Edith Cowan University Copyright Warning

You may print or download ONE copy of this document for the purpose of your own research or study.

The University does not authorize you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site.

You are reminded of the following:

- Copyright owners are entitled to take legal action against persons who infringe their copyright.
- A reproduction of material that is protected by copyright may be a copyright infringement.
- A court may impose penalties and award damages in relation to offences and infringements relating to copyright material. Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.

Development of Capillary Electrophoresis for the Analysis of Phenolics and Glucoraphanin in Brassica oleracea

Ву

Iris Sau Lan Lee BSc(Hons)

A thesis submitted in fulfillment of the requirement for the degree of

Doctor of Philosophy

Faculty of Computing, Health and Science
School of Natural Sciences
Edith Cowan University

Principal Supervisor: Dr. Mary C. Boyce

Associate Supervisor: Assoc. Prof. Michael C. Breadmore

January 2012

USE OF THESIS

This copy is the property of Edith Cowan University. However the literary rights of the author must also be respected. If any passage from this thesis is quoted or closely paraphrased in a paper or written work prepared by the user, the source of the passage must be acknowledged in the work. If the user desires to publish a paper or written work containing passages copied or closely paraphrased from this thesis, which passages would in total constitute and infringing copy for the purpose of the Copyright Act, he or she must first obtain the written permission of the author to do so.

DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

i. incorporate without acknowledgment any material previously submitted for a

degree or diploma in any institution of higher education;

ii. contain any material previously published or written by another person except

where due reference is made in the text of this thesis; or

iii. contain any defamatory material.

iv. contain any data that has not been collected in a manner consistent with ethics

approval

I also grant permission for the library at Edith Cowan University to make duplicate copies of

my thesis as required.

Signature:

1. Lee Iris Sau Lan Lee

Date:

04/01/2012

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and gratutide to the following people.

First and foremost, i wish to thank my principal supervisor, Dr. Mary Boyce for her professional guidance and encouragement during the course of my PhD studies. Mary, it was a privilege to have you as my supervisor and thank you for imparting your extensive knowledge and experiences on capillary electrophoresis, and for all our formal and informal discussions. This project would not have started or finished if it had not been for your diligence and dedication to your role as principal supervisor.

I would also like to thank my associate supervisor, Assoc. Prof. Michael Breadmore (University of Tasmania, Hobart, Australia) for all his expert advice on CE via e-mail and for the week spent in Hobart teaching me CE-mass spectrometry. Michael, thank you for responding so promptly to all my e-mail enquiries despite having a busy travel schedule.

A big thank you to all those who provided technical assistance on this project and especially to Mr Mark Bannister, Ms Nardia Bordas, Mr Jason Tranter and Mr Simon Collins and also to school officers Mrs Yvonne Garwood and Mrs Anthea Ward for their administrative assistance.

Thank you to Dr. Rob Trengove (Murdoch University, Perth, Australia) for the use of the Agilent capillary electrophoresis unit and to Dr. Renato Lori (Research Centre for Industrial Crops, Bologna, Italy) for the kind donation of the glucoraphanin standard.

I thank Edith Cowan University for the provision of a scholarship to support my studies.

Thanks to all my colleagues in the office, both past and present (you know who you are) and especially to Mr Michael Pezzaniti. Thanks for being a good friend/coffee buddy and listening to my endless whinging!

And last but not least, a big thank you to my family, my husband Alan and my children Danielle and Ethan for their endless support and understanding during the course of my studies.

LIST OF ABBREVIATIONS

ANDSA Aminonaphthalene-1,3-Disulphonic Acid

BGE Background Electrolyte

C Catechin

CD Cyclodextrin

CE Capillary Electrophoresis
CFS Continuous Flow System
CITP Capillary Isotachophoresis

CMC Critical Micelle Concentration

CTAB Cetyltrimethylammonium Bromide

CV Coefficient of Variance

CZE Capillary Zone Electrophoresis

EA Ethyl Acetate Extraction

EC Epicatechin

ED Electrochemical Detection/Detector

EDTA Ethylenediaminetetraacetic Acid

EKC Electrokinetic Chromatography

EKI Electrokinetic Injection
EOF Electro-Osmotic Flow

ESI Electrospray Ionisation

EVOO Extra Virgin Olive Oil

FASI Field Amplified Sample Injection
FASS Field Amplified Sample Stacking

HDB Hexadimetrine Bromide

HP Hydroxypropyl

HPLC High Performance Liquid Chromatography

i.d. Internal DiameterIS Internal Standard

IT Ion Trap

ITP Isotachophoresis

LIF Light Induced Fluorescence

LLE Liquid-Liquid phase Extraction

LOD Limit Of Detection

LOQ Limit Of Quantification

LVSS Large Volume Sample Stacking

MEEKC Microemulsion Electrokinetic Chromatography

MEKC Micellar Electrokinetic Chromatography

MS Mass Spectrometry/Spectrometer

NACE Non Aqueous Capillary Electrophoresis

OZT Oxazolidine-2-Thione

PSP Pseudo-Stationary Phase

RSD Relative Standard Deviation

SC Sodium Cholate

SDC Sodium Deoxycholate

SDS Sodium Dodecyl Sulphate

SPE Solid Phase Extraction

TMAB Tetramethylammonium Bromide

TMAH Tetramethylammonium Hydroxide

TOF Time Of Flight

UHPLC Ultra High Performance Liquid Chromatography

UV-vis Ultra Violet - Visible

LIST OF PUBLICATIONS

Type of publication	Number	References
Papers in refereed journals	4	1 - 4
Posters at national and international conferences	2	5 - 6

- 1. S. L. Lee, M. C. Boyce, M. C. Breadmore (2011). A rapid quantitative determination of phenolic acids in *Brassica oleracea* by capillary zone electrophoresis. *Food Chemistry*, 127, 797 801. (Chapter 2)
- 2. I. S. L. Lee, M. C. Boyce, M. C. Breadmore (2011) Extraction and on-line concentration of flavonoids in *Brassica oleracea* by capillary electrophoresis using large volume sample stacking. Food Chemistry (in press). **(Chapter 3)**
- 3. I. Lee, M. C. Boyce, M. C. Breadmore (2010). Quantitative determination of glucoraphanin in Brassica vegetables by micellar electrokinetic capillary chromatography. *Analytica Chimica Acta, 663,* 105-108. **(Chapter 4)**
- 4. I. Lee, M. C. Boyce (2011). Extraction and purification of glucoraphanin by preparative high performance liquid chromatography. *Journal of Chemical Education*, 88 (6), 832-834. **(Chapter 5)**
- 5. I. S. L. Lee, M. C. Boyce. Sensitive detection of glucoraphanin by micellar electrokinetic chromatography. *ACROSS Symposium on Advance in Separation Sciences (ASASS)*, Hobart, Australia, 8 10 December 2008.
- 6. I. S. L. Lee, M. C. Boyce, M. C. Breadmore. Determination of phenolic compounds in *Brassica oleracea* by CE. 2nd Separation Science Singapore Conference and Exhibition, Singapore, 5 6 August 2010.

ABSTRACT

Capillary electrophoresis (CE) has been used increasingly more over the last decade in the area of food analysis. Numerous food products and food components have been analysed using this technique because of its high efficiency and short separation times. However, the inherent lack of detection sensitivity in CE combined with the complex matrices present in many food samples, especially those of plant origins, is one of the contributing factors to the limited development on CE in this particular area of food analysis. In this project, the potential of CE as a tool in the analysis of vegetables belonging to the family *Brassica oleracea* was investigated.

Capillary zone electrophoresis (CZE), the most frequently used CE mode in food analysis, has been employed to quantitatively determine the phenolic acids present in vegetables of *B. oleracea*. A simple and rapid CZE method for the baseline resolution of four hydroxycinnamic acids was developed. Peak efficiencies and separation time were optimised by adjustment of the borate buffer concentration (15 mM sodium tetraborate pH 9.13) with the optimum method having a separation time of 7 min with detection limits ranging from 1.1 to 2.3 mg/kg of vegetables.

The developed CZE method was applied to resolve the key flavonoids in broccoli extracts, however, detection sensitivity was poor for these compounds. To overcome this limitation an online pre-concentration method, large volume sample stacking (LVSS), was used to enable quantitative determination of flavonols in broccoli. This LVSS-CZE method allowed for the separation of two flavonols, kaempferol and quercetin, within 8 min with average enhancement factors of approximately 20 when compared to the original CZE method, giving detection limits of 0.6 and 0.9 mg/kg of broccoli. Resolution of the two flavonols was optimised by varying the borate buffer concentration and pH (the optimum values are 10 mM sodium tetraborate pH 8.40) and by using a longer capillary (85 cm). Different LVSS parameters including stacking voltage and sample injection times were also investigated.

Micellar electrokinetic chromatography (MEKC), the second most commonly employed CE mode in food analysis, was used for the quantitative determination of glucoraphanin, a predominant glucosinolate in broccoli. A MEKC system was developed in which the surfactant, sodium cholate was used as the pseudo stationary phase and

separation of glucoraphanin was achieved in less than 5 min with detection limits ranging from 0.1 to 4 mg/100g of vegetables or seeds. Furthermore, and as a direct result of the requirement for a glucoraphanin standard, a preparative HPLC experiment was devised for the undergraduate chemistry program within Edith Cowan University.

All the developed CE methods were validated with repeatability studies and linearity measurements and then successfully applied to the quantitative determination of the target analytes in a range of *B. Oleracea* vegetables and seeds. The accuracy of the CE quantitative data was ascertained by comparison to those from HPLC analysis.

TABLE OF CONTENTS

Title	i
Use of Thesis	iii
Declaration	iv
Acknowledgements	V
List of Abbreviations	vi
List of Publications	viii
Abstract	ix
Table of Contents	xi
Chapter 1 Introduction and Literature Review	
1.1 Introduction	1
1.2 Capillary Electrophoresis (CE)	4
1.2.1 Brief Historical Background	4
1.2.2 Basic Principles	5
1.2.3 Electro-osmotic Flow (EOF)	6
1.2.4 Modes of Operation	7
1.2.4.1 Capillary Zone Electrophoresis (CZE)	7
1.2.4.2 Micellar Electrokinetic Chromatography (MEKC)	8
1.2.5 Detection in CE	9
1.2.6 Sample Preconcentration in CE	10
1.2.6.1 Field Amplified Sample Stacking (FASS) and Field A	
1.2.6.2 Large Volume Sample Stacking (LVSS)	13
1.2.6.3 Sweeping	14
1.2.7 The Role of CE in the Analysis of Food	16
1.2.7.1 Phenolics	17
1 2 7 2 Glucosinolates	31

1.3 Project Aims	35
1.4 Research Outline, Methods and Techniques	36
1.4.1 Determination of Phenolics in Brassica oleracea by CZE a	nd with an On-line Pre-
concentration Method	36
1.4.2 Determination of Gluocoraphanin in Brassica oleracea by	MEKC and Isolation of
Glucoraphanin by Preparative HPLC	38
1.5 References	39
Chapter 2 A Rapid Quantitative Determinati Acids in <i>Brassica oleracea</i> by Capillary Zone Ele	
2.1 Abstract	52
2.2 Introduction	53
2.3 Materials and Methods	55
2.3.1 Chemicals and Samples	55
2.3.2 Buffers and Solutions	55
2.3.3 Extraction and Isolation of Phenolic Acids	56
2.4 Instrumentation and Conditions	56
2.4.1 CE	56
2.4.2 HPLC	57
2.5 Results and Discussion	57
2.5.1 Comparison of Solid Phase and Solvent Extraction	57
2.5.2 Capillary Zone Electrophoresis Method Development	58
2.5.3 Validation of CE Method	59
2.5.4 Application of CZE Method to Brassica Vegetables	62
2.6 Conclusions	62
2.6 Conclusions	

Chapter 3 Extraction and On-line Concentration of Flavonoids in *Brassica oleracea* by Capillary Electrophoresis Using Large Volume Sample Stacking

3.1 Abstract	67
3.2 Introduction	68
3.3 Materials and Methods	71
3.3.1 Chemicals and Samples	71
3.3.2 Buffers and Solutions	72
3.3.3 Extraction and Isolation of Phenolic Compounds	72
3.4 Instrumentation and Conditions	73
3.4.1. CE	73
3.4.2 HPLC	73
3.5 Results and Discussion	74
3.5.1 Optimisation of Acid Hydrolysis Conditions	74
3.5.2 Method Development and Optimisation	75
3.5.2.1 CZE	75
3.5.2.2 Large Volume Sample Stacking (LVSS)	77
3.5.3 Validation of CE Method	81
3.5.4 Application of CE Method to Broccoli	84
3.6 Conclusions	85
3.7 Acknowledgements	85
3.8 References	86
Chapter 4 Quantitative Determination of Glucorar Brassica Vegetables by Micellar Electrokinetic Chromatography	
4.1 Abstract	90
4.2 Introduction	91
4.3 Materials and Methods	93

4.3.1 Chemicals	93
4.3.2 Buffers and Solutions	93
4.3.3 Food Samples	93
4.3.4 Extraction of Glucoraphanin	94
4.4 Instrumentation and Conditions	94
4.4.1 Capillary Electrophoresis	94
4.4.2 High Performance Liquid Chromatography	95
4.5 Results and Discussion	95
4.6 Conclusions	99
4.7 Acknowlegdements	99
4.8 References	. 100
Chapter 5 Extraction and Purification of Glucoraphanin	by
Preparative High-Performance Liquid Chromatography	
5.1 Abstract	. 101
5.1 Abstract	.101
5.1 Abstract	. 101 . 102
5.1 Abstract	. 101 . 102 . 104
5.1 Abstract	. 101 . 102 . 104 . 104
5.1 Abstract	.101 .102 .104 .104
5.1 Abstract	. 101 . 102 . 104 . 104 . 104
5.1 Abstract	.101 .102 .104 .104 .105
5.1 Abstract	. 101 . 102 . 104 . 104 . 105 . 105
5.1 Abstract	.101 .102 .104 .104 .105 .105 .108
5.1 Abstract	.101 .102 .104 .104 .105 .105 .108 .109
5.1 Abstract	.101 .102 .104 .104 .105 .105 .108 .109 .110
5.1 Abstract	.101 .102 .104 .104 .105 .105 .108 .109 .110

Cha	apter 6 General Conclusions and Future Directions	120
	3.5.7 Elst of Oscial Journal Articles	113
	5.9.7 List of Useful Journal Articles	110
	5.9.6 Technical Notes	.119
	5.9.5 Capillary Electrophoresis as an Alternative to Analytical HPLC	118

Chapter 1 Introduction and Literature Review

1.1 Introduction

In recent years there has been growing public awareness that the consumption of a diet rich in fruit and vegetables may be linked to reduced risks of cardiovascular disease and cancer [1-5]. Fruit and vegetables contain bioactive compounds called phytochemicals which are known to display antioxidant and anticarcinogenic activities [6-7]. The vegetables in the species Brassica *oleracea* (e.g. broccoli, cabbage, cauliflower, Brussels sprouts and kale) possess appreciable levels of phytochemicals which include phenolics and glucosinolates [8-9].

Phenolics are a large and diverse group of antioxidant compounds which occur naturally in plants. They act as antioxidants by scavenging free radicals therefore inhibiting or breaking the chain reactions which cause cellular damage. Their basic structures all contain a phenol group – an aromatic ring with at least one hydroxyl substituent. The ability of the phenol group to reduce reactive free radicals by donation of electrons makes phenolic compounds good reducing agents and thus effective antioxidants. Phenolic compounds can be categorized into two groups – simple phenols and polyphenols depending on the number of phenol subunits present.

Phenolic acids come under the category of simple phenols and these organic acids are further divided into two major classes – hydroxycinnamic and hydroxybenzoic acids (figure 1). The number and position of hydroxyl groups and other substituents (R groups) present in each acid gives rise to the different varieties of acids in each category.

$$R_4$$
 R_3
 R_2

Hydroxybenzoic acid

 R_4
 R_3
 R_1
 R_2

Hydroxycinnamic acid

Figure 1. Basic structures of phenolic acids

Of all the polyphenols which occur naturally, flavonoids are the largest group and have been studied extensively [10-11]. Flavonoids are categorised into different classes e.g. flavonols, anthocyanidins, flavones, etc. The structure of each class is based on the flavan nucleus which consists of 15 carbon atoms in a three ring arrangement (C6-C3-C6) (figure 2). As with the phenolic acids, structural variations arise from the different positioning and number of hydroxyl groups and other substituents present.

Figure 2. Structure of the flavan nucleus

Most of the studies carried out on phenolic compounds of Brassica vegetables have used broccoli florets as the model since this vegetable is cultivated and consumed in many parts of the world [8,12-17]. The main phenolic compounds found in broccoli are flavonols and hydroxycinnamic acids (as conjugated derivatives). Price *et al.* identified two major, and three minor glycosides of quercetin and kaempferol in broccoli florets [18]. Vallejo and co-workers identified four major hydroxycinnamic acid derivatives [12]. In addition four minor sinapic acid derivatives and chlorogenic acid were found in broccoli inflorescences [12,19]. On several occasions, Vallejo's group quantitatively determined the phenolic contents in broccoli [20-22]. In one study, typical values ranging from 0 to 65.4 mg/kg for flavonoids and 0 to 111 mg/kg for hydroxycinnamic acid derivatives were reported in broccoli cultivars [21].

Glucosinolates are a large group of nitrogen and sulphur containing organic compounds. They are anionic thioglucosides whose structures differ according to the attached side chain (R group) which can be aliphatic or aromatic (figure 3). Hydrolysis of glucosinolates by the plant enzyme myrosinase releases isothiocyanates, compounds known to provide chemo-protection by suppression of phase I enzymes which are responsible for activation of carcinogens and induction of phase II detoxification enzyme systems.

OH
$$O = S = 0$$
HO OH $S = R$

Figure 3. Basic structure of glucosinolate

The main source of glucosinolates is in plants of the Cruciferae family. As such, most of the studies carried out on this naturally occurring compound have been on Brassica vegetables and in many instances broccoli. Vallejo *et al.* found eleven glucosinolates in broccoli florets with the three predominant ones identified as glucoraphanin, glucobrassicin and neoglucobrassicin [12].

Phenolics and glucosinolates in the B. oleracea group have traditionally been separated by high-performance liquid chromatography (HPLC) [21,23-25]. HPLC has been the technique of choice due to its robustness, the availability of a plethora of well developed methods and sensitive detection options. Another separation technique which has been used increasingly in the last decade for food analysis is capillary electrophoresis (CE). This relatively modern separation method offers many advantages such as short analysis times, increased efficiency enhancing the resolution of difficult to separate compounds and the flexibility and versatility to rapidly change separation mechanisms simply by changing the electrolyte composition. Furthermore, it also uses minimal reagents of low toxicity leading to low running costs and more environmental friendly testing, and thus has proved to be a highly efficient analytical technique rivalling with and complementing HPLC. Despite its many advantages, CE suffers from one major drawback and that is in its inherent lack of concentration sensitivity, particularly, when ultra violet (UV) detection is employed. However, these issues with detection sensitivity have mainly been resolved either by using 1) more sensitive detectors (e.g. mass spectrometers (MS)) or 2) sample pre-concentration techniques such as solid phase extraction (SPE) or stacking. SPE is often performed as part of the extraction procedure to isolate analytes of interest where as stacking is an on-line concentration method specific to CE in which analytes are focussed in the capillary prior to analysis.

1.2 Capillary Electrophoresis (CE)

1.2.1 Brief Historical Background

Electrophoresis is defined as the differential migration of charged species under an applied electric field and in 1937, Tiselius introduced moving boundary electrophoresis as a separation technique [26-27]. His experiments on the separation of serum proteins in free solution lead to the observation that the components in a sample migrated in a direction and at a speed as dictated by their charge and electrophoretic mobility.

One of the problems encountered in these early trials of electrophoresis in free solution was band broadening, a result of diffusion and convection caused by joule heating. This negative aspect of electrophoresis could be resolved by employing anti-convective media such as paper or gels. However, one of the major drawbacks in using supporting mediums like gels is that the entire electrophoretic process is protracted and more labour intensive as the gel needs to be prepared and analysis times are, in general, much longer due to the lower voltage which can be applied for these separations. Furthermore, it is restricted for those analytes which require staining for detection which is not quantitative and has a low dynamic range as it is largely dependent on the degree of association between the analyte and staining dye.

An alternative approach to reduce band broadening effects is to perform electrophoretic separations in narrow bore tubes or capillaries. The smaller volumes of liquid that are used combined with high surface area to volume ratio of narrow bore capillaries result in minimal heat generation and improved dissipation of the heat allowing for much higher electric fields to be applied. In 1967, Hjerten constructed the first fully automated capillary free zone electrophoresis apparatus [28]. He described the use of rotating 1 - 3 mm i.d. quartz glass tubes for the separation of a diverse range of analytes (e.g. organic and inorganic ions, proteins, viruses, etc) with UV detection. Since these were the smallest bore capillaries available at that time, rotation was necessary to reduce convection during free zone electrophoresis. In the 1970's, both Virtanen and Mikkers *et al.* reported on the use of 200 μ m i.d. glass and Teflon capillaries, respectively, as a means to further reduce band broadening [29-31]. However, it was not until 1981 that the first high performance CE separations were achieved by Jorgenson and Lukacs [32]. The use of 75 μ m i.d. fused silica capillaries allowed for application of high separation voltages (up to 30 kV)

whilst still providing efficient heat dissipation and excellent separation efficiencies of over 400 000 theoretical plates were obtained. Sensitive detection of the target analytes was achieved by on-column fluorescent detection.

Since that time and with the introduction of the first fully automated commercial capillary electrophoresis system by Beckman Coulter in 1989, significant advances in CE technology has been achieved in the last two decades especially in regards to improvements on detection sensitivity via the development of on-line concentration methods and the coupling of CE instruments to mass spectrometers.

1.2.2 Basic Principles

In CE, separation of analytes is achieved by differential migration of solutes upon the application of an electric field. Figure 4 shows a schematic diagram of a typical CE apparatus. The system consists of a fused silica capillary (usually around 30 to 100 cm long with an internal diameter (i.d.) ranging from 25 to 100 µm) with both ends submerged in a buffer solution (also known as a background electrolyte (BGE)). Also immersed in the solutions at each end are electrodes which are connected to a high voltage power supply. For the majority of applications, the sample is loaded onto the anodic (positive) end of the column and when a voltage is applied, the solutes travel through the column and elute at the cathodic (negative) end, where the detector is positioned. The applied potential difference across the capillary causes a phenomenon called electro-osmotic flow (EOF).

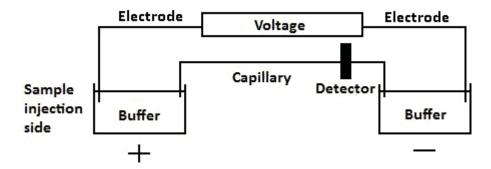


Figure 4. Schematic of a capillary electrophoresis unit

1.2.3 Electro-osmotic Flow (EOF)

Electro-osmosis plays a fundamental role in the operation of CE. It is the bulk flow of liquid along the capillary as a result of the surface charge on the interior capillary wall, and hence is given the name, electro-osmotic flow (EOF). In a fused silica capillary, this surface charge is generated in aqueous buffer from the formation of silanol (SiOH) groups. At low pH (approx. < 2.5), silanol groups are protonated i.e. they exist as neutral SiOH and the charge on the wall is nearly zero. At pH 3 and above, the wall is negatively charged due to the formation of deprotonated silanol groups i.e. SiO. The attraction of positive ions from the buffer to the negatively charged wall gives rise to an electric double layer (figure 5). This double layer is made up of the inner stem layer, which consists of positive ions held tightly to the negatively charged wall, and the outer diffused layer with mobile positive ions. On application of a voltage across the capillary, the hydrated positive ions which form the diffused layer migrate towards the cathode. This bulk movement of liquid along the column creates a dragging effect which causes the net migration of all species, regardless of charge, towards the cathode.

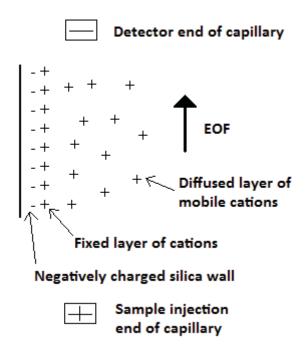


Figure 5. Electro-osmotic flow in a fused silica capillary filled with aqueous buffer

1.2.4 Modes of Operation

One of the versatile features of CE is that it can operate in numerous modes allowing separation of a diverse range of analytes (from protein macromolecules to small inorganic ions) for many different applications (food, pharmaceuticals, mining, environmental monitoring, etc). Capillary zone electrophoresis (CZE) is the simplest mode of CE and variations on this basic form gives rise to other modes such as micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF) and capillary isotachophoresis (CITP).

In the following sections, the discussion will focus on CZE and MEKC as they are the two most commonly used modes in CE and also because both methods are of relevance to this project.

1.2.4.1 Capillary Zone Electrophoresis (CZE)

In CZE, charged species, i.e. anionic and cationic solutes, are separated under the influence of an applied field and the EOF according to the vector sum of their electrophoretic mobility (μ_e) and the EOF (μ_{EOF}). The magnitude of μ_e is governed by their charge to radius ratio, while the direction is defined by the charge. Equation 1 describes the μ_e of an ion relative to its physical parameters.

For a typical CE set up using an unmodified fused silica capillary, sample is loaded onto the anodic end of the capillary and the detector placed at cathodic end. In this scenario, the EOF is towards the cathode and in accordance with equation 1, the migration order of ions will be: cations in order of decreasing mobility, neutral molecules, followed by anions in order of increasing mobility. For the fast separation of anions, it is necessary to use a reversed potential across the capillary such that the anode is at the detection end and to reverse the charge on the inner capillary wall so that the EOF is travelling in the same direction as the anions i.e. towards the detector. This may be achieved by coating the

wall with cationic surfactants, such as cetyl trimethyl ammonium bromide (CTAB), or crosslinked polymers such as polyethyleneimine. Although CZE is effective in separating ions, neutral solutes cannot be resolved by this method as their inherent electrophoretic mobility is zero and therefore they will all migrate with the EOF. To enable the separation of neutral molecules, another mode of CE, MEKC is employed.

1.2.4.2 Micellar Electrokinetic Chromatography (MEKC)

In 1984, Terabe *at al.* introduced MEKC, a CE mode depicted as a cross between electrophoresis and chromatography and has become one of the most widely used modes in CE along with CZE [33]. This technique allows for the simultaneous separation of both neutral and charged species. To facilitate the separation of neutral solutes, a surfactant is added to the BGE above its critical micelle concentration (CMC). At concentrations above their CMC, individual surfactant molecules aggregate to form micelles, which are spherical entities with hydrophobic tails positioned in the centre away from the hydrophilic buffer and charged heads located outside and towards the aqueous buffer. As the micelles are charged, they act as a pseudo-stationary phase (PSP) for the analytes and it is the partitioning of the analytes between the moving micellar and aqueous phases which effects a separation. Micelle and solute interactions are electrostatic and/or hydrophobic in nature. For a neutral species, the interactions are essentially hydrophobic where as for a charged species, they are a mixture of electrostatic and hydrophobic interactions.

The anionic surfactant sodium dodecyl sulphate (SDS) and the cationic surfactant, CTAB are commonly used in MEKC. In a typical CE set up (figure 4), anionic surfactants migrate towards the inlet end of the capillary i.e. against the EOF. However, the magnitude of the EOF is usually large enough to overcome the migration velocity of the micelles so that the net movement is in the direction of the EOF and towards the detector. For separations using cationic micelles, the surfactant coats the inner capillary wall replacing the negatively charged silanol groups with positive charges which results in reversal of the EOF, and hence the applied potential must also be reversed for the separation. In both instances, analytes with a strong electrostatic or hydrophobic interaction with the micelles will have increased migration times.

A variation of the MEKC mode is microemulsion electrokinetic chromatography (MEEKC). In MEEKC, small oil droplets along with a surfactant and a co-surfactant (usually

alcohol) are incorporated into an aqueous buffer to form a microemulsion which acts as the PSP in the separation. The separation mechanism is similar to MEKC in that analytes are partitioned between two phases but instead of micelles, the analytes are separated according to their differential interactions with the oil droplets and aqueous buffer.

1.2.5 Detection in CE

As mentioned in section 1.1, CE is a relatively new technique when compared to HPLC and offers many advantages over HPLC which includes short analysis time and high separation efficiencies. These two characteristics can be attributed to the use of narrow bore capillaries and very high voltages (10 - 30 kV) [32]. However, one negative aspect of using narrow bore capillaries with a small internal diameter is the limitation on detection sensitivity. This limitation arises for two reasons, the first because only nL of sample is injected and is restricted to 5 % or less of the total capillary volume as unless special precautions are taken, larger volumes lead to overloading and contributes to band broadening and a decrease in resolution. Second, with the exception of MS, detection in CE is performed most commonly on-capillary as part of the separation, which is advantageous because there is no band broadening caused by dead volume and mixing of separation components leading to a loss in resolution, which is typically observed in HPLC separations.

The most commonly used detector in CE is a UV absorbance detector, with detection at a fixed wavelength, multiple wavelengths or over an entire spectrum using a photo diode array. For UV transparent capillaries, direct detection is achieved through the Teflon coating and is defined by a small window in the interface in which light passes through. For polyimide coated capillaries, a small window is created by etching or burning off the outer coating of the capillary thus allowing for direct detection of analytes as they separate. The amount of light absorbed by an analyte i.e the absorbance, A, is governed by the Beer-Lambert law (equation 2).

A =
$$\epsilon$$
 C I where ϵ = molar absorptivity of the analyte
$$C = \text{concentration of the analyte} \tag{2}$$

$$I = \text{path length}$$

It is evident from equation 2 that, for any given analyte at a specific concentration, the intensity of light absorbed is dependent on the pathlength of the capillary. For CE, the optical pathlength is small and approximately equal to the i.d. of the capillary (typically 50 or 75 μ m) where as for HPLC the detection cell is usually 1 cm wide and hence a decrease in detection sensitivity of approximately 100 fold is observed for an analyte separated by CE when compared to that of HPLC.

One approach to increase sensitivity is to use more sensitive detection methods (e.g. mass spectrometry (MS), light induced fluorescence (LIF) electrochemical detection (ED)). However, these detection options have their drawbacks. For example, a time consuming sample derivatisation step is usually required for LIF detection as many substances are not naturally fluorescent and whilst MS provides both increased detection sensitivity and structural information in CE analysis, interfacing between instrument and detector is not straight forward and there is typically a dilution of the capillary effluent from a sheath flow used to increase stability of coupling CE and MS. In addition, these detectors are expensive and beyond the budget of many research and routine laboratories.

Another alternative way to increase detector response is to increase the analyte concentration within a sample prior to analysis (the C term in the Beer-Lambert law). Sample pre-concentration can be achieved off-line but over the last two decades developments in on-line concentration strategies for CE have provided reliable and alternative methods to pre-concentrate samples for both qualitative and quantitative determinations.

1.2.6 Sample Preconcentration in CE

Off-line concentration methods are performed out of the capillary and include liquid-liquid phase extraction (LLE) and SPE. Generally, LLE and SPE are methods used as a clean up step to isolate analytes from the sample matrix but simultaneously serve the purpose of also pre-concentrating analytes provided that the target analytes are eluted in a smaller volume from which they were extracted. Although off-line techniques are efficient in concentrating samples they are tedious and labour intensive involving multiple steps leading to an increase in systematic errors. For example, incomplete transfer of solute from liquid to liquid phase in LLE, sample overload or analyte adsorption onto SPE cartridges or just over handling of the sample during these processes may result in a loss of solute or

sample contamination and thus errors in qualitative and quantitative determinations. In contrast, on-line concentration is performed in the capillary prior to separation thus sample pre-treatment is limited. In some instances, a combination of off-line and on-line pre-concentration of a sample is required to obtain sufficient sensitivity.

The two most common approaches to on-line pre-concentration are stacking and sweeping. Both techniques are based on the differences in velocities of the analytes between the sample and buffer zone. Stacking involves changes in electric field strength, induced by differences in conductivity between the sample and the buffer, to manipulate the velocities of the analytes whereas sweeping relies on the picking and accumulation of analytes using a PSP and is confined to MEKC. In the following sections, three commonly used on-line pre-concentration techniques will be discussed, two of which rely on field induced changes to influence the velocity of the analytes (field amplified sample stacking (FASS)/field amplified sample injection (FASI) and large volume sample stacking (LVSS)), and the other is based on chemically induced changes on the velocity of the analytes (sweeping).

1.2.6.1 Field Amplified Sample Stacking (FASS) and Field Amplified Sample Injection (FASI)

Field amplified sample stacking (FASS) was first introduced by Mikkers *et al.* and is the simplest form of stacking. It provides a 10 to 20 fold increase in sensitivity and requires that the conductivity of the sample matrix is at least ten times lower than that of the running buffer [29]. Samples are hydrodynamically injected into the capillary and upon application of a voltage, the sample zone experiences a greater electric field (because of the differences in conductivity) and hence a greater mobility than the separation zone which causes the analytes in the sample zone to move more rapidly. When the analytes reach the sample/separation boundary, they will experience a drop in potential (and therefore a decrease in velocity) and sample stacking takes place at the boundary. This form of stacking is also referred to as normal stacking mode (NSM) and is a universal method for sample pre-concentration due to its ease of operation and in many cases, FASS is performed inadvertently because of natural conductivity differences between sample and buffer. However, the drawbacks with FASS are that it is restricted to low conductivity samples and small sample volumes. Typically, less than 5 % of the total capillary volume can

be filled with sample and using larger sample volumes induces band broadening and decrease resolution [34]. This phenomenon is caused by the mismatch in local EOFs between the sample matrix and separation buffer generating a pressure difference between these zones and resulting in laminar flow and dispersion of analytes.

Further sensitivity (generally 100-1000 fold) can be achieved by electrokinetic injection (EKI) of charged analytes. When employed under field amplified conditions (i.e. the sample has a conductivity ten times lower than that of the BGE), this technique, is known as FASI. It is similar to FASS in that field amplified conditions are employed but more analyte ions per physical volume of sample are loaded onto the capillary as analytes enter the capillary due to electrophoretic migration and EOF [35]. Large enhancements will only occur if the electrophoretic mobility of the ion is in the same direction as the EOF otherwise hydrodynamic injection is favoured. Furthermore, the electrophoretic mobility of the ions and EKI parameters (i.e. time and applied voyage) must be considered when using FASI to enhance sensitivity. The amount of analytes injected into the capillary is proportional to the electrophoretic mobility of the ion and controlled by the stacking time and voltage. Clearly, more ions with a higher mobility are injected than those with a lower mobility and similarly, the stacking efficiency is enhanced with an increase in stacking time and voltage. However, the amount of analytes that may be loaded is limited by peak band broadening and loss of resolution of solutes.

Detection sensitivity may be further improved by hydrodynamic injection of a low conductivity solvent prior to electrokinetic sample injection [36]. Low conductivity solvents provide a high field zone at the injection point where the velocity of the analytes is increased until they reach the solvent/buffer interface and stack. As a result of this field amplified zone, stacking efficiency is enhanced at the solvent/buffer boundary. Whilst FASI (with or without a solvent plug) has been used extensively over the last two decades, this stacking technique still remains popular due to its simplicity and effectiveness in concentrating analytes and a large number of recent articles have reported on the application of FASI using a solvent plug for optimised stacking [37-41]. While impressive results have been obtained with EKI, one of the most significant problems of this approach is its intolerance to variations in sample matrix. Any variation in sample matrix changes the electric field distribution which influences the migration of the ions that enter the capillary. Further, as ions preferentially migrate into the capillary as a function of their electrophoretic mobility, this can result in further reductions, making quantification via

external calibration difficult unless sample matrix variability can be eliminated (often with off-line treatment).

1.2.6.2 Large Volume Sample Stacking (LVSS)

Large volume sample stacking (LVSS), as its name implies, allows for large volumes of sample to be injected into the capillary which can then be stacked without the detrimental effects of peak band broadening associated with FASS. Following hydrodynamic injection of a large volume of sample (up to 100 % of the capillary volume), stacking is most simply achieved by polarity switching i.e. the stacking voltage applied is opposite to that of the separation voltage, enabling the sample matrix to be removed from the capillary inlet by the EOF while the analytes stack at the sample/buffer zone [42]. In this way, the band broadening mechanism is removed allowing for an increased volume of sample to be loaded onto the capillary. However, it is crucial that the applied potential is reversed and separation begins before the stacked analyte zone exits the capillary inlet. This requires the stacking current to be monitored, and the polarity to be switched when the current reaches around 95 % of the value when the capillary is completely filled with separation buffer. The conductivity and injection length of the sample must also be taken into consideration when stacking is controlled by current monitoring. For most CE instruments, the polarity switch is instigated by timing when the stacking/matrix removal step is completed which may be difficult given that the rise in electric current is rapid towards the end of stacking and thus can lead to further irreproducibilities in the migration times. Honegr et al. developed a LVSS method with polarity switching to quantitatively determine polyphenols in medicinal plant extracts [43]. The method allowed for 50 % of the capillary to be filled with sample and average enhancement factors of 90 when compared to conventional CZE. Precision of the method was satisfactory, with relative standard deviations of less than 1 and 6 % for migration times and peak areas, respectively, but an internal standard was required to correct for injection errors and fluctuation of peak areas.

An alternative approach to matrix removal in LVSS is by chemical control of the EOF to enable the transition from stacking to separation. This can be achieved by adding a dynamic EOF reversal agent to the separation buffer [44-45]. During stacking/matrix removal, the EOF reversal agent enters the capillary from the detection end until a point is reached when the EOF in the separation electrolyte is greater towards the direction of the

detector than the EOF in the sample matrix (which is moving in the opposite direction towards the inlet end) causing stacking/matrix removal to stop and the separation to begin. Compared to polarity switching, this method is simpler in that stacking and separation are chemically controlled and allows for matrix removal without manual intervention. For this approach to work, it is not necessary to completely reverse the EOF, suppressing the EOF will achieve the same result. This was initially demonstrated by He *et al.* when they used low pH buffers combined with high pH samples to provide a reduced EOF environment for stacking and separation. This method is similar to the above in that EOF is used as a pump to remove the sample matrix whilst the analytes stack and is known as LVSS with an EOF pump. EOF can also be suppressed by using other chemicals, such as diethylenetriamine or polyethylene oxide, however the downside of this approach is often elongated analysis times [46-47].

1.2.6.3 Sweeping

This mode of preconcentration was studied extensively in the late 1990's to early 2000's by Quirino and Terabe [48-57]. As demonstrated by their studies and subsequent work by other researchers, an extensive range of variations on this technique exist but all are dependent on the same basic principles. Sweeping relies on the picking and accumulation of analytes by a PSP which migrates to the sample zone upon application of voltage and only occurs when the PSP in the BGE is absent from the sample matrix [58]. In general, the sample is prepared in a matrix with the same conductivity as the BGE and loaded onto a BGE filled capillary. On application of a voltage, the PSP enter the sample zone from the BGE and the analytes are picked and accumulated (swept) into concentrated zones at the sample/BGE boundary. The length of the concentration zones i.e. swept zones (I_{sweep}) is described by equation 3.

$$I_{sweep} = I_{inj} (1 / 1 + k)$$
 where $I_{inj} = I_{inj} = I_{i$

The retention factor k, is a measure of the ratio of moles of solute in the PSP (retained solute) versus those in the aqueous phase (unretained solute). Equation 3 shows that at a fixed injection length, the swept zone of a more highly retained solute (i.e. larger

retention factor) will be narrower than that of a less retained solute. It is clear from this equation that by increasing the retention factor of the analytes, pre-concentration of the sample can be optimised. Usually, the PSP in sweeping consists of micelles formed by surfactants above their CMC and therefore this on-line pre-concentration technique was confined to MEKC. However, other PSPs such as ionic polymers and complexing agents e.g. borates have been utilised in electrokinetic chromatography [59-60].

In their initial work on this mode of pre-concentration, Quirino and Terabe explored sweeping in combination with a number of different strategies employed in other stacking methods [49,52-54,61]. For example, sweeping and LVSS with or without polarity switching or with a water plug, where the micelles enter from the inlet end of the capillary and sweep neutral analytes in order of decreasing retention factors to the sample/BGE boundary and stack whilst the sample matrix/water plug is simultaneously removed [49,54,61]. Enhancement factors of up to 100 fold were achieved using these preconcentration methods. FASI was also combined with sweeping for sample preconcentration but in these instances, the neutral analytes were prepared in a micellar matrix to impart a charge on these molecules and allow for EKI; and prior to sample injection, a water plug was injected to create a field enhanced region for the micellar analytes to pre-concentrate [52-53]. Similar to FASI in CZE where the amount of analytes injected is dependent on the electrophoretic mobility of the ions, in FASI with sweeping, analytes are preferentially loaded according to their retention factor. Concentration sensitivities similar to those obtained for sweeping with LVSS were reported for FASI with MEKC.

Palmer *et al.* investigated another approach to sweeping in which the sample, prepared in a high salt (conductivity) matrix, moved through a stacked PSP zone [62]. The high salt concentration in the matrix induces a high electric field in the low conductivity buffer zone and causes the PSP to stack before sweeping the analytes. The advantages of this method is that the stacked PSP increases the concentration of micelles available for focusing particularly for less retained solutes; and it allows for larger volumes of samples to be injected since the sample zone moves through the PSP. Quirino and Terabe proposed a similar sweeping method using a high salt sample matrix but suggested that destacking of the micellar phase occurred after sweeping, resulting in enrichment factors like those found when a sample matrix with a similar electric conductivity as the separation buffer was utilized [63]. By comparison, when using a low salt (conductivity) sample matrix, the

analytes are first swept by the PSP and then stacked at the sample to buffer boundary with pre-concentration enhanced for low to moderately retained analytes [64].

1.2.7 The Role of CE in the Analysis of Food

As stated in section 1.1, CE is currently viewed as a promising analytical tool and has been employed in many food applications. Numerous review articles have documented the use of CE to analyse a diverse range of foods (e.g. fruit juices, tea, coffee, wine, beer, oils, honey, meat, fruit, vegetables, etc) and food components (e.g. proteins and peptides, phenolic compounds, amino acids and biogenic amines, additives, organic contaminants, carbohydrates, DNAs, vitamins, inorganic and organic ions, etc) [65-87]. In addition to monitoring food quality, safety, authenticity and adulteration practices, the effects of agronomy, storage and processing on the chemical compositions of a variety of foodstuffs have also be examined by CE which clearly demonstrates the versatility of this technique [71,75,77-78,81,83,87-90].

As discussed in a recent review, Pinero et al. reported that approximately two thirds of all foods analysed by CE have used CZE as the mode of separation and the rest are separated mainly by MEKC [82]. In most instances, the analytes that have been comprehensively studied in CE are those that are present in high concentrations (e.g. catechins in tea, dyes/colorants in a variety of food and beverages) or in media which can easily be extracted from (e.g. oils and beverages). However, the antibiotic and pesticide residues in meat and vegetables which are usually present at low concentrations have also been analysed by CE using either or a combination of off- or on-line pre-concentration techniques or sensitive detectors. Phytochemicals in food, specifically phenolics and glucosinolates, have been determined by CE. Whilst tea, wine and olive oil are the main foods that have been explored for their phenolic contents, the phenolic compounds in a moderate selection of herbs and spices, cereals, legumes and nuts have also been determined by CE [80]. By comparison, only a limited number of articles have described CE separations of phenolics in fruit and vegetables. In the 1990's, several CE methods were developed for the separation and detection of glucosinolates and their degradation products but to date, applications to real samples are limited especially when compared to the volume of literature available for HPLC analyses of these compounds in food. Of those samples analysed by CE, the majority were vegetables or seeds belonging to the brassica

family (e.g. rapeseed and cabbage) as cruciferous plants are known to be rich sources of glucosinolates. The limited literature available for CE analysis of phenolics and glucosinolates on real/solid plant samples may be linked to 1) the difficulties in resolving analytes contained in more complex plant matrices resulting in the requirement to perform laborious extraction procedures to isolate the analytes of interest in order to create simpler electropherograms and 2) unlike antibiotics and pesticide residues, phenolics and glucosinolates in food are generally considered to be beneficial to health when consumed in moderation and therefore less urgency is placed on the analysis of these compounds by CE.

In the following section, some of the foods which have been analysed by CE for their phenolic and glucosinolate content will be detailed. The discussion will be mainly focused on several of the aforementioned foods or food groups which have been vigorously analysed by CE such as the phenolics in beverages and oils, and brassica glucosinolates. These examples represent and serve to provide a comprehensive insight into what has been achieved in terms of electrophoretic separations of real food matrices plus highlights the enhanced capabilities and excellent resolving power of CE as well as some of its limitations. In addition, the phenolics in a selection of the relatively few fruit and vegetables which have been determined by CE will be discussed in detail. For clarification, the term fruit will define those plants which in general taste sweet and are consumed as desserts such as berry and citrus fruits, and vegetables will refer to plants which are usually found in savoury dishes (e.g. salads) and will include tomatoes, potatoes, onions, etc.

1.2.7.1 Phenolics

A group of tea phenolics known as catechins have been extensively studied by CE [91-105]. This is because catechins are usually present in high concentrations in tea and can be easily extracted into an aqueous medium for CE analysis without any pre-concentration steps. The eight catechins found in abundance in tea are (+)-catechin (C), (-)-epicatechin (EC), (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-catechin gallate (CG), (-)-gallocatechin gallate (GCG), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) [58]. Both CZE and MEKC have been used for catechins separations but MEKC, with SDS as the micellar phase, is the preferred method because of its greater resolving power

in separating isomeric catechins (e.g. CG and ECG, and GCG and EGCG) from other components present in tea extracts which are not resolved by CZE.

Several groups have developed MEKC methods for the analysis of tea catechins [106-110]. Watanabe et al. were the first to report the separation of tea catechins by MEKC [106]. Using SDS as the micellar phase, seven catechins, caffeine and ascorbic acid in canned green and black teas were resolved within 10 min with detection limits ranging from 0.5 to 150 µg/mL. The MEKC method allowed for two groups of compounds (EGC, EC, and C, and EGCG, ECG and ascorbic acid) to be adequately separated for quantitative analysis which was not achievable by CZE and the run time for MEKC was halved when compared to that of HPLC analysis at 20 min. Bonoli and co-workers successfully separated seven catechins, three xanthines and gallic acid in under 5 min with a 10 mM potassium phosphate/8.3 mM sodium tetraborate/66.7 mM SDS buffer at pH 7 [109]. Detection sensitivity was good for this MEKC method with LODs ranging from 1.1 to 5.1 ng/mL, however, when applied to tea samples, the catechin and caffeine peaks partially comigrated and several other components were not baseline resolved. Nelson and his group used a SDS-MEKC method with a chiral selector for the separation of six catechins (EGCG, ECG, GCG, EGC, EC, and C) found in green tea [108]. Beta-cyclodextrin (θ -CD) was added to the running buffer which enabled the resolution of GCG and ECGC but at the expense of an increase in run time of over 25 min. Subsequent work by Kodama et al. with 6-O- α -Dglucosyl-CD for the chiral separation of eight catechins and caffeine also resulted in similar run times [110]. More recently, Gotti et al. quantitatively determined the catechins and methylxanthines in 24 green tea infusions using a CD-MEKC method [111]. In this case, hydroxypropyl-6-CD (HP-6-CD) was the chiral selector and eleven analytes (nine catechins and three xanthines) were resolved in 8 min with detection limits ranging from 0.05 to 0.7 µg/mL. The CD-MEKC method was also used to monitor epimerisation of the catechins under thermal treatment. In another instance, Peres et al. developed a reduced flow MEKC method for the analysis of green tea catechins [96]. Using a phosphoric acid buffer (pH 2.9) with 50 mM SDS, 0.8 % sulphated- θ -CD and 0.2 % triethylamine, five catechins (ECG, EGCG, EC, C and EGC) in six green tea samples were separated within a fast 4 min with LODs ranging from 0.02 to 0.10 μg/mL. The low pH buffer suppressed the EOF hence allowing for the fast anodic migration of the analytes as they partitioned into the negatively charged SDS micelles.

The main types of phenolic compounds found in wine are hydroxybenzoic and hydroxycinnamic acids, stilbenes, flavones, flavonols, flavanonols, flavanols and anthocyanins and the majority have been determined by CE and in particular CZE [112-126]. In most cases, LLE or SPE was used as the extraction step to isolate phenolics from the wines and HPLC as the comparative method. Garcia-Viguera and Bridle analysed non-coloured phenolic compounds in a Portuguese red wine by CZE and HPLC [116]. In total, 11 phenolic compounds were separated by CZE within 15 min using a 100 mM sodium borate buffer at pH 9.5. This CZE method was superior to HPLC in that the analysis time was halved and better peak shapes and resolution of chiral isomers were obtained. However, the wine extract was reconstituted in half the volume for the CE analysis compare to that for the HPLC analysis to achieve the same detection sensitivity but this enhanced concentration still did not allow for detection of the flavonols, myrcetin, quercetin, kaempferol and isorhamnetin by CE. This CZE method was also applied in subsequent analysis of phenolics in port, white and red wines by the same group and in these instances, a similar range of phenolics were detected with comparable analysis times of within 20 min [115,117-118].

Polyphenols in wine have also been examined in detail by MEKC [127-131]. Both Chu et al. and Prasongsidh and Skurray resolved cis- and trans-resveratrol in wines using SDS and sodium deoxycholate (SDC) as the micellar phase, respectively [127-128]. The latter group also resolved quercetin, catechin and gallic acid simultaneously and used a 150 μm bubble capillary to provide an extended optical light path for sensitive UV detection. In both cases, peak identification was difficult, particularly for cis-resveratrol mainly because of the low concentrations present in the wine samples. Sun et al. developed an efficient MEKC separation system to determine anti-carcinogenic flavonoids in wines [131]. Successful separation of six flavonoid compounds (catechin, naringenin, kaempferol, apigenin, myricetin and quercetin) was achieved in 16 minutes using SDS as the micellar phase and low detection limits of 15 to 23 ng/mL were achieved.

To enable fast analysis of phenolic anions, Hernandez-Borges and co-workers created a co-EOF environment to separate the phenolics in wine and other foodstuff [132]. A polycationic surfactant, hexadimetrine bromide (HDB), was added to the separation buffer as a dynamic coating for the capillary wall to generate a fast anodic EOF. An organic modifier, MeOH, and buffer additive, α -CD, were also included in the borate separation buffer to enhance resolution of the phenolic compounds. Although cyclodextrins are normally used in chiral separations, possible selective interactions of these compounds

with borate complexes (formed between borate buffer and analytes) may facilitate the separation of the analytes of interest. In this instance, the inclusion of α -CD in the buffer allowed for the resolution of ferulic and sinapic acids (which MeOH did not) and under these conditions and with a reverse separation potential, eight phenolics were baseline resolved within 4 min with LODs ranging from 40 to 70 ng/mL.

Several methods to enhance detection of wine phenolics have been developed [133-138]. Hamoudova et al. combined CZE with isotachophoresis (ITP) (also known as the CE mode, CITP) to pre-concentrate and separate the natural constituents in red wine [138]. Using this technique, flavonoids and phenolic acids were stacked in discrete zones between a leading and a terminating ion with high conductivity (low field zone) and low conductivity (high field zone), respectively. Low detection limits of 0.03 μg/mL for phenolic acids and ranging from 0.1 to 0.25 µg/mL for flavonoids were achieved with this CITP method, but at the expense of a long analysis time of 45 min. Du and Fung used CE coupled to an ED in the determination of polyphenolics in red wine [137]. Using a buffer composed of 20 mM sodium tetraborate and 2 mM $extit{\teta}$ -CD in 7 % MeOH at pH 9.0, five phenolics (transresveratrol, C, EC, quercetin and gallic acid) were separated within 16 min with LODs in the range of 0.031 to 0.21 µg/mL. Another positive aspect of this method was its ability to detect peak impurities which is invaluable given the complexity of some wine matrices. Hsieh and Lin introduced an on line sweeping technique coupled with low temperature fluorescence spectroscopy in the identification of trans-resveratrol in red wine [133]. Using the sweeping-MEKC method, trans-resveratrol was detected at a slightly increased time of 26 min (20 min for MEKC) but a 1500 fold enhancement in detection sensitivity was obtained when compared with the normal MEKC separation.

In addition to enhancing detection sensitivity, mass spectrometry provides structural information of target compounds. Bednar et~al. used CE-electrospray ionisation (ESI)-MS to study the fragmentation pattern of anthocyanins as a means to monitor phenolic profiles in wine and wine musts [135]. Anthocyanins are natural pigments found in many plants, and fruits such as red grapes and hence red wine contains considerable levels of these compounds, and are the sugar containing derivatives of parent anthocyanidins. In this study, six anthocyanins were detected and quantified in four wine samples using an acidic (200 mM chloroacetate-ammonium, pH 2.0) and an alkaline (200 mM borate-ammonium, pH 9) separation buffer. Whilst the acidic buffer provided lower detection limits (0.8 to $1.5~\mu g/mL$ compared to 4 to $10~\mu g/mL$ for the alkaline buffer), resolution for the

diastereomeric pair, oenin and primulin and also for the galactoside derivative, ideain was enhanced with the borate electrolyte due to selective interactions between vicinal hydroxyls and borate. The reduction in detection sensitivity with alkaline buffers can be explained by the relative stability of the anthocyanidin molecule at different pHs. Acidic buffers prevent the degradation of anthocyanidins which exists as stable red flavylium cations at below pH 2, neutral blue quinoidal species between pH 2 to 4, colourless forms at pH 5 and 6 and rapidly degrade at above pH 7 and hence a decrease in the concentration and detection of coloured phenolics occurs when using a separation buffer at high pH. From this study, it was concluded that both CZE methods were suitable for the analysis of anthocyanins in wine and furthermore, although CZE may be successfully coupled with MS provided that volatile buffers are used for the separation, this detection method is not compatible with MEKC where the separation buffer is composed of non-volatile surfactants.

Another area of CE which has largely been unexplored is the use of microchips in food analysis. This ever expanding area of miniaturised CE has been applied to a diverse range of analytes of biological, clinical and environmental significance, however to date, only a limited number of articles have been published which describes food analysis using CE microsystems and even less on separation of food phenolics by this technique. Scampicchio $et\ al.$ used a CE - ED glass microchip system for the analysis of phenolic acids in commercial red wine [139]. In this study, the glass microchip consisted of a four - way injection cross with one arm of the cross used as a sample reservoir, one channel for the running buffer and the longest channel for separation and detection and to which a screen printed carbon electrode is attached in the end channel configuration. Wine samples were loaded by EKI at 1.5 kV for 5 s and separation of four phenolic acids (chlorogenic, gentisic, ferulic and vanillic) was achieved within a very fast time of 3 min using a buffer composed of 15 mM borate buffer (pH 9.5) and 10 % MeOH and detection limits down to 0.15 μ g/mL were obtained.

In the last decade, CE analyses of phenolic compounds in olive oils and in particular, the extra virgin variety has been studied in detail by Carrasco-Pancorbo and her group in Spain [140-149]. Olive oils contain many different classes of phenolic compounds and include the simple phenols (phenolic acids and phenyl ethyl alcohols), secoiridoids, and lignans. Bendini *et al.* were the first to report the separation of phenolics in olive oil by CE [150]. Twelve phenolic acids, tyrosol, taxifolin and oleuropein were separated within 10

min using a 45 mM sodium borate buffer at pH 9.6 and detection LODs down to 1 ng/mL were obtained compared to a long separation time of 45 min and LODs down to only 1 µg/mL for HPLC analysis of the same analytes. Bonoli and co-workers applied this CZE method on two separate occasions and in one instance was able to separate 21 phenols and polyphenols in extra virgin olive oils (EVOO) within 10 minutes [151-152]. In another study, a CZE method was developed in which 26 phenolic compounds including simple phenols, secoiridoids, lignans, and for the first time flavonoids were separated in EVOO in less than 10 minutes [143].

Pancorbo et al. attempted to selectively separate 14 phenolic compounds in EVOO by controlling the EOF [153]. Similar to the above example provided for wine, HDB was added to the separation buffer to generate a reversed EOF and provide a co-EOF separation for the anionic phenolics. However, in this instance, the run time for the separation was long at over 30 min and many of the phenolics were not baseline resolved [153]. The same group also devised two new CE-MS methods, (CZE-ESI-MS and CZE-ESI-time of flight (TOF)-MS), coupled with SPE for the analysis of the phenolic fractions of EVOO [142,145]. The first method allowed for the characterization and identification of 11 phenols (3 simple phenols, 2 lignans, several complex phenols and elenolic acid) whereas in CZE-ESI-TOF-MS, 18 well known, and 28 unknown phenolic compounds were separated and detected. Since the introduction of this method, a number of publications have reported the separation and detection of phenolics in olive oils by CE-MS [146-147,154-155]. Garcia-Villalba et al. combined HPLC and CE for the analysis of phenolics in commercial olive oil samples [146]. A semi preparative HPLC step was first used to isolate phenolic fractions from EVOO extracts and in the second stage, CE was coupled to ESI-TOF-MS to separate and detect the phenolic compounds. This method allowed for tentative identification of 50 phenolic compounds present in 17 phenolic fractions of EVOO. The potential of non aqueous CE (NACE) (where the separation buffer is composed entirely of organic solvents) with ESI-TOF-MS has also been explored [147]. Gomez-Caravaca and co-workers determined the phenolic compounds in EVOO by aqueous CE and NACE [147]. Both methods detected similar phenolics in the olive oils, and whilst the separation time was faster using aqueous CE (12 min compared to 18 min for NACE), the concentration sensitivity was enhanced for the NACE method (around ten times higher) and allowed for direct injection of these hydrophobic samples.

Herbs and spices are another food group which provide a great source of phenolic antioxidants. Traditionally, some herbs are added directly to food to enhance their flavours whilst others are consumed as infusions in tea or soups and are exploited for their medicinal properties forming the basis of many traditional Chinese medicines. One of the herbs which has been most explored by CE for their phenolic contents is rosemary [156-161]. The main phenolics in rosemary are diterpenes, and these compounds are reported to be major contributors to the antioxidant activity of this herb [162-163]. Ibanez et al. initially used MEKC and HPLC for the separation of supercritical fluid extracts of rosemary [156]. Using a separation buffer of 50 mM SDC/20 mM boric acid/sodium tetraborate at pH 9 and 15 % ACN, five diterpenoids (and one unidentified compound) in the rosemary extract were resolved within 6 min compared to 24 min for HPLC although peak area reproducibility was slightly better for HPLC (2.99 % v. 4.21 % for MEKC). CZE has also been employed in the separation of rosemary phenolics [157-159]. Bonoli and co-workers quantitatively determined the carnosic and rosmarinic acid content in rosemary extracts by CZE [158]. In this study, they managed to detect seven compounds (carnosol, carnosic acid, rosmarinic acid and 4 unknowns) in unrefined rosemary extracts in an impressive time of 3 min. In addition, an excellent detection limit of 0.7 ng/mL was obtained for both carnosic and rosmarinic acids and several other possible phenolic peaks were separated in the same run. Peng et al. combined CZE with ED to separate eight active phenolic components of rosemary. Using this method, LODs in the range 0.2 to 1 µg/mL was achieved with a run time of 24 min and although electropherograms for the real extracts showed unstable baselines, satisfactory quantitative results were still obtained using this method. MS detection has also been utilised in the detection of phenolics in rosemary extracts [160]. Herrero and co-workers developed a CE-ESI-MS method to analyse the subcritical water extracts of rosemary [160]. Following pressurised liquid extraction, the rosemary fractions were separated by CE coupled to an ion trap (IT) MS which allowed for the detection of six compounds with five identified as the phenolics, isoquercitrin, carnosic acid, rosmarinic acid, homoplantagenin and gallocatechin. Although analysis times were similar for CE-MS and HPLC-MS (~ 20 min), three phenolics, carnosol, rosmanol and epirosmanol which were detected by HPLC-MS were not detected by CE-MS.

The phenolics in an extensive variety of other herbs and spices have also been investigated by CE but individually and to a lesser extent [164-176]. Numerous articles report the use of CZE or MEKC as the mode of separation for many of the herbs analysed

because most phenolics in herbs are present at considerable concentrations and therefore sufficient sensitivity is obtained using these methods. Nonetheless, a substantial number of separations involve pre-concentration techniques (e.g. CE-ITP) or sensitive detectors (e.g. EDs) for those phenolic components which are found at low or trace levels in herbs. Peng and Ye used a CE-ED method in the separation of isoflavones in Trifolium pratense (red clover) [165]. With a three electrode cell system combined with an amperometric detector and a 50 mM borate buffer at pH 9.5, three isoflavones (daidzein, genistein and biochanin A) were separated within 25 min and very low detection limits were obtained (0.02 - 0.05 μg/mL). Peng and his group also successfully applied other CE-ED methods to the separation of phenolic acids and flavonoids in Perilla frutescens (Chinese basil) and flavonoids in Hippophae rhamnoides (sea buckthorn) [168,177]. In both cases, resolution of the target analytes was achieved in ~ 20 min and whilst detection limits for the analytes in the Chinese basil were higher than those above obtained for red clover (0.2 to 1 µg/mL), excellent detection limits of 0.13 to 0.59 pg/mL were recorded for the flavonoids in sea buckthorn. Zhang and co-workers used an alternative approach to separate the same isoflavones in red clover [166]. In their study, the above isoflavones plus one other (formononetin) in red clover were resolved by MEKC with SDS and a chiral selector HP-β-CD in 11 min with detection limits of 0.03 to 0.1 µg/mL. Segura-Carretero developed a CE-ESI-MS method for the analysis of anthocyanins in Hibiscus sabdariffa [175]. The use of both IT and TOF mass spectrometers enabled the identification of five anthocyanins and chlorogenic acid in hibiscus tea extracts within 15 min.

Several ITP-CE methods have been employed for the pre-concentration and separation of phenolics in herbs [178-181]. On two separate occasions, Safra *et al.* used a column coupling configuration in which one capillary is used for the ITP pre-concentration step and the second for CZE separation with both stages performed with different BGEs [178,181]. Low detection limits were obtained in both cases (10 to 61 ng/mL for nine phenolic acids in *Herba epilobi* (willow herb) and 18 to 35 ng/mL for five phenolic acids and one flavonoid in *Melissae herba* (lemon balm)) but much like LVSS, the timing of the switch from stacking to analytical mode must be carefully monitored in order to obtained highly repeatable separations. Zhu and co-workers compared two sample pre-concentration techniques in the determination of flavonoids in the Chinese herbal medicine, *Fructus auranti* Immaturus (immature orange fruit) [167]. Using two sweeping methods described in section 1.2.6.3, LVSS and FASI with sweeping, six flavonoids (tangeretin, nobiletin,

hesperetin, naringenin, hesperidin and naringin) were separated within 20 min with enhancement factors ranging from 27 to 37 and 45 to 194 fold, respectively and LODs down to 11.5 and 2.4 ng/mL, respectively. An online electrokinetic pre-concentrating technique which relies on the accumulation of analytes at a dynamic pH junction has been used in the analysis of phenolic acids in *Majorana hortensis* (majoram) [173]. Essentially, this enhancement step involves EKI of phenolic anions in an alkaline matrix, followed by neutralisation of the analytes by an acidic buffer at the sample/buffer boundary prior to mobilisation and separation of the stacked analytes by MEKC. Detection sensitivity of up to 5560 fold and LODs down to 0.38 ng/mL was obtained for the phenolic acids with this method but injection times of 30 min were required for this level of enrichment resulting in a very long analysis time of 50 min.

One of the fruits most analysed by CE for their phenolic contents are grapes. This may be associated with the fact that grapes are commonly eaten fresh and in its dried form as raisins, and are also the main ingredient used in wine production. Most of the CE analyses on grape phenolics have been performed using MEKC or MEEKC methods [102,182-185]. Bednar et al. developed a MEKC method for the determination of anthocyanidins in wine grape skins. With a 30 mM phosphate/400 mM borate/50 mM tris(hydroxymethyl)aminomethane (TRIS) (pH 7.0) buffer, the separation of six anthocyanidin glycosides was achieved within 17 min with LODs of between 6 to 10 µg/mL. Similar to the above example provided for the detection of anthocyanins in wine, the addition of boric acid enabled the resolution of the sugar derivatives, ideain and callistephin and diastereoisomers, oenin and primulin. Herrero-Martinez and co-workers compared three MEKC systems, SDS, sodium cholate (SC) and SDS/SC, for the separation of catechins and their cysteamine derivatives in grape pomace following de-polymerisation of precursor procyanidins with cysteamine hydrochloride [184]. They established that the mixed micellar system of 40 mM SC/10 mM SDS in 50 mM phosphate at pH 7 was the optimum separation buffer in terms of resolution and migration times and were able to resolve six catechins and its derivatives within 15 min. In a subsequent study, they applied the same mixed micellar system for a similar analysis but this time cysteine hydrochloride was used in place of cysteamine hydrochloride to breakdown grape procyanidins [183]. In this instance, the products of de-polymerisation, seven catechins and their cysteinyl derivatives, were baseline resolved within 11 min with better precision for migration time (SC-SDS = 0.2 - 1.6 %, SDS = 0.7 - 4.0 %) and peak areas (SC-SDS = 1.2 - 3.8 %, SDS = 1.8 - 6.8

%), and lower detection limits (SC-SDS = $0.62 - 5.4 \,\mu g/mL$, SDS = $0.92 - 11.2 \,\mu g/mL$) when compared to a similar MEKC separation with SDS. By contrast, the same separation with HPLC was much longer at over 30 min and C and its conjugate were not resolved.

Huang and Lien reported on the use of a MEEKC method in the analysis of phenolic compounds in several food samples [102]. Under optimal separation conditions, seven phenolic acids, six flavonoids, caffeine and theophylline were baseline separated within 14 min in a microemulsion buffer containing 25 mM phosphate, 1.36 % w/v heptane, 2.89 % w/v SDS, 7.66 % w/v cyclohexanol, and 2 % w/v ACN at pH 2 and this method was successfully applied for the quantitative determination of phenolics in grapes, apples, tea leaves, tea beverages, and red wines. Further studies by this group compared the same MEEKC method with an SDS-MEKC system for the analysis of the above analytes in teas and grapes [185]. They established that for MEEKC, the SDS concentration was an important parameter in the analysis and a higher separation voltage and temperature improved separation efficiencies without affecting analyte resolution whereas for MEKC, varying the SDS concentration did not have a marked effect on the separation but an increase in separation voltage and temperature resulted in poor resolution of the analytes. Overall, the MEEKC method provided higher peak efficiencies in all the analytes (up to 1 302 000 N/m) with the exception of two catechins but lower detection limits were obtained with the SDS-MEKC system (MEKC - 0.10 to $0.45 \mu g/mL$, MEEKC - 0.35 to $1.47 \mu g/mL$).

CZE has also been used in the study of phenolic compounds in grapes [186-187]. Priego Capote *et al.* combined CZE with diode array and fluorescence detection to determine the phenolic compounds in grape skin [186]. Using a separation buffer of 50 mM sodium tetraborate with 10 % methanol (pH 8.4), ten phenolic compounds (4 anthocyanidins, 3 flavonoids, 2 catechins and resveratrol) were separated within 10 min with detection limits ranging from 0.08 to 0.86 μ g/mL. The use of both detection systems significantly improved the sensitivity of the method and allowed for the quantitative determination of a range of phenolics in extracts obtained from superheated ethanol-water leaching of grapes skin and commercial grape extracts.

A host of other berry fruits has been analysed by CE for their phenolic contents [188-197]. Fernandes *et al.* investigated a range of CZE and MEKC methods for the determination of phenolic acids and flavonoids in blackcurrant [188]. Using a separation buffer of 50 mM sodium dihydrogen phosphate/100 mM boric acid/ 9 % propanol at pH 7, they successfully resolved fifteen phenolics within 20 min with detection limits of 12 to 27

μg/mL, and demonstrated that no significant advantages were gained by using MEKC methods which generally resulted in poorer resolution and no improvement on detection sensitivity of the analytes tested. Ehala and co-workers developed a CZE method to characterise the phenolic profiles of berries grown and consumed frequently in Northern Europe. Nine phenolic analytes (5 phenolic acids, 3 flavonoids and trans-resveratrol) were separated within 12 min using a 35 mM sodium tetraborate buffer at pH 9.3 and detection limits ranging from 0.12 to 0.40 μg/mL were obtained. On average, six phenolic compounds were detected in all of the berry extracts with the exception of blackcurrant in which only caffeic acid and catechin were found in detectable quantities. The electropherograms of wild berries (cranberry, bilberry and cowberry) displayed unstable baselines and substantial increases in concentration and complexity of components present in their matrices while the peaks of cultivated berries (red and black currants and strawberry) were quite small. This, presumably, presented difficulties in determining the phenolic contents of these berries and hence only a few compounds from each extract were quantified using this method.

Several groups have reported on the separations of anthocyanidins in berries by CZE [189-190,194,196]. Bridle and Garcia-Viguera compared CZE with HPLC in the analysis of anthocyanidins in strawberries and elderberries [189]. Extracts of both fruits were separated with an alkaline buffer of 150 mM sodium borate (pH 8.0), and whilst the strawberry extract gave comparable quantitative results to HPLC analysis, larger differences were observed for the elderberry extracts due to unstable baselines most likely caused by interfering compounds present in the matrices. And although the CZE runs were faster than HPLC (8 v 25 and 13 min for strawberries and elderberries, respectively), a greater sample concentration (87 times) was required for the CZE method to achieve the same detection sensitivity as HPLC. As explained previously for the analysis of anthocyanins in wine, alkaline separation conditions degrade the stability of the coloured anthocyanidin molecules and hence a reduction in concentration sensitivity is experienced with high pH run buffers. Therefore, for sensitive detection of these compounds in the visible spectrum, acidic buffers are often employed in anthocyanidin analysis. However, run times are often compromised under acidic conditions because of a retarded EOF. For example, da Costa and co-workers used a highly acidic phosphate buffer (pH 1.5) containing 30 % ACN in the separation of anthocyanidins in blackcurrants and although all analytes were resolved with an increase in detection sensitivity (160 fold) when compared to the above CZE separation

with an alkaline buffer, the analysis time was over 30 min [190]. Similarly, Watson *et al.* and Comandini *et al.* also utilised low pH buffers (~ 2) in the determination of anthocyanidins in cranberries and strawberries, respectively and in both analysis, the analytes were resolved in around 20 min [194,196]. Although this run time was acceptable and comparable to HPLC analysis for the strawberry extracts, the HPLC separation was much shorter for the cranberry anthocyanidins at 5 min.

An online continuous flow system (CFS) coupled with CE was used in the determination of phenolic constituents in citrus fruits [198]. The CFS allowed for the cleanup of samples via a C18 minicolumn prior to introduction into the analytical column and using a separation buffer of 100 mM boric acid (pH 9.5), six phenolics (4 phenolic acids and 2 flavonoids) in a variety of oranges and grapefruits were resolved within 30 min with LODs of between 0.18 to 0.36 µg/mL. This online configuration was particularly valuable in that 1) offline sample pre-treatment was minimised and thereby reducing systematic errors associated with sample handling, etc and 2) the system allowed for two samples to be processed simultaneously i.e. one sample was cleaned up while another was analysed by CE thus, overall, resulting in a more efficient analysis in terms of reduced manual labour and time.

Chiral CE separations of fruit flavonoids have also been performed. Gel-Moreto et al. investigated the chiral separation of diastereomeric flavanone-7-O-glycosides in citrus fruit varieties including sweet/sour oranges, mandarine, grapefruit, lemon and also marmalade prepared from sour orange [199]. A range of native CDs, neutral and charged CD derivatives were tested and in all cases resolution was achieved by using a combination of chiral selectors e.g. the addition of 0.1 mM of the negatively charged sulfobutyl ether-4-6-CD to 15 mM θ -CD allowed for adequate resolution of the diastereoisomers of hesperidin and eriocitrin in lemon juice. However, in most cases, different CD combinations provided optimal separation conditions for different pairs of isomers within the same extract. For example, isomers of hesperidin in orange juice was well resolved (Rs = 1.50) using a combination of 15 mM θ -CD and 50 mM HP- θ -CD whereas no resolution was obtained for the narirutin pair in the same extract but resolution was optimised for narirutin isomers (R_s = 0.84) with a mix of 10 mM dimethyl- θ -CD and 0.5 mM sulfobutyl ether-4- β -CD while separation was not achieved for the hesperidin pair. Kofink and co-workers also used chiral selectors in the enantioseparation of C and EC in guarana (seeds and extracts) and apple juice [200]. Using 12 mM of the neutral 2-HP-γ-CD in a 100 mM borate buffer (pH 8.5),

enabled the separation of all four enantiomers of Cs and ECs in guarana extracts within 7 min.

Blasco *et al.* examined a CE - ED microchip system for the analysis of flavonoids and ascorbic acid in apples and pears [201]. A glass microchip with the same cross configuration as described above for the analysis of wine was employed in the separation of three antioxidants (arbutin, C and ascorbic acid). In this instance, both arms of the cross were used as sample reservoirs and a glassy carbon disk electrode was attached at the detection end of the separation channel. Following EKI for 5 s at 2 kV, the three analytes were separated within an impressive 4 min using a 50 mM borate buffer (pH 9.5) and a separation and detection voltage of 2000 and 1 V, respectively. Crevilllan and co-workers also exploited the use of CE microchip in the analysis of flavonoids in apples and pears but in this instance, screen-printed electrodes coated with multi-walled carbon nanotubes were used as EDs and the separation four phenolic analytes (arbutin, phloridzin, C and rutin) and ascorbic acid was achieved again within 4 min with detection limits ranging from 0.5 to 7.1 µg/mL [202].

Sawalha *et al.* developed a simple CE-ES-IT-MS method for quantification of the key phenolic compounds in sweet and bitter orange peel [203]. Although this part of the fruit is not usually eaten and is classed as plant waste, orange peels contain high concentrations of flavonoids and are a valuable source of natural polyphenols. Under optimal CE-ESI-IT-MS conditions, four flavonoids (naringin, neohesperidin, narirutin and hesperidin) in orange peel extracts were identified and quantitatively determined within 10 min with LODs of 0.23 to 1.15 µg/mL. Herrero-Martinez and co-workers employed mixed micelles for the determination of flavonoid aglycones in several food samples (orange, wine, propolis and *Ginkgo biloba*) [204]. Similar to their work on grape procyanidins detailed above, a mixed micellar system consisting of 25 mM SDS and 25 mM SC in a 50 mM phosphate buffer (pH 7) resolved ten flavonoids aglycones within 20 min and detection limits of 1.24 to 3.96 µg/mL were attained using this method. Prior to CE analysis, the flavonoids in orange pulp and peel and Ginkgo leaves were released from conjugation by acid hydrolysis.

Of all the vegetables analysed by CE for their phenolic contents, a considerable number have belonged to the Solanaceae family [205-210]. This large and diverse plant group include vegetables such as tomatoes and potatoes which are consumed worldwide and are a good source of a range of phenolic antioxidants. Helmja and her group in Estonia determined the phenolic compounds present in vegetables of the Solanaceae family by CZE

[205]. Using a 25 mM sodium tetraborate buffer (pH 9.3), the separation of various phenolics (genistein, rutin, naringenin, myrcetin, luteolin, quercetin, catechin; and the acids chlorogenic, caffeic, cinnamic and ferulic) found in skin extracts of tomato, eggplant, chilli pepper and potato was achieved in under 13 min. However, the electropherograms displayed complexity in composition of the extracts with numerous peaks present, many of which were not baseline resolved or co-migrated with other components and hence a number of compounds could not be identified in these vegetables. In two further studies, the same CZE method was applied to evaluate the antioxidant activities of tomato and eggplant respectively but in these instances, HPLC with DAD and MS was used to facilitate the identification of compounds not achievable by CE only [206-207]. Peng et al. reported the use of CE with ED for the sensitive detection of phenolic components and ascorbic acid in tomato fractions [209]. A 300 μm carbon disc electrode combined with an amperometric detector was used in the detection of five phenolics (resveratrol, naringenin, rutin, chlorogenic acid and myrcetin) and ascorbic acid present in the pulp, peel and seeds of tomatoes and detection limits in the range of 0.01 to 0.2 µg/mL were attained for all analytes. Liu and co-workers described a fast MEKC method in which the capsaicinoid content in Capsicum anuum (pepper) and other related products were determined [211]. Capsaicinoids are a group of phenolic compounds specific to the capsicum family which impart the hot/spicy flavour found in many varieties of peppers. Using a mixed micellar system of SDS and polyoxyethylene sorbitan monolaurate (Tween 20), the two main capsaicinoids (capsaicin and dihydrocapsaicin) found in hot chilli peppers were baseline resolved within 5 min with detection limits of 0.66 and 0.73 µg/mL for capsaicin and dihydrocapsaicin, respectively. Although the two capsaicinoids were fully resolved with SDS, the peak shapes were broad with considerate tailing but with the addition of Tween 20, the peak shapes became sharp and detection sensitivity was enhanced.

Another vegetable known to contain a rich source of phenolics, and in particular flavonols, are onions. Price *et al.* identified two key components of onions as quercetin-3,4'-O-diglucoside and quercetin-4'-O-glucoside (with quercetin present at low levels) by using a combination of HPLC, MS and nuclear magnetic resonance [212]. In this study, CZE was chosen to monitor the autolysis of onion tissues because of its ability to separate closely related flavonol glycosides. Caridi and co-workers also applied this technique to the quantitative analysis of quercetin glucosides in six onion varieties and because of the lack of a readily available reference standard for quercetin-3,4'-O-diglucoside, HPLC was

employed as a preparative step to isolate this compound from freeze dried onion powder and also as a comparative analytical method [213]. In their CZE method, 15 mM EDTA was added to the separation buffer of 10 mM borate/boric acid (pH 10.2) as this reduced the interaction of quercetin with the metal ions present in the buffer and hence produced sharp reproducible peaks for quercetin. The anthocyanins in onions have also been investigated by CE. Petersson *et al.* developed a CE-TOF-MS method to detect the anthocyanins in red onions [214]. Using a capillary coated with a polycationic aminecontaining polymer (poly-LA 313) combined with an acidic buffer (15 mM formic acid at pH 1.9) to prevent anthocyanin degradation and enhance ionisation process in MS mode, eight glucosides of cyanidin, peonidin and malvidin were identified in red onion extracts within 20 min with detection limits of 1.4 μ g/mL.

Other examples of vegetables investigated by CE for their phenolic profiles are cabbage and cauliflower [215]. To date, this is the only paper which describes a CE based method for the determination of a phenolic compound in the brassica family. Dadakova *et al.* applied an MEKC method to the quantitative analysis of quercetin in cabbage and cauliflower. Following acid hydrolysis of each vegetable to breakdown conjugated flavonoids to aglycones, cabbage and cauliflower extracts were resolved within 18 min using a separation buffer of 10 mM boric acid/sodium tetraborate/20 mM SDS/15 % MeOH (pH 9.2) and a detection limit down to 0.5 μ g/mL was achieved. Although the separation time of 18 min was acceptable for CE analysis, a long post conditioning time of 9 min further increased the total analysis time to 27 min. Furthermore, an internal standard (1-naphthylacetic acid) was employed to account for injection irregularities. The quercetin content in apples was also determined using this method.

1.2.7.2 Glucosinolates

Initially, the majority of CE analyses on glucosinolates were performed in MEKC mode using CTAB as the PSP [216-217]. Michaelsen *et al.* applied a CTAB-MEKC method in the determination of a large range of intact and desulpho glucosinolate standards [216]. Using 50 mM CTAB in an 18 mM borate/30 mM phosphate buffer solution (pH 7), eleven glucosinolates were successfully separated in under 15 min with high separation efficiencies of up to 566 000 theoretical plates per metre (N/m) of capillary. In addition, this group were able to elucidate the migration order of 28 plant derived glucosinolates.

Instead of CTAB, Feldl and co-workers investigated the suitability of other trimethylammonium bromides (TABs) as PSPs in the separation of indolyl glucosinolates and derivatives [218]. A series of TABs with carbon side chains from C10 to C18 were tested along with propan-2-ol as organic modifier. The most favourable surfactant and alcohol concentration for separation of the indolyl derivatives, in terms of acceptable migration times (< 30 min) and resolution, was 50 mM dodecyl trimethylammonium bromide (DTAB) and 10 % propan-2-ol respectively, resulting in peak efficiencies of up to 370 000 N/m for some of the compounds analysed.

Anion surfactants have also been employed to facilitate the separation of glucosinolates [219-224]. Several researchers in Denmark have investigated the glucosinolates, via their desulpho derivatives, in a variety of plant matrices using SC as the micellar phase in MEKC. Bjeregaard et al. developed a SC based MEKC method for the analysis of desulphoglucosinolates in plants of the order Brassicales [219]. Desulphoglucosinolates are neutral compounds which were originally produced for analysis by HPLC as the anionic glucosinolates were not well retained in reverse phase columns, an issue that has since been overcome with the use of ion-pairing reagents. Using a 250 mM SC/200 mM boric acid run buffer (pH 8.5), desulphoglucosinolates from savoy cabbage, Heperis matronalis and Reseda lutea were separated but with a considerably long run time of 50 min. To improve on detection sensitivity for the separation, a Z-cell with an extended optical pathlength of 3 mm was also investigated. A tenfold enhancement in sensitivity was observed when compared to a 75 µm i.d. capillary and peak efficiencies were slightly lower than those obtained from a 50 μ m capillary but not significantly so (N/m = 250 000 to 300 000 for the 50 µm capillary and 210 000 to 250 000 for the Z-cell). Van Eylen et al. also used the same MEKC buffer in the separation of desulphoglucosinolates in broccoli subjected to temperature and pressure treatments [223]. This method allowed for the identification and quantification of five glucosinolates (glucoiberin, glucoraphanin, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin) in broccoli florets but again the run time was quite long at over 32 min and partial co-migration occurred between the two aliphatic glucosinolates, glucoiberin and glucoraphanin.

Bellostas and her group have examined the products of glucosinolate hydrolysis by MEKC with SC in detail [221-222,225-226]. In one study, they monitored the myrosinase catalysed hydrolysis of 2-hydroxy substituted glucosinolates and the simultaneous formation of their corresponding degradation products, oxazolidine-2-thiones (OZTs) and

nitriles [221]. A glucosibarin standard was used as the model and using a separation buffer of 35 mM SC/100 mM disodium hydrogen phosphate/50 mM taurine/2 % propan-1-ol at pH 8.2, glucosibarin and its two hydrolysis products, OZT and nitrile were detected within 10 min with detection limits and peak efficiencies ranging from 7 to 48 μ g/mL and 245 000 to 264 000 theoretical plates, respectively. This method was subsequently applied to several other glucosinolate standards for the same purpose. In another study, iron (II) (Fe²⁺) was used as the catalyst in the hydrolysis of glucosibarin and again the same MEKC method was used but in this case to monitor the formation of thionamide and nitrile [222]. In total, four compounds, three expected (glucosibarin, thionamide and nitrile) and one postulated as the metal complex formed between glucosibarin and Fe²⁺ were detected within 10 min. Traces of OZT were also detected presumably as a direct result from the breakage of the thioglucoside bond in the formation of thionamide.

Paugam and co-workers combined SDS with tetramethylammonium hydroxide (TMAH) in the separation of four glucosinolate standards (glucobrassicin, methoxyglucobrassicin, glucotropaeolin, sinigrin) involved in Cruciferae resistance mechanisms [220]. TMAH acts as an ion-pairing reagent for the glucosinolates reducing its negative charge density thus allowing for increased micelle to analyte interaction between the anionic SDS and glucosinolate. With a buffer composition of 26.3 mM TMAH, 158.6 mM SDS and 24.4 % MeOH in 24 mM sodium tetraborate (pH 10), and a gradient potential of 15 kV for 6 min followed by 20 kV for 21 min, the four glucosinolates were adequately resolved (R = 1.20 to 5.00 between adjacent solutes) in 26 min with LODs of 0.40 to 0.48 μg/mL. A pre-rinse step with 95 mM borate plus the gradient voltage facilitated the reduction in analysis time by 10 min.

Karcher and his group in the US have exploited the capabilities of LIF for the indirect detection of glucosinolates in rapeseed and cabbages [227-229]. Essentially, these studies involved degradation of the parent glucosinolates by chemical (acid or base) or enzymatic (myrosinase) hydrolysis into their constituent components (e.g. carboxylic acids, isothiocyanates, etc) which are reflective of the glucosinolate present, followed by fluorescent labelling of these hydrolyzed products prior to separation and detection by CE LIF. Indirect detection is a widely used approach in the determination of glucosinolates since relatively pure degradation products of these compounds are more readily available as standards or can be easily synthesised compare to their parental counterparts and thus allowing for easy identification of parent glucosinolates via their derivatives. Using this

technique and with non ionic n-octyl- θ -D-glucoside or n-nonyl- θ -D-glucoside as the micellar phase, they were able to profile and/or quantify the glucosinolates present in red and white cabbages, and rapeseeds. In one study, they followed the LIF detection of 7-aminonaphthalene-1,3-disulphonic acid (ANDSA) labelled carboxylic acids from the acid hydrolysis of cabbage samples. Five ANDSA derivatives in red and white cabbages were detected within 15 and an increase in sensitivity of up to four orders in magnitude was observed when compared to UV detection of underivatised samples [227]. In subsequent studies by the same group on rapeseed and white cabbage, the total and individual glucosinolate contents were quantitatively determined via LIF detection of the enzymatically released glucose (as gluconic acid) and isothiocyanate derivatives (as amines), respectively [228-229]. Whilst similar run times were obtained for the ANDSA labelled gluconic acid, the analysis of the fluorescein isothiocyanate labelled amines was significantly longer at ~ 30 min.

Bringmann et al. developed a CE-MS method for the analysis of glucosinolates in Arabidopis thaliana, a crucifer often used as a model in plant science [230]. CZE was coupled to ESI-TOF-MS and using a buffer consisting of 1 M formic acid and an applied potential of - 25 kV, 14 major glucosinolates in A. thaliana seeds were detected and identified within 18 min. The highly acidic CE buffer system provided good sensitivity but did not prevent ionisation of the glucosinolates and as a result formic acid (0.2 %) was also added to the sheath liquid. Microchip CE with fluorescence detection has also been used in the analysis of glucosinolates in A. thaliana [231]. In their study, Fouad and co-workers relied on the ability of glucosinolates to form charge transfer complexes with two xanthene dyes, phloxine-B and eosin-B, as an approach to determine the glucosinolates present in A. thaliana seeds. For quantitative analysis, the decrease in signal intensity at 470 nm of the non excitable glucosinolate-phloxine-B complex was used to estimate total glucosinolate content where as the eosin-B complex was used for qualitative analysis. Under these conditions, and with a cross channel capillary configuration for complex formation and analyte separation, ten glucosinolate compounds were detected at a total concentration of 2.78 g/100g of dried seeds within a very fast time of 3 min.

Trenerry and his group quantitatively determined the concentration of the glucosinolate, glucoraphanin, in broccoli seeds and florets using the same MEKC method described by Michaelsen *et al.* but with a lower separation voltage of - 15 kV and by HPLC [216,232]. To date, this is the only example where the separation of an intact glucosinolate

in a crude vegetable extract by CE is demonstrated. For sample pre-treatment, SPE was used as a concentration step for MEKC but necessary to remove a partially co-eluting contaminant for HPLC analysis. Whilst both techniques provided very similar quantitative results (MEKC 2.1 g and HPLC 2.0 g/100 g of broccoli seeds, and MEKC 71 mg and HPLC 70 mg/100 g of broccoli florets), the glucoraphanin was resolved in five min in HPLC analysis whereas the MEKC method was considerably longer with the glucoraphanin eluting at 16 min. In addition, an internal standard (sorbic acid) was required for the CE analysis to compensate for injection irregularities and gradual changes in migration times which further increased the run time to over 20 min.

1.3 Project Aims

As demonstrated by the literature, CE displays great potential as an analytical tool for food analysis and a broad range of food components in different food products have been analysed using this technique. However, despite its demonstrated ability to resolve complex plant components and perform fast analysis, there still exist considerable areas in which the full potential of CE has not been exploited, one of these being in the analysis of the antioxidant compounds, phenolics and glucosinolates, in vegetables. As explained in the preceding sections, the limited literature available for this particular area of food analysis by CE may be a result of the frequent requirement for lengthy extraction procedures prior to analysis and also lowered priority to analyse for antioxidants when compared to compounds which are known to impart harmful effects if consumed over a long timeframe, such as antibiotic and pesticide residues. In addition, the lack of sensitivity in CE (when compared to HPLC), especially for detection of phenolic compounds which may be present at low concentrations in vegetables, may have further hindered developments in this area. For glucosinolates, whilst detection sensitivity is not usually an issue because of the high concentrations normally found in plants of the Brassica family, many of the current CE based separations are significantly long, performed only on standards or on derivatised products of glucosinolates or use sensitive detectors which are not accessible to many research and routine laboratories.

The main aim of this research was to investigate and optimise the potential of CE as a technique for the analysis of phytochemicals (phenolics and glucosinolates) of nutritional/health significance present in vegetables of *Brassica oleracea*. To achieve this

aim, strategies to allow sensitive, robust and reproducible qualitative and quantitative determinations of both compounds was investigated. CZE and MEKC, the two most commonly used CE modes, was explored to optimise the separation of key analytes. For phenolic compounds, the focal point was on developing on-line pre-concentration methods to maximise detection sensitivity for the key phenolics present at low concentrations in brassicas. For glucosinolates, the focus was on developing time efficient CE methods for the separation of the intact glucosinolate, glucoraphanin, in brassicas. And because the glucoraphanin standard was not readily available and expensive to purchase but required for identification purposes in this project, preparative HPLC was also employed as a means to obtain a pure glucoraphanin standard. The developed CE methods were applied to real samples in the Brassica family, especially broccoli which has been analysed extensively by HPLC and hence a wealth of information on phenolics and glucosinolates for this vegetable was available for comparative purposes.

1.4 Research Outline, Methods and Techniques

In the following sections, a general overview of the research framework, and the methods and techniques which have been used in this project will be presented. Whilst full details of each experiment is not supplied and can be found in succeeding chapters, this outline serves to provide a summary of the research structure and links to some of the chapters in this thesis. The discussion will be presented in order of the chapter by which they appear in the thesis.

1.4.1 Determination of Phenolics in *Brassica oleracea* by CZE and with an On-line Pre-concentration Method

Chapter 2 describes the development of a robust CZE method for the analysis of phenolic acids, one of the main phenolic groups identified in *Brassica oleracea* from the literature. The CZE method was developed using a borate separation buffer and a base hydrolysed broccoli extract from which the four key hydroxycinnamic acids (sinapic, ferulic, p-coumaric and caffeic) were detected. Parameters such as buffer concentration was optimised in order to obtain the best possible run times and peak efficiencies and as the phenolic compounds that characterize a vegetable will vary, the ruggedness or

transferability of the method was investigated by the determination of phenolic acids in a range of *B. oleracea* vegetables (broccoli, broccolini, Brussels sprout, cabbage and cauliflower) and the quantitative results were validated by HPLC. Prior to quantitative analysis, the method was validated by intraday repeatability studies on a base hydrolysed broccoli extract and the linearity of the detector response to varying concentrations of phenolic acid standards. In addition, SPE of the phenolic acids was introduced after hydrolysis of the crude extracts as a means to isolate and concentrate the analytes of interest, and compared to the frequently used LLE.

In Chapter 3, following the successful application of the above described CZE method for the determination of phenolic acids in B. oleracea vegetables, the same method was employed in the separation of flavonoids (quercetin and kaempferol), the other class of phenolics found in broccoli. Subsequent to pH adjustment of the separation buffer, six phenolic compounds (two flavonoids and four phenolic acids known to be present in acid hydrolysed broccoli extracts) in a standard mix were resolved using this method. However, when this modified CZE method was applied to acid hydrolysed broccoli extracts, detection sensitivity for the flavonoids was poor and as a result, an on-line pre-concentration technique was employed in an attempt to enhance sensitivity and allow for quantification of these compounds in broccoli. Knowing the extent of enrichment required for UV detection of these two flavonoids, LVSS was investigated as a potential method for preconcentration of these compounds. In doing so, the above optimised CZE method was further refined for on-line concentration through optimisation of LVSS parameters such as stacking voltage and sample injection times. The combined LVSS-CZE method was applied to the separation of phenolics in an acid hydrolysed broccoli extract. Following minor adjustments to stacking times, the LVSS-CZE method was used to quantitatively determination the flavonoid contents in broccoli extracts and the results were validated by HPLC. To ensure method precision, intraday and interday validation studies were performed on both the standard mix and an acid hydrolysed broccoli extract in addition to monitoring the linearity of the detector response to varying concentrations of flavonoids standards prior to quantitative analysis. Furthermore, the acid hydrolysis conditions were optimised for the crude broccoli extract, and under these conditions, the suitability of acid hydrolysis to isolate phenolic acids along with flavonoids (so that both groups can be analysed simultaneously by CE) was also investigated.

1.4.2 Determination of Gluocoraphanin in *Brassica oleracea* by MEKC and Isolation of Glucoraphanin by Preparative HPLC

In Chapter 4, CZE and MEKC methods were examined for the analysis of glucoraphanin, one of the key glucosinolates found in broccoli. In this study, the separation of glucoraphanin in crude broccoli extracts was performed using four different run buffers (for CZE, sodium borate and formic acid and for MEKC, SC and SDS both in sodium borate solutions) and resolution of glucoraphanin was achieved only with the micellar SC system. Thus, parameters for this SC-MEKC method such as micelle concentration was optimised in order to obtain the best possible run times and peak efficiencies and since the method was developed using crude broccoli extracts, the ruggedness or transferability of the method was investigated by determination of glucoraphanin in other cruciferous samples (two broccolis, broccoli seeds and Brussel sprouts) and the quantitative results were validated by HPLC. The method was validated in a similar manner to the CZE method for separation of phenolic acids but with a crude broccoli extract and varying concentrations of glucoraphanin standards for repeatability and linearity measurements, respectively.

As explained in section 1.3, since glucosinolates standards are not readily available and very expensive to purchase, preparative HPLC was employed to produce a pure standard for identifying glucoraphanin in the above work. Although we managed to isolate a sufficient quantity for qualitative purposes, the amount of glucoraphanin required for quantification was too time consuming to generate with the instruments available in our laboratory and hence we relied on the donation of glucoraphanin from another laboratory for our quantitative work (see acknowledgements). However, as a direct result of having to adapt an existing preparative HPLC method suitable to isolate glucoraphanin in our laboratory environment, we used the opportunity to design a preparative HPLC experiment for the undergraduate chemistry course with the aim of introducing another element to HPLC analysis and to increase important laboratory skills which are deemed valuable by employers, from performing extractions to operating the HPLC instrument both as an analytical and preparative tool. A version of this experiment is now currently running in our second year undergraduate analytical chemistry unit. Full details of this experiment are provided in Chapter 5 of the thesis.

1.5 References

- [1] Perez-Lopez, F. R., Chedraui, P., Haya, J., Cuadros, J. L., Maturitas 64 (2009) 67-79.
- [2] Tang, L., Zirpoli, G. R., Guru, K., Moysich, K. B., Zhang, Y. S., Ambrosone, C. B., McCann, S. E., Cancer Epidemiology Biomarkers & Prevention 17 (2008) 938-944.
- [3] Bendinelli, B., Masala, G., Saieva, C., Salvini, S., Calonico, C., Sacerdote, C., Agnoli,
 C., Grioni, S., Frasca, G., Mattiello, A., Chiodini, P., Tumino, R., Vineis, P., Palli, D.,
 Panico, S., American Journal of Clinical Nutrition 93 (2011) 275-283.
- [4] Genkinger, J. M., Platz, E. A., Hoffman, S. C., Comstock, G. W., Helzlsouer, K. J., American Journal of Epidemiology 160 (2004) 1223-1233.
- [5] Lee, S. A., Fowke, J. H., Lu, W., Ye, C. Z., Zheng, Y., Cai, Q. Y., Gu, K., Gao, Y. T., Shu,X. O., Zheng, W., American Journal of Clinical Nutrition 87 (2008) 753-760.
- [6] Pfannhauser, W., Fenwick, G. R., Khokhar, S., *Biologically active phytochemicals in food Analysis, metabolism, bioavailability and function*, The Royal Society of Chemistry, UK, 2001.
- [7] Kushad, M. M., Masiumas, J., Smith, M. A. L., Kalt, W., Eastman, K., Horticultural reviews 28 (2003) 125-185.
- [8] Borowski, J. J., Szajdek, A., Borowska, E. J., Ciska, E., Zielinski, H., European Food Research and Technology 226 (2008) 459-465.
- [9] Heimler, D., Vignolini, P., Dini, M. G., Vincieri, F. F., Romani, A., Food Chemistry 99 (2006) 464-469.
- [10] Stalikas, C. D., Journal of Separation Science 30 (2007) 3268-3295.
- [11] Wang, S. F., Zhang, J. Y., Chen, X. G., Hu, Z. D., Chromatographia 59 (2004) 507-511.
- [12] Vallejo, F., Tomas-Barberan, F.A., Garcia-Viguera, C., Journal of Agricultural and Food Chemistry 51 (2003) 3029-3034.
- [13] Vallejo, F., Tomas-Barberan, F.A., Garcia-Viguera, C., Journal of the Science of Food and Agriculture 82 (2002) 1293-1297.
- [14] Vallejo, F., Gil-Izquierdo, A., Perez-Vicente, A., Garcia-Viguera, C., Journal of Agricultural and Food Chemistry 52 (2004) 135-138.
- [15] Singh J., R. M., Upadhyay A.K., Bahadur A., Chaurasia S.N.S., Singh K.P., Journal of Food Science and Technology 43 (2006) 391-393.
- [16] Kaur, C., Kumar, K., Anil, D., Kapoor, H.C., Journal of Food Biochemistry 31 (2007) 621-638.

- [17] Vallejo, F., Tomas-Barberan, F.A., Garcia-Viguera, C., Journal of the Science of Food and Agriculture 83 (2003) 1511-1516.
- [18] Price, K. R., Casuscelli, F., Colquhoun, I.J., Rhodes, M.J.C., Journal of the Science of Food and Agriculture 77 (1998) 468-472.
- [19] Price, K. R., Casuscelli, F., Colquhoun, I.J., Rhodes, M.J.C., Phytochemistry 45 (1997) 1683-1687.
- [20] Vallejo, F., Garcia-Viguera, C., Tomas-Barberan, F. A., Journal of Agricultural and Food Chemistry 51 (2003) 3776-3782.
- [21] Vallejo, F., Tomas-Barberan, F. A., Garcia-Viguera, C., Journal of the Science of Food and Agriculture 82 (2002) 1293-1297.
- [22] Vallejo, F., Tomas-Barberan, F. A., Garcia-Viguera, C., European Food Research and Technology 216 (2003) 395–401.
- [23] Ferreres, F., Sousa, C., Vrchovska, V., Valentao, P., Pereira, J. A., Seabra, R. M., Andrade, P. B., European Food Research and Technology 222 (2006) 88-98.
- [24] Vallejo, F., Tomas-Barberan, F. A., Gonzalez Benavente-Garcia, A., Garcia-Viguera, C., Journal of the Science of Food and Agriculture 83 (2003) 307-313.
- [25] Gliszczynska-Swiglo, A., Ciska, E., Pawlak-Lemanska, K., Chmielewski, J., Borkowski, T., Tyrakowska, B., Food Additives and Contaminants 23 (2006) 1088-1098.
- [26] Tiselius, A., The Biochemical Journal 31 (1937) 313-317.
- [27] Tiselius, A., The Biochemical Journal 31 (1937) 1464-1477.
- [28] Hjerten, S., Chromatographic Reviews 9 (1967) 122-219.
- [29] Mikkers, F. E. P., Everaerts, F. M., Verheggen, T. P. E. M., Journal of Chromatography 169 (1979) 11-20.
- [30] Virtanen, R., Acta Polytechnica Scandinavica Chemical Technology Series 123 (1974) 1-67.
- [31] Mikkers, F. E. P., Everaerts, F. M., Verheggen, T. P. E. M., Journal of Chromatography 169 (1979) 1-10.
- [32] Jorgenson, J. W., Lukacs, K. D., Analytical Chemistry 53 (1981) 1298-1302.
- [33] Terabe, S., Otsuka, K., Ichikawa, K., Tsuchiya, A., Ando, T., Analytical Chemistry 56 (1984) 111-113.
- [34] Burgi, D. S., Chien, R. L., Journal of Microcolumn Separations 3 (1991) 199-202.
- [35] Breadmore, M. C., Electrophoresis 28 (2007) 254-281.
- [36] Zhang, C.-X., Thormann, W., Analytical Chemistry 68 (1996) 2523-2532.

- [37] Hu, X. L., Cui, S. Y., Liu, J. Q., Chromatographia 72 (2010) 993-997.
- [38] Zinellu, A., Sotgia, S., De Murtas, V., Cossu-Rocca, P., De Miglio, M. R., Muroni, M. R., Mura, A., Uras, M. G., Contini, M., Deiana, L., Carru, C., Analytical and Bioanalytical Chemistry 399 (2011) 1181-1186.
- [39] Li, J. M., Jiang, Y., Biomedical Chromatography 24 (2010) 186-194.
- [40] Hou, X. L., Deng, D. L., Wu, X., Lv, Y., Zhang, J. Y., Journal of Chromatography A 1217 (2010) 5622-5627.
- [41] Xu, L., Basheer, C., Lee, H. K., Journal of Chromatography A 1217 (2010) 6036-6043.
- [42] Chien, R.-L., Burgi, D. S., Analytical Chemistry 64 (1992) 1046-1050.
- [43] Honegr, J., Safra, J., Polasek, M., Pospisilova, M., Chromatographia 72 (2010) 885-891.
- [44] Burgi, D. S., Analytical Chemistry 65 (1993) 3726-3729.
- [45] See, H. H., Hauser, P. C., Ibrahim, W. A. W., Sanagi, M. M., Electrophoresis 31 (2010) 575-582.
- [46] Mallampati, S., Wolfs, K., Pendela, M. M., Hoogmartens, J., Van Schepdael, A., Journal of Liquid Chromatography & Related Technologies 33 (2010) 802-817.
- [47] Al-Ghobashy, M. A., Williams, M. A. K., Laible, G., Harding, D. R. K., Chromatographia 73 (2011) 1145-1153.
- [48] Quirino, J. P., Terabe, S., Journal of Capillary Electrophoresis 4 (1997) 233-245.
- [49] Quirino, J. P., Terabe, S., Journal of Chromatography A 791 (1997) 255-267.
- [50] Quirino, J. P., Terabe, S., Journal of Chromatography A 781 (1997) 119-128.
- [51] Quirino, J. P., Terabe, S., Science 282 (1998) 465-468.
- [52] Quirino, J. P., Terabe, S., Analytical Chemistry 70 (1998) 1893-1901.
- [53] Quirino, J. P., Terabe, S., Journal of Chromatography A 798 (1998) 251-257.
- [54] Quirino, J. P., Terabe, S., Analytical Chemistry 70 (1998) 149-157.
- [55] Quirino, J. P., Terabe, S., Journal of High Resolution Chromatography 22 (1999) 367-372.
- [56] Quirino, J. P., Terabe, S., Analytical Chemistry 71 (1999) 1638-1644.
- [57] Quirino, J. P., Terabe, S., Analytical Chemistry 72 (2000) 1023-1030.
- [58] *Electrokinetic Chromatography Theory, Instrumentation & Applications*, Wiley, 2006.
- [59] Quirino, J. P., Terabe, S., Chromatographia 53 (2001) 285-289.
- [60] Palmer, C. P., Electrophoresis 30 (2009) 163-168.

- [61] Quirino, J. P., Otsuka, K., Terabe, S., Journal of Chromatography B 714 (1998) 29-38.
- [62] Palmer, J., Munro, N. J., Landers, J.P., Analytical Chemistry 71 (1999) 1679-1687.
- [63] Quirino, J. P., Terabe, S., Bocek, P., Analytical Chemistry 72 (2000) 1934-1940.
- [64] Quirino, J. P., Terabe, S., Journal of Chromatography A 965 (2002) 357-373.
- [65] Cifuentes, A., Electrophoresis 31 (2010) 2091-2091.
- [66] Cheung, R. H. F., Marriott, P. J., Small, D. M., Electrophoresis 28 (2007) 3390-3413.
- [67] Escarpa, A., Gonzalez, M. C., Crevillen, A. G., Blasco, A. J., Electrophoresis 28 (2007) 1002-1011.
- [68] Garcia-Canas, V., Cifuentes, A., Electrophoresis 28 (2007) 4013-4030.
- [69] Kvasnicka, F., Electrophoresis 28 (2007) 3581-3589.
- [70] Escarpa, A., Gonzalez, M. C., Gil, M. A. L., Crevillen, A. G., Hervas, M., Garcia, M., Electrophoresis 29 (2008) 4852-4861.
- [71] Font, G., Ruiz, M. J., Fernandez, M., Pico, Y., Electrophoresis 29 (2008) 2059-2078.
- [72] McGlinchey, T. A., Rafter, P. A., Regan, F., McMahon, G. P., Analytica Chimica Acta 624 (2008) 1-15.
- [73] Asensio-Ramos, M., Hernandez-Borges, J., Rocco, A., Fanali, S., Journal of Separation Science 32 (2009) 3764-3800.
- [74] Garcia-Campana, A. M., Gamiz-Gracia, L., Lara, F. J., Iruela, M. D., Cruces-Blanco, C., Analytical and Bioanalytical Chemistry 395 (2009) 967-986.
- [75] Ravelo-Perez, L. M., Asensio-Ramos, M., Hernandez-Borges, J., Rodriguez-Delgado,M. A., Electrophoresis 30 (2009) 1624-1646.
- [76] Robledo, V. R., Smyth, W. F., Electrophoresis 30 (2009) 1647-1660.
- [77] Chen, X. J., Yang, F. Q., Wang, Y. T., Li, S. P., Electrophoresis 31 (2010) 2092-2105.
- [78] Herrero, M., Garcia-Canas, V., Simo, C., Cifuentes, A., Electrophoresis 31 (2010) 205-228.
- [79] Herrero, M., Simo, C., Garcia-Canas, V., Fanali, S., Cifuentes, A., Electrophoresis 31 (2010) 2106-2114.
- [80] Hurtado-Fernández, E., Gómez-Romero, M., Carrasco-Pancorbo, A., Fernández-Gutiérrez, A., Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 1130–1160.
- [81] Vallejo-Cordoba, B., Gonzalez-Cordova, A. F., Electrophoresis 31 (2010) 2154-2164.
- [82] Pinero, M. Y., Bauza, R., Arce, L., Electrophoresis 32 (2011) 1379-1393.

- [83] Rodriguez-Ramirez, R., Gonzalez-Cordova, A. F., Vallejo-Cordoba, B., Analytica Chimica Acta 685 (2011) 120-126.
- [84] Klampfl, C. W., Buchberger, W., Haddad, P. R., Journal of Chromatography A 881 (2000) 357-364.
- [85] Boyce, M. C., Electrophoresis 22 (2001) 1447-1459.
- [86] Klampfl, C. W., Electrophoresis 28 (2007) 3362-3378.
- [87] Cifuentes, A., Electrophoresis 27 (2006) 283-303.
- [88] Kvasnicka, F., Journal of Separation Science 28 (2005) 813-825.
- [89] Cifuentes, A., Bartolome, B., Gomez-Cordoves, C., Electrophoresis 22 (2001) 1561-1567.
- [90] Boyce, M. C., Electrophoresis 28 (2007) 4046-4062.
- [91] Bonoli, M., Pelillo, M., Toschi, T. G., Lercker, G., Food Chemistry 81 (2003) 631-638.
- [92] Lee, B. L., Ong, C. N., Journal of Chromatography A 881 (2000) 439-447.
- [93] Nelson, B. C., Thomas, J. B., Wise, S. A., Dalluge, J. J., Journal of Microcolumn Separations 10 (1998) 671-679.
- [94] Weiss, D. J., Anderton, C. R., Journal of Chromatography A 1011 (2003) 173-180.
- [95] Horie, H., Kohata, K., Journal of Chromatography A 802 (1998) 219-223.
- [96] Peres, R. G., Tonin, F. G., Tavares, M. F. M., Rodriguez-Amaya, D. B., Food Chemistry 127 (2011) 651-655.
- [97] Larger, P. J., Jones, A. D., Dacombe, C., Journal of Chromatography A 799 (1998) 309-320.
- [98] Arce, L., Rios, A., Valcarcel, M., Journal of Chromatography A 827 (1998) 113-120.
- [99] Horie, H., Mukai, T., Kohata, K., Journal of Chromatography A 758 (1997) 332-335.
- [100] Aucamp, J. P., Hara, Y., Apostolides, Z., Journal of Chromatography A 876 (2000) 235-242.
- [101] Worth, C. C. T., Wiessler, M., Schmitz, O. J., Electrophoresis 21 (2000) 3634-3638.
- [102] Huang, H. Y., Lien, W. C., Electrophoresis 26 (2005) 3134-3140.
- [103] Huang, H. Y., Huang, I. Y., Liang, H. H., Lee, S., Electrophoresis 28 (2007) 1735-1743.
- [104] Zhang, H. H., Zhou, L., Chen, X. G., Electrophoresis 29 (2008) 1556-1564.
- [105] Kartsova, L. A., Ganzha, O. V., Russian Journal of Applied Chemistry 79 (2006) 1110-1114.
- [106] Watanabe, T., Nishiyama, R., Yamamoto, A., Nagai S., Terabe, S., Analytical Sciences 14 (1998) 435-438.

- [107] Barroso, M. B., van de Werken, G., Journal of High Resolution Chromatography 22 (1999) 225-230.
- [108] Nelson, B. C., Thomas, J. B., Wise, S. A., Dalluge, J. J., Journal of Microcolumn Separations 10 (1998) 671-679.
- [109] Bonoli, M., Colabufalo, P., Pelillo, M., Toschi, T. G., Lercker, G., Journal of Agricultural and Food Chemistry 51 (2003) 1141-1147.
- [110] Kodama, S., Yamamoto, A., Matsunaga, A., Yanai, H., Electrophoresis 25 (2004) 2892-2898.
- [111] Gotti, R., Furlanetto, S., Lanteri, S., Olmo, S., Ragaini, A., Cavrini, V., Electrophoresis 30 (2009) 2922-2930.
- [112] Dobiasova, Z., Pazourek, J., Havel, J., Electrophoresis 23 (2002) 263-267.
- [113] Pazourek, J., Gonzalez, G., Revilla, A. L., Havel, J., Journal of Chromatography A 874 (2000) 111-119.
- [114] Guadalupe, Z., Soldevilla, A., Saenz-Navajas, M. P., Ayestaran, B., Journal of Chromatography A 1112 (2006) 112-120.
- [115] Gil, M. I., Garcia-Viguera, C., Bridle, P., Tomas-Barberan, F. A., Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung 200 (1995) 278-281.
- [116] Garcia-Viguera, C., Bridle, P., Food Chemistry 54 (1995) 349-352.
- [117] Andrade, P., Seabra, R., Ferreira, M., Ferreres, F., Garcia-Viguera, C., Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung a-Food Research and Technology 206 (1998) 161-164.
- [118] Andrade, P. B., Oliveira, B. M., Seabra, R. M., Ferreira, M. A., Ferreres, F., Garcia-Viguera, C., Electrophoresis 22 (2001) 1568-1572.
- [119] Saenz-Lopez, R., Fernandez-Zurbano, P., Tena, M. T., Journal of Chromatography A 1052 (2004) 191-197.
- [120] Pazourek, J., Gajdosova, D., Spanila, M., Farkova, M., Novotna, K., Havel, J., Journal of Chromatography A 1081 (2005) 48-54.
- [121] Wang, S. P., Huang, K. J., Journal of Chromatography A 1032 (2004) 273-279.
- [122] Saenz-Lopez, R., Fernandez-Zurbano, P., Tena, M. T., Journal of Chromatography A 990 (2003) 247-258.
- [123] Peres, R. G., Micke, G. A., Tavares, M. F. M., Rodriguez-Amaya, D. B., Journal of Separation Science 32 (2009) 3822-3828.

- [124] Woraratphoka, J., Intarapichet, K. O., Indrapichate, K., Food Chemistry 104 (2007) 1485-1490.
- [125] Minussi, R. C., Rossi, M., Bologna, L., Cordi, L., Rotilio, D., Pastore, G. M., Duran, N., Food Chemistry 82 (2003) 409-416.
- [126] Berzas Nevado, J. J. B., Salcedo, A. M. C., Penalvo, G. C., Analyst 124 (1999) 61-66.
- [127] Prasongsidh, B. C., Skurray, G. R., Food Chemistry 62 (1998) 355-358.
- [128] Chu, Q. Y., O'Dwyer, M., Zeece, M. G., Journal of Agricultural and Food Chemistry 46 (1998) 509-513.
- [129] Rodriguez-Delgado, M. A., Perez, J. P., Sanchez, M. J., Montelongo, F. J. G., Chromatographia 52 (2000) 289-294.
- [130] Rodriguez-Delgado, M. A., Perez, M. L., Corbella, R., Gonzalez, G., Montelongo, F. J.G., Journal of Chromatography A 871 (2000) 427-438.
- [131] Sun, Y., Fang, N., Chen, D. D. Y., Donkor, K. K., Food Chemistry 106 (2008) 415-420.
- [132] Hernandez-Borges, J., Borges-Miquel, T., Gonzalez-Hernandez, G., Rodriguez-Delgado, M. A., Chromatographia 62 (2005) 271-276.
- [133] Hsieh, M. C., Lin, C. H., Electrophoresis 25 (2004) 667-682.
- [134] Peng, Y. Y., Chu, Q. C., Liu, F. H., Ye, J. N., Journal of Agricultural and Food Chemistry 52 (2004) 153-156.
- [135] Bednar, P., Papouskova, B., Muller, L., Bartak, P., Stavek, J., Pavlousek, P., Lemr, K., Journal of Separation Science 28 (2005) 1291-1299.
- [136] Moreno, M., Arribas, A. S., Bermejo, E., Zapardiel, A., Chicharro, M., Electrophoresis 32 (2011) 877-883.
- [137] Du, F. Y., Fung, Y. S., Electrophoresis 31 (2010) 2192-2199.
- [138] Hamoudova, R., Urbanek, M., Pospisilova, M., Polasek, M., Journal of Chromatography A 1032 (2004) 281-287.
- [139] Scampicchio, M., Wang, J., Mannino, S., Chatrathi, M. P., Journal of Chromatography A 1049 (2004) 189-194.
- [140] Carrasco-Pancorbo, A., Gomez-Caravaca, A. M., Cerretani, L., Bendini, A., Segura-Carretero, A., Fernandez-Gutierrez, A., Journal of Agricultural and Food Chemistry 54 (2006) 7984-7991.
- [141] Caravaca, A. M. G., Pancorbo, A. C., Diaz, B. C., Carretero, A. S., Gutierrez, A. F., Electrophoresis 26 (2005) 3538-3551.

- [142] Carrasco-Pancorbo, A., Arraez-Roman, D., Segura-Carretero, A., Fernandez-Gutierrez, A., Electrophoresis 27 (2006) 2182-2196.
- [143] Carrasco-Pancorbo, A., Gomez-Caravaca, A. M., Cerretani, L., Bendini, A., Segura-Carretero, A., Fernandez-Gutierrez, A., Journal of Separation Science 29 (2006) 2221-2233.
- [144] Carrasco-Pancorbo, A., Gomez-Caravaca, A. M., Segura-Carretero, A., Cerretani, L., Bendini, A., Fernandez-Gutierrez, A., Journal of the Science of Food and Agriculture 89 (2009) 2144-2155.
- [145] Carrasco-Pancorbo, A., Neususs, C., Pelzing, M., Segura-Carretero, A., Fernandez-Gutierrez, A., Electrophoresis 28 (2007) 806-821.
- [146] Garcia-Villalba, R., Carrasco-Pancorbo, A., Vazquez-Martin, A., Oliveras-Ferraros, C., Menendez, J. A., Segura-Carretero, A., Fernandez-Gutierrez, A., Electrophoresis 30 (2009) 2688-2701.
- [147] Gomez-Caravaca, A. M., Carrasco-Pancorbo, A., Segura-Carretero, A., Fernandez-Gutierrez, A., Electrophoresis 30 (2009) 3099-3109.
- [148] Menendez, J. A., Vazquez-Martin, A., Garcia-Villalba, R., Carrasco-Pancorbo, A., Oliveras-Ferraros, C., Fernandez-Gutierrez, A., Segura-Carretero, A., BMC Cancer 8 (2008).
- [149] Pancorbo, A. C., Cruces-Blanco, C., Carretero, A. S., Gutierrez, A. F., Journal of Agricultural and Food Chemistry 52 (2004) 6687-6693.
- [150] Bendini, A., Bonoli, M., Cerretani, L., Biguzzi, B., Lercker, G., Toschi, T. G., Journal of Chromatography A 985 (2003) 425-433.
- [151] Bonoli, M., Montanucci, M., Toschi, T. G., Lercker, G., Journal of Chromatography A 1011 (2003) 163-172.
- [152] Bonoli, M., Bendini, A., Cerretani, L., Lercker, G., Toschi, T. G., Journal of Agricultural and Food Chemistry 52 (2004) 7026-7032.
- [153] Pancorbo, A. C., Carretero, A. S., Gutierrez, A. F., Journal of Separation Science 28 (2005) 925-934.
- [154] Nevado, J. J. B., Penalvo, G. C., Robledo, V. R., Martinez, G. V., Talanta 79 (2009) 1238-1246.
- [155] Nevado, J. J. B., Penalvo, G. C., Robledo, V. R., Talanta 82 (2010) 548-554.
- [156] Ibanez, E., Cifuentes, A., Crego, A. L., Senorans, F. J., Cavero, S., Reglero, G., Journal of Agricultural and Food Chemistry 48 (2000) 4060-4065.

- [157] Saenz-Lopez, R. R., Fernandez-Zurbano, P., Tena, M. T., Journal of Chromatography 953 (2002) 251-256.
- [158] Bonoli, M., Pelillo, M., Lercker, G., Chromatographia 57 (2003) 505-512.
- [159] Crego, A. L., Ibanez, E., Garcia, E., Rodriguez de Pablos, R., Senorans, F.J., Reglero,G., Cifuentes, A., European Food Research and Technology 219 (2004) 549-555.
- [160] Herrero, M., Arraez-Roman, D., Segura, A., Kenndler, E., Gius, B., Raggi, M. A., Ibanez, E., Cifuentes, A., Journal of Chromatography A 1084 (2005) 54-62.
- [161] Peng, Y. Y., Yuan, J. J., Liu, F. H., Ye, J. N., Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 431-437.
- [162] Richheimer, S. L., Bernart, M. W., King, G. A., Kent, M. C., Bailey, D. T., Journal of the American Oil Chemists Society 73 (1996) 507–514.
- [163] Cuvelier, M. E., Richard, H., Berset, C., Journal of the American Oil Chemists Society 73 (1996) 645–652.
- [164] Cheung, H. Y., Zhang, Q. F., Journal of Chromatography A 1213 (2008) 231-238.
- [165] Peng, Y. Y., Ye, J. N., Fitoterapia 77 (2006) 171-178.
- [166] Zhang, Y., Chen, J., Zhao, L., Shi, Y. P., Biomedical Chromatography 21 (2007) 987-992.
- [167] Zhu, J. H., Yu, K., Chen, X. G., Hu, Z. D., Journal of Chromatography A 1166 (2007) 191-200.
- [168] Peng, Y. Y., Ye, J. N., Kong, J. L., Journal of Agricultural and Food Chemistry 53 (2005) 8141-8147.
- [169] Baskan, S., Oztekin, N., Erim, F. B., Food Chemistry 101 (2007) 1748-1752.
- [170] Fonseca, F. N., Tavares, M. F. M., Horvath, C., Journal of Chromatography A 1154 (2007) 390-399.
- [171] Wang, X. K., He, Y. Z., Qian, L. L., Talanta 74 (2007) 1-6.
- [172] Kocevar, N., Glavac, I., Injac, R., Kreft, S., Journal of Pharmaceutical and Biomedical Analysis 46 (2008) 609-614.
- [173] Petr, J., Vitkova, K., Ranc, V., Znaleziona, J., Maier, V., Knob, R., Sevcik, J., Journal of Agricultural and Food Chemistry 56 (2008) 3940-3944.
- [174] Yang, H. P., Yue, M. E., Shi, Y. P., Chromatographia 63 (2006) 449-452.
- [175] Segura-Carretero, A., Puertas-Mejia, M. A., Cortacero-Ramirez, S., Beltran, R., Alonso-Villaverde, C., Joven, J., Dinelli, G., Fernandez-Gutierrez, A., Electrophoresis 29 (2008) 2852-2861.

- [176] Zhang, S., Dong, S. Q., Chi, L. Z., He, P. G., Wang, Q. J., Fang, Y. Z., Talanta 76 (2008) 780-784.
- [177] Chu, Q. C., Qu, W. Q., Peng, Y. Y., Cao, Q. H., Ye, J. N., Chromatographia 58 (2003) 67-71.
- [178] Safra, J., Pospisilova, M., Spilkova, J., Chromatographia 64 (2006) 37-43.
- [179] Urbanek, M., Blechtova, L., Pospisilova, M., Polasek, M., Journal of Chromatography A 958 (2002) 261-271.
- [180] Hamoudova, R., Pospisilova, M., Spilkova, J., Electrophoresis 27 (2006) 4820-4826.
- [181] Safra, J., Pospisilova, M., Honegr, J., Spilkova, J., Journal of Chromatography A 1171 (2007) 124-132.
- [182] Bednar, P., Tomassi, A. V., Presutti, C., Pavlikova, M., Lemr, K., Fanali, S., Chromatographia 58 (2003) 283-287.
- [183] Herrero-Martinez, J. M., Rafols, C., Roses, M., Bosch, E., Lozano, C., Torres, J. L., Electrophoresis 24 (2003) 1404-1410.
- [184] Herrero-Martinez, J. M., Rafols, C., Roses, M., Torres, J. L., Bosch, E., Electrophoresis 24 (2003) 707-713.
- [185] Huang, H. Y., Lien, W. C., Chiu, C. W., Journal of Separation Science 28 (2005) 973-981.
- [186] Priego Capote, F., Luque Rodriguez, J. M., Luque de Castro, M. D., Journal of Chromatography A 1139 (2007) 301-307.
- [187] Berli, F., D'Angelo, J., Cavagnaro, B., Botini, R., Wuilloud, R., Fernanda Silva, M., Journal of Agricultural and Food Chemistry 56 (2008).
- [188] Fernandes, J. B., Griffiths, D. W., Bain, H., Phytochemical Analysis 7 (1996) 97-103.
- [189] Bridle, P., GarciaViguera, C., Food Chemistry 59 (1997) 299-304.
- [190] da Costa, C. T., Nelson, B. C., Margolis, S. A., Horton, D., Journal of Chromatography A 799 (1998) 321-327.
- [191] Ichiyanagi, T., Hatano, Y., Matsugo, S., Konishi, T., Chemical & Pharmaceutical Bulletin 52 (2004) 434-438.
- [192] Ichiyanagi, T., Hatano, Y., Matsuo, S., Konishi, T., Chemical & Pharmaceutical Bulletin 52 (2004) 1312-1315.
- [193] Ichiyanagi, T., Kashiwada, Y., Ikeshiro, Y., Hatano, Y., Shida, Y., Horie, M., Matsugo,S., Konishi, T., Chemical & Pharmaceutical Bulletin 52 (2004) 226-229.

- [194] Watson, D. J., Bushway, A. A., Bushway, R. J., Journal of Liquid Chromatography & Related Technologies 27 (2004) 113-121.
- [195] Ehala, S., Vaher, M., Kaljurand, M., Journal of Agricultural and Food Chemistry 53 (2005) 6484-6490.
- [196] Comandini, P., Blanda, G., Cardinali, A., Cerretani, L., Bendini, A., Caboni, M. F., Journal of Separation Science 31 (2008) 3257-3264.
- [197] Fukuji, T. S., Tonin, F. G., Tavares, M. F. M., Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 430-438.
- [198] Kanitsar, K., Arce, L., Rios, A., Valcarcel, M., Electrophoresis 22 (2001) 1553-1560.
- [199] Gel-Moreto, N., Streich, R., Galensa, R., Electrophoresis 24 (2003) 2716-2722.
- [200] Kofink, M., Papagiannopoulos, M., Galensa, R., European Food Research and Technology 225 (2007) 569-577.
- [201] Blasco, A. J., Barrigas, I., Gonzalez, M. C., Escarpa, A., Electrophoresis 26 (2005) 4664-4673.
- [202] Crevillen, A. G., Pumera, M., Gonzalez, M. C., Escarpa, A., Lab on a Chip 9 (2009) 346-353.
- [203] Sawalha, S. M. S., Arraez-Roman, D., Segura-Carretero, A., Fernandez-Gutierrez, A., Food Chemistry 116 (2009) 567-574.
- [204] Herrero-Martinez, J. M., Oumada, F. Z., Roses, M., Bosch, E., Rafols, C., Journal of Separation Science 30 (2007) 2493-2500.
- [205] Helmja, K., Vaher, M., Gorbatsova, J., Kaljurand, M., Proceedings of the Estonian Academy of Sciences Chemistry 56 (2007) 172–186.
- [206] Helmja, K., Vaher, M., Pussa, T., Kaljurand, M., Journal of Chromatography A 1216 (2009) 2417-2423.
- [207] Helmja, K., Vaher, M., Pussa, T., Raudsepp, P., Kaljurand, M., Electrophoresis 29 (2008) 3980-3988.
- [208] Fernandes, J. B., Griffiths, D. W., Bain, H., Fernandes, F. A. N., Phytochemical Analysis 7 (1996) 253-258.
- [209] Peng, Y. Y., Zhang, Y. W., Ye, J. N., Journal of Agricultural and Food Chemistry 56 (2008) 1838-1844.
- [210] Kvasnička, F., Čopíková, J., Ševčík, R., Krátká, J., Syntytsia, A., Voldřich, M., Central European Journal of Chemistry 6 (2008) 410-418.

- [211] Liu, L. H., Chen, X. G., Liu, J. L., Deng, X. X., Duan, W. J., Tan, S. Y., Food Chemistry 119 (2010) 1228-1232.
- [212] Price, K. R., Rhodes, M. J. C., Journal of the Science of Food and Agriculture 74 (1997) 331-339.
- [213] Caridi, D., Trenerry, V. C., Rochfort, S., Duong, S., Laugher, D., Jones, R., Food Chemistry 105 (2007) 691-699.
- [214] Petersson, E. V., Puerta, A., Bergquist, J., Turner, C., Electrophoresis 29 (2008) 2723-2730.
- [215] Dadakova, E., Prochazkova, E., Krizek, M., Electrophoresis 22 (2001) 1573-1578.
- [216] Michaelsen, S., Moller, P., Sorensen, H., Journal of Chromatography 608 (1992) 363-374.
- [217] Morin, P., Villard, F., Quinsac, A., Dreux, M., Journal of High Resolution Chromatography 15 (1992) 271-275.
- [218] Feldl, C., Moller, P., Otte, J., Sorensen, H., Analytical Biochemistry 217 (1994) 62-69.
- [219] Bjergegaard, C., Michaelsen, S., Moller, P., Sorenson, H., Journal of Chromatography A 717 (1995) 325-333.
- [220] Paugam, L., Menard, R., Larue, J. P., Thouvenot, D., Journal of Chromatography A 864 (1999) 155-162.
- [221] Bellostas, N., Sorensen, J. C., Sorensen, H., Journal of Chromatography A 1130 (2006) 246-252.
- [222] Bellostas, N., Sorensen, A. D., Sorensen, J. C., Sorensen, H., Journal of Natural Products 71 (2008) 76-80.
- [223] Van Eylen, D., Bellostas, N., Strobel, B. W., Oey, I., Hendrickx, M., Van Loey, A., Sorensen, H., Sorensen, J. C., Food Chemistry 112 (2009) 646-653.
- [224] Bellostas, N., Sorensen, A. D., Sorensen, J. C., Sorensen, H., Journal of Molecular Catalysis B-Enzymatic 57 (2009) 229-236.
- [225] Bellostas, N., Sorensen, J. C., Sorensen, H., (2007) http://orgprints.org/11399.
- [226] Bellostas, N., Bjergegaard, C., Jensen, S. K., Sorensen, H., Sorensen, J. C., Sorensen, S., (2007) http://orgprints.org/11397.
- [227] Karcher, A., El Rassi, Z., Journal of Liquid Chromatography & Related Technologies 21 (1998) 1411-1432.
- [228] Karcher, A., Melouk, H. A., El Rassi, Z., Journal of Agricultural and Food Chemistry 47 (1999) 4267-4274.

- [229] Karcher, A., Melouk, H. A., El Rassi, Z., Analytical Biochemistry 267 (1999) 92-99.
- [230] Bringmann, G., Kajahn, I., Neususs, C., Pelzing, M., Laug, S., Unger, M., Holzgrabe, U., Electrophoresis 26 (2005) 1513-1522.
- [231] Fouad, M., Jabasini, M., Kaji, N., Terasaka, K., Tokeshi, M., Mizukami, H., Baba, Y., Electrophoresis 29 (2008) 2280-2287.
- [232] Trenerry, V., Caridi, D., Elkins, A., Donkor, O., Jones, R., Food Chemistry 98 (2006) 179-187.

Chapter 6 General Conclusions and Future Directions

In this project, the ability of CE to determine the phytochemicals present in edible plants of *Brassica oleracea* has been demonstrated. Methods were developed for two fundamental CE modes, CZE and MEKC and were successfully applied to the quantitative determination of phenolic and glucosinolate compounds in a variety of brassica vegetables and seeds.

The key phenolics in brassicas, phenolic acids and flavonoids, which are representative of their conjugated forms, were separated by CZE using sodium borate, a buffer typically employed in CE separations. This simple and rapid CZE method allowed for the baseline resolution of four hydroxycinnamic acids within 7 min. For the flavonoids, sensitive detection was achieved by sample enrichment using the online pre-concentration technique, LVSS. The combination of LVSS and CZE enabled quantitative determination of the two predominant flavonols in broccoli with a separation time of less than 8 min. For both CE methods, analysis times were significantly faster when compared to existing traditional HPLC analysis of brassica phenolics (40 to 100 min) which highlights a major advantage of this electrophoretic technique.

Since the phenolics in *B. oleracea* occur naturally as conjugated derivatives, prior to analysis, the brassica extracts were chemically hydrolysed and the target analytes were isolated and concentrated by SPE in order to create simplified electropherograms for identification and quantification of the key phenolics. For future work, the hyphenation of CE to MS would be beneficial for the identification of native phenolic species in brassicas. This CE-MS coupling would 1) provide enhanced detection sensitivity, and structural information for the phenolic derivatives present in brassicas and 2) eliminate the requirement for the hydrolysis step (which, at present, is necessary for quantification with UV detection) thereby reducing offline pre-treatment and handling of the sample resulting in less manual labour and time, and systematic errors. Another approach worth considering for the analysis of brassica phenolics would be multi capillary/channel CE systems such as CFS or microchip CE. As described in previous sections, these CE arrangements exploit the use of two or more interlinking capillaries/channels for multiple functions from sample clean up to complex formation to analyte separation. The possibility to develop methods for integration of the sample pre-treatment steps (hydrolysis or isolation/pre-

concentration) into an on-line format, especially for the ever expanding research area of CE microsystems where usually sensitive detectors (fluorescent or EDs) are employed and analysis times are very short, may make this an attractive option for phenolic analysis of brassica vegetables in the near future.

Glucoraphanin, one of the pre-dominant aliphatic glucosinolates in B. oleracea, was successfully resolved by a MEKC method. With SC as the micellar phase, the separation of glucoraphanin from crude extracts of brassica vegetables and seeds was achieved within 5 min. This SC-MEKC method is superior to previous CE methods used in glucosinolate analysis of brassica matrices in that 1) no derivatisation procedures were involved and the intact glucosinolate extracts were loaded directly onto the capillary and 2) the run times were considerably shorter (by over 15 min). For future work, this method could be extended to other glucosinolates in B. oleracea. However, as our present SC-MEKC system was modelled on glucoraphanin, the interaction of aromatic or indeed other aliphatic glucosinolates with the relatively polar SC cannot be predicted. Modification of the method e.g. by using other anionic surfactants (SDS) or mixed micelle systems (SC-SDS) may be necessary to ensure adequate micelle to analyte interaction and hence satisfactory resolution and analysis times for a suite of glucosinolates in crude brassica extracts. In any case, for many routine and research laboratories, the issues with the lack of availability and prohibitive costs of many glucosinolates must be resolved before any further attempts can be made to identify and quantify these compounds by CE. This provides a future opportunity for the development of small scale preparative HPLC methods for glucosinolates and whilst fractionation is possible with CE, the amount required for analytical purposes would be difficult to generate given the small volume of sample which can be loaded onto a capillary and hence this is not a viable alternative to preparative HPLC.

Finally, as the phenolic and glucosinolate characteristics between brassica families are very similar, the CE methods developed in this project could generally be used for the analysis of these compounds in crucifers outside the *B. oleracea* group.