METHOD DEVELOPMENT FOR THE ANALYSIS OF PROTEINS AND SMALL
MOLECULES BY CE-LIF, CE-MS, AND HPLC-ELSD

BY

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ABSTRACT

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METHOD DEVELOPMENT FOR THE ANALYSIS OF PROTEINS AND SMALL MOLECULES BY CE-LIF, CE-MS, AND HPLC-ELSD

Dissertation under the direction of Christa L. Colyer, Professor of Chemistry

Analytical separations in conjunction with powerful detection methods make possible the quantitation of large and small molecules important in environmental science, food science, pharmaceutical, and medical matrices. Many of these separations utilize instrumental methods such as capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) to alleviate the complexity of these various matrices. Fluorescence, laser-induced fluorescence (LIF), evaporative light scattering detection (ELSD), and mass spectrometry (MS) are detection methods commonly employed in conjunction with these separation strategies. Three distinct projects to develop and exploit such analytical technologies are described. First, few biological molecules are natively fluorescent, so they often must be labeled with a fluorescent tag in order to be detected via LIF. Furthermore, protein separations using CE-LIF may be complicated by protein interactions with the negatively charged surface of the inner capillary wall, arising from the ionization of surface silanol groups. Presented here is a method for quantitation of protein-dye complexes using a self assembled, surfactant-coated capillary and on-column protein labeling with a non-covalent squarylium dye, Red-1c. Second,
seasonal sugar levels are relevant to the relationship between winter anthocyanin production and drought stress in angiosperm evergreen species, and so the development of a method for rapid analysis of sugars in seasonal tree leaf tissue is needed. An HPLC-ELSD method was developed for the quantitation of three sugars, glucose, fructose, and sucrose. The method was subjected to standard addition validation and was shown to provide precise and accurate measurement of all three analytes in real samples.

Third, in addition to their many uses as solvents, ionic liquids (IL) have been utilized as highly effective substrates for complexation and subsequent quantitation of anions by electrospray ionization mass spectrometry (ESI-MS) and high performance liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS). The present work provides an alternative method utilizing capillary electrophoresis coupled with electrospray ionization mass spectrometry (CE-ESI-MS). This method offers advantages over LC-MS in that CE, in general, provides shorter separation times, requires less reagent and solvent, and needs less sample volume. Mono- and divalent anionic analytes, were complexed with di- and trivalent cationic liquids. The complexation reaction was undertaken in a variety of modes, including pre-column, on-column and post-column, and the resulting complexes, which were positively charged and of higher mass than the native analytes, were subsequently analyzed by MS. This method allowed for greater sensitivity than could be achieved by direct detection.
CHAPTER 1

INTRODUCTION

Analytically quantitative separations are required for complex mixtures that include biologically relevant molecules such as proteins, saccharides, and organic and inorganic ions. Many biological separations utilize instrumental methods such as high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) to alleviate the complexity of various matrices. Fluorescence, laser-induced fluorescence (LIF), evaporative light scattering detection (ELSD), and mass spectrometry (MS) are detection methods commonly employed in conjunction with these separation strategies and will be discussed in detail. Several biological and environmentally relevant analytical applications will be defined and explored in this body of work.

Capillary Electrophoresis

Capillary electrophoresis is used to separate ionic species by their charge and frictional forces arising due to their hydrodynamic radius and, as a result, is the most efficient analytical separation technique available for both large and small molecules. The origins of CE date to the turn of the 20th century when Kohlrauch derived a theory for ionic migration that described electrophoretic principles of separation.¹ In 1937, Arne Tiselius first described moving boundary electrophoresis to separate proteins.² Although this effort was limited by incomplete separation, requirement of large sample volumes, low electric fields, and long separation times, Tiselius was subsequently awarded the Nobel Prize for this work in 1948. The advent of CE brought with it reduced sample solvent and waste, improved precision and better resolution as solutions to some of the
problems encountered by Tiselius. The first narrow bore tube CE was described by Hjerten in 1967. The evolution of CE in the later part of the 20th century included the use of even narrower bore capillaries and the movement from Pyrex glass to fused silica. The hallmark benefits of CE, namely resolution, efficiency, sample volume and waste reduction, were greatly improved by CE pioneers Jorgenson, Lukacs, Mikkers and co-workers.

A CE apparatus of the modern era consists of a high voltage supply (typically +/-30 kV), a fused silica capillary (typically 10-100 μm inner diameter), two buffer reservoirs, two platinum electrodes, and an on-column detector (see Figure 1). The fused silica capillary is externally coated with polyamide to make it stronger and more flexible. The sample is introduced for analysis by controlling the injection voltage (electrokinetic injection) or the injection pressure (hydrodynamic injection), with one end of the capillary in a sample vial. CE is an advantageous analytical technique because of fast analysis times, its use of very little sample, and its lower cost than chromatography or conventional electrophoresis.

Electrophoresis is the migration of dispersed, charged species relative to a fluid under the influence of a spatially uniform electric field. The electrophoretic mobility of an ion is proportional to the charge on the ion and inversely proportional to any impeding forces, for example, ion size and viscosity of the solution in which it is migrating (see Figure 2). Therefore, two ions of the same charge and in the same solution will migrate according to size, with the larger ion traveling slower than the smaller ion. Electroosmosis refers to the bulk movement of the buffer in the capillary. When the surface of the capillary is presented with a buffer above pH 2, the surface of the capillary
is negatively charged due to the ionization of surface silanol groups. The negative surface charges are counter balanced by positive ions in the buffer forming an electric double layer. As the positive charges migrate toward the cathode they drag along their waters of hydration. This migration is referred to as electroosmotic flow (EOF).

A unique feature of capillary electrophoresis relative to other bulk flow analytical separation techniques, such as HPLC, is the flat flow profile resulting from the EOF. When utilizing a pressure driven system such as HPLC, the frictional forces at the liquid-solid interfaces, such as the packing and the walls of the tubing, result in substantial pressure drops. In an open tube, the frictional forces are enough to result in a laminar, or parabolic, flow profile. A consequence of parabolic flow is a flow velocity that is highest in the middle of the tube and approaches zero at the tubing wall, thus resulting in substantial band broadening. A cross-sectional flow profile of electroosmotic flow and laminar flow are shown in Figure 3. The unique, electrically driven flat flow profile of CE results in a uniform velocity across the entire diameter of the tubing, except for the most extreme edges at the wall of the tubing, and this leads to reduced band broadening and hence, increased separation efficiency.

The apparent mobility of a solute or analyte is the sum of the electrophoretic mobility and the electroosmotic mobility. Solutes (and/or analytes) with neutral charge migrate with the electroosmotic flow while the positive ions move ahead of this and negative ions lag behind this with affinity towards the anode (see Figure 2). Joule heating results from the resistance of the solution to the flow of the current. If this generated heat is not dissipated, the resulting increases in temperature and density gradients dramatically reduce the separation efficiency. As a result of the large surface
area-to-volume ratio of a capillary when compared to a slab gel, the capillary dissipates the joule heat more efficiently, therefore providing a more efficient separation and allowing for a higher voltage to be applied for the separation. A higher voltage will result in a decrease in the overall analysis time.

When considering capillary electrophoresis as an analytical tool, it is important to discuss sample introduction. There are three general mechanisms used to inject a sample onto a capillary column. Electrokinetic injection involves placing the inlet electrode and capillary into the sample vial with the outlet electrode and capillary in the run buffer and applying a potential for a fixed period of time. This technique can provide biased injections due to differing mobilities of the analytes and sample matrices. A second procedure, pressure injection, involves placing the inlet end of the capillary and electrode into the sample vial, and introducing positive air pressure to the inlet end of the capillary, or by applying a vacuum to the outlet end of the capillary to draw the sample into the capillary. When siphoning, the inlet end of the capillary is elevated relative to the outlet end, forcing a siphon into the capillary under force of gravity. Each method of injection requires very little sample volume, thus separations with nanoliter amounts of sample can be injected into microliter quantities of buffer.

The selection of buffer for a CE separation, analogous to selection of mobile phase for liquid chromatography, is crucial in method development. The buffer should not affect the detection of analyte, which requires it to be non-absorbing in the wavelength region of the analyte of interest. Also, the buffer should have good buffering capacity to prevent pH changes in the capillary, as well as maintaining a pH conducive to adequate separation. Buffer concentration must also be considered. Higher buffer
concentrations minimize wall interactions and generally slows EOF for better separation of analytes, although broader peaks may result.

**CE Modes**

There are several modes of capillary electrophoresis to be considered including capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary electrochromatography (CEC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP). Capillary zone electrophoresis (CZE) is the most common mode and is the primary mode used for separations described in this work. CZE uses a constant buffer composition and separates analytes by forming zones in which differently charged particles migrate. By manipulating buffer selection, additives, and capillary wall treatment, CZE is easily adapted to one of the many other modes of CE.

Terabe described micellar electrokinetic chromatography (MEKC) in the early 1980s. MEKC implementation has allowed for the separation of neutral molecules as well as charged molecules by the utility of micelles in the separation buffer. Micelles are aggregates of amphiphilic (having both hydrophilic and lipophilic regions) monomers called surfactants. When surfactant molecules exceed their critical micelle concentration (CMC) they are drawn together by the polar medium with the hydrophobic tails pointing inward and the hydrophilic head-groups outwardly facing. Micelles serve as pseudostationary phases that solutes can interact with during the separation. Charged micelles can also participate in the separation through their own inherent migration. The partitioning of the solutes between the micelles and buffer can be controlled by the concentration of the surfactant, buffer additives, temperature, pH, and ionic strength.
The surfactant also helps to minimize the adsorption of compounds to the negatively charged silanol groups of the capillary walls. Adsorption to the walls can lead to sample loss, peak broadening, poor resolution, unstable EOF, and increased migration times. Another use of long-chained ionic surfactants as semi-permanent capillary coatings will be discussed in this work.

Capillary electrochromatography (CEC) is a hybridized form of CE and chromatography. The capillary is packed with a stationary phase or the capillary is coated with a stationary phase, into which analytes can partition. Capillary isoelectric focusing (CIEF) separates zwitterionic compounds such as proteins, peptides, and amino acids based on their isoelectric points, with separation achieved by utilizing a pH gradient across the capillary. In capillary gel electrophoresis (CGE), a capillary is filled with a porous gel or polymer matrix. The pores contain buffer, and the separation of molecules that differ in size but not necessarily charge, such as DNA, proteins, and macromolecules, is possible with this technique. Capillary isotachophoresis (CITP) relies on two electrolytes, one a leading electrolyte with a higher mobility than the analyte, the other a trailing electrolyte with a slower mobility than the analyte, between which the sample is injected. CITP can be used to separate a mixture of cations or a mixture of anions, but not both simultaneously.

**Liquid Chromatography**

High performance liquid chromatography (HPLC) is an analytical technique in which the mobile phase is a liquid and the separation is carried out on a stationary phase, usually on a packed column. HPLC generally utilizes very small packing particles, 2.0-10.0 microns, and a relatively high back pressure, 500-4,000 p.s.i. When a column
packing is used with a particle size smaller than 2.0 microns and back pressures range between 4000-15,000 p.s.i., this is referred to as ultra performance liquid chromatography, or UPLC. HPLC can efficiently separate a mixture of compounds, and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture. HPLC utilizes different types of stationary phase, a pump that moves the mobile phase and analyte through the column, and a detector that provides a characteristic retention time for the analyte. The detector may also provide other characteristic information, such as UV/Vis spectroscopic or mass spectral data for an analyte if so equipped. Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio and composition of solvents used, and the flow rate of the mobile phase.

In HPLC, the sample is forced through a column that is packed with irregularly or spherically shaped particles, a porous monolithic layer (stationary phase) or a porous membrane by a liquid (mobile phase) at high pressure. HPLC is typically divided into two different sub-classes based on the polarity of the mobile and stationary phases. Separations in which the stationary phase is more polar than the mobile phase (for example, hexane as the mobile phase and silica as the stationary phase) are termed “normal phase liquid chromatography” and the opposite (for example, a mixture of water-methanol as the mobile phase and octadecylsilyl (C18) as the stationary phase) is termed “reversed phase liquid chromatography”. Normal phase typically has fewer applications and reverse phase is therefore used considerably more, especially for quantitative analysis. A schematic diagram of a typical HPLC system is shown in Figure 4.
Chromatography was first described in 1903 by the Russian botanist Mikhail Tswett and subsequently published in 1906. His pioneering studies focused on separating leaf pigments, extracted from plants using a solvent, in a column packed with particles. Tswett filled an open glass column with particles, either powdered chalk, or calcium carbonate, and alumina. He transferred his liquid sample, a solvent extract of homogenized plant leaves, onto the column and allowed it to move through the particle bed, followed by addition of pure solvent. As the sample passed down through the column by gravity, different colored bands were observed because some components were moving faster than others. He determined the differently colored bands to be the different compounds that were originally contained in the sample. He developed an analytical separation of these compounds based on the differing strength of each compound’s chemical attraction to the packing particles. The compounds that were more strongly attracted to the particles migrated slowly, while other compounds more strongly attracted to the solvent moved faster. The compounds contained in the sample are distributed, or partitioned, differently between the moving solvent, called the mobile phase, and the particles, called the stationary phase. This causes each compound to move at a different speed, thus creating a separation of the compounds. Tswett conceived the name chromatography, from the Greek words *chroma*, meaning color, and *graph*, meaning writing, to describe his experiment.

In 1941, Martin and Synge published their Nobel prize winning work in which they described liquid-liquid, or partition chromatography, which also included plate theory. Plate theory describes the chromatography system, consisting of the mobile and stationary phases, as being in equilibrium and the partitioning of species throughout this
equilibrium. The next evolution of chromatography in the mid 1900s brought the technique of plane chromatography to the forefront. Paper was initially used as the plane support, or stationary phase. Then, thin layers of silica gel replaced paper as thin-layer chromatography (TLC) became a preferred technique. Rate theory was also developed in the mid-20th century by chromatography pioneers such as van Deemter et al.\textsuperscript{20} and Giddings.\textsuperscript{21} Rate theory describes the process of peak dispersion (band spreading) and allows the calculation of the variance per unit length of a column (the height equivalent of the theoretical plate, HETP) in terms of the mobile phase velocity and other physical-chemical properties of the solute and distribution system.\textsuperscript{20,21}

The sample to be analyzed by HPLC is introduced in small volume to the stream of mobile phase. The analyte motion through the column is slowed by specific chemical or physical interactions with the stationary phase as it traverses the length of the column. How much the analyte is slowed depends on the nature of the analyte and on the compositions of the stationary and mobile phases. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time. Under particular conditions, the retention time is considered a reasonably unique identifying characteristic of a given analyte. The use of smaller particle size column packing (which creates higher backpressure) increases the linear velocity giving the components less time to diffuse within the column, leading to improved resolution in the resulting chromatogram. Common solvents used include any miscible combination of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the analyte components, or compounds such as trifluoroacetic acid, which acts as an ion pairing agent.
A further refinement to HPLC has been to vary the mobile phase composition during the analysis, also known as gradient elution. A typical gradient for reversed phase chromatography might start at 5% methanol and progress linearly to 50% methanol over 25 minutes; the gradient chosen depends on how hydrophobic the analyte is. The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase composition relative to the stationary phase. This partitioning process is similar to that which occurs during a liquid-liquid extraction but in the case of gradient elution LC, is continuous, not step-wise. In the situation described above using a water/methanol gradient, the more hydrophobic components will elute (come off the column) when the mobile phase consists mostly of methanol, which is a relatively more hydrophobic mobile phase). The more hydrophilic compounds will elute under conditions of relatively low methanol/high water content. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte. Often a series of tests are performed on the analyte and a number of trial runs may be processed in order to find the HPLC method that gives the best separation of peaks.

**Detection of Analytes**

The detection of analytes of interest is an important factor to be considered when developing a separation method. Quantitative detection should be over a large dynamic range with a high sensitivity for the analyte. There have been numerous detection methods employed with CE and/or HPLC including UV/Vis absorbance, fluorimetry, laser-induced fluorimetry, mass spectrometry, amperometry, conductivity, NMR, refractive index, radiometry, Raman spectrometry, and evaporative light scattering detection (ELSD). The present work will concentrate on CE in conjunction with laser-
induced fluorimetry (LIF) and mass spectrometry (MS), and HPLC in conjunction with ELSD and MS. A summary of each detection method and corresponding detection limit is given in Table 1.

Fluorescence is the emission of light from the transition of an electronically excited molecule in the singlet state to its electronic ground state. The molecule is excited by absorbing a photon of an appropriate wavelength and thus promoting a non-bonding electron to an anti-bonding orbital. A finite, limited number of molecules in the excited state will emit fluorescence because of the competition of internal conversion and inter-system crossing. A good fluorophore must possess a high fluorescence quantum yield, meaning that it should have a rate constant for fluorescence comparable to those of transitions without radiation. It should also have a high molar absorptivity. With laser based techniques, the fluorescence signal is proportional to the detection path length and the irradiance.22

LIF is the second-most widely used detection method (behind UV-Vis absorption) for CE because of its high sensitivity and selectivity.10,23 LIF is performed by using a laser as the excitation source. One main disadvantage is that many molecules are not natively fluorescent with sufficient quantum yields at easily measured wavelengths. Also, the cost of some gas and dye laser systems can be high.22 Another disadvantage is increased sample preparation and handling should fluorescent derivatization be required, and in some cases, the time for sample preparation can exceed the time required to conduct the separation.24

There are two ways to detect an analyte that does not have native fluorescence. The first is indirect fluorescence detection, in which a running buffer containing a
fluorescent probe is used and the analyte displaces or pairs with the fluorescent molecules. This technique is based on charge displacement. The second is by derivatization.

In principle, almost any detection method can be combined with a derivatization procedure. Fluorescence is the most favored choice as it is more sensitive and selective than UV detection. It is also applicable to a wider variety of solvents than electrochemical detection, and is more robust than chemiluminescence and electrochemical detection. LIF offers selective excitation, small detection volumes, and high signals. Besides improved sensitivity, other reasons to derivatize a sample in order to facilitate LIF detection include: improved resolution and improved electrophoretic behavior. Typically, the limit of detection for samples is controlled by the concentration of analyte necessary for derivatization, not by the capability of the detector. Dilute samples are difficult to derivatize; therefore, dilution needs to take place after derivatization. The two types of derivatization are covalent and noncovalent. Covalent derivatization occurs when a covalent bond is formed between the analyte and reagent. Noncovalent derivatization includes ion-pair formation, hydrogen bonding, intercalation, and/or electrostatic interactions. Most derivatization schemes use covalent labels; however, covalent labeling can require strict control of solution pH and temperature. This could mean that derivatization may not be accomplished at the physiological conditions needed for the preservation of some biological samples. Covalent labeling can also be very time consuming, and as a result, noncovalent labeling techniques aid in making fluorescence studies easier.
Derivatization, when coupled with a separation process, can be performed by three possible modes: pre-column, on-column, or post-column. The most suitable approach is dictated by the reason for derivatization, the number of samples, and the chemical properties of the reagent and analyte. Post-column and on-column are better suited for automated systems because they can be done on-line, whereas pre-column procedures must be done off-line.\(^2\) Post-column is also advantageous because there is no change in the analyte’s mobility due to the label. Disadvantages of post-column derivatization are loss of efficiency and analyte, dilution of analyte, incomplete reactions, and higher baseline noise.\(^2\) On-column labeling provides a simpler protocol than post-or pre-column labeling with no sample pretreatment required. Limits of detection may also be improved for on-column labeling when compared to post- or pre-column labeling because during on-column labeling the derivatizing agent’s concentration is essentially constant throughout the capillary and the equilibrium between the free agent and the complex formed with the analyte is readily established and maintained throughout the CE separation. Table 2 contains a list of advantages and disadvantages of the three derivatization schemes mentioned.

When choosing a fluorescent label to derivatize an analyte, certain aspects must be taken into account. First, the reaction conditions: the analyte must be stable under the chosen conditions and it must dissolve in the required solvent. One must also account for the possibility of side reactions as well as the fluorescence sensitivity of the derivatives. Also to be considered is the difference in spectroscopic properties between the label and its reaction products, and, finally, the purity, stability, availability, and price of the label. Ideally, a derivatization should be fast, occur under mild conditions, form a single, stable,
Labels can be chosen with specific reactions to reduce interferences in the electropherogram, and careful control of labeling conditions can be achieved, resulting in a single tagged analyte with sharper peaks and improved efficiencies. LIF detections works with CE because LIF can be done on-column with a window burned to remove the opaque polyamide coating. Also, the laser can be focused to a narrow spot suitable for small diameter capillaries. A wide variety of lasers are available that can match source wavelengths with excitation wavelengths of the analytes.

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules. MS principle consists of ionizing chemical compounds to generate charged molecules or molecular fragments and measurement of their mass-to-charge ratios. Separation techniques often coupled to MS include gas chromatography (GC), HPLC, and CE, though MS can be performed independent of separation in some instances. Coupling requires ionization at the interface between separation and MS detection. Some of the more common ionization sources are shown in Table 3, including mass limits and ion type. Table 4 shows several types of MS analyzers including upper mass range and resolution. In this work, electrospray ionization was employed with an ion trap mass spectrometer and is discussed, *vida infra*.

Electrospray ionization (ESI) is a technique used in mass spectrometry to produce ions. Ionization is achieved by transferring effluent typically from a separation (i.e. CE or HPLC) containing the analyte(s) of interest so that it is dispersed by
nebulization into a fine aerosol and charged by a voltage applied at the spray source. Because the ion formation involves extensive solvent evaporation, the typical solvents for electrospray ionization are prepared by mixing water with volatile organic compounds (i.e. methanol, acetonitrile). To decrease the initial droplet size, compounds that increase the conductivity (i.e. acetic acid) are customarily added to the solution. Large-flow electrosprays can benefit from additional nebulization by an inert gas such as nitrogen. The aerosol is sampled into the first vacuum stage of a mass spectrometer through a capillary, which can be heated to aid further solvent evaporation from the charged droplets.

Evaporative light scattering detection (ELSD) is based on the ability of particles to scatter light when they pass through a beam of light. The detector responds to compounds less volatile than the mobile phase, which is nebulized upon introduction into the detector. This technique was first introduced in 1980s for the determination of lipid concentrations, which were difficult to obtain with an absorbance detector. The ELSD consists of three basic elements: the nebulizer, drift tube, and scattering chamber. The solvent stream first enters the nebulizer where it is nebulized and the droplets formed are entrained in a flow of gas. The droplets are evaporated in the drift tube and the dry particles that remain are carried in the flowing gas and solvent vapor stream. These dry particles scatter light, which is measured and the intensity of this light is a function of the size and number of particles present. ELSDs are not spectrometric detectors and do not obey Beer’s Law and so response curves are non-linear. The overall detector response is a mixture of all scattering types such as Rayleigh, Mei, refraction,
reflection, etc. Calibration curves produced by an ELSD are best fit by quadratic equations.

### Applications

#### Coated Capillaries

Protein separations by CE-LIF are complicated not only by the need for fluorescent derivatization but also by protein interactions with the charged surface of the inner capillary wall, arising from the ionization of surface silanol groups. These interactions reduce the separation efficiency and degrade detection limits due to peak broadening and inconsistent migration times. Often, these factors lead to poor resolution and identification of proteins because of consequent peak overlap and/or reduced peak areas. In an attempt to avoid protein-wall interactions, a capillary wall coating can be used, but these can be time-consuming to prepare, irreproducible in quality and coverage, can degrade rapidly upon use, and may be viable only within a very limited pH range. Yassine and Lucy has recently demonstrated the feasibility of using special two-tailed surfactant molecules to create a fast, inexpensive and effective capillary coating, as depicted in Figure 5.

Yassine and Lucy found that by flushing a capillary with a buffer solution containing a double-chained cationic surfactant they could generate a semi-permanent coating of hydrophobic molecules on the capillary wall, which would repel proteins or simply prevent adsorption to the otherwise negatively charged wall and thus improve separations. Their coatings generated a positively-charged wall coating, resulting in a reversed EOF and so a reversed polarity (that is, cathode at the inlet end of the capillary) was employed in their studies. Yassine and Lucy employed surfactants such as
dimethylditetradecylammonium bromide (2C_{14}DAB), dihexadecylammonium bromide (2C_{16}DAB) and dimethyldioctadecylammonium bromide (2C_{18}DAB) to create coatings which lasted for 60 trials over 12 days without having to regenerate the coating.\textsuperscript{39} The migration times between trials varied by less than 2.3\%.

The advantages gained by this coating procedure were demonstrated for unlabeled proteins detected by UV absorbance. However, in order to achieve greater sensitivity and selectivity, it is sometimes desirable to employ laser induced fluorescence (LIF) detection for protein analytes that are themselves natively fluorescent or have been rendered fluorescent by some derivatization or labeling process. Work in the Colyer laboratory has established a number of alternative methods exploiting noncovalent labeling to facilitate protein determination by CE-LIF.\textsuperscript{40,41,42} The potential for interactions between a fluorescent probe molecule and the surfactant capillary coating may be in competition with interactions between the probe and protein analytes, thus reducing the sensitivities that can be achieved by noncovalent labeling methods with surfactant-based coatings. Potential degradation of the coating by the presence of a fluorescent probe molecule in the running buffer, as required by on-column, noncovalent labeling procedures, is also of concern. Hence, the study presented in Chapter II was conducted to evaluate the utility of double-chained cationic surfactant coatings for CE-LIF assays of noncovalently labeled proteins.

The label of interest in this work is a squarylium dye Red-1c (Figure 6), which belongs to the class of 1,3-disubstituted compounds synthesized from squaric acid and two aromatic and/or heterocyclic compounds.\textsuperscript{43} This class of dyes has found applications as photoconductors in copying devices, organic solar cells, optical recording media, and as
optical sensors for metal determination. Squarylium dyes are attracting the attention of analysts for their long excitation and emission wavelengths and resistance to photodegradation.

**Saccharide Analysis**

The analysis of specific sugars in complex matrices by HPLC has been described numerous times and is of great utility. However, many methods have long analysis times due to isocratic separations in long columns and the necessity for a consistent mobile phase for refractive index (RI) detection. Shorter columns and new detection instrumentation allow carbohydrate analysis times of less than 8 minutes per injection. Environmental samples require stringent sampling regimes and many replicates so as to provide credible statistics. This leads to rather large quantities of samples to be analyzed, so high throughput and ease of sample preparation are necessary in method development. Demonstrated in Chapter III is a study of “Comparative Water Relations Of Angiosperm Evergreen Species Differing In Synthesis Of Anthocyanin During Winter”, which includes a new method employing HPLC with evaporative light scattering detection specifically developed to quantitatively determine sugars in various leaf species. Details of the method development portion of this work are given in Appendix 1.

**Singly Charged and Divalent Anions**

In addition to their many uses as solvents, ionic liquids (IL) have been utilized as highly effective substrates for complexation and subsequent quantitation of anions by electrospray ionization mass spectrometry (ESI-MS) and high performance liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS).
Proposed here is an alternative method utilizing capillary electrophoresis coupled with electrospray ionization mass spectrometry (CE-ESI-MS). This method offers advantages over LC-MS in that CE, in general, provides shorter separation times, requires less reagent and solvent, and needs less sample volume when sample may be limited. Using CE-ESI-MS, various complexation schemes, including pre-column, post-column (IL in sheath liquid), and on-column are compared for the separation and identification of anions in terms of analysis time, resolution, and sensitivity.

Experimentation shown in Chapter IV involved using dicationic liquids to quantitatively determine singly charged anions in a mixture. The four anions to be separated were perfluoroocanate (PFOA⁻, C₈H₁₅O₂⁻, 414.1 g/mol), benzenesulfonate (BZSN⁻, C₆H₅O₃S⁻, 180.2 g/mol), trifluoromethanesulfonimide (NTF2⁻, C₈H₁₅O₂⁻, 414.07 g/mol) and monochloroacetate (MCA⁻, C₂H₂ClO₂⁻, 93.5 g/mol). These analytes were chosen to allow a direct comparison to the separation shown in previous work by Armstrong et al.57 MCA is a known disinfectant byproduct and is under regulation by the EPA. Additionally, these particular analytes are of their relevance to human environmental health. Various benzenesulfonates including benzenesulfonate (BZSN) are used in numerous industrial processes.59 Perfluoro-octanoic acid (PFOA) is also an environmental contaminant compound.60 The new CE-ESI-MS method described herein provided baseline resolution of these 4 anions, which was not observed with the previously reported LC-ESI-MS method.57

The dicationic compound XVI (Figure 7) used in this work was kindly provided by Dan W. Armstrong. It was anion exchanged to the fluoride form to maximize complex formation between the dication complexing agent and the injected anionic
analyte. Briefly, this procedure involves and anion solid phase extraction (SPE) type exchange in conjunction with NaF (sodium fluoride). The fluoride form demonstrated much greater signal-to-blank and signal-noise ratios in ESI-MS experiments than other dihalide-dication salt solutions such as bromine, chlorine, and iodine.\(^{58}\) It is assumed that the mass of fluorine is well below the mass spectrometer’s conventional analysis range and is exhausted to high vacuum more easily than the other halides, thereby limiting the impact on ion suppression.

Complex formation between a doubly charged cation, provided by the dicationic liquid, and each of the singly charged analyte anions will result in a net +1 charge on the resulting complexes. Therefore, positive mode electrospray ionization is employed. Negative mode ESI-MS could be used for direct determination of the anions, but sensitivity in negative mode is greatly reduced.\(^{61,62}\)

A similar approach using tricationic liquids and doubly charged analyte anions \([(+3)_{\text{IL}} + (-2)^{\text{analyte}} = (+1)^{\text{net, complex}}]\) was explored in Chapter V following completion of the dicationic liquid studies. One in particular – trication 1,3,5-1-butyl-3-methyl-1H-imidazol-3-ium-2,4,6-trimethylbenzene, which possesses a trimethyl benzene core and butyl imidazolium charged groups (Fig. 8) – was the best to analyze for sulfate while also performing well for other anions (especially thiosulfate and \(o\)-benzenedisulfonate) used in this study. Development of new analytical methods for the rapid and reproducible separation and identification of some anions is important for future biological and environmental research.

The sensitivity and efficiency of the new anion analysis methods introduced herein depends on the mode by which the cationic reagent is introduced to the sample
solution, for example, by three possible modes – pre column (before injection and CE separation); on-column (by incorporation of the dication in the CE separation buffer); or post-column (by introduction of the dication to the separated analytes via a sheath liquid necessary to the ESI process). Review articles have critically examined the performance of CE-ESI-MS relative to LC-ESI-MS\textsuperscript{63}, and the utility of CE-ESI-MS as a quantitative tool\textsuperscript{64}, and as a tool for bioanalysis.\textsuperscript{65}
REFERENCES


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<table>
<thead>
<tr>
<th>Method</th>
<th>Detection Limit (M)</th>
<th>Advantages/Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-Vis Absorption</td>
<td>$10^{-5}$-$10^{-8}$</td>
<td>universal; diode array offers spectral information</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>$10^{-7}$-$10^{-9}$</td>
<td>sensitive; sample derivatization required</td>
</tr>
<tr>
<td>Laser-Induced Fluorescence</td>
<td>$10^{-14}$-$10^{-16}$</td>
<td>very sensitive; sample derivatization required; expensive</td>
</tr>
<tr>
<td>Mass Spectrometry (Tandem MS)</td>
<td>$10^{-8}$-$10^{-9}$</td>
<td>sensitive and offers structural information; complicated CE-MS interface</td>
</tr>
<tr>
<td>Evaporative Light Scattering (ELSD)</td>
<td>$10^{-3}$-$10^{-5}$</td>
<td>Carbohydrate analysis; macromolecules, and non-UV/Vis absorbers; no selectivity and not sensitive</td>
</tr>
<tr>
<td>Amperometry</td>
<td>$10^{-10}$-$10^{-11}$</td>
<td>sensitive; selective; but useful only for electroactive analytes; requires special electronics and capillary modification</td>
</tr>
<tr>
<td>Conductivity</td>
<td>$10^{-7}$-$10^{-8}$</td>
<td>universal; selective; but useful only for electroactive analytes</td>
</tr>
<tr>
<td>Indirect UV/fluorescence/ amperometry</td>
<td>various</td>
<td>universal; lower sensitivity than direct methods</td>
</tr>
<tr>
<td>Labeling Strategy</td>
<td>Advantages</td>
<td>Disadvantages</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Pre-Column</td>
<td>Better control of reaction conditions; many reagents available; extra instrumentation not needed</td>
<td>Need to remove or separate excess reagent; derivatives aren’t always stable</td>
</tr>
<tr>
<td>Post-Column</td>
<td>On-line, automated; no change in analyte mobility;</td>
<td>Loss of efficiency, dilution of analyte, baseline noise, incomplete reaction</td>
</tr>
<tr>
<td>On-column</td>
<td>On line, automated; no pretreatment; improved limit of detection; no interference from excess reagent</td>
<td>Not necessarily suited for covalent labels; not many reagents available; possible elevated background</td>
</tr>
</tbody>
</table>
Table 3: Mass Spectrometry Ionization Sources

<table>
<thead>
<tr>
<th>Ionization Method</th>
<th>Upper Mass Limit (Daltons)</th>
<th>Molecular Ion (M) or Fragmentation (F)</th>
<th>Separation Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron Impact (EI)</td>
<td>1,000</td>
<td>F</td>
<td>GC</td>
</tr>
<tr>
<td>Chemical Ionization (CI)</td>
<td>1,000</td>
<td>M</td>
<td>GC</td>
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<tr>
<td>Particle Beam</td>
<td>10,000</td>
<td>F</td>
<td>HPLC</td>
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<tr>
<td>Thermospray (TSI)</td>
<td>2,000</td>
<td>M</td>
<td>HPLC</td>
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<tr>
<td>Electrospray (ESI)</td>
<td>200,000</td>
<td>M</td>
<td>HPLC, CE</td>
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<td>Atmospheric Pressure (API)</td>
<td>10,000</td>
<td>M</td>
<td>HPLC</td>
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<tr>
<td>Atmospheric Pressure Photoionization (APPI)</td>
<td>10,000</td>
<td>M</td>
<td>HPLC</td>
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<tr>
<td>Atmospheric Pressure Chemical (APCI)</td>
<td>10,000</td>
<td>M</td>
<td>HPLC</td>
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<tr>
<td>Fast Atom Bombardment (FAB)</td>
<td>10,000</td>
<td>F</td>
<td>HPLC</td>
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<tr>
<td>Matrix-Assisted Laser Desorption (MALDI)</td>
<td>500,000</td>
<td>M</td>
<td>HPLC</td>
</tr>
<tr>
<td>Analyzers</td>
<td>Mass Range (a.m.u.)</td>
<td>Resolution</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>---------------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>Sectors (Magnetic or Electric)</td>
<td>$10^2$-$10^4$</td>
<td>$10^2$-$10^4$</td>
<td></td>
</tr>
<tr>
<td>Quadrupole</td>
<td>$50$-$10^4$</td>
<td>$10^2$-$10^4$</td>
<td></td>
</tr>
<tr>
<td>Ion Trap</td>
<td>$50$-$10^5$</td>
<td>$10^3$-$10^4$</td>
<td></td>
</tr>
<tr>
<td>Time-of-flight (TOF)</td>
<td>$10^2$-$10^3$</td>
<td>$10^4$-$10^5$</td>
<td></td>
</tr>
<tr>
<td>Fourier transform ion cyclotron resonance (FT-ICR)</td>
<td>$10^2$-$10^4$</td>
<td>$10^4$-$10^6$</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Schematic diagram of a typical CE instrument setup.
Figure 2. Electroosmotic flow.
Figure 3. Cross section of a) laminar flow and b) flat flow profiles characteristic of HPLC and CE, respectively.
Figure 4. Schematic diagram of a typical HPLC instrument setup.
**Figure 5.** A depiction of the inside of a capillary coated with a double-chained cationic surfactant.
Figure 6. Structure of Red-1c dye (C$_{30}$H$_{34}$N$_2$O$_4$, MW=486.6)
Figure 7. Structure of dicationic liquid XVI (C_{21}H_{44}N_{2}, MW=324.6).
Figure 8. Structure of tricationic liquid reagent ($C_{33}H_{51}N_6$, MW=531.8).
CHAPTER II

DIMETHYLDITETRADECYLAMMONIUM BROMIDE (2C₁₄DAB) AS A SELF-ASSEMBLED SURFACTANT COATING FOR DETECTION OF PROTEIN-DYE COMPLEXES BY CE-LIF.

Anthony R. Gerardi, Jennifer L. Lubbeck, and Christa L. Colyer

The following manuscript was published in Journal of Solid State Electrochemistry, Volume 29, pages 1044-1053, November 2008 and is reprinted with permission. Stylistic variations are due to formatting requirements of the journal. Lubbeck performed initial CE experiments and Gerardi performed method development, capillary electrophoresis experiments, and prepared the manuscript. Colyer served in advisory and editorial capacities.
Abstract

A self-assembled column coating for capillary electrophoresis in conjunction with laser induced fluorescence detection (CE-LIF) has been evaluated for the separation and quantitation of protein-dye complexes. This semi-permanent coating, composed of dimethylditetradecylammonium bromide (2C\textsubscript{14}DAB), is inexpensive and easily assembled onto the column and it allows for better peak resolution and greater control over electroosmotic flow (EOF). The versatility of long chained surfactant coatings was determined particularly with respect to their use with fluorescent probes, different pH buffers, and different proteins. Studies were performed to determine the stability of the coating under various pH- and buffer conditions. Red-1c, a red luminescent squarylium dye, was used for on-column protein labeling concurrently with the surfactant coating and LIF detection. Protein – Red-1c complexes were excited with a 650 nm diode laser and their emission detected by a photomultiplier tube with a 664 nm filter. A comparison of pre-column labeling and on-column labeling of a two model protein system (human serum albumin (HSA) and β-lactoglobulin A (BLG-A)) revealed higher efficiencies and greater sensitivities for both proteins using on-column labeling and coated columns. A linear relationship between peak height and protein concentration was obtained by CE-LIF for this on-column labeling method with 2C\textsubscript{14}DAB coated columns and the Red-1c probe.
Introduction

Protein separations by CE-LIF are complicated not only by the need for fluorescent derivatization but also by protein interactions with the charged surface of the inner capillary wall, arising from the ionization of surface silanol groups. These interactions reduce the separation efficiency and degrade detection limits due to peak broadening [1,2] and reduced or inconsistent migration times [3]. Often, these factors lead to poor resolution and identification of proteins because of consequent peak overlap and/or reduced peak areas. To avoid protein-wall interactions, a capillary wall coating can be used, but these can be time-consuming to prepare, irreproducible in quality and coverage, degrade rapidly upon use [4], and may be viable only within a very limited pH range [5]. Work by Yassine and Lucy has recently demonstrated the feasibility of using special two-tailed surfactant molecules to create a fast, inexpensive and effective capillary coating [6].

Yassine and Lucy found that by flushing a capillary with a buffer solution containing a double-chained cationic surfactant they could generate a semi-permanent coating of hydrophobic molecules on the capillary wall, which would repel proteins and thus improve separations [6]. Their coatings generated a positively-charged wall coating, resulting in a reversed EOF and so a reversed polarity (that is, cathode at the inlet end of the capillary) was employed in their studies. Yassine and Lucy employed surfactants such as dimethylditetradecylammonium bromide (2C_{14}DAB), dihexadecylammonium bromide and dimethyldioctadecylammonium bromide (2C_{18}DAB) to create coatings
which lasted for 60 trials over 12 days without having to regenerate the coating [6]. The migration times between trials varied by less than 2.3%.

The advantages gained by this coating procedure were demonstrated by Yassine and Lucy for unlabeled proteins detected by UV absorbance. However, in order to achieve greater sensitivity and selectivity, it is sometimes desirable to employ laser induced fluorescence (LIF) detection for protein analytes that are themselves natively fluorescent or have been rendered fluorescent by some derivatization or labeling process. Work in our own laboratory has established a number of alternative methods exploiting noncovalent labeling to facilitate protein determination by CE-LIF [7,8,9]. The potential for interactions between a fluorescent probe molecule and the surfactant capillary coating may be in competition with interactions between the probe and protein analytes, thus reducing the sensitivities that can be achieved by noncovalent labeling methods with surfactant-based coatings. Potential degradation of the coating by the presence of a fluorescent probe molecule in the running buffer, as required by on-column, noncovalent labeling procedures, is also of concern. Hence, this study was conducted to evaluate the utility of double-chained cationic surfactant coatings for CE-LIF assays of noncovalently labeled proteins.

Noncovalent interactions between proteins and dyes have made possible the development of analytical methods that demonstrate high sensitivity when quantifying proteins in complex matrices. Noncovalent labeling of proteins is an attractive alternative to covalent derivatization due to the fact that it entails minimal sample
preparation, is fast, and is feasible at biological pHs. Pre-column, post-column, and on-column labeling methods can each be employed with noncovalent probes. The last of these methods, by which the dye is incorporated into the separation buffer, thus encountering the analyte throughout the separation as it migrates through the capillary, eliminates the need for sample derivatization prior to injection and separation and does not result in any sample dilution during labeling. A recent comparison of pre-column and on-column labeling of proteins with squarylium dyes [10] revealed higher efficiencies and greater sensitivities for on-column labeling, and so the compatibility of a double-chained cationic surfactant coating with this method is of interest.

The label of interest in this work is a squarylium dye Red-1c, which belongs to the class of 1,3-disubstituted compounds synthesized from squaric acid and two aromatic and/or heterocyclic compounds [11]. This class of dyes has found applications as photoconductors in copying devices, organic solar cells, optical recording media, and as optical sensors for metal determination [12-18]. Squarylium dyes are attracting the attention of analysts for their long excitation and emission wavelengths and resistance to photodegradation. Specifically, the absorbance maximum for Red-1c is 607 nm in the absence of protein (HSA), and 642 nm in the presence of HSA [10], making the excitation of its protein complexes feasible with an inexpensive and robust diode laser. Because of the inherently low fluorescence of this dye in its free state, Red-1c has been successfully used as an on-column label for proteins in CE-LIF studies [7,8,10]. The quantum yield of Red-1c is very low in the absence of HSA, but increases significantly, from 0.03 to 0.92, when noncovalently bound to HSA [19]. To improve separation
efficiencies for protein mixtures labeled by this (or any related) dye without sacrificing assay sensitivities, we herein describe a comparison of pre- and on-column labeling methods employing 2C_{14}DAB coated capillaries.

**Materials and Methods**

*Reagents, Buffers, and Sample Solutions*

Red-1c was synthesized by Nakazumi and Yagi, as previously described [19,20]. Proteins (HSA and β-lactoglobulin A (BLG-A), formic acid, ammonium formate, sodium hydroxide and boric acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Buffers were prepared to nominal concentrations of 25 mM or 50 mM by dissolving the appropriate amount of reagent in Milli-Q distilled, deionized water (Millipore, Bedford, MA, USA) and adjusting the pH by the addition of formic acid (formate buffer) or sodium hydroxide (borate buffer). An adjusted pH of 4.5 (formate buffer) or 9.5 (borate buffer) was employed unless stated otherwise. Buffers were filtered through 0.2 μm nylon syringe filters (Corning, NY, USA) before use. A stock solution of Red-1c dye was prepared in methanol (Burdick and Jackson, MI, USA) to a concentration of 2.1 x 10^{-5} M and stored in the dark at 4°C when not in use. Working solutions of the dye were prepared just prior to use by dilution of the stock to the final desired concentration with Milli-Q water or buffer. Stock protein solutions were prepared to a concentration of 0.5 - 6.0 x10^{-4} M in water and stored at 4°C in the dark. Pre-column mixtures of protein with dye were prepared by adding the proper volume of protein stock solution to diluted dye solution with thorough mixing.
Dimethylditetradecylammonium bromide (2C_{14}DAB) surfactant was purchased from Sigma-Aldrich. The surfactant was dissolved in filtered running buffer to a concentration of 0.1 mM through a series of sonication cycles consisting of 20 minutes of sonication at the surfactant’s melting point (45°C) followed by ten minutes of stirring. The cycles were repeated twice or until the surfactant was dissolved. Mesityl oxide was used as a neutral EOF marker at a concentration of 0.1 mM in Milli-Q water. Mesityl oxide is sparingly soluble in water and so it was sonicated for an hour and then left to stir overnight to ensure complete dissolution.

**Instrumentation**

Most capillary electrophoresis experiments discussed herein were carried out on a Beckman P/ACE CE system (Fullerton, CA, USA) with an LIF detector, which was equipped with a 650 nm external diode laser (Oz Optics, Carp, Canada) and a 664 nm DF20 filter (Omega Optical, Brattleboro, VT, USA). Separations were conducted in uncoated and surfactant-coated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA), with 50 µm I.D., 365 µm O.D., 30.0 cm total length, and 24.5 cm effective length. An Agilent HP3DCE system (Waldbronn, Germany) equipped with a UV absorbance detector at 254 nm was used for unlabelled protein separations on a coated column. A home-built CE system with absorbance detection was also used to determine EOF with the neutral marker, mesityl oxide.
**Capillary Coating**

A modified method similar to Yassine and Lucy’s coating procedure [6] was developed and incorporated on the instrument used. A new 50 µm I.D., 30 cm section of fused silica capillary was flushed with 1 M NaOH for 15 minutes under high pressure (20 p.s.i.) and then with deionized water for 4 minutes at 20 p.s.i. After the preconditioning steps, the capillary was coated by rinsing with 0.1 mM 2C_{14}DAB surfactant in 50 mM formate buffer (pH 4.5) for 15 minutes at 20 p.s.i. Finally, the capillary was rinsed with the formate buffer alone for 6 minutes at 0.5 p.s.i. The resulting coated capillary was stored filled with the formate buffer between analyses.

**Results and Discussion**

Semi-permanent capillary coatings afford analysts increased flexibility in the realm of method development for large biomolecules such as proteins, which might otherwise experience significant wall adsorption and hence significantly reduced separation efficiencies and sensitivities. However, the coatings themselves must be compatible with other aspects of the analysis, particularly fluorescent derivatization and derivatizing agents in the case of LIF detection. To this end we have carefully examined the performance of a self-assembled cationic surfactant coating prepared from the two-chained surfactant 2C_{14}DAB in CE-LIF experiments with on-column and pre-column labeling of model proteins with the noncovalent squarylium probe Red-1c. An assessment of EOF modification, coating stability, and separation efficiencies for uncoated versus coated capillaries will allow us to evaluate the benefits of this procedure.
EOF in Surfactant-Coated Capillaries.

The electroosmotic mobility $\mu_{\text{eof}}$ of a system depends on many system-specific variables, such as the zeta potential $\zeta$ and solution viscosity and permittivity. Furthermore, $\zeta$ itself is dependent upon the surface charge density on the capillary wall, and the double-layer thickness at the capillary wall, which, in turn, is dependent upon the magnitude of ionic charge and concentration. Hence, EOF or $\mu_{\text{eof}}$ can be affected by any of the following parameters: buffer concentration, ionic strength, pH, viscosity, permittivity, and capillary wall composition. This study will focus on effecting a change in the surface charge on the capillary wall (and hence, the EOF) by application of a semipermanent $2\text{C}_{14}\text{DAB}$ surfactant coating. The positive surface charge imposed by the presence of this bilayer surfactant coating ensures that positively charged analytes will not be adsorbed to the capillary surface and hence, this should lead to better separations for large, cationic proteins, for example [5,6,21].

To determine the stability of the coating during a normal week of use, a 0.1 mM mesityl oxide sample was repeatedly injected (3 sec at 0.29 p.s.i.), to serve as a neutral marker of EOF. The running buffer in these stability studies was 50 mM formate (pH 4.5), and a separation voltage of $-10$ kV was applied. In this way we were able to monitor any change in EOF over the lifetime of the capillary by simply measuring changes in the retention time of the mesityl oxide. Observed changes in EOF would be indicative of a breakdown in the coating because the charge on the wall would change.
The average $\mu_{cof}$ found for $2C_{14}$DAB-coated column with 50 mM formate buffer (pH 4.5) was $(7.98 \pm 0.06) \times 10^{-4} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$ ($N = 10$) over a 1-week period. This value is comparable to that demonstrated by Yassine and Lucy [6]. The less than 1% variability observed in electroosmotic mobility for the coated capillary over the course of a week is excellent relative to the variation in $\mu_{cof}$ observed for a comparable uncoated capillary in use over the course of a week $(8.21 \pm 0.13) \times 10^{-4} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$ ($N = 10$). It should be noted that the EOF in the case of the coated column was in the direction of (towards) the anode (or positive electrode), while in the case of the uncoated column, it was towards the cathode, but in both cases the magnitude of the EOF was comparable. It should also be noted that data from the first ten trials using a newly coated capillary were not included in the average measured $\mu_{cof}$ value, since these were considered “conditioning” runs – to ensure excess surfactant molecules were fully removed and a reproducible surface charge was achieved. Additionally, newly coated capillaries were allowed to sit overnight filled with running buffer before prior to their first usage, since this was observed to produce a more stable EOF.

These experiments were conducted under acidic pH conditions, which are not incompatible with normal procedures for the analysis of proteins by CE, but to ensure maximum utility of the surfactant coating, studies at other pHs were also conducted. These include the analysis of proteins, ribonuclease-A, lysozyme, and $\alpha$-chymotrypsinogen-A using 50 mM borate buffer (pH 9.5) and 50 mM tris buffer (pH 7.0) with a coated capillary prepared from related surfactant $2C_{18}$DAB (data not shown). The coating was found to be stable under these conditions; however, with 50 mM tris (pH
10) and 50 mM citrate (pH 3.2), the surfactant itself was less soluble and the resulting dynamic coating was less reproducible. As such, the recommended pH range for these coatings with acidic proteins is from 4.5 – 9.5. All subsequent experiments reported in this paper were conducted at pH 4.5.

Resolution of Proteins Using a 2C14DAB Coated Capillary

Figure 1 depicts electropherograms of individual cationic proteins α-chymotrypsinogen A, ribonuclease A, and lysozyme as well as a mixture of these three proteins. Complete resolution of these proteins in less than 6 min serves to demonstrate the effectiveness of the 2C14DAB coating. In comparison, this same protein mixture separated on an uncoated capillary yielded broader peaks and a significant reduction in average resolution from 2.5 to 1.6 relative units (data not shown). It seems apparent that the double-layer surfactant coating is preventing protein adsorption and hence greatly improving the quality of the separation of free (unlabeled) proteins. However, it still remained to be established that the coating could be equally effective for the separation of fluorescently labeled proteins, as described next.

Effect of Dye in Running Buffer on 2C14DAB Coating.

In order to determine if the dynamic surfactant coating would be appropriate for the analysis of noncovalent protein-dye complexes, it was necessary to first assess the effect of the presence of dye in the running buffer (as is characteristic of on-column,
noncovalent labeling protocols) on the observed EOF in coated capillaries. To this end, mesityl oxide samples were again employed as markers of EOF, but in these experiments the reproducibility of mesityl oxide transport (or $\mu_{\text{eof}}$) over the course of 5 days using a coated capillary was evaluated with Red-1c present in the running buffer at a concentration of $9.35 \times 10^{-6} \ \text{M}$. The average $\mu_{\text{eof}}$ found for 2C$_{14}$DAB-coated column with 25 mM formate buffer (pH 4.5) containing $9.35 \times 10^{-6} \ \text{M}$ Red-1c was $(6.16 \pm 0.05) \times 10^{-4} \ \text{cm}^2\text{V}^{-1}\text{s}^{-1}$ ($N = 10$) over a one-week period. The average $\mu_{\text{eof}}$ found for a 2C$_{14}$DAB-coated column with 25 mM formate buffer (pH 4.5) containing no Red-1c was $(6.67 \pm 0.12) \times 10^{-4} \ \text{cm}^2\text{V}^{-1}\text{s}^{-1}$ ($N = 10$) over a one-week period. The less than 1% variability observed in the electroosmotic mobility for the coated capillary with dye present in the separation buffer compares favorably to the earlier measurement of electroosmotic mobility for coated columns in the absence of dye. Given this indicator of stability, it is reasonable to conclude that the dye does not inadvertently affect the coating and so the use of 2C$_{14}$DAB coated capillaries for dye-protein assays seems warranted. It should be noted that the absolute value of $\mu_{\text{eof}}$ ($6.67 \times 10^{-4} \ \text{cm}^2\text{V}^{-1}\text{s}^{-1}$) for the coated column in the absence of Red-1c in this particular study differs from $\mu_{\text{eof}}$ reported earlier in this work under similar conditions, and this difference may be attributed to such factors as changes in wall surface charge on new pieces of capillary and minor differences in coating protocols. These differences in $\mu_{\text{eof}}$ for different coated capillaries mimic the differences seen in $\mu_{\text{eof}}$ for different uncoated capillaries, but it should be emphasized that the reproducibility in $\mu_{\text{eof}}$ for any one given coated capillary (day-to-day) was high.
The presence of the Red-1c noncovalent protein probe in the running buffer had several potential impacts on the method. First, it contributed to the overall ionic strength of the buffer solution and so was expected to reduce the rate of EOF. This was observed \((6.16 \pm 0.05) \times 10^{-4}\) cm²V⁻¹s⁻¹ with dye present and \((6.67 \pm 0.12) \times 10^{-4}\) cm²V⁻¹s⁻¹ without dye present. Second, the dye itself is hydrophobic and so may partition into or otherwise associate with the double-layer coating, thus affecting the coating integrity and, in turn, the reproducibility of EOF. Given the high degree of reproducibility observed for \(\mu_{\text{eof}}\) in the presence of Red-1c, there is no concern about the dye negatively impacting coating integrity. Third, any interactions between the dye and coating may serve to enhance the fluorescence of the dye, and in the case of on-column labeling this would increase the background fluorescence signal, thus degrading the sensitivity of the assay. Calibration curves were constructed to evaluate the theoretical limit of detection for this method (see next section). And fourth, given that the dye is insoluble in purely aqueous solution, the presence of 0.4% (v/v) methanol in the final dye+buffer solution filling the capillary, which was necessary for solubility, may also affect coating integrity. However, recent work by Diress et al. [22] demonstrated the compatibility of double-chain cationic surfactant coatings with up to 60% (v/v) methanol-buffer mixtures and so although the presence of some organic solvent in the buffer system would affect the EOF, it is not likely (especially at the low organic levels present in these experiments) to compromise the coating itself.

*On-Column, Noncovalent Protein Labeling with Red-1c in 2C₁₄DAB-Coated Capillaries.*
Having established the compatibility of the coating with Red-1c dye, it remained to be shown that a mixture of proteins could be separated and labeled on-column in this same system. To this end, we studied HSA and BLG-A samples. Repeated injections of a mixture composed of 10 µM HSA and 10 µM BLG-A onto a surfactant coated capillary filled with 25 mM formate buffer (pH 4.5) with 9.35 x 10^{-6} mM Red-1c over a period of 10 days demonstrated excellent reproducibility for the retention times of the noncovalent protein-dye complexes, as seen in Fig. 2. The average retention times for HSA and BLG-A in a mixture over this measurement period translate into net mobilities of $(2.35 \pm 0.09) \times 10^{-4}$ cm$^2$V$^{-1}$s$^{-1}$ and $(1.89 \pm 0.08) \times 10^{-4}$ cm$^2$V$^{-1}$s$^{-1}$, respectively. The capillary was stored filled with deionized water between days and was flushed at low pressure (0.5 p.s.i.) at the beginning of each new day of experiments with 0.1 M NaOH for 3 minutes, deionized water for 3 minutes, 0.1 M surfactant solution for 5 minutes, and finally with deionized water again for 5 minutes before use. This ensured a consistent coating from day-to-day by regenerating rather than completely stripping and recoating the capillary each day. This procedure proved sufficient to allow 10 analyses per day while maintaining retention time and EOF reproducibility from day-to-day.

Quantitation of these model proteins was possible by constructing a calibration curve for each. Figure 3 shows on-column labeling with Red-1c for increasing concentrations of HSA and BLG-A. A subsequent plot of peak area for the protein-dye complex as a function of protein concentration (for a fixed dye concentration of 9.35 x 10^{-6} M) resulted in regression equations of $(\text{Peak Area}) = 9.87 \times 10^9(\text{Protein Concentration}, \text{M}) + 4005$, and $(\text{Peak Area}) = 7.89 \times 10^9(\text{Protein Concentration}, \text{M}) +$
3204, with correlation coefficients of 0.984 and 0.979 for HSA and BLG-A, respectively. Based on these standard curves and the standard deviation in the baseline of the corresponding electropherograms, computed limits of detection (3σ) of 60 nM HSA and 90 nM BLG-A were obtained for this method. The mean resolution for these two analytes was R=2.4, and the mean column efficiency for both HSA and BLG-A was $2 \times 10^5$ plates/meter. These figures of merit can be compared to corresponding figures obtained for the same protein-dye complexes using an uncoated capillary, as summarized in Table 1.

**Conclusions**

To be able to employ dynamic surfactant coatings to increase the resolution and separation efficiency of protein separations by CE is an important outcome of this work, and indeed, we were able to demonstrate a nearly 2-fold improvement both in terms of resolution and efficiency in 1/3-less analysis time for model protein analysis in a coated versus uncoated column. However, to achieve these improvements without compromising LIF detection sensitivity is an even more important advance in method development. The two-chained surfactant coatings developed by Yassine and Lucy have been shown to be compatible with on-column, noncovalent labeling protocols employing the squarylium dye Red-1c developed in our own lab. As such, the range of protein-dye complexes that can be effectively analyzed by CE-LIF should be extended by the simple incorporation of a 2C$_{14}$DAB coated capillary in the procedure. However, the utility of this coating to facilitate more efficient separations even in the presence of fluorescent
probe molecules is likely to be realized for a host of labeled analytes and should not be considered to be limited to proteins only, nor to on-column derivatization procedures or noncovalent probe-analyte interactions.

**Acknowledgements**

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References

**Figure 1:** Electropherograms (from top to bottom) of 0.1 mg/mL α-chymotrypsinogen A, 0.1 mg/mL ribonuclease A, 0.1 mg/mL lysozyme samples, and a mixture of all three proteins. Separation conditions include: 50 mM formate buffer (pH 4.5), -9V separation voltage, 20 s hydrodynamic injection, 50 µm ID x 44.7 cm total length (34.7 cm effective length) 2C_{14}DAB-coated capillary, absorbance detection at 214 nm.

**Figure 2.** Day-to-day reproducibility in retention times for on-column, noncovalently labeled HSA and β-lactoglobulin A samples, both at a concentration of 1.0 x 10^{-5} M, on a surfactant coated capillary over a 10 day period. Experimental conditions include: -9 kV separation voltage, 3 s injection at 0.5 p.s.i., 50 µm id x 30 cm (24.5 cm effective length) capillary with 2C_{14}DAB coating, 25 mM formate buffer (pH=4.5), excitation λ=650 nm, emission λ=664nm.

**Figure 3.** On-column labeling of mixtures of HSA and β-lactoglobulin A in 25 mM formate buffer (pH 4.5) with 9.35 x 10^{-6} M Red-1c. Other experimental conditions include: -9 kV separation voltage, 3 sec injection at 0.5 p.s.i., 50 µm id x 30 cm (24.5 cm effective length) capillary with 2C_{14}DAB coating. Protein concentrations range from 1.0 x 10^{-6} M to 3.0 x 10^{-5} M for both proteins.
Table 1: Figures of merit for CE-LIF separation of on-column, Red-1c labeled proteins in 30-cm uncoated and surfactant-coated capillaries. Separation conditions as stated in the text, with an applied separation voltage of +9 kV for the uncoated column and -9 kV for the 2C14DAB coated column.

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CHAPTER III

COMPARATIVE WATER RELATIONS OF ANGIOSPERM EVERGREEN
SPECIES DIFFERING IN SYNTHESIS OF ANTHOCYANIN DURING WINTER

Nicole M. Hughes, Keith S. Reinhardt, Taylor S. Feild, Anthony R. Gerardi, and William K. Smith

The following manuscript was published in Journal of Experimental Botany, Volume 61 Issue 6, pages 1699-1709, April 2010 and is reprinted with permission. Stylistic variations are due to formatting requirements of the journal. Gerardi performed all HPLC-ELSD and prepared the HPLC-ELSD methods section of the manuscript. Reinhardt and Field performed sample collection and water potential measurements. Hughes performed sample collection, performed other analyses and prepared the manuscript. Smith served in advisory and editorial capacities.
Abstract

Leaves of many evergreen angiosperm species turn red under high light during winter due to production of anthocyanin pigments, while leaves of other species remain green. There is currently no explanation for why some evergreen species exhibit winter color change while others do not. Conditions associated with low leaf water potentials (e.g. drought, high salinity, sugar treatments) have been shown to induce reddening in many plant species. Because evergreen species differ in susceptibility to water stress during winter, we hypothesized that species which undergo winter color change correspond with those that experience/tolerate the most severe daily declines in leaf water potentials during winter. Consistent with this hypothesis, red-leafed species as a group had significantly lower leaf water potentials during mid-day in winter than green-leafed species, but not during the summer when all leaves were green. However, there was much overlap in mid-day water potentials between red and green-leafed species, inconsistent with a “threshold” induction of anthocyanin synthesis. Pressure-volume curves measured one month before color change and one month after also showed some evidence of adaptation to more negative water potentials by red-leafed species (e.g. more negative osmotic potential at full turgor, and greater cell wall hardening during), but again, much overlap existed between red and green-leafed species, and some of the least drought-tolerant species were red-leafed. There was no observed difference in transpiration during winter between red and green-leaved species. These results suggest that greater declines in daily water potentials alone are not sufficient to explain winter reddening in angiosperm evergreen species.
Introduction

The potential functional significance of anthocyanin pigments in leaves has received substantial attention in the recent literature (see recent reviews by Manetas et al. 2006, Archetti et al. 2009). However, comparatively less attention has been given to the question of why only certain species change leaf color from green to red during certain ontogenetic stages or seasons while others do not. During winter, leaves of many evergreen angiosperms turn a range of red to purple colors in response to sunlight, corresponding with synthesis of anthocyanin pigments (Oberbauer and Starr, 2002; Hughes and Smith 2007; Kytridis et al. 2008). In general, leaves with high chlorophyll content appear purplish, while leaves experiencing chlorophyll bleaching appear more red (Hughes, personal observation). In some species, leaf color change may be winter-transient, with leaves metabolizing anthocyanins to become green again with the return of springtime warming. Leaves of other winter-red species may senesce while still red at winter’s end, concomitant with a new flush of leaves (Hughes, personal observation). In contrast, other evergreen angiosperms maintain leaves that are entirely green throughout the winter. Many of these winter-green species do synthesize anthocyanins in other tissues or during different ontogenetic stages, such as in juvenile leaves, flowers, stems, roots, senescing leaves, and/or in response to pathogen infection. Their lack of anthocyanin in winter leaves suggests that anthocyanins are not beneficial for these species during the winter season. However, this assumption has yet to be supported, and why some evergreen species synthesize anthocyanin in winter leaves while others do not is currently unknown (Hughes and Smith, 2007).
An explanation for redness versus greenness during winter is complicated by a lack of consensus among plant physiologists regarding the physiological function of anthocyanins in leaf tissues (see reviews by Manetas 2006; Archetti et al. 2009). Most research seeking to determine a functional role of anthocyanins in evergreen leaves has focused on their putative roles in photoprotection (e.g. Hughes et al. 2005; Hughes and Smith 2007; Kytridis et al. 2008). Winter leaves are especially vulnerable to high light stress, as low temperatures reduce the rate at which leaves may process energy for photosynthesis, thereby resulting in an imbalance of energy capture versus processing. This imbalance may lead to an increase in light energy that is transferred from chlorophyll to oxygen, resulting in the production of reactive oxygen species (ROS) and tissue damage (Huner et al. 1998, Adams et al. 2004). Anthocyanins are thought to minimize photo-oxidative damage by either absorbing green light, thereby reducing energy available for absorption by chlorophyll (Feild et al., 2001; Lee and Gould, 2002; Hughes et al. 2005), and/or through neutralizing ROS directly as antioxidants (Gould et al. 2002; Nagata et al. 2003; Kytridis and Manetas 2006). The idea that winter redness reflects an increased need for photoprotection has been supported in some studies (Kytridis et al. 2008), but not others (Hughes et al. 2007). Much evidence also exists counter to a photoprotective function in senescing (Lee et al 2003), young (Manetas et al 2003; Dodd et al. 1998; Karageorgou and Manetas 2006), and mature (Kyparissis et al 2007, Kytridis et al. 2008) leaves, rendering additional explanations for winter color change timely and necessary.

In addition to the well-described relationship between anthocyanins and high light stress, there also exists some correlative evidence for a relationship between anthocyanins
and water stress (see reviews by Chalker-Scott 1999, 2002). Anthocyanin synthesis is known to be inducible under conditions linked to osmotic stress, including high salinity (Dutt et al. 1991 Ramanjulu et al. 1993; Kaliamaorthy and Rao, 1994; Eryilmaz 2006), drought (Zhi-min et al. 2000; Allen et al. 1999; Bills et al. 1994; Spyropoulos and Mavormmatis 1978), and sugar treatment (Tholakalabavi et al. 1997, Sakamoto et al. 1994; Suzuki, 1995, and others). Furthermore, species with high levels of foliar anthocyanin seem to be common in environments characterized by low soil moisture (Spyropoulos and Mavormmatis, 1978; Schemske and Bierzychudek, 2001), and are more tolerant of drought conditions (Diamantoglou et al. 1989, Knox 1989; Beeson, 1992; Paine et al. 1992). Because winter conditions are often accompanied by environmental and physiological factors that promote water stress (e.g. low vapor pressure deficit (VPD) of air, low soil moisture, and reduced hydraulic conductivity due to freeze-thaw embolisms), it is possible that anthocyanin synthesis during winter may correspond with relative differences in water stress in some species compared to others. Indeed, evergreen species are known to significantly differ in vulnerability to water stress during winter months, due to differences in xylem cavitation, solute accumulation, cell wall hardening, freezing damage, and transpirational and cuticular water loss (Davis et al. 1999; Uemera and Steponkus 1999; Verhoeven et al. 1999; Adams et al. 2002; Taneda and Tateno 2005). Because anthocyanin synthesis is known to be inducible by lower leaf water potentials, perhaps those species experiencing the greatest leaf water deficits during the day (due to any combination of the above) would correspond with those that synthesize anthocyanin. Though this hypothesis does not directly test a physiological
function of anthocyanin *per se*, identifying a unifying stress factor that corresponds with its appearance could be helpful answering this question as well.

The objective of this study was to test the hypothesis that species which synthesize anthocyanin during winter correspond with those which experience (i.e. tolerate) the most extreme declines in daily water potentials. We test this hypothesis first directly, by measuring seasonal pre-dawn and mid-day water potentials, and second, by examining cell characteristics indicative of adaptation to prolonged water stress (e.g. osmotic adjustment, cell wall hardening).

**Materials and methods**

*Sites and species*

Species used in this study were mature field plants growing along sun-exposed road-sides in Jonas Ridge, NC, USA (35° 57’ 20” N, 81° 53’ 55”W; altitude: c. 1200 m) on south or southeast-facing sites receiving >6 h full sunlight (i.e. >1350 µmol m⁻² s⁻¹ on a horizontal surface) per day during both summer and winter months. Measurements were taken on clear sunny days, with little or no cloud cover. Winter red-leafed species included: *Rhododendron spp.* (a horticultural azalea), *Galax urceolata* (Poir.) Brummitt, *Lonicera japonica* (Thunb.), *Gaultheria procumbens* (L.), *Leucothoe fontanesiana* (Steud.).

*Sleumer,* and *Hedera helix* (L.); winter green-leafed species included *Kalmia latifolia* (L.), *Rhododendron spp.* (a horticultural azalea), *Rhododendron maximum* (L.), *Vinca minor* (L.), and *Rhododendron catawbiense* (Michx.). Detailed descriptions of
most of these species are given in Hughes and Smith (2007). Field temperatures were recorded by a field station 3 km from the study site.

Field water potential measurements

Water potential measurements ($\Psi_w$) were made on four winter days—December 14, 2007 (max: 13°C; min: 6°C), January 27, 2008 (max: 3°C; min: -3°C), February 25, 2008 (max: 14°C; min: 0°C), and March 2, 2008 (max: 13°C; min: -4°C), and one autumn day before leaves had changed color—September 30 (max: 21°C; min: 6°C). Leaf water potentials were measured for all species except *V. minor*, and *L. japonica* (green and red-leafed species respectively), which did not have leaf petioles; for these species, stem water potentials with 5 attached leaves were used instead. Leaves or stems from at least five separate individuals of each species were excised in the field both predawn (500-700) and mid-day (1100-1300, kept in plastic baggies from which air had been removed, and stored on ice until measurement within 4 hours. Measurements were made using a Scholander-type pressure chamber (Model 1000, PMS Instrument Company, Corvallis, OR) with nitrogen gas (Turner 1981). Very cold conditions sometimes resulted in $\Psi_w$ values which were more negative than could be read by the instrument (i.e. < -6 MPa), most likely reflecting significant xylem cavitation; as such, only values which were measurable were used, and values < -60 were discarded. First year individuals were used in all measurements. Measurements were also made in the field and compared to measurements made after 4 hours on ice, to ensure no significant change in water potentials occurred between harvest and measurement.
**Pressure-volume curves**

Pressure-volume curves for each species were measured using leaf or stem material excised one month before color change (October) and one month after (December). First year leaves were used in all cases. Three to five leaves were excised from separate individuals in the field, stored in sealed plastic bags, and transported on ice. Petioles were re-cut underwater, and leaves were hydrated overnight. The following day, pressure-volume curves were derived using methodology described in Turner (1981). Briefly, leaf water potential ($\Psi_w$) was measured periodically as leaves transpired freely; leaf mass was determined immediately following each measurement. At least five points on the curve were derived for each leaf, and at least five points on the line following turgor loss. Dry mass was determined following completion of the curve by drying leaves in an oven at 80°C until a constant mass was achieved. Water relation parameters derived from graphical and linear regression analyses included: osmotic potential at full turgor ($\Psi_{\pi,100}$), osmotic potential at the turgor loss point ($\Psi_{\pi,0}$), relative water content at the turgor loss point (%RWC$_0$), symplastic water fraction (SWF), and the bulk modulus of elasticity ($\varepsilon$) between 95% and 98% RWC. It should be noted that leaves of some species required up to 14 hours for a complete curve to be derived (e.g. *Rhododendron catawbiense*).

**Stomatal conductance**

Winter photosynthesis measurements were taken between 4 December 2005 and 4 March 2006, and 15–17 December 2007. All measurements were made on first-year leaves under full ambient sunlight (>1350 μmolm$^{-2}$ s$^{-1}$) at mid-day (1100 until 1500
hours) on warm days (daily high temperature > 18°C), in order to get maximum winter gas exchange values. Plants were sampled via a standard random walk procedure. A LI-COR model Li-6400 (Li-Cor, Inc., Lincoln, NE, USA) was used to measure leaf gas exchange, with red/ blue LED set to 1500 μmol m⁻² s⁻¹.

**Sugars**

Sugar concentrations in leaves were determined by High Pressure Liquid Chromatography (HPLC), using a Waters Alliance 2695 system. Two hundred milligrams of freeze-dried leaf tissue was weighed into a 10 ml disposable borosilicate test tube. Four milliliters of deionized water was added and the test tube was shaken at 350 rpm on an orbital shaker for 30 min. Standard reagents of sucrose, glucose, and fructose were obtained from Sigma Aldrich and were dissolved in distilled, deionized water to a concentration of 3.0 mg ml⁻¹. Subsequent dilutions of the stock were prepared to 1.5, 0.9, and 0.3 mg ml⁻¹ for 4-point quadratic calibration curves. Separations were carried out on a 7353 mm Altech Prevail Carbohydrate ES Rocket column maintained at 50 °C, using an isocratic flow of 2.0 ml min⁻¹, an injection volume of 2 μl, and an analysis time of 6 min.

The mobile phase consisted of 75% acetonitrile and 25% water. Sugars were detected with a Waters 2420 evaporative light scattering detector (ELSD) with a drift tube temperature of 50 °C; N2 as the nebulizer gas at 50 psi; and the nebulizer heater set to 40%.

**Statistics**
All data except sugar analyses were transformed by log_{10} for normality (determined as $p < 0.05$ by Shapiro-Wilks test). The effects of leaf color on pre-dawn and mid-day water potentials were assessed for each measurement day separately using a nested MANOVA with identity contrast (with species nested within color). The change in water potential between pre-dawn and mid-day was calculated for each species using average pre-dawn and mid-day values (as random plants were used in each, and could therefore not be compared using individual plants as replicates); a one-tailed Student’s t-test was used to compare the change in water potential for the red and green-leafed species each month. A one-tailed Student’s t-test with unequal variance was used to compare summer and winter pre-dawn and mid-day water potential values for each species individually; for winter values, all months were pooled together. The effects of leaf color on $\Psi_{\pi,100}$, $\Psi_{\pi,0}$, RWC$_0$, SWF, and $\varepsilon$ were analyzed using a nested MANOVA with identity contrast. The effects of leaf color on sucrose, glucose, fructose, and total soluble sugars were analyzed using a nested standard least squares test for each sugar type separately. Significance was determined as $p < 0.05$ for all tests. Seasonal comparisons (i.e. summer versus winter) for soluble sugars and pressure/volume curve data were compared using a one-tailed Student’s t-test with equal variance. Winter gas exchange parameters (photosynthesis, stomatal conductance, and transpiration) for red and green-leafed species were compared by nested MANOVA.

**Results**

*Seasonal Water Potentials*
During September (before color change had occurred), summer green leaves of winter-red species had significantly lower pre-dawn water potentials compared to those of perennially green-leafed species ($\bar{X} = -0.45$ MPa for red, -0.38 for green; $p = 0.04$); during mid-day the reverse was observed—leaves of green-leafed species had significantly lower water potentials during those of red-leafed species ($\bar{X} = -0.88$ MPa for red, -1.03 for green; $p < 0.01$) (Figure 1A). There was no significant difference in mean change in water potential between pre-dawn and mid-day in September between the two groups ($p > 0.05$) (Figure 1B).

Winter pre-dawn water potentials were significantly lower for red-leafed species compared to green-leafed species in January ($p < 0.0001$), while during December and February, there was no difference between groups ($p = 0.56$ and 0.52, respectively); during March, green-leafed species had significantly lower pre-dawn water potentials compared to red ($p < 0.0001$) (Figures 2, 3). When data for all winter months were pooled, red and green-leafed species did not significantly differ with regards to pre-dawn water potentials ($p = 0.83$). During mid-day, red-leafed species had significantly lower water potential values compared to green-leafed species during all months except for February ($p < 0.0001$ for Dec, Jan, and March; for February, $p = 0.37$). When all data for winter months were pooled, red-leafed species had significantly lower mid-day water potential values compared to green-leafed species ($\bar{X} = -2.2$ MPa for red, -1.3 for green, $p < 0.0001$). There was no significant change in daily water potential between red and green-leafed species in December, January, or February ($p = 0.15, 0.48, 0.35$); in March, red-leafed species had a significantly greater mean decline in water potential compared to green-leafed species ($\bar{X} = 1.2$ MPa for red, 0.13 for green; $p = 0.01$); when all winter
months were pooled, red-leafed species exhibited a significantly greater daily decline on average \((p = 0.05)\) (Figure 3D).

Most species had significantly lower pre-dawn and mid-day water potentials during winter compared to summer \((p < 0.05)\); exceptions included the winter green-leafed \textit{V. minor}, which had similar pre-dawn and mid-day water potential values during summer and winter \((p = 0.45\) for pre-dawn; 0.16 for mid-day), \textit{L. japonica}, which had significantly less negative pre-dawn water potentials during winter compared to summer \((p = 0.04)\), and the red-leafed \textit{Rhododendron spp}, which had similar mid-day water potential values between summer and winter \((p = 0.14)\) (Figure 2).

\textit{Pressure-Volume Curves}

Seasonal pressure volume curves are shown in Figure 4A (red species) and 4B (green species), with calculated parameters shown in Table 1 and Figure 5. There was no significant difference in \(\Psi_{\pi,100}\) during summer between summer leaves of green and red species \((p = 0.29)\), but during winter, red-leafed species had significantly more negative \(\Psi_{\pi,100}\) than green-leafed species \((\bar{x} = -1.6\) for green and -2.0 for red; \(p < 0.01)\). SWF at full turgor, and bulks modulus of elasticity at 96.5\% RWC were significantly higher for red-leafed species compared to green during both summer (SWF = 0.57 for green 0.66 for red; \(\varepsilon = 12.9\) for green and 6.5 for red; \(p < 0.05\) in both cases) and winter (SWF = 0.34 and 0.42 for green and red; \(\varepsilon = 15.3\) and 19.1 for red and green respectively; \(p < 0.01\) for both).

During the summer, leaves of species that would remain green during winter exhibited significantly more negative water potential at the turgor loss point \((\Psi_{\pi,0})\) and
lower relative water content at the turgor loss point (RWC₀) than leaves of species that would turn red ($\Psi_{π,0}$ $\bar{X} = -2.1$ MPa for green, -1.8 for red; $p < 0.0001$; %RWC₀ $\bar{X} = 0.87$ for green, 0.91 for red $p < 0.0001$); however, the groups did not differ during winter, after color change had occurred ($\Psi_{π,0}$ $\bar{X} = -2.6$ and -2.7 for green and red respectively, $p = 0.23$; %RWC₀ = 0.87 for green, 0.89 for red; $p = 0.1$).

Sugars

All red and green-leafed species showed significant increases in the combined amounts of glucose + fructose + sucrose during winter, with the exception of the red-leafed G. procumbens (Table 1, Figure 6). Seasonal levels of total sugars (glucose + fructose + sucrose) were not significantly different between red and green-leafed species during summer ($p = 0.66$) or winter ($p = 0.23$). All red-leafed species increased glucose content during winter (significant at $p < 0.05$ for all but H. helix), and most exhibited significant increases in fructose and sucrose (Table 1). Half of green-leafed species measured did not show significant increases in fructose or sucrose content during winter, though most significantly increased glucose (only exception being K. latifolia). Red-leafed species had significantly higher sucrose content during the summer than green-leafed species ($\bar{X} = 9.7$ mg/g for green, 22 for red; $p<0.0001$), but during winter, green-leafed species had significantly higher sucrose content ($\bar{X} = 53$ mg g⁻¹ for green, 39 for red; $p<0.0001$) (Figure 6, Table 1). Green-leafed species had significantly higher glucose and fructose content during summer than red leaves ($p < 0.0005$ for both), but red-leafed species had significantly higher glucose during winter ($p < 0.01$); red and green-leafed species did not differ in fructose content during winter ($p = 0.78$).
Photosynthetic Gas Exchange

Red and green-leaved species did not significantly differ in any gas exchange parameters during winter (Figure 7). Mean winter photosynthesis was 4.2 μmol CO₂ m⁻² s⁻¹ for red and 3.6 for green (p = 0.14); mean winter stomatal conductance (g) was 43 mmol m⁻² s⁻¹ for red and 49 mmol m⁻² s⁻¹ (p = 0.12); and mean winter transpiration (e) was 79 mmol m⁻² s⁻¹ for red and 93 mmol m⁻² s⁻¹ for green (p = 0.16).

Discussion

Consistent with our hypothesis, red-leafed species had significantly more negative mid-day water potentials during winter compared to green-leafed species during three of the four winter measurement days (Figure 2 and 3), as well as when all winter days were pooled (Figure 3C). Furthermore, pressure volume curves showed that red-leafed species, as a group, were more likely to exhibit physiological features characteristic of adaptation to prolonged water stress. Red-leafed species had significantly more negative osmotic potential at full turgor (Ψπ,100) and greater cell wall hardening (ε) than green-leafed species (Table 1, Figure 5D and 5B); the groups did not differ in these characteristics during the summer when all leaves were green (Table 1). Low osmotic potential at full turgor indicates increased accumulation of solutes (i.e. osmotic adjustment), which is a strategy for retaining water osmotically during periods of water stress (Verslues et al, 2006). Solutes most commonly used in osmotic adjustment are sugar alcohols, monosaccharides, amino acids, and inorganic ions (commonly K⁺) (Handa et al. 1983; Ranney et al. 1991; Wang and Stutte 1992). Additionally, cell walls
of red-leafed species as a group were significantly harder than green-leafed species during summer and winter (Table 1). A less elastic cell wall results in a rapid loss of turgor pressure as water is lost, and a faster decline in $\Psi_w$ accordingly (as positive cell wall pressure, $\Psi_p$, is not maintained); this drop in water potential allows the cell to avoid further water loss due to a less steep water potential gradient between the cells and the air (Verslues 2006). Together, the loss of turgor pressure and increased osmotic adjustment appeared to account for relative declines in mid-day water potentials seen in both red and green-leafed species, as well as stomatal closure (Table 2, Figure 7).

Although red-leafed species as a group were more likely to have lower mid-day water potentials, higher $\epsilon$, and more negative $\Psi_{\pi,100}$ than green-leafed species, it should be noted that these attributes were not mutually exclusive. For example, the species with the highest $\epsilon$ and lowest $\Psi_{\pi,100}$ during winter was a green-leafed evergreen (Vinca minor), and several red-leafed evergreens exhibited $\epsilon$ and $\Psi_{\pi,100}$ which were comparable to those of green-leafed evergreens during winter (Table 1, Figure 5). Similarly, although red-leafed species as a group did exhibit significantly lower mid-day water potentials than green-leafed species, some red-leafed species (L. fontanesiana and Rhododendron spp.) exhibited only very mild declines in mid-day water potential, similar to, or milder than, those of some green-leafed species (Figure 3). When the data are combined to examine individual species holistically, it becomes clear that both red and green-leafed groups contain species exhibiting a broad range of drought tolerance (Table 2). Therefore, although red-leafed species are more likely to correspond with those that tolerate the most negative water potentials during winter, this is not a satisfactory explanation for winter color change as a general rule.
In addition to examining the relationship between leaf water status and reddening, we also used this opportunity to examine possible proximate explanations for winter reddening in the species examined. Anthocyanin synthesis is known to be inducible by negative water potentials, and also by accumulation of specific solutes, especially sugars (see introduction); either of these might therefore function as a proximate mechanism for anthocyanin induction in evergreens. Our results are not consistent with the explanation that osmolarity alone is responsible for inducing reddening in angiosperm evergreens. We found that the species with the most negative osmotic potential at full turgor during winter was a green-leafed specie (*V. minor*), and there was some degree of overlap observed between green-leafed species’ osmotic potentials with those of some red-leafed species during winter (i.e. the green-leafed *K. latifolia* and *R. maximum* with the red-leafed *H. helix* and *Rhododendron* spp.; Table 1). This suggests that concentrations of osmolytes alone are not sufficient to induce anthocyanin synthesis in evergreen systems. Because sugars commonly play a role in osmotic adjustment, and are also known to be effective in inducing anthocyanin synthesis in many species (e.g. Do and Cormier, 1991; Neta-Sharir et al. 2000, Nagira and Ozeki 2004, Teng et al. 2005, Murakami et al. 2008), we measured levels of fructose, glucose, and sucrose of all species during summer and winter. During winter, we found no difference in fructose concentrations between red and green-leafed species, a significantly greater amount of glucose in red-leafed species, and a significantly greater amount of sucrose in green-leafed species (Figure 5). Because of the significant overlap in relative amounts of these sugars, there does not appear to be a threshold effect with these particular solutes in these particular species. However, it is
possible that other sugars or sugar alcohols may be affecting anthocyanin synthesis as well, which were not examined here.

Lastly, it has been suggested that anthocyanins may be directly involved in osmotic adjustment by functioning as an osmolyte (Chalker-Scott 1999, 2000). Our results are generally not consistent with this explanation. If anthocyanins were contributing significantly to the osmotic pool, we might expect red-leafed species to consistently exhibit more negative Ψπ,100 compared to green-leafed species, and anthocyanin content to negatively correlate with Ψπ,100 within individual species; neither of these were evidenced in this study. As previously mentioned some green-leafed species had more negative Ψπ,100 without anthocyanin, and some red-leafed species had Ψπ,100 similar to those of green-leafed species (Figure 5D). Furthermore, when anthocyanin concentration was plotted against Ψπ,100 for individual species, a negative correlation was only observed in one species (data not shown). Instead, we observed (anecdotally) that anthocyanin concentration was more strongly dictated by sun exposure, within individual leaves and species, rather than Ψπ,100.

**Conclusion**

When taken as a whole, these data suggest that winter redness can not be explained solely on the basis of leaf water stress. Only three of the six red-leafed species we observed appeared adapted to very negative leaf water potentials (*G. urceolata*, *G. procumbens*, and *L. fontanesiana*), as did one green-leafed species entirely lacking anthocyanin in winter leaves (*V. minor*). Instead, we observed redness to be more
strongly coupled with light environment of individual leaves, rather than $\Psi_W$, consistent with a light-related function (e.g. photoprotection, antioxidants).

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Figure 1. (A) Mean pre-dawn and mid-day summer water potentials for species that either turn red (left half) or remain green (right half) during winter. (B) Delta water potential between pre-dawn and mid-day. Bars represent means of 5-10 replicates; error bars represent standard deviation. Measurements made September 30, 2008 (High: 21°C, Low 5°C).
Figure 2. Mean pre-dawn (A) and mid-day (B) water potential values of red-leafed species (solid lines, black symbols) and green-leafed species (dashed lines, white symbols) from September through March. Note that during September, leaves of all species were green. Points represent means of 5-10 replicates; error bars represent standard deviation. For dates and temperature details, refer to Field Water Potential Measurements in Materials and Methods.
Figure 3. Winter water potential values for red (left half of graphs) and green-leafed species (right half). Monthly mean pre-dawn (A) and mid-day (B) water potentials; (C) average winter pre-dawn and mid-day water potential values; (D) average delta water potentials between pre-dawn and mid-day. Bars represent means of 5-10 replicates; error bars depict standard deviation (A, B, D) and standard error (C). For dates and temperature details, refer to Field Water Potential Measurements in Materials and Methods.
Figure 4A. Seasonal pressure/volume curves for red-leafed angiosperm evergreen species. Open circles with dashed lines represent winter measurements (after leaf color change had occurred), solid circles represent summer measurements (prior to leaf color change). Curves were derived from leaves of 3-5 separate individuals.
Figure 4B. Seasonal pressure/volume curves for green-leafed angiosperm evergreen species. Open circles with dashed lines represent winter measurements, solid circles represent summer measurements. Curves were derived from leaves of 3-5 separate individuals.
Figure 5. Data derived from winter pressure-volume curves. Left column, from top to bottom: (A) percent relative water content at turgor loss point, (B) symplastic water fraction at full turgor, and (C) bulks modulus of elasticity between 95% and 98% RWC. Right column, from top to bottom: (D) osmotic potential at full turgor, (E) osmotic potential at turgor loss point. Bars represent means of 3-5 replicates, error bars are standard deviation.
Figure 6. Seasonal sugar content of winter-red (left column) and winter-green (right column) angiosperm evergreens. Bars represent means of 5 replicates, ± SD.
Figure 7. Winter gas exchange of winter-red (left column) and winter-green (right column) leafed angiosperm evergreens. Photosynthesis is illustrated in (A), stomatal conductance, (B), and transpiration, (C). Bars represent means of 10-20 replicates ± SD.
CHAPTER IV

CE-ESI-MS ANALYSIS OF SINGLY CHARGED INORGANIC AND ORGANIC ANIONS USING A DICATIONIC REAGENT AS A COMPLEXING AGENT.

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The following manuscript was published in Electrophoresis, Volume 30 Issue 22, pages 3918-3925, November 2009 and is reprinted with permission. Stylistic variations are due to formatting requirements of the journal. Gerardi and Lin performed capillary electrophoresis experiments and wrote portions of the manuscript. Breitbach and Armstrong synthesized complexing reagents and served in an advisory capacity. Colyer served in advisory and editorial capacities.
ABSTRACT

A dicationic ion pairing reagent, N, N'-dibutyl 1,1'-pentylenedipyrrolidinium, was used to form complexes with singly charged anions for their subsequent analysis by CE-ESI-MS in positive ion mode. This methodology offers the advantages of greater versatility and sensitivity relative to direct detection of the anions in negative ion mode, and it can be realized by a number of possible complexation strategies, including pre-column, on column, and post-column modes. Four model anions, perfluorooctanoate (PFOA), benzenesulfonate (BZSN), monochloroacetate (MCA), and trifluoromethanesulfonimide (NTF2) were amenable to complexation with the dicationic reagent, yielding singly charged cations with greater m/z ratios. By optimizing various parameters, including the CE separation buffer composition and pH, the concentration of the dicationic reagent, the mode of complexation, the nebulizing gas pressure, and the sheath liquid composition, it was possible to develop a robust CE-ESI-MS method appropriate for the analysis of anions in a tap water sample. By this method, limits of detection were found to be 20.9 and 1.31 ng/mL for MCA and BZSN, respectively.

INTRODUCTION

The diverse fields of environmental, pharmaceutical, and food science, amongst others, share the common need for methods capable of determining anions with great sensitivity. Established methods that permit the direct detection of anions without incorporating any prior separation techniques include mass spectrometry,[1-3] spectrophotometry, [4,5] ion-selective potentiometry, and other electrochemical techniques. [6,7] However, complex sample matrices often necessitate the incorporation of separation methods in
conjunction with anion detection. Ion chromatography is the most common separation method used in this context.[8-11] Gas chromatography,[12] reverse-phase liquid chromatography [13] and capillary electrophoresis (CE) [14,15] methods for anion analysis have also been demonstrated, and each of these can be routinely coupled to MS for anion detection. The detection of anions by these coupled techniques can be complicated by the need for derivatization into volatile species (GC-MS) or by the higher background and poorer electrospray stability experienced in negative ion mode.[12,16] Also, very small anionic analytes may fall below the low mass cutoff of the spectrometer and thus be undetectable in their native state, while other (detectable) low mass anionic analytes reside in a region of high chemical noise[5] and may suffer from reduced sensitivity relative to larger mass ions.[5] To overcome these limitations, small quantities of large, chaotropic, organic dicationic or tricationic ion-pairing agents can be added to pair with singly-charged or doubly-charged anions, yielding positively charged complexes of higher m/z.[17-21] The trace analysis of perchlorate in various samples first demonstrated the success of this approach.[5] Armstrong and coworkers [2] introduced the use of an imidazolium-based dicationic reagent as a complexing agent for 34 singly-charged anions. This allowed for the detection of these anions in the more stable and sensitive positive-ion mode ESI-MS. The analysis of a tap water sample by this method revealed the presence of five anions (chloride, nitrate, bromide, monochloroacetate (MCA), and benzenesulfonate (BZSN)), which were also quantitated. A mixture of five other selected anions (thiocyanate (SCN), triflate (TFO), BZSN, perfluorooctanoate (PFOA), and trifluoromethanesulfonimide (NTF2)) were separated by HPLC prior to a comparison of detection in both positive and negative selected ion
monitoring (SIM) mode. The results proved the ultra-sensitivity of this technique and demonstrated its compatibility with HPLC.

The structure and nature of the dicationic reagent can have significant effects on the reagent’s affinity for anionic analytes and stability. A recent study by Remsburg et al. [19] compared 23 dicationic salts to evaluate their efficacies as pairing agents for anion analysis in positive ion mode. From these, four dications were recommended for general use in subsequent anion analysis by this ion-pairing method. One in particular – dication N, N'-dibutyl 1,1'-pentylenedipyrrolidinium, which possesses a pentane linkage and butylpyrrolidinium charged groups (Fig. 1) – was the best to analyze for cyanate while also performing well for other anions (especially iodide and nitrate).

Capitalizing on this earlier work, and recognizing the inherent advantages of CE as a separation technique (including reduced solvent/reagent consumption, reduced sample requirements, and high efficiencies relative to LC), we have developed a CE-ESI-MS method using the dication N, N'-dibutyl 1,1'-pentylenedipyrrolidinium fluoride as a complexing agent for anion analysis in the positive-ion mode. Review articles have critically examined the performance of CE-ESI-MS relative to LC-ESI-MS,[22] and the utility of CE-ESI-MS as a quantitative tool,[23] and as a tool for bioanalysis.[24] The sensitivity and efficiency of the new anion analysis method introduced herein depends on the mode by which the dicationic reagent is introduced to the sample solution, and so a comparison of three possible modes – pre-column (before injection and CE separation); on-column (by incorporation of the dication in the CE separation buffer); or post-column (by introduction of the dication to the separated analytes via a sheath liquid necessary to the ESI process) – is presented. Finally, a practical application of our CE-ESI-MS
method with dication pairing is demonstrated by the detection of two environmental contaminants (BZSN and MCA) in a tap water sample. The rapid and reproducible separation, quantitation, and identification of singly-charged anions permitted by this CE-ESI-MS method with dication pairing is important for future biological and environmental research.

EXPERIMENTAL

Materials. All chemicals and solvents used were of analytical grade or HPLC quality. Perfluoro-octanoic acid (PFOA), sodium benzenesulfonate (BZSN), monochloroacetic acid (MCA), and lithium trifluoromethanesulfonimide (NTF2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of each anion were prepared in distilled, deionized water (Millipore, Bedford, MA, USA) to the appropriate concentration. N, N'-dibutyl 1,1'-pentylenedipyrrolidinium dication (Fig. 1) was synthesized as its bromide salt according to a previously published procedure from Armstrong and coworkers.[19] To maximize complex formation between the dicationic reagent and singly-charged analyte anions, the dication was subjected to ion exchange to produce a stock solution (10 mM) of the fluoride salt in water according to the procedure described by Martinelango et al.[25] The dicationic reagent solution was either added to the anion sample, the CE separation buffer, or to the sheath flow liquid in the appropriate concentration according to the method being studied. Ammonium acetate (>99.99%), ammonium formate, ammonium carbonate (ACS reagent), ammonium bicarbonate (ACS reagent), ammonium hydroxide (28 – 30%, ACS reagent) and formic acid ( > 99.9%, ACS reagent) were from Sigma-Aldrich, and were used to prepare CE separation buffers.
Working buffer solutions were prepared to the required concentrations and their pH values were adjusted by adding ammonium hydroxide or acetic acid as appropriate. Buffers were filtered through 0.2 μm nylon syringe filters (Corning, NY, USA) before use.

**CE-ESI-MS Analysis.** A HP3DCE capillary electrophoresis system (Agilent Technologies, Palo Alto, CA, USA) equipped with a UV absorbance diode array detector was used for CE-MS coupling. CE-MS experiments were performed with a 75 cm (21.5 cm to the UV absorbance detector), 48 μm i.d. fused-silica capillary (Polymicro Technologies, Phoenix, AZ). The capillary temperature was controlled at 25oC inside the CE safety interlock compartment. The capillary was conditioned prior to its first use by consecutively flushing with H2O for 10 min, 0.1 M NaOH for 10 min, H2O for 10 min, and the electrophoresis buffer for 20 min. After each sample run, the capillary was rinsed with the electrophoresis buffer for 2 min. The buffer in the inlet reservoir was renewed after every three runs for improved reproducibility. The separation voltage was 20 kV. Analytes were hydrodynamically injected at 50 mbar for 10 s. The CE system was coupled to an Esquire 3000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an orthogonal electrospray ionization (ESI) source. The CE-ESI-MS coupling was facilitated by a coaxial sheath liquid interface (Agilent Technologies). The ion trap mass spectrometer was used in the positive ion mode, and the capillary voltage was set at 3.5 kV. Dry nitrogen gas was heated to 325oC and delivered at a flow rate of 5 L/min, while the nebulizing gas (N2) pressure was 15 psi. The lenses and block voltages were fixed using the tuning software, assuming the compound
stability to be 100%. The ion trap was operated in ion charge control mode to accumulate 30000 ions, for a maximum accumulation time of 200 ms. The sheath liquid (50:50, v/v; MeOH/H2O) was delivered at 0.4 mL/min by a pump equipped with a 1:100 splitter (thus providing sheath liquid to the CE-MS interface at 4 μL/min).

RESULTS AND DISCUSSION

Optimization of CE separation buffer and pH. Optimization of the CE separation buffer with regard to analyte migration behavior, analysis time, peak shape, and resolution is complicated by coupling to MS due to the stringent requirements of the latter for buffer volatility. Commonly used CE-MS buffers are based on the ammonium salts of acetate, formate, carbonate, and bicarbonate. Fortuitously, these typical CE-MS buffer anions are relatively poorly complexed by the dicationic pairing reagent,[2] and as such, are well suited for the dicationic reagent approach to anion analysis by CE-ESI-MS. A comparison of the electropherograms obtained using each of the four common CE buffers, prepared with the addition of 20 μM of N, N'-dibutyl 1,1'-pentylenedipyrrolidinium dication, for a mixture of four anionic analytes (PFOA, BZSN, NTF2 and MCA) is shown in Fig. 2. Although baseline resolution of the four anions was easily achieved using each of the four buffers and “on-column” complexation with the N, N'-dibutyl 1,1'-pentylenedipyrrolidinium dication, the sensitivity of the method towards MCA was significantly diminished in the carbonate and biocarbonate buffers, especially relative to the acetate buffer. This is likely due to the fact that acetate is not efficiently complexed by the dication and so poses relatively less interference than the other buffer anions. Although the relative sensitivity of the method obtained with the formate buffer
was comparable to that obtained with the acetate buffer (and was even better in the case of BZSN with formate), migration times increased and peak shapes worsened, and so the ammonium acetate buffer was employed in subsequent experiments. The net mobility of the cationic complexes that were formed with the four model anions increased as the pH of the ammonium acetate buffer was increased over the range from 5.0 to 8.9, as seen in Fig. 3. Electroosmotic flow increases with increasing pH, and it is likely this effect that dominates the change in net mobility observed in this study. Since baseline resolution was achieved at all pHs studied, it was possible to optimize the pH on the basis of analysis time (eliminating the use of pH 5.0 based on its increased analysis time) and signal intensity (eliminating the use of pH 8.9 based on its reduced sensitivity). Thus, pH 6.68 was determined to be optimal for the acetate buffer in these studies.

**Effect of dicationic reagent concentration.** It is necessary to use a sufficient quantity of the dicationic reagent to ensure complete complexation of the analyte anions; however, there is likely no good reason to add additional dicationic reagent beyond this point. A large excess of the dicationic reagent could have the effect of contaminating the ion trap, thus degrading overall sensitivities. To determine a suitable concentration of the dicationic reagent, electropherograms were recorded for the standard four anion sample mixture in 30 mM ammonium acetate buffer (pH 6.68) with either 10.0, 20.0, or 40.0 μM N, N'-dibutyl 1,1'-pentylenedipyrrolidinium added to the buffer for on-column complexation (Fig. 4). As can be seen in Fig. 4, peak heights increased (increasing sensitivity) with increasing concentration of the dicationic reagent added to the separation buffer up to 20 μM of added N, N'-dibutyl 1,1'-pentylenedipyrrolidinium, while a
subsequent reduction in signal was observed in the case of 40 μM of added N, N'-dibutyl 1,1'-pentylene-1,1'-pentylenedipyrrolidinium. Thus, the optimum concentration for the dicationic reagent in these studies was determined to be 20 μM.

Effect of the composition of sheath liquid composition. The optimization of the ESI parameters is crucial to achieve MS signal for any analyte, and this is especially true in CE-ESI-MS, where a sheath liquid is typically employed to make up the necessary volume flow from the capillary to the electrospray interface as well as to serve as a place in which to provide a ground for the separation voltage. In all cases, the sheath liquid affects the transfer of analyte from the liquid phase into the gas phase, thus having a significant impact on the resulting MS signal (and sensitivity). It has been reported that small amounts of formic acid, acetic acid, ammonium formate, or ammonium acetate can be added to the sheath liquid to enhance ESI-MS in positive ion mode. [26,27] Furthermore, incorporation of an organic solvent in the sheath liquid can improve the efficiency of ion evaporation.[28] Juan-García et al.[29] investigated the effect of methanol and isopropanol as sheath liquids and found that methanol gave the most stable and highest MS signal. Thus, methanol was employed as a component of the sheath liquid in the present studies.

The concentration of methanol in the sheath liquid was optimized by comparing the sensitivity and stability of the MS signal resulting from experiments conducted with three different concentrations of methanol (20, 50, 80% v/v) in water as the sheath liquid. As can be seen in Fig. 5, peak areas (sensitivities) for the four analytes (PFOA, NTF2, BZSN, and MCA) were reduced when 20/80 methanol/water was employed as the sheath liquid. Increasing the methanol content of the sheath liquid up to 50% resulted in
markedly improved sensitivities (and peak shapes); however, increasing the methanol content further still (up to 80%) resulted in higher background noise, which degraded overall sensitivities. Thus, a 50/50 (v/v) methanol/water mixture was employed as the sheath liquid in subsequent experiments.

The effect of formic acid concentration (0.0, 0.1%, 0.5%) in the sheath liquid was also investigated. Fig. 6 shows that peak areas (sensitivities) for the four analytes (PFOA, NTF2, BZSN, and MCA) were reduced as the formic acid concentration was increased, and when the formic acid concentration was 0.5%, BZSN and MCA could no longer be detected. Longer migration times were observed when the highest concentration of formic acid (0.5%) was employed in the sheath liquid. This result was unexpected but reproducible, and may be attributed to alteration of the electrophoresis buffer composition by the presence of higher concentrations of acid in the sheath liquid, which, in turn, could reduce electroosmotic flow and hence, increase migration times.[30] Since the greatest sensitivity (and no alteration of migration times) was achieved with no added formic acid, the simple 50/50 (v/v) methanol/water sheath liquid was determined to be optimum.

The effect of instrumental parameters: Sheath liquid flow rate, drying gas flow rate, and N₂ pressure. In order to examine the effect of the flow rate of the sheath liquid, the flow rate was increased from 0.2 to 1.0 mL/min (although it should be recognized that the actual sheath flow at the CE-MS interface was 100-times lower due to the use of a pump with a 1:100 split ratio, as described previously). When the flow rate was increased from 0.2 to 0.4 mL/min, a gradual increase in peak intensity was observed (data not shown). However, when the flow was increased from 0.4 to 0.6 mL/min, no
further increase in sensitivity was found. Furthermore, at flow rates greater than 0.6 mL/min, a gradual decrease in peak intensity was observed. The decrease in sensitivity may be a consequence of the sample diluting effect and decreased ionization efficiency at higher flow rates. Thus, 0.4 mL/min was selected as the optimal flow rate of sheath liquid.

The drying gas in ESI is generally used to accelerate desolvation, to increase sensitivity, and to avoid the entry of undesirable ions into the mass spectrometer. Drying gas flow rates in the range of 4 -10 L/min were tested, with no affect on peak height (data not shown). A drying gas flow rate of 5 L/min was used in these experiments.

The effect of nebulizing gas pressure on CE-ESI-MS performance was examined for N2 pressures of 10, 15, and 20 psi. Sensitivity was found to increase with increasing nebulizing gas pressure, but this came at the expense of CE resolution (data not shown), presumably because the N2 can have the effect of siphoning or drawing the contents of the separation capillary more rapidly toward the outlet. To retain sufficient resolution to permit the separation of many potential analytes in a complex mixture with enhanced sensitivity, a nebulizing gas pressure of 15 psi was selected.

**Comparison of modes of complexation of singly-charged anions with N, N'-dibutyl 1,1'-pentylenedipyrrolidinium.** In this work, the purpose of forming ion-pair complexes of singlycharged analyte anions with the N, N'-dibutyl 1,1'-pentylenedipyrrolidinium dicationic reagent was to increase the overall m/z of the species being detected and to render them positively charged, thus permitting their detection in positive ion mode with greater sensitivity than could be achieved by direct detection of the analyze anions in negative ion mode. To demonstrate this, Fig. 6 compares the
detection of four anions, complexed and uncomplexed, in positive and negative polarity modes, respectively. The peak areas and signal-to-noise ratios of the four anions when complexed with N, N'-dibutyl 1,1'-pentylenedipyrrolidinium and detected in positive ion mode (Fig. 6(b)) were significantly larger than those of the uncomplexed ions in negative ion mode (Fig. 6(a)). In fact, the anion MCA could not even be detected at concentrations of 100 \( \mu \text{g/mL} \), in the negative mode. Clearly, complexation with the dicationic reagent led to the enhanced sensitivity and versatility that was sought. In particular, the signals for BZSN and MCA showed the greatest increases when detected in the positive ion mode, while NTF2 showed the greatest sensitivity overall. This is due to the NTF2 anion being quite chaotropic and more surface active,[31] allowing for detection at low levels in both positive and negative ion mode.

However, it should be noted that the mode of complexation (of the anions with N, N'-dibutyl 1,1'-pentylenedipyrrolidinium) can also have an impact on detectability. Ion pairing can take place in the “pre-column” mode, whereby the dicationic reagent is mixed with the sample prior to injection and analysis by CE-ESI-MS; or in the “on-column” mode, whereby the dicationic reagent is added to the electrophoresis buffer so that the sample ions have a chance to undergo ion pairing throughout the duration of the separation and their migration through the capillary towards the detector; or in the “post-column” mode, whereby the dicationic reagent is added to the sheath liquid so that the sample ions undergo separation in their native state prior to the formation of ion-pair complexes and introduction to the MS detector. A comparison of pre-column, on-column and post-column complexation modes is shown in Fig. 6(b), (c) and (d). On-column complexation resulted in greater sensitivities for all four analyte anions, and was
characterized by shorter migration times compared with pre-column. In CE-LIF studies employing noncovalent fluorescent labeling of proteins with dyes, enhanced sensitivities are likewise achieved for on-column labeling relative to pre-column labeling.[32] This can be attributed to the presence of complexing agent (or dye) at a constant concentration throughout the capillary, so that the equilibrium between free and bound complexing agent (or dye) can be readily established and maintained throughout the separation. Also, variations in analyte migration times (observed for pre-column versus on-column labeling or complexation methods) are likely due to variations in electroosmotic flow caused by alteration of the buffer composition upon the addition of complexing agents.

A further comparison of the on-column mode of complexation and the post-column mode for the test mixture of four analyte anions with \( N, N'\)-dibutyl 1,1'-pentylenedipyrrolidinium is shown in Fig. 6. (c) and (d). In these experiments, 20.0 \( \mu \text{M} \) \( N, N'\)-dibutyl 1,1'-pentylenedipyrrolidinium was either added to the separation buffer (Fig. 6(c)) or to the sheath liquid (Fig. 6(d)). Post-column complexation resulted in greater sensitivity (greater peak height) only in the case of MCA, while the other peak sizes were diminished relative to on-column complexation. It should be noted that the sensitivity for all analytes gradually decreased with repeated runs using the post-column complexation methodology (due to some as-yet unknown cause), and so it was determined that the on-column complexation method would provide optimal sensitivity for a tap water analysis, as described presently.

**Tap water analysis.** A tap water sample was collected from the cold-water tap of a laboratory sink at Wake Forest University. The water was allowed to run for 15 min before collection. For the determination of anions in the tap water sample by CE-ESI-MS
with dicationic reagent complexation, the optimized experimental conditions, as previously determined, were used (namely: a separation buffer consisting of 30 mM ammonium acetate with 20 μM N, N'-dibutyl 1,1'-pentylenedipyrrrolidinium (pH 6.68) for on-column complexation; a 50/50 (v/v) methanol/water sheath liquid delivered at 0.4 mL/min; and a 5 L/min flow of N2 nebulizing gas at 15 psi and 325° C). Quantitation of individual anions was performed using the extracted ion electropherogram mode, which allows for the extraction of signal corresponding to a given m/z ratio from the total ion current. The limit of detection for anions BZSN and MCA was calculated based on 3s/m, where s is the standard deviation in the baseline (approximated as 1/5 of the peak-to-peak noise in the blank signal); and m is the slope or sensitivity of a three-point calibration curve constructed from peak area versus injected anion standard concentration. Determination of ions in tap water samples by this CE-ESI-MS method with on-column dicationic reagent complexation, as summarized in Table 1, revealed the presence of MCA and BZSN at levels similar to those found in tap water samples analyzed by LC-MS. [2] This new CE-ESI-MS method thus provides sufficient sensitivity to permit the rapid and accurate analysis of anions in water samples, as may be necessitated by environmental, health, industrial, or processing applications.

CONCLUSIONS

In this paper, a CE-ESI-MS method was developed for the separation and identification of four anions in the positive ion mode using a dicationic ion-pairing reagent. Compared to LC-ESI-MS methodologies, this CE-ESI-MS method has several advantages, such as higher separation efficiencies and lower sample and solvent
consumption. Method optimization revealed that increasing concentrations of dicationic reagent in the electrophoresis buffer led to increasing sensitivities for singly-charged anionic analytes, but this held true only up to 20 μM added N, N'-dibutyl 1,1'-pentylenedipyrrolidinium. Additionally, a comparison of complexation modes – pre-column, on-column, and post-column – revealed that on-column complexation (achieved by adding the dicationic reagent directly to the electrophoresis buffer) was most effective. Finally, our results showed that CE-ESI-MS could be applied to quantitative water analyses, with limits of detection for MCA and BZSN comparable to those obtained by LC-ESI-MS.

ACKNOWLEDGEMENTS

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REFERENCES


Table 1. Quantifiable Anions in Winston-Salem Tap Water Sample

<table>
<thead>
<tr>
<th>Anion</th>
<th>Concentration&lt;sup&gt;a)&lt;/sup&gt; (ng/mL)</th>
<th>Regression Equation</th>
<th>R²</th>
<th>LOD (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCA</td>
<td>47 (+8)</td>
<td>y=(2.30 x 10^7)x-(7.86 x 10^4)</td>
<td>0.9947</td>
<td>21</td>
</tr>
<tr>
<td>BZSN</td>
<td>BDL</td>
<td>y=(1.05 x 10^8)x+(4.85 x 10^5)</td>
<td>0.9903</td>
<td>11</td>
</tr>
</tbody>
</table>

<sup>a)</sup> Expressed as the average of six replicate runs; +/- the standard deviation
Figure 1. Structure of the dicationic complexing reagent
Figure 2. Electropherograms showing the effect of electrophoresis buffer composition on the separation of four anions (20 μg/mL PFOA, 1 μg/mL NTF₂, 10 μg/mL BZSN, and 100 μg/mL MCA) by CE-ESI-MS. Each buffer contains 20 μM N, N'-dibutyl 1,1' pentylenedipyrrolidinium for on-column ion-pairing of the analytes. (a) 30 mM ammonium acetate, pH 8.93; (b) 30 mM ammonium carbonate, pH 8.85; (c) 30 mM ammonium bicarbonate, pH 7.79; (d) 30 mM ammonium formate, pH 7.50. Other CE and ESI-MS parameters are as stated in the Experimental section.
Figure 3. Electropherograms showing the effect of electrophoresis buffer pH on the separation of four anions (20 μg/mL PFOA, 1 μg/mL NTF₂, 10 μg/mL BZSN, and 100 μg/mL MCA) by CE-ESI-MS. Each buffer contains 30 mM ammonium acetate with 20 μM N, N'-dibutyl 1,1'-pentylendipyrrolidinium for on-column ion-pairing of the analytes. (a) pH 5.0; (b) pH 6.68; (c) pH 8.93. Other CE and ESI-MS parameters are as stated in the Experimental section.
Figure 4. Electropherograms resulting from varying the concentration of dicationic reagent added to a 30 mM ammonium acetate separation buffer (pH 6.68) for on-column complexation and CE-ESI-MS analysis of four anions (20 μg/mL PFOA, 1 μg/mL NTF₂, 10 μg/mL BZSN, and 100 μg/mL MCA). (a) 10.0 μM N, N'-dibutyl 1,1'-pentylenedipyrrolidinium; (b) 20.0 μM N, N'-dibutyl 1,1'-pentylenedipyrrolidinium; (c) 40.0 μM N, N'-dibutyl 1,1'-pentylenedipyrrolidinium. Other CE and ESI-MS parameters are as stated in the Experimental section.
Figure 5. The effect of sheath liquid composition on the separation of four anions (20 μg/mL PFOA, 1 μg/mL NTF₂, 10 μg/mL BZSN, and 100 μg/mL MCA) by CE-ESIMS with on-column complexation by 5.0 μM N, N'-dibutyl 1,1'-pentylenedipyrrolidinium in a 30 mM ammonium acetate separation buffer (pH 6.68). (a) 50/50 (v/v) methanol/water; (b) 80/20 (v/v) methanol/water; (c) 20/80 (v/v) methanol/water. Other CE and ESI-MS parameters are as stated in the Experimental section.
Figure 6. Electropherograms showing the effects of separation mode (positive ion vs. negative ion mode; and pre-column vs on-column vs. post-column ion-pairing mode) on the analysis of four anions (20 μg/mL PFOA, 1 μg/mL NTF₂, 10 μg/mL BZSN, and 100 μg/mL MCA) by CE-ESI-MS employing a 30 mM ammonium acetate separation buffer (pH 6.68). (a) Negative ion mode with no ion pairing; (b) positive ion mode with precolumn complexation of the anions by mixing the sample with 20 μM N,N'-dibutyl 1,1'-pentylendipyrroloidium prior to injection; (c) positive ion mode with on-column complexation with 20 μM N,N'-dibutyl 1,1'-pentylendipyrroloidium in the separation buffer; (d) positive ion mode with post-column complexation with 20 μM N,N'-dibutyl 1,1'-pentylendipyrroloidium in the sheath liquid. Other CE and ESI-MS parameters are as stated in the Experimental section.
CHAPTER V

CE-ESI-MS ANALYSIS OF DIVALENT ORGANIC AND INORGANIC ANIONS
USING A TRICATIONIC REAGENT AS A COMPLEXING AGENT.

Anthony R. Gerardi, Xiuli Lin, Zachary S. Breitbach, Daniel W. Armstrong, and Christa L. Colyer

The following chapter is prepared for submission to Electrophoresis. Stylistic variations are due to formatting requirements of the journal. Gerardi performed the capillary electrophoresis experiments and prepared the manuscript. Lin performed some additional capillary electrophoresis experiments. Breitbach and Armstrong provided tricationic reagent samples and served in an advisory capacity. Colyer served in advisory and editorial capacities.
ABSTRACT

A tricationic ion pairing reagent, 1,3,5-1-butyl-3-methyl-1H-imidazol-3-ium-2,4,6-trimethylbenzene, was used to form complexes with doubly charged anions for their subsequent analysis by CE-ESI-MS in positive ion mode. This methodology offers the advantages of greater versatility and sensitivity relative to direct detection of the anions in negative ion mode, and it can be realized by a number of possible complexation strategies, including pre-column, on-column, and post-column modes. Three model anions, sulfate [SO₄²⁻], thiosulfate [TSFA, S₂O₃²⁻], and benzenedisulfonate [BZDS, C₆H₄(SO₃)₂²⁻] were amenable to complexation with the tricationic reagent, yielding singly charged cations with greater m/z ratio than the native analytes. By utilizing optimized parameters obtained through previous work with dicationic reagents and singly charged anions (including the CE separation buffer composition and pH, the concentration of the dicationic reagent, the mode of complexation, the nebulizing gas pressure, and the sheath liquid composition) it was possible to develop a robust CE-ESI-MS method appropriate for the analysis of divalent anions in a mixture such as environmental or biological samples. By this method, limits of detection were found to be 23.8, 68.4, and 52.3 ng/mL for BZDS, TSFA, and sulfate, respectively.

INTRODUCTION

The ability to analyze anions with great sensitivity is important to diverse fields such as environmental science, geochemistry, the pharmaceutical industry, and food science. The analysis of sulfates, in particular, is important to food science in regards to
the practice of using sulfur containing food preservatives with antibiotic and antioxidant activity [1]. Various established methods, which permit the direct detection of anions without incorporating any prior separation techniques, include flow injection analysis mass spectrometry [2,3,4], spectrophotometry [5,6], ion-selective potentiometry, and other electrochemical techniques [7,8].

With complex sample matrices, it is often necessary to incorporate separation methods in conjunction with anion detection. Ion chromatography is the most prevalent separation method used for anions [9,10,11,12,13], followed by other separation methods such as gas chromatography [14], reverse-phase liquid chromatography [15] and capillary electrophoresis (CE) [16,17] methods, and each of these can be routinely coupled to MS for anion detection. The detection of anions by these various coupled techniques can be complicated by the need for derivatization into volatile species (GC-MS) or by the higher background and poorer electrospray stability experienced in negative ion mode [14,18]. Divalent anions present a further problem as negative ion mode detection would reduce the observed mass by a factor of 2 due to mass/charge spectrometry principles. This would result in small anionic analytes falling below the low mass cutoff of the spectrometer, thus making them undetectable in their native state, while other (detectable) low mass anionic analytes would reside in a region of high chemical noise [6] and may suffer from reduced sensitivity relative to larger mass ions.[6]

To overcome these limitations, small quantities of large, chaotropic, organic dicationic or tricationic ion-pairing agents can be added to pair with singly-charged or doubly-charged anions, yielding positively charged complexes of higher m/z [19,20,21,22,23]. The trace analysis of perchlorate in various samples first demonstrated
the success of this approach.[6] Armstrong and coworkers [3] introduced the use of an imidazolium-based dicationic reagent as a complexing agent for 34 singly-charged anions. This allowed for the detection of these anions in the more stable and sensitive positive-ion mode ESI-MS. This approach was subsequently applied to divalent anions using tricationic ion pairing reagents. [4] This practice could then be applied to higher valence anions and multi-charged cations as shown in recent work. [24]

The structure and nature of the tricationic reagent can have significant effects on the reagent’s affinity for anionic analytes and stability of resulting complexes. A recent study by Soukup-Hein et al. [4] compared 17 tricationic salts to evaluate their efficacies as pairing agents for anion analysis in positive ion mode. From these, 8 trications were recommended for general use in subsequent anion analysis by this ion pairing method due to their sensitivity and highly effective ion pairing. One in particular – trication 1,3,5-1-butyl-3-methyl-1H-imidazol-3-iium-2,4,6-trimethylbenzene, which possesses a trimethyl benzene core and butyl imidazolium charged groups (Fig. 1) – was the best to analyze for sulfate while also performing well for other anions (especially thiosulfate and o-benzenedisulfonate).

Capitalizing on this earlier work, and recognizing the inherent advantages of CE as a separation technique (including reduced solvent/reagent consumption, reduced sample requirements, and high efficiencies relative to LC), we have developed a CE-ESI-MS method using 1,3,5-1-butyl-3-methyl-1H-imidazol-3-iium-2,4,6-trimethylbenzene fluoride as a complexing agent for anion analysis in the positive-ion mode. Review articles have critically examined the performance of CE-ESI-MS relative to LC-ESI-MS
[25], as well as the utility of CE-ESI-MS as a quantitative tool [26] and as a tool for bioanalysis [27].

The sensitivity and efficiency of the new anion analysis method introduced herein depends on the mode by which the tricationic reagent is introduced to the sample solution. Analyte complexation may occur by any of one of three possible modes – pre column (before injection and CE separation); on-column (by incorporation of the trication in the CE separation buffer); or post-column (by introduction of the dication to the separated analytes via a sheath liquid necessary to the ESI process). Previous studies indicated that a dicationic reagent paired best with singly charged anions using the on-column complex formation technique for analysis by CE-ESI-MS. [28]

Recently, we have successfully used dicationic reagents to detect singly charged anions in the positive mode by CE-ESI-MS [28]. The dicationic reagent paired with the anion in the gas phase and enabled detection in the positive mode using common LC and/or CE solvents. Additional benefits to the CE-ESI-MS approach include moving anions to a higher mass range out of the low-mass region dominated by chemical noise, increasing sensitivity for divalent anions with masses near the low mass cutoff of quadrupole instruments, and discriminating against interferences with the same mass-to-charge ratio. The success of dicationic reagents to detect singly charged anions in the positive mode has encouraged a similar approach for the detection of doubly charged anion in the positive mode in CE-ESI-MS using a tricationic reagent in the present work.

**EXPERIMENTAL**

**Materials.** All chemicals and solvents used were of analytical grade or HPLC quality. Sulfate [SO$_4^{2-}$], thiosulfate [TSFA, S$_2$O$_3^{2-}$], and benzenedisulfonate [BZDS,
C₆H₄(SO₃)₂²⁻ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of each anion were prepared in distilled, deionized water (Millipore, Bedford, MA, USA) to the appropriate concentration. 1,3,5-1-butyl-3-methyl-1H-imidazol-3-ium-2,4,6-trimethylbenzene trication (Fig. 1) was synthesized as its bromide salt according to a previously published procedure from Armstrong and coworkers.[19] To maximize complex formation between the tricationic reagent and divalent analyte anions, the trication was subjected to ion exchange to produce a stock solution (10 mM) of the fluoride salt in water according to the procedure described by Martinelango et al.[25] The tricationic reagent solution was either added to the anion sample, the CE separation buffer, or to the sheath flow liquid in the appropriate concentration according to the method being studied. Ammonium acetate (>99.99%), ammonium formate (ACS reagent), ammonium hydroxide (28 – 30%, ACS reagent) and formic acid (> 99.9%, ACS reagent) were from Sigma-Aldrich, and were used to prepare CE separation buffers. Working buffer solutions were prepared to the required concentrations, and their pHs were adjusted by adding ammonium hydroxide or acetic acid as appropriate. Buffers were filtered through 0.2 μm nylon syringe filters (Corning, NY, USA) before use.

**CE-ESI-MS Analysis.** A HP3DCE capillary electrophoresis system (Agilent Technologies, Palo Alto, CA, USA) equipped with a UV absorbance diode array detector was used for CE-MS coupling. CE-MS experiments were performed with a 75 cm (21.5 cm to the UV absorbance detector) X 48 μm i.d. fused-silica capillary (PolymicroTechnologies, Phoenix, AZ). The capillary temperature was controlled at 25°C inside the CE safety interlock compartment. The capillary was conditioned prior to its first use by consecutively flushing with H₂O for 10 min, 0.1 M NaOH for 10 min, H₂O
for 10 min, and the electrophoresis buffer for 20 min. After each sample run, the capillary was rinsed with the electrophoresis buffer for 2 min. The buffer in the inlet reservoir was renewed after every three runs for improved reproducibility. The separation voltage was 20 kV. Analytes were hydrodynamically injected at 50 mbar for 10 s. The CE system was coupled to an Esquire 3000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an orthogonal electrospray ionization (ESI) source. The CE-ESI-MS coupling was facilitated by a coaxial sheath liquid interface (Agilent Technologies). The ion trap mass spectrometer was used in the positive ion mode, and the capillary voltage was set at 3.5 kV. Dry nitrogen gas was heated to 325°C and delivered at a flow rate of 5 L/min, while the nebulizing gas (N₂) pressure was 15 psi. The lenses and block voltages were fixed using the tuning software, assuming the compound stability to be 100%. The ion trap was operated in ion charge control mode to accumulate 30000 ions, for a maximum accumulation time of 200 ms. The sheath liquid (50:50, v/v; MeOH/H₂O) was delivered at 0.4 mL/min by a pump equipped with a 1:100 splitter (thus providing sheath liquid to the CE-MS interface at 4 μL/min).

RESULTS AND DISCUSSION

It was determined in previous studies that several parameters must be optimized in order to develop a robust and sensitive CE-ESI-MS method for the analysis of monovalent anionic analyte complexes with an ion-pairing reagent. An initial assessment of parameters successfully optimized for dicaticonic paring with singly charged analytes [28] was performed for tricaticonic pairing with divalent anions. Slight deviations from the previous optimization were sequentially applied to determine the most favorable ion
pairing and separation parameters for the tricationic reagent tested. An evaluation of the CE separation buffer and pH was essential, as this affects both the CE separation and MS detection. Next, a comparison of pre-column and on-column complexation was performed. Instrumental parameters such as nebulizing gas and drying gas flow rates and temperatures and MS trap voltages, previously evaluated for dicationic pairing, were largely employed herein, since these parameters were within normal, recommended default ranges. A detailed assessment of method optimization follows.

**Optimization of CE Separation Buffer and pH**

CE separation buffer optimization with consideration for analyte migration behavior, analysis time, peak shape, and resolution, is complicated by coupling to MS due to the stringent requirements of the latter for buffer volatility. Commonly used CE-MS buffers are based on the ammonium salts of acetate, formate, carbonate, and bicarbonate. Unfortunately, these typical CE-MS buffer anions may be unintentionally involved in complex formation with the tricationic pairing reagent. Though not examined in ESI-MS studies by Soukup-Hein et al.[19], it is apparent that the tricationic reagents may form complexes with singly charged anions such as those in typical CE-MS buffer systems. Possible complexation scenarios are shown in Fig. 2. A comparison of the electropherograms obtained using each of two common CE buffers, prepared with the addition of 20 μM of 1,3,5-1-butyl-3-methyl-1H-imidazol-3-iun-2,4,6-trimethylbenzene, for a mixture of three divalent anionic analytes (sulfate [SO₄²⁻], thiosulfate [S₂O₅²⁻], and benzenedisulfonate [C₆H₄(SO₃)₂²⁻]) is shown in Fig. 3. Baseline resolution of the three anions was easily achieved using each of the two buffers and “on column” complexation with the 1,3,5-1-butyl-3-methyl-1H-imidazol-3-iun-2,4,6-trimethylbenzene trication.
The sensitivity of the method towards sulfate was somewhat diminished in the formate buffer relative to the acetate buffer. This is likely due to the fact that acetate is not efficiently complexed by the trication and so poses relatively less interference than the formate buffer anion. Since the sensitivity of the method obtained with the formate buffer was less than obtained with the acetate buffer and migration times increased and peak shapes worsened with formate, the ammonium acetate buffer was employed in subsequent experiments.

The net mobility of the cationic complexes that were formed with the three model divalent anions increased as the pH of the ammonium acetate buffer was increased over the range from 5.0 to 8.9, as seen in Fig. 4. Electroosmotic flow increases with increasing pH, and it is likely this effect that dominates the change in net mobility observed in this study. Since baseline resolution was achieved at all pHs studied, it was possible to optimize the pH on the basis of analysis time (eliminating the use of pH 5.0 based on its increased analysis time) and signal intensity (eliminating the use of pH 8.9 based on its reduced sensitivity). Thus, pH 6.68 was determined to be optimal for the acetate buffer in these studies.

**Effects of tricationic reagent concentration, sheath liquid composition, and other MS parameters**

The tricationic reagent should be in excess concentration relative to the divalent anions to be analyzed so as to ensure complexation. Also to be considered is the effect of too much tricationic reagent, which poses the risk of a higher background, or lower signal-to-noise ratio thereby, lowering analytical sensitivity. Though the scan range employed (m/z from 610-820) effectively removes possible interferences from the
uncomplexed tricationic reagent, it is possible to experience degraded signal due to carryover (of the reagent to the source), space charge effects, and trap volume interference from excess reagent. It was determined from previous work with a dicationic reagent that 20.0 μM concentration of dicationic reagent in a 30 mM ammonium acetate buffer solution was adequate for complexation of analyte anions [28]. This optimum concentration provided for the best electromobility of the complexes while maintaining a suitable sensitivity. This parameter was assumed optimal for the tricationic reagent used.

The optimization of the ESI parameters is critical to attain MS signal for any analyte, and this is particularly true in CE-ESI-MS, where a sheath liquid is typically employed to make up the necessary volume flow from the capillary to the electrospray interface as well as to serve as a place in which to provide a ground for the separation voltage. In all cases, the sheath liquid affects the transfer of analyte from the liquid phase into the gas phase, thus having a significant impact on the resulting MS signal (and sensitivity). It has been reported that small amounts of formic acid, acetic acid, ammonium formate, or ammonium acetate can be added to the sheath liquid to enhance ESI-MS in positive ion mode [29,30]. Furthermore, incorporation of an organic solvent in the sheath liquid can improve the efficiency of ion evaporation.[31] Juan-García et al. [32] investigated the effect of methanol and isopropanol as sheath liquids and found that methanol gave the most stable and highest MS signal. Thus, methanol was employed as a component of the sheath liquid in the present studies.

The concentration of methanol in the sheath liquid was optimized in previous work by comparing the sensitivity and stability of the MS signal resulting from
experiments conducted with three different concentrations of methanol (20, 50, 80% v/v) in water as the sheath liquid [28]. It was determined that a 50/50 (v/v) methanol/water mixture was optimum and so was employed as the sheath liquid in subsequent experiments. This optimum was assumed for the tricationic reagent used in this study as well. Addition of formic or acetic acid modifiers to the sheath liquid proved detrimental in previous work and so modifiers were not employed here. The optimum flow rate of 0.4 mL/min was used in all experiments with a N₂ drying gas flow optimum of 5 L/min.

**Comparison of modes of complexation of divalent anions with 1,3,5-1-butyl-3-methyl-1H-imidazol-3-ium-2,4,6-trimethylbenzene.**

In this work, the purpose of forming ion-pair complexes of divalent analyte anions with the 3,5-1-butyl-3-methyl-1H-imidazol-3-ium-2,4,6-trimethylbenzene tricationic reagent was to increase the overall m/z of the species being detected and to render them positively charged, thus permitting their detection in positive ion mode with greater sensitivity than could be achieved by direct detection of the analyte anions in negative ion mode. To demonstrate this, Fig. 5 compares the detection of three divalent anions, complexed and uncomplexed, in positive and negative polarity modes, respectively. The peak areas and signal-to-noise ratios of the four anions when complexed with 3,5-1-butyl-3-methyl-1H-imidazol-3-ium-2,4,6-trimethylbenzene and detected in positive ion mode (Fig. 5(b)) were significantly larger than those of the uncomplexed ions in negative ion mode (Fig. 5(a)).

However, it should be noted that the mode of complexation (of the anions with 3,5-1-butyl-3-methyl-1H-imidazol-3-ium-2,4,6-trimethylbenzene) can also have an impact on detectability. Ion pairing can take place in the “pre-column” mode, whereby
the tricationic reagent is mixed with the sample prior to injection and analysis by CE-ESI-MS; or in the “on-column” mode, whereby the tricationic reagent is added to the electrophoresis buffer so that the sample ions have a chance to undergo ion pairing throughout the duration of the separation and their migration through the capillary towards the detector. “Post column” mode, whereby the tricationic reagent is added to the sheath liquid so that the sample ions undergo separation in their native state prior to the formation of ion-pair complexes and introduction to the MS detector was not performed. A comparison of pre-column and on-column complexation modes is shown in Fig. 5(b) and (c). On-column complexation resulted in greater sensitivities for all three analyte anions, and was characterized by shorter migration times compared with pre-column. In CE-LIF studies employing noncovalent fluorescent labeling of proteins with dyes, enhanced sensitivities are likewise achieved for on-column labeling relative to pre-column labeling.[33] This can be attributed to the presence of complexing agent (or dye) at a constant concentration throughout the capillary, so that the equilibrium between free and bound complexing agent (or dye) can be readily established and maintained throughout the separation. Also, variations in analyte migration times (observed for pre-column versus on-column labeling or complexation methods) are likely due to variations in electroosmotic flow caused by alteration of the buffer composition upon the addition of complexing agents.

Quantitation of individual anions was performed using the extracted ion electropherogram mode, which allows for the extraction of signal corresponding to a given m/z ratio from the total ion current. The limit of detection [LOD] for anions BZDS, TSFA and sulfate was calculated based on 3s/m, where s is the standard deviation in the
baseline (approximated as 1/5 of the peak-to-peak noise in the blank signal); and $m$ is the slope or sensitivity of a three-point calibration curve constructed from peak area versus injected anion standard concentration. Limit of Quantitation [LOQ] was calculated by multiplying the LOD by 3.3. Quantitative results, summarized in Table 1, revealed LOD/LOQ at levels similar to LC-MS. [4] This new CE-ESI-MS method thus provides sufficient sensitivity to permit the rapid and accurate analysis of anions in environmental, health, industrial, or processing applications.

Conclusions

In this paper, a CE-ESI-MS method was developed for the separation and identification of three divalent anions in the positive ion mode using a tricationic ion-pairing reagent, 1,3,5-1-butyl-3-methyl-1H-imidazol-3-ium-2,4,6-trimethylbenzene. Compared to LC-ESI-MS methodologies, this CE-ESI-MS method has several advantages, such as higher separation efficiencies and lower sample and solvent consumption. A comparison of complexation modes – pre-column and on-column revealed that on-column complexation (achieved by adding the tricationic reagent directly to the electrophoresis buffer) was most effective. Finally, our results showed that CE-ESI-MS could be applied to quantitative analyses, with limits of detection for the three model analytes comparable to those obtained by LC-ESI-MS.
Figure 1. Structure of the tricationic complexing reagent.
Figure 2. Anion complexation strategies for use with a tricationic liquid reagent.
Figure 3. Electropherograms showing the effect of electrophoresis buffer composition on the separation of three anions (10 µg/mL BZDS, 100 µg/mL TSFA, 50 µg/mL Sulfate) by CE-ESI-MS. Each buffer contains 20 mM 1,3,5-1-butyl-3-methyl-1H-imidazol-3-ium-2,4,6-trimethylbenzene for on-column ion pairing of the analytes. (A) ammonium acetate, pH 8.92; (B) ammonium formate, pH 7.48.
Figure 4. Electropherograms showing the effect of electrophoresis buffer pH on the separation of three anions (10 μg/mL BZDS, 100 μg/mL TSFA, 50 μg/mL Sulfate) by CE-ESI-MS. Each buffer contains 30mM ammonium acetate with 20 mM 1,3,5-1-butyl-3-methyl-1H-imidazol-3-iium-2,4,6-trimethylbenzene for on-column ion pairing of the analytes. (A) pH 8.92; (B) 6.68; (C)pH 5.0.
Figure 5. Electropherograms showing the effects of separation mode (positive ion vs. negative ion mode; and pre-column vs on-column ion-pairing mode) on the analysis of three anions (10 μg/mL BZDS, 100 μg/mL TSFA, 50 μg/mL Sulfate) by CE-ESI-MS employing a 30 mM ammonium acetate separation buffer (pH 6.68). (a) Negative ion mode with no ion pairing; (b) positive ion mode with pre-column complexation of the anions by mixing the sample with 20 μM 1,3,5-1-butyl-3-methyl-1H-imidazol-3-ium-2,4,6-trimethylbenzene; (c) positive ion mode with on-column complexation with 20 μM 1,3,5-1-butyl-3-methyl-1H-imidazol-3-ium-2,4,6-trimethylbenzene in the separation buffer. Other CE and ESI-MS parameters are as stated in the Experimental section.
### Table 1. Quantitative Results for Ion-Paring of Model Divalent Analytes with Trication 1,3,5-1-butyl-3-methyl-1H-imidazol-3-iium-2,4,6-trimethylbenzene

<table>
<thead>
<tr>
<th>Divalent Anion</th>
<th>Regression Equation</th>
<th>R²</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BZDS</td>
<td>y = (2.05 x 10⁻⁸)x - (3.751 x 10⁵)</td>
<td>0.9911</td>
<td>24</td>
<td>79</td>
</tr>
<tr>
<td>TSFA</td>
<td>y = (3.52 x 10⁻⁷)x - (6.064 x 10⁵)</td>
<td>0.9985</td>
<td>68</td>
<td>224</td>
</tr>
<tr>
<td>Sulfate</td>
<td>y = (6.14 x 10⁻⁷)x + (3.075 x 10⁴)</td>
<td>0.9962</td>
<td>52</td>
<td>171</td>
</tr>
</tbody>
</table>
## References:


CHAPTER VI

CONCLUSIONS

The field of analytical chemistry has evolved into a discipline that provides necessary qualitative and quantitative determination in fields such as forensics, bioanalysis, clinical analysis, environmental analysis, and materials analysis. Analytical chemistry is focused on the development of new tools for the measurement of chemical species, both large and small. Method development in analytical chemistry must focus on the separation and selective detection of the analytes of interest. Some of the most common separation techniques in modern analytical chemistry, as discussed herein, are capillary electrophoresis and high performance liquid chromatography. CE has continued to be developed as a viable alternative to other techniques due to decreased analysis times, reduced sample and solvent requirements, high efficiency and resolution. When coupled to LIF or MS detection, method selectivity and sensitivity for CE are relatively high.

Many biological molecules of interest, in particular proteins, are not natively fluorescent in the visible or near-IR region. Therefore, they must be tagged with a fluorescent label in order to be detected with LIF detection. This can lead to more sample preparation and may be difficult with very dilute samples. Also, band broadening during the separation can result from a derivatized sample. To circumvent these problems, fluorescent dyes that form a non-covalent interaction with the analyte can be
used. The utility of using a non-covalent fluorescent probe in the presence of a semi-
permanent surfactant coating was assessed in this work.

The semi-permanent coating, composed of dimethylditetradecylammonium bromide (2C14DAB), has previously been shown to be of utility for the determination of proteins by CE coupled to absorbance detection. Presented here was an extension of this method, to permit 2C14DAB coated capillaries to be used with LIF detection of non-
covalently labeled proteins complexed with Red-1c, a red luminescent squarylium dye. This novel progression provided a separation with a nearly 2-fold improvement both in terms of resolution and efficiency in 1/3-less analysis time for model protein analysis in a coated versus uncoated column while maintaining LIF detection sensitivity.

Future efforts should be designed with regards to different surfactant coatings and how well they perform with other non-covalent dyes, with a goal to achieve more efficient, faster separations while maintaining detector sensitivity. The utility of these coatings to facilitate more efficient separations in the presence of fluorescent probe molecules is likely to be realized for a variety of labeled analytes and should not be considered to be limited to proteins only, nor to on-column derivatization procedures or noncovalent probe-analyte interactions.

Mass spectrometry (MS) is an analytical technique used for identification of chemical structures, determination of mixtures, and quantitative elemental analysis that measures the mass-to-charge ratio of charged particles. Mass spectrometry is both very selective and sensitive and can be used on a wide range of molecules, from low-mass, small molecules to large proteins. Analytes are introduced into the mass spectrometer with a charge that, in conjunction with applied voltage and frequency, guides the analytes
or fragments to the detector. A common interface between an analytical separation and the mass spectrometer is an electrospray ionization (ESI) source. Ionization is achieved in ESI by transferring effluent from a separation (i.e. CE or HPLC) containing the analyte(s) of interest so that they are dispersed by nebulization into a fine aerosol and are charged by the applied voltage and pH of the effluent at the spray source. The nebulized analytes are desolvated by heat from the source and the charged ions enter the MS.

CE-ESI-MS is a powerful hyphenated analytical methodology that combines the efficient separations of CE with the selectivity and sensitivity of MS. Since ESI relies on the pH for solution phase ionization, it is easily compatible with the buffer system needed for CE separations. In this case, acetic and formic acid and salts of these are the preferred buffers for the CE separation as these are well matched to MS. Larger acids and their respective analogues can deposit on, clog and corrode the ion transfer tube at the MS interface. Though limiting to CE, these buffers allow for a range of pHs suitable for separation. The advantages of CE-ESI-MS are higher separation efficiencies and lower sample and solvent consumption versus HPLC-ESI-MS.

Several parameters were optimized for the analysis of monovalent anions with a dicationic complexing reagent, N, N'-dibutyl 1,1'-pentylenedipyrrolidinium. Optimized parameters revealed that increasing concentrations of dicationic reagent in the electrophoresis buffer led to increasing sensitivities for singly-charged anionic analytes, but this held true only up to 20 μM added reagent. Also, a comparison of complexation modes – pre-column, on-column, and post-column – revealed that on-column complexation (achieved by adding the dicationic reagent directly to the electrophoresis buffer) was most effective. An analysis of tap water revealed that CE-ESI-MS could be
applied to quantitative water analyses, with limits of detection for MCA and BZSN comparable or better than those obtained by LC-ESI-MS. Again, the advantages are higher separation efficiency, less solvent and less sample needed for the analysis with CE-ESI-MS.

As the conclusions above were favorable for the analysis of singly charged anions with divalent cationic liquids, it was logical to apply CE-ESI-MS analysis to higher valence analytes and cationic reagents. In this case, three divalent anions were chosen as model analytes and the tricationic reagent, 1,3,5-1-butyl-3-methyl-1H-imidazol-3-ium-2,4,6-trimethylbenzene, was used as the complexing reagent. Once again, the CE-ESI-MS method offered several advantages, such as higher separation efficiencies and lower sample and solvent consumption compared to HPLC-ESI-MS. A comparison of complexation modes – pre-column and on-column revealed that on-column complexation (achieved by adding the tricationic reagent directly to the electrophoresis buffer) was most effective. Finally, our results showed that CE-ESI-MS could be applied to quantitative analyses, with limits of detection for the three model analytes comparable to those obtained by LC-ESI-MS.

Future work with cationic liquids as complexing reagents for the analysis of anions using CE-ESI-MS should concentrate on both analytes and reagents of higher valences or mixtures with several valences. For example, using a cationic reagent that has a charge of +4 could be used to complex anions with a charge of -3, -2, or -1 which would result in complexes with a net charge from +1 to +3. A mass spectrometer using electrospray ionization can be used to analyze species at any m/z, where m is the mass and z is the charge and the ratio is between the values of m/z +/-50-3000 for the ion trap
instrument employed in these studies. Depending on the type of sample, an analytical strategy best suited for the greatest number of analytes of interest would be developed.

Utilizing high efficiency separations also allowed us to better understand leaf color change in winter for some evergreen species by making robust measurements of sugars in plant leaf material. A rapid HPLC-ELSD method was developed for the determination of three sugars (glucose, sucrose, and fructose) in plant leaves. HPLC separations are ideal for sugar molecules since the separations can be done at neutral pH and no derivatization is required, unlike with GC separations of sugars. ELSD is a relatively novel detection method that is fast becoming a staple for analytes that do not contain chromophores, as is the case for sugars. ELSD is based on the ability of particles to scatter light when they pass through a beam of light and relies on the analyte being less volatile than the mobile phase. A four-point quadratic standard curve was obtained by the analysis of sugar standards of known concentration prepared with a mixture of three different sugars: fructose, glucose, and sucrose. The regression coefficient ($R^2$) for the standard curve was greater than 0.99 for all three analytes. The recovery from standard addition experiments showed recovery of approximately 99% for all three sugars.

Future studies involving the determination of sugars in leaf material should expand the sugar profile to include other common plant mono- and disaccharides. Starch breakdown products such as dextrin or other polysaccharides of glucose could be evaluated in the same runs. Also, this methodology could be adapted for a UPLC (ultra pressure liquid chromatograph) system, in which higher back pressures and smaller particle size columns make possible faster analysis times while maintaining chromatographic efficiency and analyte resolution.
METHOD DEVELOPMENT FOR SUGAR DETERMINATION BY HPLC-ELSD

The analysis of specific sugars in complex matrices by HPLC has been described numerous times and is of great utility. However, many methods have long analysis times due to isocratic separations in long columns and the necessity for a consistent mobile phase for refractive index (RI) detection. Shorter columns and new detection instrumentation allow carbohydrate analysis times of less than 8 minutes per injection. Environmental samples require stringent sampling regimes and many replicates so as to provide credible statistics. This leads to large numbers of samples to be analyzed, so high throughput and ease of sample preparation are necessary in method development.

Evaporative light scattering detection (ELSD) is based on the ability of particles to scatter light when they pass through a beam of light. The detector responds to compounds less volatile than the mobile phase, which is nebulized upon introduction into the detector. This technique was first introduced in the 1980s for the determination of lipid concentrations, which were difficult to obtain with an absorbance detector. The ELSD consists of three basic elements: the nebulizer, drift tube, and scattering chamber. The solvent stream first enters the nebulizer where it is nebulized and the droplets formed are entrained in a flow of gas. The droplets are evaporated in the drift tube and the dry particles that remain are carried in the flowing gas and solvent vapor stream. These dry particles scatter light, which is measured and the intensity of
this light is a function of the size and number of particles present. ELSDs are not spectrometric detectors and do not obey Beer’s Law and so response curves are non-linear. The overall detector response is a mixture of all scattering types such as Rayleigh, Mei, refraction, reflection, etc. Calibration curves produced by an ELSD are best fit by quadratic equations.

William Smith, Professor of Biology at Wake Forest University, studies the functional significance of plant accessory pigments such as anthocyanins, which are commonly found in newly developing leaves on growing tips in a wide variety of plant species. These red leaves contrast sharply with the mature leaves found at older nodes lower on the branch. Some species have leaves that turn red when exposed to high sunlight levels and a large number of evergreen species show greater leaf redness during the winter [11,12]. These pigments are believed to have a direct correlation to the free sugar found in the leaf biomass. Hence, the development of an HPLC-ELSD method was pursued in order to provide the leaf sugar measurements necessary to make or refute such correlations.

**Materials and Methods**

**Extraction**

200 mg of freeze-dried leaf tissue was weighed into a 10 mL disposable borosilicate test tube. 4.0 mL of deionized water was added and the test tubes were shaken on an orbital shaker for 5, 10, 20, and 30 minutes at 350 rpm to determine optimized extraction time. This same procedure was performed using ethanol, and 50:50 (v:v) ethanol:water mixture. A 30 minute agitation at 30 rpm using deionized water was determined to be the optimum extraction protocol.
Standard Reagent Preparation

Standard reagents of sucrose, glucose, and fructose were obtained from Sigma Aldrich (St. Louis, MO, USA) and were dissolved in distilled, deionized water to a concentration of 3.0 mg/mL. Subsequent dilutions were made from this stock to achieve concentrations of 1.5, 0.9, and 0.3 mg/mL. These four concentrations were used for the 4-point quadratic calibration curves (*ELSD response vs. sugar standard concentration*).

HPLC Analysis

The HPLC used was a Waters (Milford, MA, USA) Alliance 2695 HPLC system with isocratic flow of 2.0 mL/min. Separations were carried out on a 7 x 53 mm Altech (Deerfield, IL, USA) Prevail Carbohydrate ES Rocket column, which was maintained in a column heater at 50° C. The injection volume was 2 μL and the analysis time was 6 minutes. The mobile phase consisted of 75% acetonitrile and 25% water. A Waters 2420 evaporative light scattering detector was used for detection of fructose, glucose, and sucrose. The ELSD settings were as follows: drift tube temperature was 50° C; nebulizer gas was N₂ at 50 psi; and the nebulizer heater was set to 40%.

Recovery from Spiked Samples

Dried and ground magnolia leaves (picked in the winter) were extracted according to the procedure described earlier into 12 different test tubes labeled as three replicate sets of four different standard addition levels. Prior to shaking, 10 μL of each of the 3.0, 1.5 and 0.9 mg/mL standards were added to one test tube in each of the three replicate sets, with one test tube in each repetition being the baseline control (no added standard). Standard addition curves for each of the three replicate groups were generated after chromatographic analysis to determine recovery and accuracy of the analysis.
Results

A typical chromatogram of the high sugar standard is seen in Figure 1. Fructose, glucose and sucrose are adequately resolved ($R_s>1.5$) for all three analytes. Sucrose is the last analyte to elute at 5.16 minutes. The total analysis time is 6 minutes as the column re-equilibrates at the 2.0 mL/min flow rate.

**Figure 1.** ELSD chromatogram of a 3.0 mg/mL sugar standard obtained using aforementioned procedure. Fructose elutes at 3.05 min, glucose at 3.50 min, and sucrose at 5.16 min. Experimental parameters as described in “Materials and Methods”.

Figure 2 shows a representative 4-point quadratic standard curve for fructose used for quantitation of fructose. The limits of detection were obtained by determining the concentration that produced a signal-to-noise ratio of 10 and dividing that concentration by 3. The limits of detection so obtained for fructose, glucose, and sucrose in leaves were 0.1%, 0.03%, and 0.02% by dry weight, respectively.
A measure of precision was performed by injecting three different leaf sample extracts from the same vial 10 times each and calculating the percent relative standard deviation (%RSD) for each of the three analytes for each of the three leaf samples. The results showed that the %RSD for each analyte within each leaf sample was less than 1.0 %. A standard addition recovery study was performed. The area corresponding to the sample with no added standard (control) was subtracted from the total area for each sugar measured in the standard addition experiment. The added concentration was compared to the calculated concentration at the different levels. The average recovery for all levels was calculated. This resulted in average recoveries of 99.1%, 98.7% and 99.7% for fructose, glucose and sucrose, respectively.
Five replicates of 27 samples of tree leaves, corresponding to various collection times and locations on the tree (Table 1), were freeze-dried, ground, extracted and analyzed by HPLC-ELSD as described. Several of the samples were collected during the winter and summer so as to compare seasonal influence on saccharide storage and utilization. In several instances, mature leaves were compared to younger leaves.

Table 1. Sugar levels of 12 different leaf types from both summer and winter collections.

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<tr>
<th>SAMPLE ID</th>
<th>FRUCTOSE</th>
<th>GLUCOSE</th>
<th>SUCROSE</th>
<th>TOTAL SUGARS IN DRY TISSUE</th>
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BDL = Below Detectable Limits

**Discussion and Conclusions**

Based on the HPLC-ELSD results presented in Table 1, for leaf sugar content it was possible to reliably assess the importance of factors such as season, leaf age, and leaf color on sugar production. The ecological and plant physiological effects of these factors are discussed fully in Chapter III. The method development herein was beneficial to this plant study for several reasons. One advantage of this method is that it is more specific than colorimetric determinations used in previous studies, since individual sugars were analyzed. Another advantage is that no derivatization is required for sugar analysis as is the case for GC sugar methods. Also, this method incorporates a simple extraction and sample preparation, thereby decreasing sample throughput time. Future work should focus on utilizing smaller particle size chromatography columns in conjunction with higher pressure UPLC systems.
References


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Anthony R. Gerardi and William M. Coleman.: “Qualitative and Relative Quantitative Determination of Carbon-Centered Free Radicals in Whole Smoke from Various Cigarette Types”. Presentation at 2010 CORESTA Congress in Edinburgh, Scotland and at the 2010 Tobacco Science Research Conference in Hilton Head, SC.