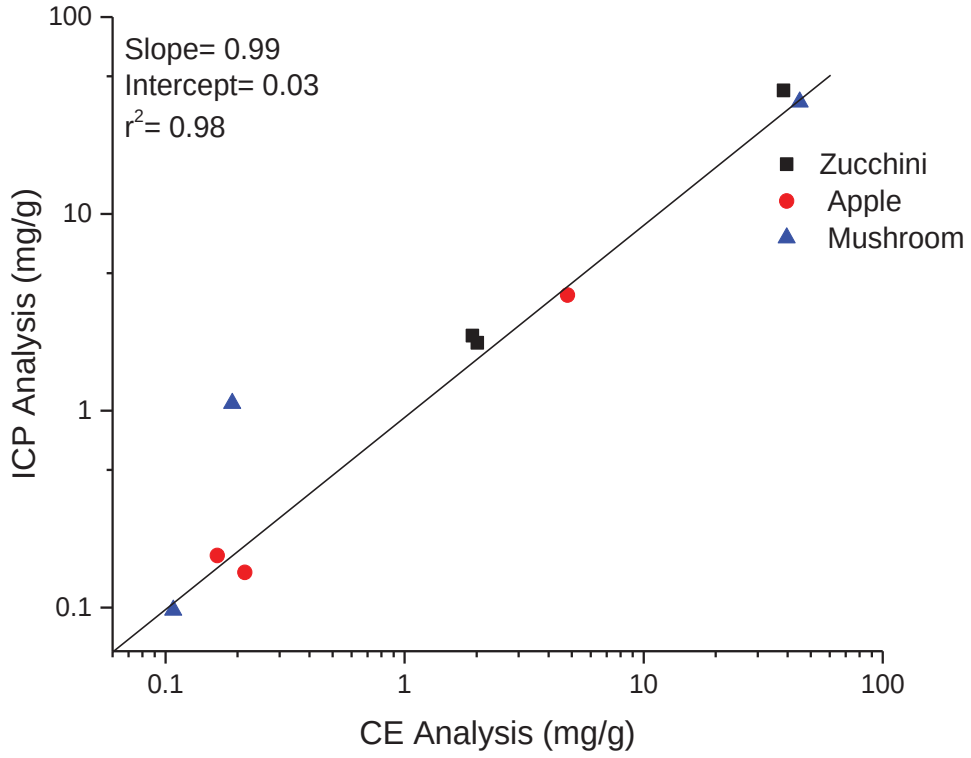


Fig 5. Comparison of average ICP-MS and CE concentrations of K^{+}, $Ca^{2+,}$ and Mg^{2+} in zucchini, apple and mushroom (n=3).

Table 2. Comparison of K, Ca and Mg concentrations (mg/g dry weight, 1g fresh weight = 0.25g dry weight) in four varieties of apples determined using external standards prepared from each variety.

		Calibration Matrix Type			
		Amount, mg/g (Accuracy %)			
Sample	Analyte	Red delicious	Fuji	Pink lady	Royal gala
Red delicious	K^+	8.40 (-)	9.60 (14)	9.20 (11)	8.80 (5.4)
	Ca^{2+}	0.0156 (-)	0.0168 (6.1)	0.0156 (0.5)	0.0212 (26)
	Mg^{2+}	0.244 (-)	0.208 (-16)	0.248 (1.7)	0.204 (-19)
Fuji	K^+	6.40 (-10)	7.20 (-)	6.80 (-6.7)	6.40 (-9.8)
	Ca^{2+}	0.352 (29)	0.248 (-)	0.320 (22)	0.292 (15)
	Mg^{2+}	0.224 (14)	0.192 (-)	0.228 (15)	0.188 (-1.1)
Pink lady	K^+	5.60 (2.5)	5.60 (7.9)	5.20 (-)	5.20 (-0.5)
	Ca^{2+}	ND*	ND*	ND*	ND*
	Mg^{2+}	0.224 (3.4)	0.184 (-17)	0.216 (-)	0.184 (-17)
Royal gala	K^+	9.20 (-7.2)	10.8 (9.6)	10.4 (5.7)	9.60 (-)
	Ca^{2+}	0.720 (-15)	0.52 (-17)	0.68 (22)	0.60 (-)
	Mg^{2+}	0.328 (19)	0.284 (7.3)	0.344 (16)	0.264 (-)

*ND= Not detected or below limits of detection

5.5. Conclusion

A novel, fast and inexpensive method for the determination of cations from the direct injection of fruits and vegetables into a capillary electrophoresis system is demonstrated. The approach has broad applicability to a range of fruits and vegetables, and comparison of the concentration of three cations (K^+, Ca^{2+}, Mg^{2+}) in three different matrices (apple, mushroom, zucchini) with quantitative data found to correlate well with ICP-MS. Differences between sample matrix mean that a matched calibration must be used, with quantitation between the same type of fruit/vegetable possible. This approach has potential applicability

for quantitative analysis of other analytes in a wide range of tissue samples. For example, determination of antioxidants in a variety of fruits and vegetables, pesticides and herbicides in plants, ascorbic acid concentration in citrus fruits and amines in fish. However, the applicability of this approach is limited to analytes which can be charged on application of voltage, as this technique considers electrokinetic injection only. The simplicity of this approach shows promises for implementation in a portable device for on-site food analysis.

5.6. References

1. Tůma, J.; Skalický, M.; Tůmová, L.; Bláhová, P.; Rosůlková, M. *Plant Soil Environ.* **2004**. 50, 18-26.
2. Genccelep, H.; Uzun, Y.; Tuncturk, Y.; Demirel, K. *Food Chem.* **2009**. 113, 1033-1036.
3. Tucker, K. L.; Hannan, M. T.; Chen, H. L.; Cupples, L. A.; Wilson, P. W. F.; Kiel, D. P. *Am. J. Clin. Nutr.* **1999**. 69, 727-736.
4. Jodral-Segado, A. M.; Navarro-Alarcon, M.; de la Serrana, H. L. G.; Lopez-Martinez, M. C. *Sci. Total Environ.* **2003**. 312, 47-58.
5. Greer, F. R.; Krebs, N. F. *Pediatrics* **2006**. 117, 578-585.
6. Song, Y. Q.; Manson, J. E.; Buring, J. E.; Liu, S. M. *Diabetes Care* **2004**. 27, 59-65.
7. Lavedrine, F.; Ravel, A.; Villet, A.; Ducros, V.; Alary, J. *Food Chem.* **2000**. 68, 347-351.
8. Metson, A. J.; Saunders, W. M. H. *New Zeal. J. Agri. Res.* **1978**. 21, 341-353.
9. Zekri, M.; Parsons, L. R. *Plant Soil* **1992**. 147, 171-181.
10. Kmiecik, W.; Lisiewska, Z.; Korus, A. *Eur. Food Res. Technol.* **2007**. 224, 573-579.
11. Nour, V.; Trandafir, I.; Ionica, M. E. *Not. Bot. Horti. Agrobot. Cluj. Napoca* **2010**. 38, 228-233.
12. Fredrikson, M.; Carlsson, N. G.; Almgren, A.; Sandberg, A. S. *J. Agric. Food Chem.* **2002**. 50, 59-65.
13. Kvasnicka, F. *J. Sep. Sci.* **2005**. 28, 813-825.
14. Sa´decka´, J.; Polonsky, J. *J. Chromatogr. A* **1999**. 834, 401-417.
15. Nascentesa, C. C.; Kornb, M.; Arruda, M. A. Z. *Microchem. J.* **2001**. 69, 37-43.
16. Fukushi, K.; Takeda, S.; Wakida, S.; Higashi, K.; Hiiro, K. *J. Chromatogr. A* **1997**. 759, 211-216.
17. Reyzer, M. L.; Hsieh, Y. S.; Ng, K.; Korfmacher, W. A.; Caprioli, R. M. *J. Mass Spectrom.* **2003**. 38, 1081-1092.
18. Seeley, E. H.; Caprioli, R. M. *Proc. Natl. Acad. Sci.* **2008**. 105, 18126–18131.

19. Metzner, R.; Schneider, H. U.; Breuer, U.; Schroeder, W. H. *Plant Physiol.* **2008**. 147, 1774-1787.

20. Zhang, H.; Gu, H.; Yan, F.; Wang, N.; Wei, Y.; Xu, J.; Chen, H. *Sci. Rep.* **2013**. 3, 1-6.

21. Oguri, S.; Nomura, M.; Fujita, Y. *J. Chromatogr. B-Anal. Technol. Biomed. Life Sci.* **2006**. 843, 194-201.

22. Wang, Y.; Hong, J.; Cressman, E. N. K.; Arriaga, E. A. *Anal. Chem.* **2009**. 81, 3321-3328.

23. Cecala, C.; Sweedler, J. V. *Analyst* **2012**. 137, 2922-2929.

24. Karel Klepárník ; František Foret. *Anal. Chim. Acta* **2013**. 800, 12-21.

25. Francois, C.; Morin, P.; Dreux, M. *J. Chromatogr. A* **1995**. 706, 535-553.

26. Gibert, C.; Chadouef, J.; Vercambre, G.; G´enard, M.; Lescourret, F. *J. Am. Soc. Hortic. Sci.* **2007**. 132, 583-591.

27. Gibert, C.; Génard, M.; Vercambre, G.; Lescourret, F. *Funct. Plant Biol.* **2009**. 37, 264–274

28. Shabala, S.; Schimanski, L. J.; Koutoulis, A. *Annal. Bot.* **2002**. 89, 221-226.

29. Koyyalamudi, S. R.; Jeong, S.-C.; Manavalan, S.; Vysetti, B.; Pang, G. *J. Food Compost. Anal.* **2013**. 31, 109-114.

30. Györfi, J.; Geösel, A.; Vetter, J. *J. Med. Food* **2010**. 13, 1510-1514.

31. Uzun, Y.; Genccelep, H.; Kaya, A.; Akcay, M. E. *Ekoloji* **2011**. 20, 6-12.

32. Martínez-Valdivieso, D.; Font, R.; Gómez, P.; Blanco-Díaz, T.; Río-Celestino., M. D. *J. Sci. Food Agric.* **2014**3171–3180.

33. Moggia, C.; Yuri, J. A.; Pereira, M. *Acta Hortic.* **2006**. 721, 265−271.

Chapter 6 Direct Injection of Amino Acids in Fruits and Vegetables for CE Analysis

6.1. Abstract

The potential of direct injection method was explored for the analysis of amino acids in zucchini. The electrokinetic injection of amino acids was carried in to the capillary without any sample preparation. Separation was achieved by CZE using 2.5 M acetic acid as the BGE and a capacitively-coupled contactless conductivity detector was used for detection. The uncontrolled hydrodynamic injection of tissue fluid into the capillary was minimised by using a BGE consisting of 5 % poly(ethylene oxide). Using this polymer the RSD between replicates (n=3) of zucchini was ≥10 %. Both poor sensitivity due to inherent low concentrations of amino acids in zucchini and poor peak efficiency were addressed by using a pre-concentration technique, isotachophoresis. The peak efficiencies were successfully improved for two amino acid standards i.e. histidine and valine from 2,300 and 13,000 to 112,500 and 234,375/meter, respectively, by using HCl as the leading electrolyte and hydroxyproline as the terminating electrolyte.

6.2. Introduction

Amino acids are organic compounds of biological significance consisting of an amine (-NH_2) and carboxylic acid (-COOH) functional group [1]. There are a variety of roles performed by amino acids of which the most important is their role in the synthesis of other molecules. For example, tryptophan, an amino acid, is required for synthesis of serotonin [2]; similarly, phenylalanine is used for synthesising various phenylpropanoids, which play important role in plant metabolic processes [3] and arginine is a precursor of nitric oxide which is vital for a variety of biological processes [4]. Humans and animals cannot synthesis all the amino acids required for essential biological processes and these amino acids are obtained through the consumption of a plant-based diet [5]. Therefore, the concentration of amino acids is often measured to estimate the nutritional value of fruits and vegetables.

Amino acid analysis in food has been achieved by a number of techniques including; colorimetry [6], gas chromatography [7], high performance liquid chromatography [8] and capillary electrophoresis (CE) [9-13]. CE analysis of amino acids has been reported extensively in a wide range of samples [9-13]. Analysis has been achieved by both CZE [14-16] and MEKC [17-19] in combination with a variety of detectors such as UV [20], LIF [15, 17], capacitively-coupled contactless conductivity detectors (C^4D) [15, 21], amperometric [11] and MS [22]. However, UV detection is the mode of choice due to ease of use, inexpensive analysis and availability of a wide range of well-developed methods. As amino acids [23] lack a chromophore, derivatization to impart UV absorbing characteristics is necessary for sensitive detection. The derivatising agents that have been used for amino acids include; 9-fluoroenylmethyl chloroformate (FMOC) [24, 25], dabsyl chloride [26], naphthalene-2,3-Dicarboxyaldehyde (NDA), *o*-phthaldehyde (OPA) [27], Phenylisocyanate [28] and fluorescamine, 2,4-dinitrophenyl(DNP), dansyl chloride (DNS), 6-ammoquinolyl-N-hydroxysuccinimidylcarbamate (AQC) [29]. However, derivatization is a complicated procedure and often results in the formation of unstable derivatives, side products or heat and light sensitive derivatives.

An alternative detector, C^4D, has become increasingly popular for simple and sensitive analysis without the need for derivatization. In C^4D, the electrode is placed outside the capillary and detection is dependent on differences in the conductivity of the analyte and background electrolyte solutions passing through the capillary. For C^4D, it is not important for the electrode to be in touch with the solution as it can sense the solutions inside the capillary without coming in direct contact [30]. C^4D has also been increasingly applied for the analysis amino acids with a variety of methods reported [21, 31-34]. In addition to using C^4D detection, the sensitivity can be further improved by pre-concentration of analytes before analysis. Isotachophoresis (ITP) has been successfully applied for the analysis of biologically significant analytes [35]. ITP provides excellent sensitivity enhancement and large sample volume loading it offers outstanding potential for quantification using C^4D [36].

In ITP, the sample is sandwiched between a highly mobile leading electrolyte (LE) and a very slow terminating electrolyte (TE). The analytes possess a high mobility in LE and slow in TE, therefore, when analyte ions enter from a highly mobile LE zone into less mobile TE zone they experience a decrease in speed and are stacked at TE/ LE interface [37]. As a result, all ionic analytes migrate with the same speed and form sharp boundaries of analytes. Therefore, not only improvement in sensitivity is observed but also better peak shapes are obtained.

Independent of which method is selected for determination of amino acids, the sample preparation step is always complicated and time consuming. This step usually involves freezing, grinding or crushing of leaves, extraction with a solvent, centrifugation and filtration of the extracted analytes [14]. For example; Warren and Adam (2000) extracted amino acids (for 30 min) from plant leaves using hot water, followed by centrifugation (5 min) prior to analysis by CE [12]. The sample pre-treatment can be avoided by directly injecting sample from fruit and vegetables. In Chapter 5, a CE method with indirect UV detection that allowed the electrokinetic injection of minerals directly from fruits and vegetable without any sample pre-treatment was developed. This method has the potential to be applied for direct injection of amino acids from fruits and vegetables. However, given the low concentration of amino acids in comparison to minerals, indirect detection is

unlikely to provide sufficient sensitivity. An alternative more sensitive detection mode, such as C4D, is preferred. This aim of this work was to investigate the potential for direct injection and analysis of amino acids in fruit and vegetables by CE.

6.3. Materials and Methods

6.3.1. Chemicals

Tryptophan, valine, proline, methionine, glutamine, histidine, glycine, glutamic acid, alanine, arginine, hydroxyproline and poly(ethylene oxide) (average, Mv Ca 600,000 inhibited 200-500ppm with BHT) were obtained from Sigma Aldrich Sydney, Australia. Threonine was obtained from Fluka, Buchs, Germany. Cysteine and glacial acetic acid were purchased from BDH Chemicals, Poole, England. HCl was purchased from Merck KGaA Darmstadt, Germany.

6.3.2. Instrumentation

Hewlett Packard 3D CE (Waldbron, Germany) instrument consisting of an on column diode array UV/ Vis detector was used for all analyses. On capillary detection was also achieved using a TraceDec®C^4D cell (Innovative Sensor Technologies, Innsbruck, Austria) which was placed inside the capillary cassette. The detector was operated at −12 Db and a gain of 150%; the filter function was kept off. Untreated fused silica capillary (Polymicro, Phoenix, AZ, USA) with a 50 µm internal diameter and a total length of 40 cm (effective length to UV detector= 31.5 cm and length to C^4D detector= 25 cm), was used for separation. For collecting the C^4D signals an Agilent 35900E analogue-to-digital convertor (Agilent Technologies, Waldbronn, Germany) was used throughout the study. Integration and processing of signals was achieved using 3D-CE Chem Station software.

The separation voltage was set at +20 kV and all separations were achieved with the cassette temperature set at 30 ^{o}C. The sample was injected electrokinetically at 5 kV for 60 s. These conditions (voltage, temperature and electrokinetic injection parameters) were kept constant throughout the analysis unless otherwise stated.

New capillaries were flushed with NaOH, then MilliQ water and finally BGE, each for 15 mins. The capillary was purged with BGE for 3 min between runs. At the end of each day, the capillary was flushed with NaOH and then milliQ water, each for 5 min.

6.3.3. Preparation of Solutions

The stock solutions (1000 ppm) of amino acids were prepared in milli Q water. From these stock solutions, standards of amino acids in the range of 10— 100 ppm were prepared and used for identification and calibration.

The background electrolyte, 2.30 M acetic acid (pH= 2.00), was prepared by adding 13.14 mL of glacial acetic acid (17.5 M) to the 100 mL volumetric flask and filling to the mark with milliQ water.

To prepare BGEs containing 3 %, 4 % and 5 % polymeric solutions, 3 g, 4 g and 5 g of polymer was added to the 100.0 mL of BGE and mixed with a magnetic stirrer for 30 min or until the polymer was completely dissolved. The viscous polymer solution was sonicated for 30 min or until all the bubbles were completely removed.

Leading electrolyte containing 0.05 M HCl was prepared in MilliQ water by adding 0.41 mL of 12.00 M HCl to 99.59 mL of water. For terminating electrolyte, 0.06 M HCl was prepared by adding 0.5 mL 12 M HCl to 99.50 mL of MilliQ water. To this solution, 0.26 g of hydroxyproline was added to make a 0.02 M solution.

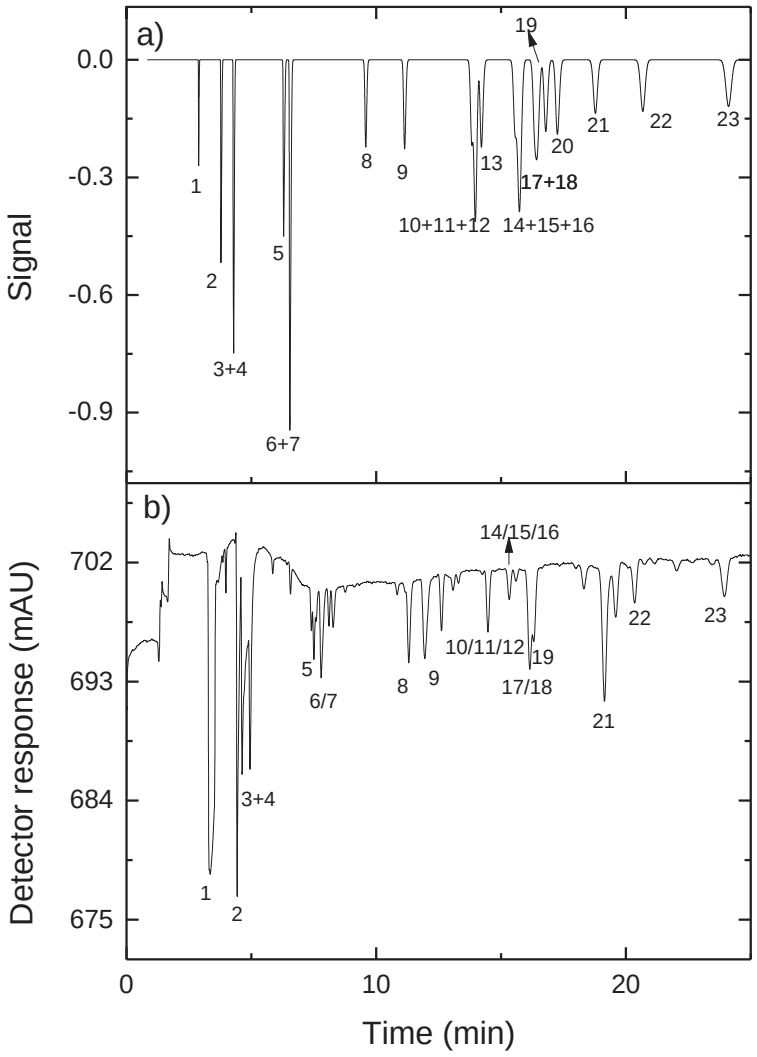
6.4. Results and Discussion

Sample pre-treatment is a complicated process and in some cases it takes more time than the actual analysis step. As demonstrated in Chapter 5, sample preparation can be avoided by injecting sample from plant tissues directly. In this study, the potential of this direct injection method was evaluated for the analysis of amino acids. As zucchini proved to be an excellent sample matrix, it was chosen again as a representative for direct injection of amino acids. For direct injection, a piece of zucchini was place inside the CE vial and sample was injected electrokinetically.

6.4.1. Direct Injection of Amino Acids from Zucchini

Preliminary experiments were carried to investigate what amino acids were likely to be extracted from zucchini and to obtain a rough estimate of their concentration. For that purpose, a reported method for determination of amino acids using C^4D was used with slight modifications [21]. Briefly, 2.3 M acetic acid was used as BGE without pH adjustment. The injection was carried electrokinetically from a piece of zucchini at +20 kV for 10 s. A longer injection (10 s) and a higher voltage (+20 kV) was attempted so as to maximise the number of analytes and their amount into the capillary. The resulting electropherogram showed the presence of approximately 28 analytes (Fig 1b). Peak Master (http://web.natur.cuni.cz/~gas/) was used to determine expected migration time for amino acids separated under the given conditions. The amino acids were predicted to elute in the region between 5 and 25 minutes which coincided with the elution of a large number of analytes. As anticipated the large peaks eluting early were attributed to the mineral cations (Fig 1a).

The previous work on direct injection (Chapter 5) of mineral ions showed the need to use a viscous buffer to minimise non-reproducible hydrodynamic injection due to pushing of tissue fluid into the capillary as soon as it enters the sample flesh [38, 39]. The addition of 2% HPMC to the buffer was sufficient to increase viscosity and minimise hydrodynamic injection. HPMC (2 %) was added to the acetic acid buffer, however, it gave unstable baselines and currents (Fig 2). It was suspected that the unstable current profile was because of hydrolysis of the HPMC polymer chain under acidic conditions [40]. Therefore, the preparation of HPMC polymer in a less acidic BGE (0.5 M instead of 2.3 M) was trialled. Unfortunately, decreasing the acid concentration of BGE did not improve the stability of the current.



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2 **Fig 1.** (a) Simulation from Peak master and (b) electropherogram for direct injection of
3 zucchini. Conditions: 2.3 M acetic acid, 20 kV, 10 s injection, +30 kV separation voltage, 50
4 µm, 60 cm capillary, 51.5 cm to UV and 42 cm to C^4D detector. Peak identification: 1. K^+, 2.
5 Ca^+, 3. Na^+, 4. Mg^{+2}, 5. lysine, 6. histidine, 7. arginine, 8. glycine, 9. alanine, 10. valine, 11.
6 isoleucine, 12. serine, 13. leucine, 14. threonine, 15. aspartic acid, 16. tryptophan, 17.
7 methionine, 18. glutamine, 19. glutaminc acid, 20. phenylalanine, 21. proline, 22. cysteine,
8 23. hydroxyproline.

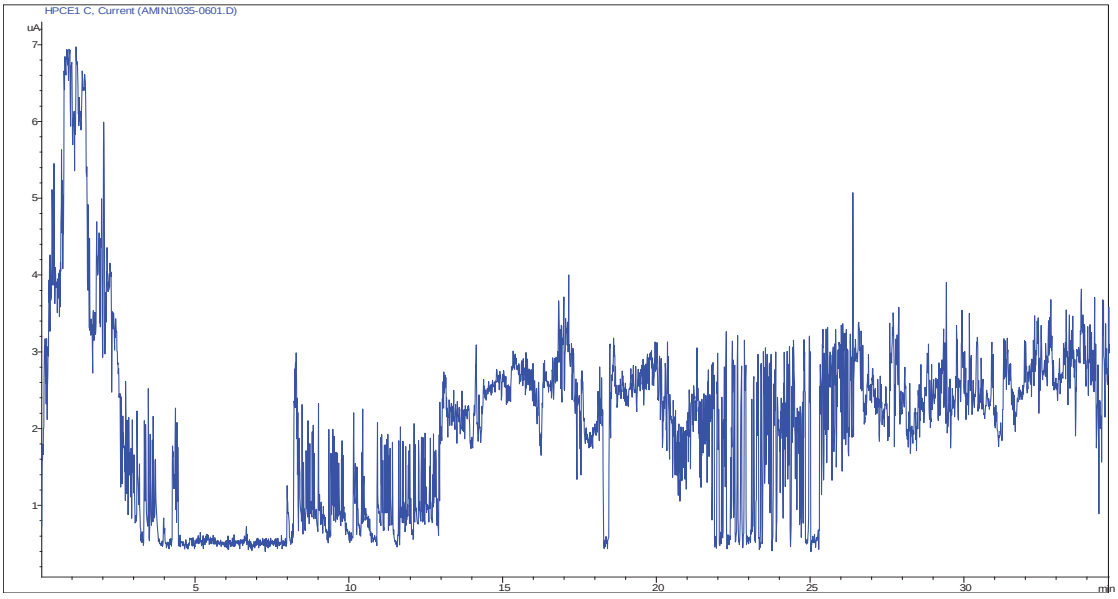


Fig 2. Unstable current profile using 2 % HPMC added to BGE.

6.4.2. Selection of Polymer

A number of polymers were tested for their ability to minimise hydrodynamic injection and provide stable baseline and current profile. Poly(ethylene oxide) (PEO) provided a stable current and hence a stable baseline. Initially, 3% PEO was used to achieve a constant current and baseline as the solution appeared reasonably viscous. As highly acidic conditions were suspected to be responsible for degradation of polymer, the concentration of BGE containing acetic acid was also kept at 0.5 M. Having achieved a stable baseline with PEO, experiments were then conducted to determine the optimum concentration of PEO required to minimise the hydrodynamic pushing of sample in to the capillary.

The concentration of PEO in BGE was varied from 3- 5 %. Above 5 % concentration, blockage of the capillary was observed. The best peak shapes and peak areas were obtained using 5% PEO in the BGE (Table 1). To observe consistency in peak shapes and peak area, three replicates were run from three pieces of zucchini using 5 % PEO concentration in the BGE.

Fig 3 shows that consistent peak shapes and peak area for all the amino acids for three replicates of zucchini. The variation in peak area for all the amino acids in three replicates was determined using the optimum PEO concentration (5%). The variation in peak area for the amino acids was found to be less than 10 % (Table 1). To identify the peaks in zucchini, standards of amino acids were run using 2.5 M acetic acid prepared in 5 % PEO. Thirteen amino acids standards available in our lab including; glycine, cysteine, valine, alanine, glutamine, glutamic acid, methionine, arginine, serine, threonine, proline, histidine, tryptophan were chosen for analysis. The electropherogram showing the migration time of all the amino acids is presented in Fig 4. It is evident that the amino acids peaks are broad, and this is reinforced by the theoretical plate numbers for the peaks (Table 2). The poor sensitivity [41] and peak shapes [42, 43] may be improved by pre-concentration of the sample prior to analysis. Isotachophoresis was chosen as a pre-concentration technique due to its ability to both improve peak shapes and sensitivity at the same time.

Table 1. Mean (n=3) and standard deviation in peak area of amino acid replicates using 5 % PEO.

	3 % PEO		4 % PEO		5 % PEO	
Amino Acid	Mean	RSD	Mean	RSD	Mean	RSD
Histidine	14.7	14.3	9.53	85.1	2.58	9.97
Arginine	8.69	99.5	5.47	35.2	2.83	7.41
Glycine	5.59	48.7	23.1	57.1	3.69	10.1
Alanine	10.6	46.7	25.3	96.5	8.09	7.78
Valine	5.61	47.3	5.99	68.5	6.42	8.34
Methionin/ Threonine	61.7	68.2	23.9	10.7	20.7	6.94

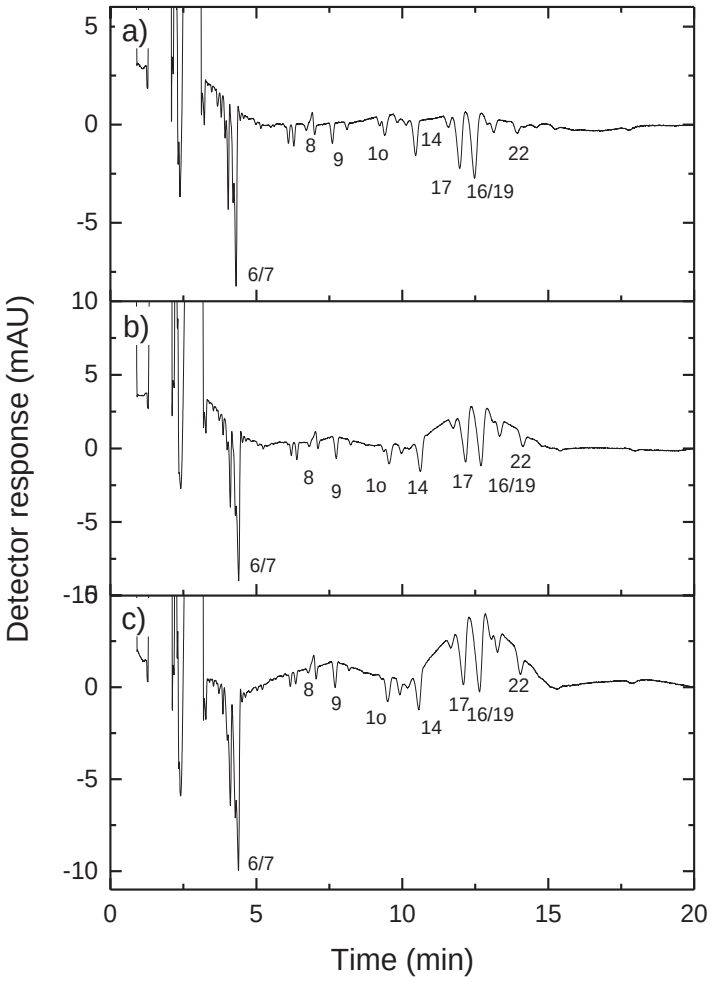
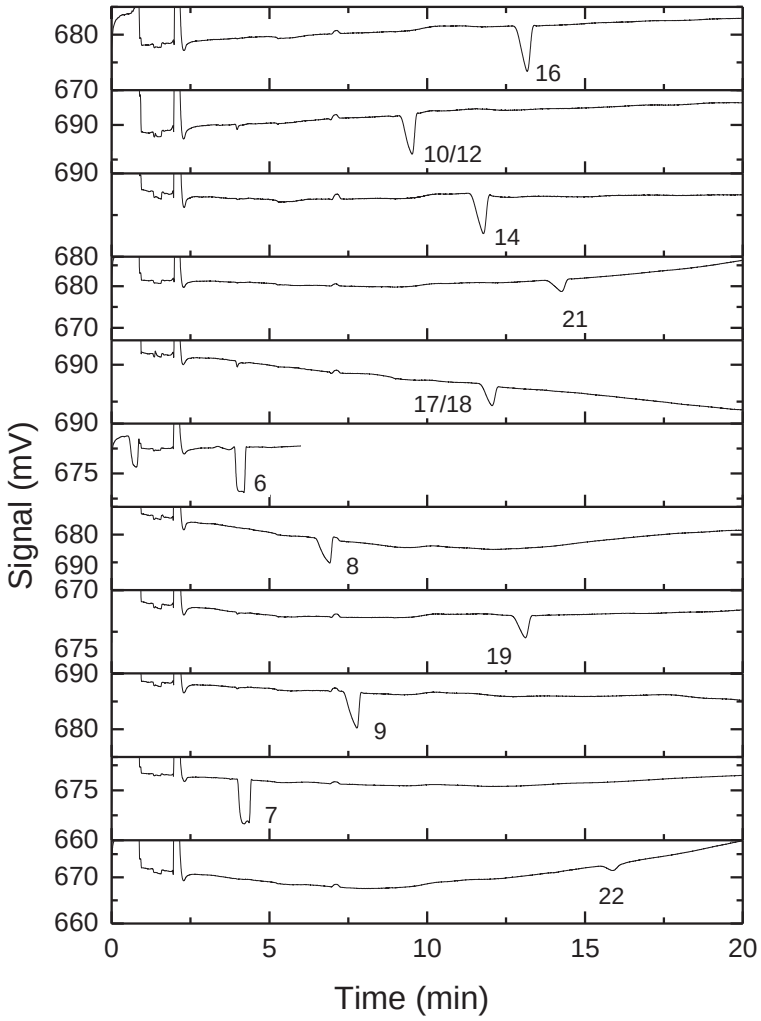


Fig 3. Electropherogram for direct injection three replicates (a, b, c) of zucchini. BGE; 0.5 M acetic acid containing 5 % PEO, +5kV, 60 s injection, +20 kV separation voltage, 50 µm, 40 cm capillary, 31.5 cm to UV and 25 cm to C^4D detector. Peak identification: 1. K^+, 2. Ca^{2+}, 3. Na^+, 4. Mg^{2+}, 5. lysine, 6. histidine, 7. arginine, 8. glycine, 9. alanine, 10. valine, 11. isoleucine, 12. serine, 13. leucine, 14. threonine, 15. aspartic acid, 16. tryptophan, 17. methionine, 18. glutamine, 19. glutaminc acid, 20. phenylalanine, 21. proline, 22. cysteine, 23. hydroxyproline.



1

2 **Fig 4.** Electropherograms for the amino acids standards (100 µg/ L). BGE; 0.5 M acetic acid
3 containing 5 % PEO, +5kV, 60 s injection, +20 kV separation voltage, 50 µm, 40 cm capillary,
4 31.5 cm to UV and 25 cm to C^4D detector. Peak identification: 6. histidine, 7. arginine, 9.
5 alanine, 10. valine, 12. serine, 14. threonine, 16. tryptophan, 17. methionine, 18. glutamine,
6 19. glutaminc acid, 21. proline, 22. cysteine.

7

Table 2. Theoretical plates and LODs for amino acids using 2.5 M acetic acid consisting of 5% PEO concentration.

Amino Acids	Theoretical plates/meter	LOD (ppm)
Alanine	7,000	0.02
Arginine	1,670	0.11
Cysteine	22,390	0.01
Glutamic acid	19,096	0.01
Glycine	5,532	0.03
Hipstidine	2,300	0.08
Methionine	18,280	0.01
Threonine	16,382	0.01
Proline	20,500	0.01
Tryptophan	21,776	0.01
Valine	13,000	0.01

6.4.3. Isotachophoresis of Amino Acids

For isotachophoresis of amino acids, the LE and TE were chosen from the literature. Gebauer *et al*. (1989) reported isotachophoresis of amino acids using hydroxyproline as a terminating electrolyte [37]. The simulations on peak master also indicated that hydroxyproline to be the slowest of all the amino acids at pH 2.5 (Fig 1a) and should be a suitable TE for pre-concentration of amino acids. Two amino acid standards, valine and histidine, were chosen for carrying out the pre-concentration experiments. The LE consisted of 0.05 M HCl and TE contained 0.02 M hydroxyproline in 0.06 M HCl. Initially, the LE was injected at 4 bar for 0.2 mins followed by sample (5 kV for 60 s) and TE at 4 bar for 0.2 mins. Using these injection parameters, improvements in peak shapes were obtained. The peak width for histidine and valine was reduced by half (0.14 and 0.18 to 0.05 and 0.0737 respectively) and is highlighted in a visual comparison between peak shapes for a separation

with and without isotachophoresis (Fig 5). Furthermore, the peak efficiency as measured using theoretical plates improved 12 times for histidine and 7 times for valine when isotachophoresis was performed (Table 3).

The injection time of LE and TE was optimised from 0.2 to 0.8 mins at 4 bar. The LOD and theoretical plates for injection times are given in Table 3. As it can be seen in Table 3 that maximum efficiency was obtained by injecting the LE and TE for 0.4 mins. The injection of LE and TE above 0.8 mins was not tested as injection longer than 0.8 min resulted in co-elution of histidine with LE peak and furthermore no significant improvement in peak efficiency was observed (Fig 7).

Table 3. Peak efficiency data for histidine and valine for optimisation of LE and TE injection varying from 0.0 min to 0.8 min carried at 4 bar.

Injection time (LE & TE)	Efficiency (plates/m)	
	Histidine	Valine
0.0 min	9,375	31,250
0.2min	75,000	181,250
0.4min	112,500	234,375
0.6min	81,250	181,250
0.8min	100,000	131,250

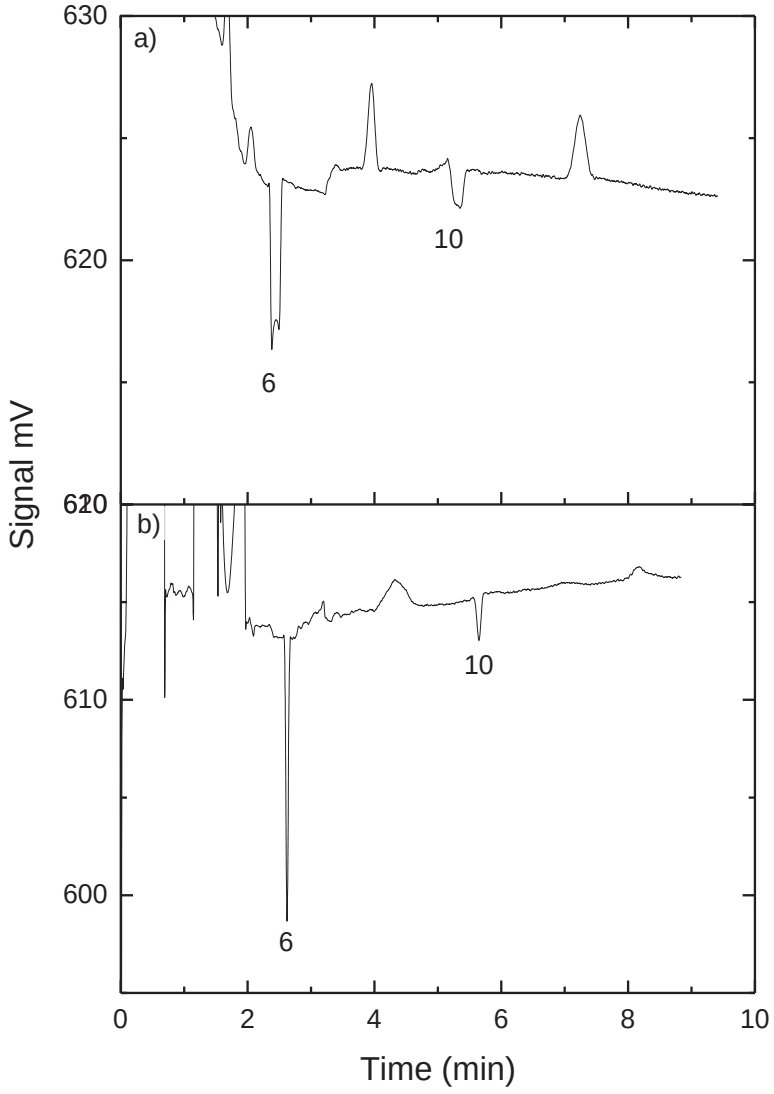


Fig 5. Electropherograms for separation of histidine and valine a) without stacking b) with stacking. Conditions; BGE comprised of 0.5 M acetic acid (pH not adjusted) in 5% PEO, LE was injected for 0.2 min at 4 bar followed by sample at 5 kV for 1 min and TE for 0.2 min at 4 bar, 50 µm, 40 cm capillary and +30 kV separation voltage. Peak identification: 6. histidine, 10. Valine.

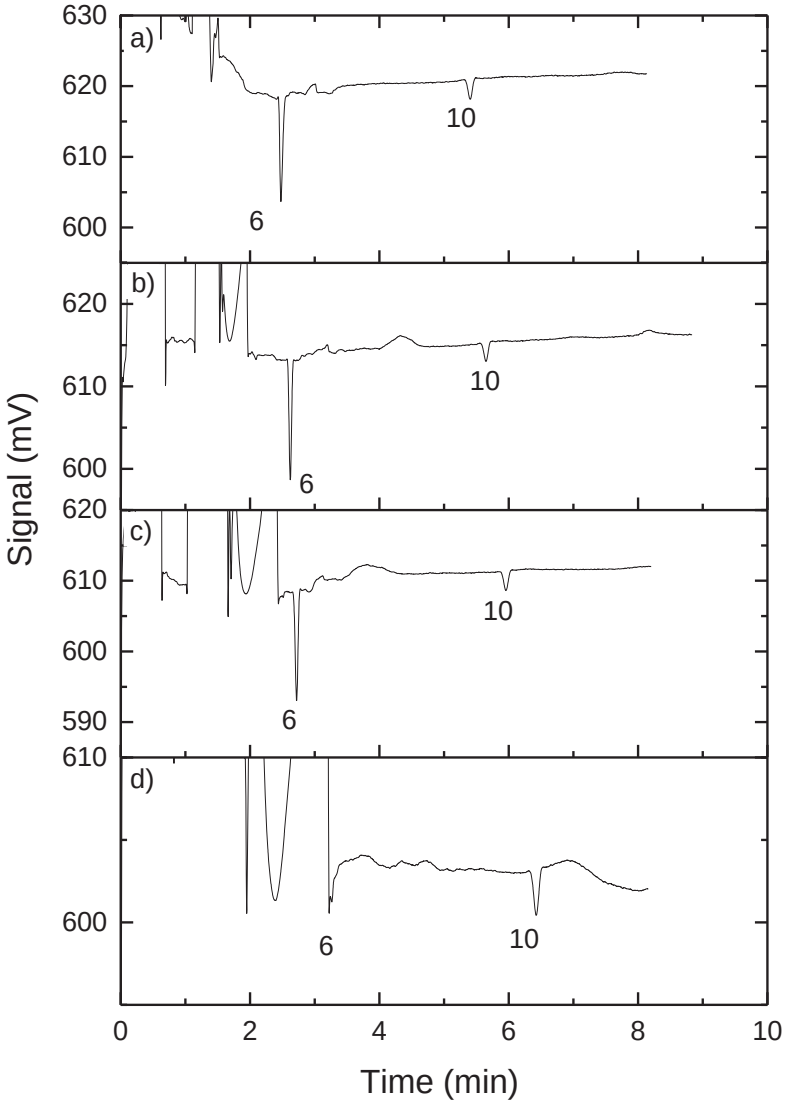


Fig 6. Electropherograms for separation of histidine and valine (10 µg/ L) a) Injection; LE= 0.2 min at 4 bar, sample= 5 kV, 1 min, TE= 0.2 min at 4 bar b) Injection; LE= 0.4 min at 4 bar, sample= 5 kV, 1 min, TE= 0.4 min at 4 bar c) Injection; LE= 0.6 min at 4 bar, sample= 5 kV, 1 min, TE= 0.6 min at 4 bar d) Injection; LE= 0.8 min at 4 bar, sample= 5 kV, 1 min, TE= 0.8 min at 4 bar. Conditions; BGE comprised of 0.5 M acetic acid (pH not adjusted) in 5% PEO, 50 µm, 40 cm capillary and +30 kV separation voltage.

6.5. Conclusion

The developed DI method for mineral was successfully applied for the electrokinetic injection of amino acids from zucchini. Using this method, it was possible to identify amino acids in zucchini. Isotachophoresis was successfully used to improve the peak shapes for two amino acid standards.

6.6. References

[1] Wagner, I.; Musso, H. *Angew. Chem. Int. Ed. Engl.* **1983**, 22, 816-828.

[2] Savelieva, K.V.; Zhao, S.; Pogorelov, V. M.; Rajan, I.; Yang, Q.; Cullinan, E.; Lanthorn, T. H. *Plos One* **2008**, 3.

[3] Ritter, H.; Schulz, G. E. *Plant Cell* **2004**, 16, 3426-3436.

[4] Tejero, J. S.; Biswas, A.; Wang, Z. Q.; Page, R. C.; Haque, M. M.; Hemann, C.; Zweier, J. L.; Misra, S.; Stuehr, D. J. *J. Biol. Chem.* **2008**, 283, 33498-33507.

[5] Kirma, M.; Araujo, W. L.; Fernie, A. R.; Galili, G. *J. Exp. Bot.* **2012**, 63, 4995-5001.

[6] Magne, C.; Larher, F. *Anal. Biochem.* **1992**, 200, 115-118.

[7] Molnar-Perl, I. *J. Chrom. A* **2000**, 891, 1-32.

[8] Sanchez-Machado, D.I.; Lopez-Cervantes, J.; Lopez-Hernandez, J.; Paseiro-Losada, P.; Simal-Lozano, J. *Chromatographia* **2003**, 58, 159-163.

[9] Wakayama, M.; Aoki, N.; Sasaki, H.; Ohsugi, R. *Anal. Chem.* **2010**, 82, 9967-9976.

[10] Zahou, E.; Jornvall, H.; Bergman, T. *Anal. Biochem.* **2000**, 281, 115-122.

[11] Guo, Y.; Colon, L. A.; Dadoo, R.; Zare, R. N. *Electrophoresis* **1995**, 16, 493-497.

[12] Warren, C. R.; Adams, *J. Exp. Bot.* **2000**, 51, 1147-1157.

[13] Arlt, K.; Brandt, S.; Kehr, J. *J. Chrom. A* **2001**, 926, 319-325.

[14] Chen, Z. L.; Warren, C. R.; Adams, M. A. *Chromatographia* **2000**, 51, 180-186.

[15] Cheng, Y. F.; Dovichi, N. J.; Subattomole Amino Acid Analysis by Laser Induced Fluorescence- The Sheath Flow Cuvette Meets Capillary Zone lectrophoresis. *Laser Techniques in Luminescence Spectroscopy*. Eds, Vodinh, T. Eastwood, D. L. Vol. 1066. 1990. 151-159.

[16] Vcelakova, K.; Zuskova, I.; Kenndler, E.; Gas, B. *Electrophoresis* **2004**, 25, 309-317.

[17] Carlavilla, D.; Moreno-Arribas, M. V.; Fanali, S.; Cifuentes, A. *Electrophoresis* **2006**, 27, 2551-2557.

[18] Viglio, S.; Fumagalli, M.; Ferrari, F.; Iadarola, P. *Electrophoresis* **2010**, 31, 93-104.

[19] Jaworska, M.; Szulinska, Z.; Wilk, M.; Anuszewska, E. *Talanta* **2010**, 83, 513-520.

[20] Soga, T.; Ross, G. A. *J. Chrom. A* **1999**, 837, 231-239.

[21] Coufal, P.; Zuska, J.; van de Goor, T.; Smith, V.; Gas, B. *Electrophoresis* **2003**, 24, 671-677.

[22] Olivares, J. A.; Nguyen, N. T.; Yonker, C. R.; Smith, R. D. *Anal. Chem.* **1987**, 59, 1230-1232.

[23] Zunic, G.; Jelic-Ivanovic, Z.; Colic, M.; Spasic, S. *J. Chrom. B* **2002**, 772, 19-33.

[24] Han, Y.; Chen, Y. *Electrophoresis* **2007**, 28, 2765-2770.

[25] Ptolemy, A. S.; Britz-McKibbin, P. *Analyst* **2005**, 130, 1263-1270.

[26] Sato, Y.; Bounoshita, M.; Iwaya, K.; Miyaji, T.; Saito, M. *Bunseki Kagaku* **2010**, 59, 823-838.

[27] Ptolemy, A. S.; Tran, L.; Britz-McKibbin, P. *Anal. Biochem.* **2006**, 354, 192-204.

[28] Montero, C. M.; Dodero, M. C. R.; Sánchez, D. A. G.; Barroso, C. G. *Chromatographia* **2004**, 59, 15-30.

[29] Underberg, W. J. M.; Waterval, J. C. M. *Electrophoresis* **2002**, 23, 3922-3933.

[30] Guijt, R. M.; Evenhuis, C. J.; Macka, M.; Haddad, P. R. *Electrophoresis* **2004**, 25, 4032-4057.

[31] Elbashir, A. A.; Aboul-Enein, H. Y. *Biomed. Chrom.* **2012**, 26, 990-1000.

[32] Johns, C.; Yang, W. C.; Macka, M.; Haddad, P. R. *J. Chrom. A* **2004**, 1050, 217-222.

[33] Mori, M.; Kaseda, M.; Yamamoto, T.; Yamada, S.; Itabashi, H. *Anal. Bioanal. Chem.* **2012**. 402, 2425-2430.

[34] Doan, T. K.; Kubáň, P.; Kubáň, P.; Kiplagat, I. K.; Boček, P.; *Electrophoresis* **2011**. 32, 464-471.

[35] Chiu, T.-C. *Anal. Bioanal. Chem.* **2013**, 405, 7919-7930.

[36] Kler, P. A.; Huhn, C. *Anal. Bioanal. Chem.* **2014**, 406, 7163-7174.

[37] Gebauer, P.; Krivankova, L.; Bocek, P. *J. Chrom.* **1989**, 470, 3-20.

[38] Wang, Y.; Hong, J.; Cressman, E. N.; Arriaga, E. A.; *Anal. Chem.* **2009**, 81, 3321-3328.

[39] Oguri, S.; Nomura, M.; Fujita, Y. *J. Chrom. B-Anal. Technol. Biomed. Life Sci.* **2006**. 843, 194-201.

[40] Dong, Z.; Choi, D. S. *Amer. Assoc. Pharm. Sci.* **2008**. 9, 991-997.

[41] Reinhoud, N. J.; Tinke, A. P.; Tjaden, U. R.; Niessen, W. M. A.; Vandergreef, J. *J. Chrom.* **1992**. 627, 263-271.

[42] Takeda, S.; Fukushi, K.; Chayama, K.; Nakayama, Y.; Tanaka, Y.; Wakida, S. *J. Chrom. A* **2004**, 1051, 297-301.

[43] Quirino, J. P.; Kim, J. B.; Terabe, S. *J. Chrom. A* **2002**, 965, 357-373.

Chapter 7 Discussion, Conclusions, and Directions for Future Work

This chapter will look at the findings of each study with regards to the research questions raised in Chapter 1, make conclusions on the basis of the results of each study and highlight possibilities for future research.

In this thesis, the ability of capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) to investigate three sets of plant analytes including plant osmoregulants, minerals, and amino acids was investigated. Firstly, the study focused on method development for analysing three commonly explored osmoregulants i.e. proline, betaine and mannitol. A review of the literature highlighted the use of separate methods to analyse each osmoregulant, therefore, this study focused on developing a method for simultaneous analysis of all three osmoregulants. Simultaneous analysis was challenging for CE as at high pH proline and mannitol only can be charged and betaine remains neutral, while at low pH proline and betaine are positively charged and mannitol is neutral and cannot be resolved from the matrix. Therefore, a capillary zone electrophoresis (CZE) method for the analysis of two commonly explored osmoregulants i.e. proline and betaine was developed. Using CZE in combination with indirect detection, allowed simultaneous and sensitive analysis of proline and betaine. This simple and fast method with baseline separation of proline and betaine in 10 min provided an attractive alternative to derivatisation. The developed method was successfully applied for separation and quantitiation of osmoregulants in spinach and beetroot ethanolic extracts.

Although the CE method provided superior sensitivity and rapid analyses of osmoregulants compared to previous reported CE methods, the separation mechanism in CE relies solely on mobility of analyte which means it is not possible to differentiate co-migrating species. However, the identification of the analytes could be verified using mass spectrometry (MS) detection. Therefore, for future work, it will be worth investigating MS detection coupled to

CE for sensitive and accurate detection of the osmoregulants. Furthermore, using MS detection, it would be possible to identify mannitol from other analytes on the basis of molecular mass even if it remained unresolved, thus making the simultaneous analysis of three osmoregulants possible.

Another approach that has potential to simultaneously determine all three omosregulants is a dual-capillary sequential injection-capillary electrophoresis (SI-CE) configuration that has been used for the simultaneous determination of cations and anions [1]. This unit has two capillaries in parallel, one at low pH and other at high pH, allowing the separation of cations and anions simultaneously. There is a possibility that the three osmoregulants can be analysed simultaneously using this simple and novel configuration. The SI-CE unit has only been used for the separations of inorganic anions and cations and a method for simultaneous determination of osmoregulants will provide an additional difficult and relevant application of the system. This method will allow the biologist studying water logging and salinity to analyse the osmoregulants in minimum time and cost when three of them are studied together.

The HPLC method described in chapter 4 demonstrates a quick and novel method for concurrent analysis of proline, betaine and mannitol. The combination of HPLC with evaporative light scattering detection (ELSD) resulted in enhanced sensitivity for three analytes compared to traditional available HPLC methods. The quick analyses and baseline resolution of three analytes under 20 min makes the current method superior compared to existing HPLC methods (40 min). The developed method was successfully applied for the quantitative analysis of osmoregulants in three halophytes including *Stylosanthes guianensis*, *Atriplex cinerea* and *Rhagodia baccata*. However, the universal nature of ELSD detection does mean that identification of the analyte is dependent on the retention time and in real samples co-elution of analytes is a real issue. Keeping that in mind, an alternative method using a C_{18} column with a completely different mechanism of separation was used for confirmation of results. As for the CE method, using a MS detector would overcome coelution issues.

Secondly, the study aimed at development of methods to address sample preparation challenges. In doing so, chapter 5 describes a method for direct injection of inorganic mineral cations from plant tissues. Using this method, it was possible to analyse inorganic mineral cations from plants without the need for extensive sample preparation procedures. The method was effectively applied for quantitation of inorganic mineral cations from a variety of fruits and vegetables including; zucchini, apple and mushroom. The direct injection method reduces the sample preparation to a minimum without any need to digest or extract the analytes from the matrix. Previously, there is no method available for direct analyses of inorganic minerals and there are only few methods available on minimising the sample preparation using CE. Furthermore, there are only two papers reported on direct injection from tissues using CE. These methods are either qualitative or involve complicated and time consuming procedures to prepare the capillary for direct injection from tissues. However the current CE method for direct injection does not require precise cutting of sample and allows simple and direct electrokinetic injection of cations from the whole fruits or vegetables without destroying the integrity of samples. The limitation of the described CE method is that it requires preparation of external standards for quantitation of mineral cations and the calibration curve is not useful for analyses of cations across different fruits. However, efforts have been made to broaden the application of the external calibration to analyse cations across different varieties of a fruit which makes it useful for studies investigating maturity, ripeness and heterogeneity. The potential of direct injection was also explored for amino acids (Chapter 6). The applicability of this method needs to be investigated for a range of real samples. More broadly, the direct method can be applied for determination of a diverse range of analytes in plants such as; antioxidants, ascorbic acid and other analytes. In addition to this, the potential of this approach to analyse compounds in other matrices such as fish, meat, and cheese should also be investigated.

In conclusion, the CE and HPLC methods developed and outlined in this thesis for the determination of osmoregulants will be useful to biologists studying water logging and salinity. A novel CE direct injection method has been developed and its use in the determination of cations and amino acids demonstrated, however, its potential application is much broader both with respect to analyte and sample.

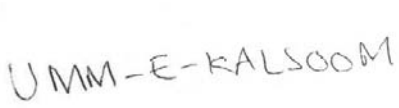
7.1. References

[1] Kuban, P.; Karlberg, B.; Kuban, P.; Kuban, V. *J. Chrom. A* **2002**, 964, 227-241.

Appendix A: Statement of Contribution

To Whom It May Concern,

I, Umme Kalsoom, was the major contributor (>50 %) for each of the publications listed below.

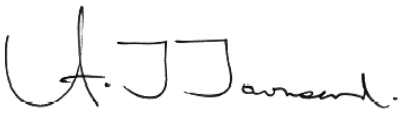


Signed: Umme Kalsoom (Date 10-12-14)

I, as a Co-Author, endorse that this level of contribution by the candidate

Signed: Mary Boyce (Edith Cowan University, Australia) (Date)

Signed: Michael Breadmore (University of Tasmania, Australia) (18-12-2014)



Signed: Ashley Townsend (University of Tasmania, Australia) (18-12-2014)

Signed: Rob Haselberg (University of Tasmania, Australia) (Date 10-12-2014)

Signed: Rosanne Guiljt (University of Tasmania, Australia) (Date)

List of Publications

1. Kalsoom, U.; Boyce M. C. *J. Agric Food Chem*. 2015. (Manuscript submitted) **(Chapter 2)**
2. Kalsoom, U.; Breadmore, M. C.; Guijt, R. M.; Boyce, M. C. *Electrophoresis*. 2014, *35,* 3379-3386. **(Chapter 3)**
3. 3. Kalsoom, U.; Boyce, M. C.; Bennett, I. J.; Veraplakorn, V. *Chromatographia* 2013, *76*, 1125-1130 **(Chapter 4)**.
4. 4. Kalsoom, U.; Guijt, R. M.; Boyce, M. C.; Townsend, A. T.; Haselberg, R.; Breadmore, M. C. *Anal. Chem*. 2015 (Manuscript submitted) **(Chapter 5)**

Appendix B: Published Paper 1 (Chapter 3)

Electrophoresis 2014, *35*, 3379–3386

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Research Article

Evaluation of potential cationic probes for the detection of proline and betaine

Osmoregulants are the substances that help plants to tolerate environmental extremes such as salinity and drought. Proline and betaine are two of the most commonly studied osmoregulants. An indirect UV CE method has been developed for simultaneous determination of these osmoregulants. A variety of reported probes and compounds were examined as potential probes for the indirect detection of proline and betaine. Mobility and UV-absorption properties highlighted sulfanilamide as a potential probe for indirect analysis of proline and betaine. Using 5 mM sulfanilamide at pH 2.2 with UV detection at 254 nm, proline and betaine were separated in less than 15 min. The LODs for proline and betaine were 11.6 and 28.3 μM, respectively. The developed method was successfully applied to quantification of these two osmoregulants in spinach and beetroot samples.

Keywords:
Betaine / Cationic probes / Indirect detection / Osmoregulants / Proline
DOI 10.1002/elps.201400303

1 Introduction

Environmental stresses such as salinity, drought, temperature extremes, and water logging effect the growth, productivity, and quality of plants [1]. To tolerate these stresses plants produce low molecular weight metabolites such as amino acids and quaternary ammonium compounds that are generally known as osmoregulants [2]. Proline is the most commonly studied amino acid osmoregulant [2] and glycine betaine (betaine) is the most commonly explored quaternary ammonium osmoregulant [3]. These osmoregulants protect plants in stressed environments by performing several functions including suppression of free radicals, regulation of osmotic balance, and storage of nitrogen and carbohydrates [4]. This basic understanding of the role of osmoregulants has resulted in an increased interest in the application to plants in order to increase yield and quality [5]. For this purpose, osmoregulants are applied externally [6] or plants rich in osmoregulants are selected for breeding by traditional means or by genetic engineering [7]. Therefore, the concentration of these osmoregulants is often studied to estimate a plant's ability to survive in stressed conditions or to determine the success of the new breeds.

There are a variety of methods reported for analysis

and CE. Furthermore, HPLC methods described vary in terms of sample preparation (e.g. derivatizing agents used) and detection mode [3, 9–12]. Similarly, CE analysis of proline has been reported with a variety of detection modes with UV and LIF being the most common ones. The commonly reported labeling agents for UV detection include 1-(9-fluorenyl)ethyl chloroformate, fluorescamine, 9-fluorenyl-methylchloroformate, o-phthalaldehyde, and phenylisothiocyanate and for LIF are fluorescein isothiocyanate, dansyl chloride, and o-phthalaldehyde [13].

Similarly, betaines have been analyzed both by HPLC and CE [14–21]. Using HPLC the betaines have been determined by ion exchange separation and UV detection at low wavelength [14–16]. Derivatization of betaines to impart UV-absorbing abilities and the use of C18 columns to improve retention have also been reported [18, 19]. CE analysis of *p*-bromophenacyl esters of betaines with UV detection using CZE [20] and MEKC [21] separation have been reported.

When a study involves both osmoregulants, that is proline and betaine, each analyte is usually determined by an individual method [22, 23]. However, there are some HPLC methods reported for simultaneous determination of proline and betaine. For example, Naidu reported HPLC-UV analy-

Appendix C: Published Paper 2 (Chapter 4)

Chromatographia (2013) 76:1125–1130
DOI 10.1007/s10337-013-2494-7

ORIGINAL

Simultaneous Determination of Key Osmoregulants in Halophytes Using HPLC–ELSD

Umme Kalsoom · Mary C. Boyce · Ian J. Bennett · Varaporn Veraplakorn

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Abstract Osmoregulants are the substances produced by plants that assist in tolerating environmental stresses. Three commonly analysed osomoregulants include mannitol, betaine and proline. A simple, sensitive and rapid HPLC–ELSD method has been developed for the simultaneous analysis of these common osmoregulants in plant extracts. Osmoregulants were extracted using 80 % ethanol and separated on an NH_2 column using 0.1 % formic acid and acetonitrile as the mobile phase. Retention time repeatability was 0.85, 1.50, and 0.93 % for mannitol, betaine and proline, respectively. The limit of detection (μmol) was 1.43×10^{-4}, 7.81×10^{-5} and 1.08×10^{-4} for mannitol, betaine and proline, respectively. The developed method was applied to three different plant extracts, *Stylosanthes guianensis*, *Atriplex cinerea* and *Rhagodia baccata*. A second method using a C18 column with 0.1 % heptafluorobutyric acid and acetonitrile as the mobile phase proved to be a useful complementary method for verifying tentative peak identifications.

Keywords Column liquid chromatography · ELSD ·

Introduction

Environmental stresses such as drought, salinity and temperature extremes adversely affect the growth and development of plants. To cope with these environmental factors, plants produce secondary metabolites including sugars, sugar alcohols, amino acids and quaternary ammonium salts which are collectively referred to as osmoprotectants or osmoregulants [1]. The three most commonly analysed osmoregulants are proline [1], mannitol [2], and glycine betaine (betaine) [3].

These osmoregulants increase the plant's tolerance to stress by performing various functions, such as, facilitating osmotic adjustments in water-stressed plants [4], scavenging of free radicals [5, 6], stabilization of the sub-cellular structures [7], storage of nitrogen and carbohydrates [3], and regulation of co-enzymes. Therefore, osmoregulants are often studied to estimate the tolerance of plants during environmental stress [8].

A number of methods have been reported for quantification of each osmoregulant. For example, proline has been