ABSTRACT

Title of Document:
THE ESTABLISHMENT, EVALUATION, AND VALIDATION OF OPERATING AND WORKING PARAMETERS FOR DEVELOPING AN ANALYTICAL METHOD TO QUANTIFY POLYPHENOLIC COMPOUNDS USING AN ELECTROCHEMICAL APPARATUS.

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Polyphenols have health-beneficial antitumorigenic and antioxidative effects. This study evaluated the spectroelectrochemical responses to polyphenols from an electrochemical apparatus for the development of an analytical polyphenolic method. Operating parameters of 0.5 V for 1000 seconds were chosen after evaluations of cyclic voltammograms and durational response. Linearity was measured (R^2 = 0.96 caffeic acid, 0.94 protocatechuic acid, 0.93 gallic acid over concentration range 0.5 - 5.0 mM), as were effects of ion strength (no baseline effect by buffer less than 1.0 M), and solvent composition (influence detected in non-aqueous solutions). The effect of ascorbic or monohydroxyphenolic acid on polyphenolic determinations by electrochemical apparatus was compared determinations by Folin Ciocalteu (FC) method. Ascorbic acid exerted significant interference on electrochemical and FC measurements.

Monohydroxyphenolic acids did not interfere with electrochemical polyphenol measurements but significantly interfered with FC quantifications. This electrochemical apparatus may offer potential for the development of a quantitative assay for polyphenols.

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DEVELOPING AN ANALYTICAL METHOD TO QUANTIFY

POLYPHENOLIC COMPOUNDS USING AN

ELECTROCHEMICAL APPARATUS.

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DEDICATION

This work is dedicated to Tom Dechand, who continually stressed the importance of the mental focus over physical endurance – there may be something to that. I am indebted to Tom for his steadfast support, encouragement, and love. This work is the least I could offer in return.

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CHAPTER 1: LITERATURE REVIEW

Introduction

Phenolic compounds have attracted considerable attention for their health-beneficial properties, having been linked to lower incidences of cardiovascular disease, cancer, obesity, and diabetes when part of regular dietary consumption (Hertog et al., 1993, Stitch, 1991, Beecher, 2003, Yang et al., 2001, Ross and Kasum, 2002, van Praag et al., 2009, Hernanz et al., 2007, Yang et al., 2008, Sajilata et al., 2008, Brown et al., 2009, Crozier, et al., 2009.) Phenolics occur in many common foods including grains, legumes, tea, vegetables, fruits, wine, cocoa, and spices.

Recent interest has focused on certain types of phenolic compounds, the polyphenols, as a subgroup of phenolic chemicals that may have a higher chemical activity and may offer a greater health benefit than other non-polyphenolic phenols (Scheepens, Tan, and Paxton, 2010, Stevenson and Hurst, 2007, Manach, 2004). These polyphenolic compounds (polyphenols) are defined to be chemicals with two or more hydroxyl groups attached to one or more benzene rings as opposed to their single-hydroxyl counterparts. In order to make comparisons between polyphenols and (what will be referred to as) monophenols and their effects on health, a method of quantification is needed.

The conventional method for the quantification of phenolics is an oxidative-reductive analysis called the Folin Ciocalteu (FC) method (Singleton and Rossi, 1965) and it does not differentiate between monophenols and polyphenols. The active reagent in the FC method is a corrosive, acidic solution that contains molybdenum and tungsten. There have also been recent shortages in the world supply of the reagent. An assay that could quantify the polyphenolic content of a given sample might be of use considering the new interest in polyphenols.

The objective of this research was to study the signals of polyphenols and monophenols in a recently-developed electrochemical apparatus that was shown to be selective for the oxidation of phenolics. This electrochemical apparatus involved the anodic oxidation of a solution over a chitosan-coated electrode. Two signals from the apparatus, an electrochemical and an optical, were measured to investigate parameters of operation, influence, and interference, to assess the suitability of development of an analytical method using this device.

1.1 Effects of Dietary Phenolics on Health

1.1.1 Benefits of polyphenolics

Over the last 30 years, oxidative stress has been identified and incorporated into theories of aging and disease development (Rizvi, 2010, Ames, Shigenaga, and Hagen, 1993, Sai, Takagi, Umemura, Hasegawa, and Kurokawa, 1992, Stadtman, 1992, Harman, 1981). Another aspect of oxidative stress research occurred in the early 1990s, when the "French Paradox" (Renaud, 1992) and the "Zutphen Elderly Study" (Hertog, et al., 1993) became the landmarks for the antioxidant research community. Since that time, many articles have reported on the health benefits of the consumption of polyphenols and reviews are regularly published (Crozier, et al., 2009, Moon, et al., 2009, Ramprasath & Jones, 2009, Yang, et al., 2008, Stevenson & Hurst, 2007).

The consumption of polyphenols is generally regarded as a healthy habit (Schini-Kerth, et al., 2010, Halliwell, 2007, Kondratyuk, et al., 2004). The intake of fruits and vegetables is associated with a lower risk of heart disease (D'Archivio, 2010, Vinson, 2001). In a review of 156 studies on cancer, an inverse relation between consumption of polyphenolics and incidence of cancer was present in 128 of the studies (Vinson, 2001). But cancer and heart disease aren't the only chronic diseases diminished in populations who consume a significant amount of

polyphenolics. Lowered incidence of obesity, diabetes, and other chronic diseases are observed in conjunction with polyphenolic compounds (American Dietetic Association, 2008, King, 2005).

1.1.2 Presence of polyphenolics

Polyphenolics are commonly found in vegetables, fruits, herbs and spices, tea, chocolate, and wine (Su, et al., 2007, Ross & Kasum, 2002). Polyphenolic compounds may be evenly distributed throughout all of the plant tissues or heavily concentrated in a certain tissue (Velioglu, et al., 1998). Phenolic compounds often originate as secondary metabolites in plants. Some of their original functions involve hormone signaling, pigmentation, insect attraction, and plant defense (Pereira, 2009, Vermerris & Nicholson, 2006). In plants, phenolics are abundant and are also rapidly turned over, indicating a metabolism of the compound as opposed to having the status of a by product (Vickery and Vickery, 1981).

Some polyphenolics are synthesized via the Shikimate and phenylpropanoid pathways (Vermerris and Nicholson, 2006), which are essential metabolic pathways in plants, but alternative pathways of production also exist. For example, the production of gallic acid is thought to begin in the same manner as the Shikimic acid pathway but to diverge at an intermediate stage (Vermerris and Nicholson, 2006), and many flavonoids are further-developed than the "final" materials from the phenylpropanoid pathway such as *p*-coumaroyl-Coenzyme A (Pereira, 2009, Vermerris and Nicholson, 2006). The existence of many pathways for polyphenol production may indicate an integral place polyphenolics hold in plant biology.

Presence in vegetables

Extracts of vegetable phenolics showed stronger protective activity vs. antioxidant vitamins C and E when tested by *in vitro* methods (Vinson, 1998). Kale, cabbage, and Brussels sprouts, often have levels upwards of 250 µg/g of hydroxybenzoic and hydroxycinnamic acids while potatoes may have amounts (of mono- and di-esters between caffeic and quinic acid, often

collectively referred to as chlorogenic acids) on the order of 1200 µg/g of dry matter (Clifford, 1999).

Presence in grains

Phenolic compounds are often produced as secondary metabolites (Appel, et al., 2001, Vermerris, 2006) and are nearly ubiquitous amongst known flora such that they are one of the most-studied chemicals within plants (Appel et al., 2001, Schultz, 1988). Whole wheat is an excellent source of phenolic acids (Clifford, 1999) as are most cereals (Fardet, 2010).

Presence in fruits

Fruits vary in the concentration of polyphenolics contained in a given portion, in the types (flavonols, polyphenolic acids, etc.) and forms (glycosylated vs. aglycone) of the polyphenols, and in the distribution of the polyphenolics contained within them (e.g. peel vs. flesh). The frequency of consumption and portion size of a given fruit affects the its contribution to an overall dietary polyphenolic intake.

For example, cranberries have some of the highest measured antioxidative activity of commonly-consumed fruits at 6.8 mg/g Catechin Equivalents (Vinson et al., 2001). However, the smaller portion size (55 g, 373 mg CE/portion) of cranberries coupled with a lower frequency of consumption in comparison to apples and bananas makes cranberries a modest contributor of polyphenolics in the diet. Apples (1.9 mg/g Catechin Equivalents) have a lower concentration of polyphenolics in the flesh than in the peel of the fruit (Vieira, et al., 2009) and a lower concentration of polyphenols in comparison to cranberries, but the frequency of consumption combined with a larger portion size (138 g, 256 mg CE/portion) makes them a major dietary source of flavonols in a typical U.S. diet (Vinson, 2001).

Presence in beverages

Coffee, tea, wine, and cocoa beverages are all esteemed for their high polyphenolic content and the anti-carcinogenic activity they may confer to human health when consumed regularly (Yang, 2009, Cooper, et al., 2008, Seeram, 2008, Stevanato, 2004, Rechner, 2002). Polyphenol-containing beverages may also exert positive effects on the cardiovascular health of individuals when consumed regularly (Ramprasath and Jones, 2010, Khan, 2007, Gil, et al., 2000). Polyphenol-rich beverages (such as green tea) have also been shown to decrease the oxidative stress resulting from exercise (Panza et al., 2008). In short, beverage sources of polyphenols are known to offer a variety of health benefits to the consumer.

1.1.3 Antioxidative effects of polyphenolic compounds

The antioxidative activity of polyphenolic compounds is not necessarily a radical-scavenging mechanism. While there is a general correlation between the number of hydroxyl groups on the benzene ring and the antioxidant activity of a polyphenol (Pereira, 2009), there is no exact antioxidative "potential" that can be assessed from a known chemical structure (Li, 2005, Tang, 2003, Bors, 2001, Lien, 1999). This is, of course, due to the environs and metabolism of a polyphenolic compound (Cheng, 2007), among other factors.

There is ongoing discussion of whether polyphenolics act to quench free radicals or prevent their generation altogether. Pereira (2009) mentions the chelation of free-radical producing metal ions by phenolic species, thus stopping the oxidative process before it starts. In some cases the chelation of metals by polyphenolics may increase the activity of the metal (Pereira, 2009) as noted that they may, "[reduce] metals, thus increasing their ability to form free radicals."

Another mechanism by which polyphenolics exert an antioxidant effect may be through the denaturation of an oxidative enzyme. The hydroxyl groups of a polyphenol may engage in

hydrogen bonding over polar surfaces of a protein and/or the benzyl rings may associate over the less polar areas. If the polyphenolic concentration is high enough and the interactions are extensive, polyphenols may denature a protein to a certain degree. Denaturation of a lipoxygenase or cyclooxygenase may prevent the activity of the oxidative enzyme and constitute antioxidative activity (Pereira, 2009).

There is also discussion that the antioxidative activity of polyphenols may occur by mechanisms other than an electron transfer. Fardet (2008) mentions that the *in vivo* activity of polyphenols in cereals was not necessarily entirely due to radical scavenging because of the low bioavailability of the already small fractions of the grain and cites a publication by Scalbert and Williamson (2000) to say as much. It seems that Fardet article moreso means to cite the idea evidenced in Pereira (2009) that an antioxidant must act directly to stop or reverse oxidation, rather than preventing oxidation several steps before it occurs. Fardet surmises that the health beneficial antioxidant activity is the activation or repression of certain genes or transcription factors as do Na & Surh (2006) who suggest there is not a single mechanism by which polyphenolics act. The introduction of such a topic of debate in this literature review is not to tackle theoretical issues of 'antioxidant' but to stress the many ways by which polyphenols could possibly affect our health and make a case for the need to quantify polyphenolic compounds. This is especially important when considering effects that may be detrimental to health.

1.1.4 Toxicology of phenolic compounds

Polyphenolic compounds are present in nearly all plant tissues. The same antioxidative and protective activities of polyphenols cited in the previous section may be toxic at certain levels or under certain exposures.

For example, some polyphenolic flavonoids in plants are known to be protective against infection (Havsteen, 2002). However, for many larger animals plants may contribute the majority

of energy in the diet and an abundance of these protective compounds in the diet could potentially be poisonous to larger animals as well.   While consumption of a polyphenol-rich plant diet may have helped to select for animals with a higher tolerance to toxicity from certain polyphenolics over time, animals on a diet of little variety such as feed (as opposed to animals who forage for their food) may be more susceptible to a potential overdose of polyphenolic compounds. Indeed, polyphenolics have been shown to be antinutritious in animal feed (De Vincenzi, et al., 2006). With respect to human diets, some dark colored legumes have been found to reduce the bioavailability of proteins (Khandelwal, Udipi and Ghugre, 2010).

The same associations between a polyphenol and a protein noted in section 1.1.3 can be antinutritive or even toxic when protein-denaturing interactions occur between a nutritive or necessary protein and render it non-digestible (Thomson, 1993). Some studies have indicated that a negative effect on the absorption of vitamin C or other micronutrients could be impeded by the presence of polyphenols (Silberberg, 2006). A widespread use of supplements in the United States, some of which may be potent sources of polyphenols, may make it possible for a person to consume a polyphenol dose with toxicological effects (Timbo, 2006, Halsted, 2003).

There exist limits to the effectiveness of polyphenols as antioxidants and tolerance of polyphenols. Substances that exert a positive effect at a certain dose and a negative effect at higher doses may obey a hermetic effect or j-shaped relation. Calabrese (2010) writes, "[a] biphasic dose-response relationship (i.e., hormesis) displays low-dose stimulation and a high-dose inhibition ... dietary polyphenols also act hormetically, displaying cytoprotective effects at low doses. However, excessive nutritional supplementation (i.e., high doses) can have negative consequences."

Many polyphenolics also feature a slight hormonal activity (Miksicek, 1995, Rudel, et al., 1998), which may increase the likelihood of cancer genesis (Khandelwal, Udipi and Ghugre, 2010, Longstaff, 1992, Alzueta, 1992), while some polyphenolic compounds (apigenin, kaempferol, and naringenin) may exert an anti-estrogenic effect at certain dosage levels (Kundu

et al., 2003, Mikcesek, 1995). The boundaries between the non-effective, antioxidative, stimulatory, and toxic levels of exposure to polyphenols need to be established and an easily-determined quantification of dosage is needed to carry out such toxicity studies.

Some research into the limits of toleration of polyphenol consumption has been conducted at the organism level. A study on the subchronic toxic level of oligonol in rats (Fujii, et al., 2008) found no adverse effects at levels up to 1000 mg/kg body weight per day. The toxicology of green tea extracts was tested in rats and found to not be lethal at concentrations up to 2000 mg/kg body weight/day (Chengelis, et al., 2008). A third study of the subchronic effects of gallic acid in rats found that male and female rats could tolerate gallic acid up to 119 and 128 mg/kg/day, respectively, with no adverse reaction (Niho, et al., 2001).

This information coupled with increasing sales of polyphenolic compound supplements merit the monitoring of polyphenolic consumption in general nutritional studies. However, in order to quantify dietary consumption, supplemental dosages, and nutritional or toxic effects, a rapid and reproducible assay is needed.

1.2 Chemical Structure of Phenolic Compounds

A polyphenolic compound is one with at least one benzylic group and at least two hydroxyl groups (Crozier, et al., 2009, Pereira, 2009, Stevenson and Hurst, 2007). Multiple benzene rings are not a requirement for polyphenols; catechol (1,2-dihydroxyphenol) is a polyphenol. In this document, both "polyphenol" and "polyphenolic chemical/compound" will be used to refer to chemicals satistfying the criteria of at least one benzyl and two hydroxyl groups while the term "monophenol" (monophenolic compounds) will be used to refer to phenolic compounds that do not satisfy the criteria for polyphenol.

Polyphenols occur chiefly as secondary metabolites in plants. The synthesis of polyphenols overlaps the Shikimate and phenylpropanoid pathways (Vermerris and Nicholson,

2006). Phenylalanine is often cited as the building block for the biosynthesis of polyphenolic compounds. Plants deaminate the amino acids tyrosine and/or phenylalanine to make phenolic building blocks and then unite two or more phenolic units to make a variety of polyphenols (Pereira, 2009). There are several different end-product classes of polyphenolic compounds, when categorizing polyphenols by the various pathways of biosynthesis: flavonoids, chalcones, stilbenes, and anthocyanins (Stevenson and Hurst, 2007). Because of the simplicity of phenolic acids and their incidence in the earlier stages of polyphenolic synthesis pathways, a brief review of select polyphenols will begin with phenolic acids.

There are many ways to categorize phenolic compounds. One way is by chemical activity – in general, polyphenols exhibit greater activity than monophenols. Along with grouping by activity or biosynthetic pathways, one may organize phenolic compounds according to the number of carbons in a skeletal subunit of the compound's chemical structure. This classification was proposed by Harborne and Simmonds (1964), has been observed by many (Crozier, et al., 2009, Bravo, 1998, Vermerris & Nicholson, 2006), and will be referred to when describing the structure of polyphenolic compounds. For example, phenol would fit into the C_6 category, while benzoic acid would be C_6-C_1. Within a group of chemicals sharing a common C_x-C_x-C_x backbone, finer distinctions can be made. One method of organization is that of ring condensation. This can be used to distinguish two compounds that might otherwise be unrecognizably the same. For example, a chain of carbons denoted by C_3 using the Harbone and Simmonds notation might exist as a bridge between two phenols in one chemical while the C_3 subunit in a different chemical may be present as a portion of a ring, but couldn't be a ring on its own (e.g. if the C_3 formed a ring in conjunction with an adjacent C_6 phenol ring such as occurs in the formation of a chroman ring). A presentation of some phenolic compounds follows.

Phenolic acids

A phenolic acid is a carboxylic acid of phenol. The basic chemical structure of a phenolic acid is a phenol with a carboxyl group linked to the benzene ring. The carboxyl group may be immediately attached to the benzylic ring, or there may be a chain of carbons between the phenol ring and the carboxy. Using the Harborne and Simmonds designations, the most abundant types of phenolic acids found in plant tissue, as cited in the literature, are the C_6-C_1 and C_6-C_3 structures of the hydroxybenzoates (C_6-C_1) and the hydroxycinnamates (C_6-C_3). The carboxyl group of the hydroxybenzoates is attached directly to the phenolic ring while the hydroxycinnamic acids have a double bond between C2 and C3 of the 3-carbon arm (Pereira, 2009). The double bond on the acid end of the hydroxycinnamic acids extends the conjugation system beyond the benzylic ring. In comparison to the hydroxybenzoates, the hydroxycinnamates are more stable compounds. Pictured below are examples of a hydroxycinnamate and a hydroxybenzoate.
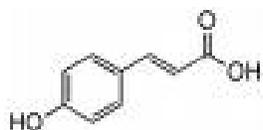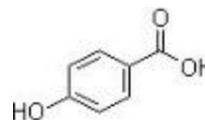
p-Hydroxycoumaric acid   **p-Hydroxybenzoic acid**

Figure 1.1: para-Hydroxy members of the cinnamic and benzoic acid families.

Hydroxybenzoates and hydroxycinnamates are two very general forms of phenolic acids. A variety of functional groups may be attached to the phenol ring and may alter the activity of the compound. There is no exact relation between the chemical structure and the activity of the compound, but there are some general trends between the relation between structure and activity (Heim, et al., 2002). For example, the oxidative potential of a dihydroxyphenolic acid is diminished when the hydroxyl groups are vicinal to each other. Thus 3,4-dihydroxybenzoic acid is generally more reactive than is 2,5-dihydroxybenzoic acid. There are many other ways in which a structural change to a phenolic acid could change the activity of the compound. Other

differences of structure may alter the functionality of the chemical, but may be considered as a different form of a single chemical rather than as a different phenolic acid with different activities. This is often the case for phenolic acids glycosylated or esterified to a sugar moiety (Crozier, et al., 2009, Bravo, 1998). Overall, functional groups and attachments of a phenolic acid to another compound may alter the solubility, ease of metabolism, or reactivity of the phenolic acid.

Phenolic acids do not always exist in a pure form in plant tissue. Chlorogenic acid might be an example of an altered compound that is considered a variation of an original phenolic acid. Chlorogenic acid is is the most abundant phenolic acid in coffee (Crozier, et al., 2009). Structurally chlorogenic acid is 3,4-dihydroxycinnamic acid esterified to quinic acid. Chlorogenic acid may sometimes be part of discussions of caffeic acid (3,4-dihydroxycinnamic acid) just as much as it may be discussed on its own, in part because there are two near-distinct parts to the molecule. The oxidative activity of the unit chlorogenic acid is similar to the activity of the aglycone caffeic acid (3,4-dihydroxycinnamate), but the attachment to quinic acid alters the solubility of the phenolic acid.

Chlorogenic acid   **Caffeic acid**
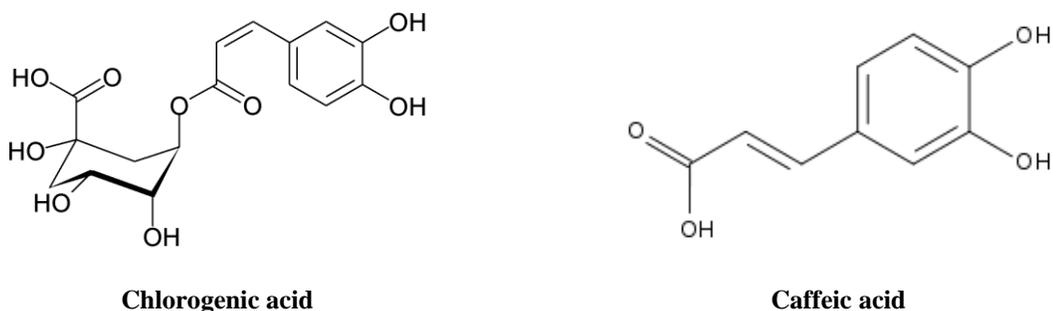
Figure 1.2: Example of glycosylated (chlorogenic) and aglycone (caffeic) forms of a cinnamic acid.

Lignans and Lignins

Lignans are polyphenolic compounds with a C6-C4-C6 backbone and are formed from the dimerization of two phenylpropanoids.
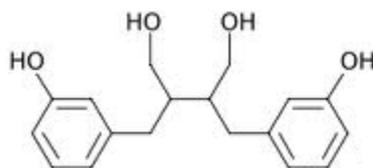
Figure 1.3: Enterodiol, a lignan from two p-hydroxycinnamoyl alcohols joined at the the β-β' carbons.

Lignans are often formed from two hydroxycinnamoyl alcohols (Pereira, 2009).There are two main classes of lignans, the classical lignans and the neolignans (Pan et al., 2009). Classical lignans (Pan, 2009) result from the dimerization of two phenylpropanoids at the 8 and 8' carbons of each monomer, while neolignans are formed from the union of two phenylpropanoids but are not necessarily joined at a C8-C8' juncture.

Longer chains of phenolic alcohols are known as lignins (Pereira, 2009, Bravo, 1998) and are the structural framework to which cellulose is attached in the cell walls of plants (Thevenot, et al., 2010). Lignins are formed from the polymerization of p-hydroxycinnamyl alcohols (p-coumaryl, coniferyl and sinapyl) as are lignans, but involve greater numbers of phenylpropanoid monomers.

Stilbenes

Stilbenes are the dimers of carboxylic acids with a C_6-C_2-C_6 skeleton (Pereira, 2009). One of the best-known stilbenes is resveratrol (3,5,4'-trihydroxytransstilbene). The 2-carbon chain between the phenyl rings of stilbenes is a double bond and the phenyl groups may be trans- or cis- to each other. The double bond increases the stability of the chemical.
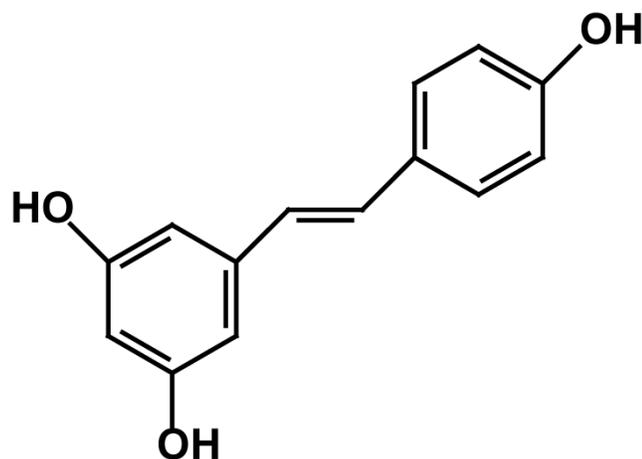
Figure 1.4: Resveratrol

Flavonoids

The Harborne & Simmonds structure of flavonoids is a C_6-C_3-C_6 carbon sequence. In the following illustration, the basic skeleton of a flavonoid is shown. Two phenyl rings and a pyran form the backbone of the flavonoid. The pyran ring and adjacent phenyl ring are together called a chroman arrangement (chromone group). It is the placement of the hydroxyl and other functional groups (not pictured) that is used to subclassify flavonoids.
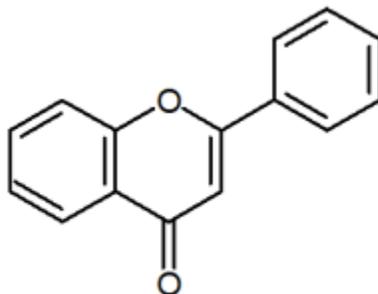
Figure 1.5: Flavonoid Ring Structure
Flavonoids are an example of a condensed phenolic compound due to the formation of the third ring from the carbon bridge (Pereira, 2009). The letters referring to the three rings are are depicted in the next illustration along with the numbering of the carbons in the flavonoid backbone. The original phenol is termed the A ring, the second 6-carbon phenyl ring is referred to as ring B and the benzylpyran ring comprised from the condensation of the 3-carbon bridge

between A and B is termed ring C. (Pereira, 2009, Vermerris & Nicholson, 2006). The pyran ring

is constructed from some of the carbons in one of the phenol rings, along with the alcoholic

oxygen from that ring and 3 carbons from the benzo-phenyl bridge (Beecher, 2003).

Figure 1.6: Labeled carbons of the flavonoid structure. (courtesy bioinformatics.cs.vt.edu,

Jonathan Watkinson)

Most of the subgroups of flavonoids are categorized by the arrangement of hydroxyl

groups attached to the flavonoid backbone. The isoflavonoid subgroup differs from the basic

flavonoid backbone in that the B ring is attached to the C3 of the chromone and a hydroxyl group

is attached to C2.

Overall, polyphenolic compounds come in many different forms, but share chemical

stability due to their common polyhydroxyphenolic structure.

1.3 Detection and Quantification of Phenolic Compounds

There are many procedures for detecting phenolic compounds (Magalhaes, et al., 2008,

Bonanni, 2007, Huang, 2005, Robbins, 2003). Many procedures oxidize the phenolic chemical in

the course of detection and quantification. Some assays oxidize the phenolics through a chemical

electron transfer (FC method) while others do so electrochemically (diode array detectors coupled

with liquid chromatography). Each method has its limitations and these limitations are often mechanism-related. In this section of the literature review, a brief summary of some of the methods for phenolic determination are presented. Following a short survey of polyphenolic determination techniques, a sketch of a new electrochemical technology for the sensing of phenolics and its potential use in quantitative polyphenolic analysis will be presented.

1.3.1 Folin-Ciocalteu method

One of the most widely used methods in the food industry for detecting and quantifying phenolic compounds is the Folin-Ciocalteu (FC) method (Weingerl, 2009).

The FC method was established by Singleton and Rossi and published in the American Journal of Enology in 1965. The method was an improvement upon the AOAC official method for phenolic quantification and worked by coupling the reducing activity of phenolic wine tannins with the color producing reduction of the Folin-Ciocalteu phenol reagent (Singleton and Rossi, 1965). It should be noted that some papers still use the Folin-Denis reagent and continue to encounter its well-known problems of precipitation, etc. that were lessened by Singleton and Rossi's modifications (Appel et al., 2001). The FC method was developed over a variety of conditions (temperatures, pHs and duration) and yielded several advantages over the previous assay with the Folin-Denis reagent with respect to precipitaiton. The reagent is oxidized by a reducing species (a phenolic antioxidant), yields a chemical complex that absorbs light in the 600 to 800 nm range. Although originally designed for use with the quantification of phenolic compounds in wine, the rugged and straightforward assay has become a convention for quantifying phenolics in much more than just wines: the FC method has been adopted in the quantification of phenolics in fruits & vegetables, herbs & spices, honey, tea, and many other foods ever since Singleton and Rossi's published modifications.

Procedure of FC method

There are many variations of the procedure (Cicco, et al., 2009, Medina-Remon, et al., 2009; Luther, et al., 2007) but the general method is to mix the sample and the Folin Ciocalteu reagent (FCR) and let react for a few minutes. During this time the mixture is acidic and the kinetics of the H^+ transfer between a phenol and the FC reagent are slower than under alkaline conditions. The pH of the reaction is adjusted alkaline and the solution is allowed to stand for a preset time. With a pH greater than 8, the kinetics of the reaction are much faster, but the stability of the FC reagent is compromised. During this time of reaction, the majority of the electron transfer reaction occurs and any excess FCR decays. Visually, the oxidized form of FCR is yellow. Upon reduction the FCR turns blue but the degree of reduction is measured by the solution's absorbance in the red region of 650-800nm. If the FCR is not reduced by the sample, the alkaline pH degrades the reagent and the solution becomes colorless. Following the predetermined reaction time the absorbance of the solution is measured in the range 650 and 800 nm and the reducing capacity of the sample is calculated in correlation to the absorbance of a phenolic standard.

Development of the Folin-Ciocalteu reagent

The chemical reaction that occurs in the FC method is an an oxidation-reduction reaction between the Folin Ciocalteu (FC) reagent and a phenolic (or other) reducing agent. Hence, although the FC method is said to quantify phenolics, it can also be said to measure the reducing capacity of a sample (Huang, Ou, and Prior, 2005). The FCR itself was theorized, designed, and improved in articles published by Otto Folin and others throughout the 1910s and 1920s (Folin and Denis, 1915, Singleton and Rossi, 1965). The FC reagent was designed to react with phenolics (Singleton, 1999) and was first used in protein quantification assays and later applied to the sensing of phenolic compounds in general.

The reagent contains phosphotungstic/phosphomolybdic acid that accepts a reducing electron (Singleton, 1999, Folin and Ciocalteu, 1927) to form a product that maximally absorbs light between 700-800 nm. The source of this reducing agent need not be phenolic. The alkaline pH of the reaction facilitates the electron transfer from a phenolic species to the phosphotungsten-molybdenum active compound because at alkaline pHs greater proportion of phenolics exist as phenolate ions, and the availability of a compound to be reduced is more favorable for the electron transfer than it would be between a phenolic compound and water. Ultimately this affects the kinetics of the reaction: at an alkaline pH, the transfer of an electron from the phenolic compound to the FCR proceeds more quickly than it would under acidic conditions (when a smaller percentage of phenolics are thermodynamically predicted to be in an ionic state) (Singleton, 1999).

This Folin-Ciocalteu reagent was an improvement upon the Folin-Denis reagent that was liable to deviate from the Beer-Lambert law or to form a precipitate under the alkaline conditions of its use (Singleton, 1999). Folin and Ciocalteu's modifications increased the solubility of the phosphomolybdate and rendered the reagent more responsive to phenolics (Folin and Ciocalteu, 1927, Singleton and Rossi, 1965). The FCR is not stable in alkaline conditions and is destroyed during the reaction (Folin and Ciocalteu, 1927, Singleton, 1999) hence the double advantage of the FCR over the Folin-Denis reagent: a more stable reagent requires less reagent in the assay and the dissolved reagent reacts with the sample to a greater extent (Singleton and Rossi, 1965).

Prior to Singleton & Rossi's report in 1965, the conventional phenolic detection assay was a similar colorimetric method that used the Folin-Denis reagent (Singleton and Rossi, 1965, Swain and Hillis, 1959). The assay was subject to deviations from the Beer-Lambert law or the formation of a precipitate under the alkaline conditions of the experiment that further reduced the repeatability of the assay (Singleton and Rossi, 1965).

Limitations of the FC method

Many factors affect the reaction between a polyphenolic compound to the FCR, and not many phenolic compounds reduce the FCR to the same degree. Nor is there an exact mapping of a structure-based understanding of what influences are known as of yet there is no concise relationship between the reactivity of a chemical to the structure, degree of polymerization, and redox potential, etc. (Swain and Goldstein, 1964). With this in mind, the standard used in the FC method is best when its chemical activity is similar to the predominant chemical in the sample of interest (Appel et al., 2001, Singleton et al., 1999). Standards are a scientifically accepted aspect of a general assay. So that even if a theory linking structure to activity (Bors, 2001) may not be codified at this time, this is not a serious limitation to an assay unless the method is construed to measure, e.g., polyphenolics alone as has been the case (Acosta, Perez, and Vaillant, 2009).

Other chemicals (antioxidants) that exhibit an electron transfer mechanism may interfere with the FC method in a way that creates a false positive.

1.3.2 Biosensors with amperometric detection and quantification

There are many bio-electrical methods for the detection of phenolic compounds (Abhijith, Kumar, Kumar, Thakur, 2007, Dall'Orto et al., 2005, McMahon, Doyle, O'Connor, 2005, Stevanato, Fabris, Momo, 2004, Brett and Ghica, 2003, Abd-El-Haleem, et al., 2002, Capannesi et al., 2000, Mosca et al., 2000, Cliffe, 1994). These methods for the determination of phenolic compounds couple an enzyme (which converts a phenolic compound into a more-easily oxidize polyphenolic or quinone form) to an electric oxidation source. The enzyme is considered the biological part of the determination and the anodic oxidation is used for quantification. One reason for measuring the oxidation of phenolic compounds under anodic oxidation is that electrical processes may mimic physiological processes. As Zielinska et al. (2008), have reported, "electroanalytical methods are very useful for determination of bioactive compounds

due to their sensitivity, selectivity and accuracy as well vital information on electrochemical mechanisms which are sometimes comparable to those occurring in metabolic processes in living organisms." In addition to the similarities to biological processes and accuracy of the detection, the use of electrical quantification can be very precise and measure the oxidation down to the electron. A variety of biosensing methods exists. Many of the differences between methods are those of method of oxidation (voltammetry, coulometry), choice of enzyme and extent of use (to convert a phenolic compound into a polyphenol or to convert a polyphenol into a quinone), material of electrode, method of introducing sample to enzyme and electrode, and of course the individual oxidation parameters (Abhijith, Kumar, Kumar, Thakur, 2007).

The enzyme polyphenol oxidase is often used in conjunction with a potentiostat in a fluid device. Liquid sample flows past the enzyme which is stabilized at a location in/on the device. The sample flows past at a constant flow rate. Phenolic compounds in the sample are converted to easily-oxidized quinones which flow downstream to an electrode where they are oxidized and the charge is measured as a quantification of the phenolic compounds (Serra, et al., 2003)

Polyphenol oxidase (tyrosinase) is an enzyme with two-catalytic functions. The first action of the enzyme converts a monophenolic compound to an ortho-diphenolic compound and the second operation converts the o-diphenolic to a quinone (Cosnier and Innocent, 1993). Once a quinone, the chemical is easily oxidized using a mild voltage (~0.2 V) in comparison to the catechol or mohopenol in the sample prior to the enzymatic activity (which may require a voltage >0.5 V for oxidation). The electrical signal from the oxidation can be counted by an amperometer or electrochemical analyzer.

Tyrosinase is an enzyme found in a variety of plants. Because the polyphenol oxidase is selective for phenolics, the majority of the electrical signal can be attributed to what was originally a phenolic compound. Different sources exhibit varied activity and introduce potential for the standard of measurement to vary in these biosensing methods (Grönhut, Palomeque, Lista, and Band, 2007). The source of the polyphenol oxidase also plays a role in the reproducibility of

the assay; different plant sources of tyrosinase may react to different phenolic compounds with varying to specificity (McMahon, Doyle, O'Connor, 2005). However, the greatest impediment to the measurement of polyphenols is selectivity of the enzyme –polyphenol oxidases act on monophenols as well as polyphenols.

Polyphenol oxidase can be incorporated into many different experimental setups such as: carbon paste electrodes (Cosnier and Innocent, 1993) combination glassy carbon and functionalized clay electrodes (Mbouguen, Ngameni, and Walcarius, 2007) graphite electrodes with epoxy to give hardness and yield polishing (Önnerfjord, Emnéus, Marko-Varga, Gorton, Ortega, and Dominguez, 1995) carbon foams (Pena, Reviejo, and Pingarrón, 2001) sol-gel films (ElKaoutit, Naranjo-Rodriguez, Hernandez-Artiga, Bellido-Milla, and Hidalgo-Hidalgo-de-Cisneros, 2008, Wang, Zhang, and Dong, 2000) with varying degrees of success. Tyrosinase-doped biosensors are subject to fouling (Önnerfjord, Emnéus, Marko-Varga, Gorton, Ortega, and Dominguez, 1995) and may have a limited shelf life (Tembe, Inamdar, Haram, Karve, and D'Souza, 2007).

The variety of polyphenol oxidase sources and experimental arrays can be an impediment to communication and establishment of standards across scientific investigations into phenolic quantification.

1.3.3 High performance liquid chromatography

There are many high performance liquid chromatography (HPLC) protocols for the separation of phenolic compounds (Gruz, et al., 2007). The detection methods to accompany the chromatography are as varied as the separation methods.

Ayaz, et al. (2005) used HPLC in conjunction with a mass spectrophotometric (MS) detection for blueberry phenolics, while La Torre et al. (2008), contrasted an MS detector against an evaporative light-scattering detector to analyze the polyphenols in marsala wines. Some

investigators have used several detectors for HPLC separated phenolic samples. For example, Mertz, et al. (2007) used a diode array detector (DAD) as well as an MS detector and a spectrophotometric analysis and found that the UV-VIS detector was helpful to quickly differentiate amongst major functional groups on the phenolic ring, but more specific structural information was best found through MS.

1.3.4 Mechanisms of polyphenolic sensing assays

Debate over the mechanism of the reaction in the total polyphenolic content assay (single electron transfer, or hydrogen transfer) has been recently published through several review articles (Karadag, et al., 2009, Apak et al., 2007, Huang et al., 2005, MacDonald-Wicks et al., 2006, Prior et al. 2005, Robbins, 2003, Singleton, et al., 1999). While the exact mechanism is unknown, there is also some disagreement as to the type of mechanism that occurs (Keradag, 2009). Some researchers draw parallels between the chemical mechanisms that may occur in assay such as the FC method and the activity of polyphenolic chemicals in the body (Heim, et al., 2002) while other phenolic sensing assays are enzyme-mediated and are may be familiar to metabolic processes for some organisms.

1.3.5 Need for other detection methods

New frontiers in phenolic detection

The amount of polyphenolic chemical in a given portion of a single food is not easily measured. Such analysis requires access to a lab facility. There are also many studies that have investigated polyphenolic profiles of selected foods, but these studies often focus on a single polyphenolic compound rather than a total polyphenol quantity. Polyphenolic content is known to fluctuate based on the stress of a plant and the freshness of a product. Compositional analysis may require many standards for individual polyphenols, and good sample preparation can be

crucial to certain assays. Instead of searching out individual polyphenolics (Clifford, 1999), there may be value in measuring the total polyphenolic content of a food.

Electrochemical detection of phenolics

Electrochemical oxidation for the identification of phenolic compounds is not new. Available electrochemical equipment is precise enough to count single electrons. However, a quantification of electrons does not provide any information about the identitfy of the chemical itself, it only measures total oxidation. Further, a count of the electrons is not necessarily a stoichiometric count of the number of molecules oxidized (Hotta, et al., 2001). Separation and/or selective conditions may be required in order to account a measured charge from the oxidation of a sample to a phenolic component. This has been seen in the previous sections of this literature review in examples of tandem use of an enzyme with an electrochemical quantification following bioconversion of a phenolic compound (section 1.3.2) or when an electrical oxidation follows a physical separation as in conjunction with high performance liquid chromatography (section 1.3.3) (Brenes, et al., 2000, Woodring, et al., 1990, Mahler, et al., 1988).

It was recently shown that the anodic oxidation of phenolic compounds over a chitosan-coated electrode renders a change to the absorbance spectrum of the chitosan film (Liu et al., 2008). The apparatus used an electrochemical analyzer to record and quantify the charge transferred during oxidation and a spectrophotometer to observe changes to the absorption spectrum in the chitosan film laid over the working electrode (following the oxidation).

Hence, two signals were presented for the investigation of the oxidation of phenolic compounds using the bimodal sensing apparatus as published by Liu et al. (2008): an optical and an electrical signal. The optical signal was contained within the permeable chitosan film spread across the surface of the working electrode and was seen to be selective for phenolic compounds. A positive correlation was demonstrated between the intensity of optical changes and the electrical charge transferred from an oxidation. The change in absorbance in the chitosan film

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could indicate the formation of a complex between the (oxidized) phenolic species and the chitosan film laid over the electrode. Using this approach, any oxidation from the sample contributed to the overall (non-selective) electrochemical signal and the optical signal was noted only in relation to the oxidation of phenolic compounds. However, relations between the two outputs were not investigated in the article.

The experimental apparatus: A glass slide coated with transparent indium tin oxide (ITO) is coated with a film of transparent chitosan (estimated thickness 1 um, Liu et al., 2008). A rubber cylinder clamped on top of the chitosan-coated slide formed in which a liquid sample can be oxidized anodically. An electrochemical analyzer measured the charge transferred across the electrode by the oxidized chemical in solution, and the absorbance of the chitosan film that had been in contact with the oxidized solution could be measured by inserting it into the measuring beam of a spectrophotometer since the ITO was transparent. Chitosan is soluble in an acidic medium, but insoluble in pH greater than 5.

Liu, et al. (2008) was the first to report explorations into the color change in chitosan following exposure to post-electrode oxidized phenolics as a potential source of information, but it had been noted in other electrochemical experiments; just not as attributed to a reaction with the chitosan film (Pasanphan, et al., 2008, Abdullah, 2006). Other researchers used measurements of charge from an oxidized substance in combination with lengthier HPLC separations. Some of the informative experimental conditions that help to identify an unknown chemical were the potential used to oxidize the sample.

The novelty of the electrochemical apparatus is the elicitation of two signals. The information of the electrical signal could potentially be compared or contrasted with absorbance changes in the chitosan and such contrasts may provide more information about the sample than the use of either signal alone. Further investigation into the quantification of the spectroelecric signals using an electrochemical analyzer and spectrophotometer could prove useful for the

development of an analytical method for the measurement of phenolic species using this electrochemical apparatus.

Electrochemical analysis of polyphenolics: the electrochemical analyzer

In the experimental setup, an electrochemical analyzer (potentiostat) is used to control the oxidative conditions and measure the oxidation at the same time. In the bimodal sensing technique the potential is set and held constant and the total charge over the duration of the reaction becomes the electrical measurement. The potential can be set to any given voltage, and the oxidation that occurs under a given potential may influence the chemicals that do oxidize due to their oxidation-reduction potential. A chemical may have a threshold voltage that it can withstand before it will appreciably oxidize, and it may also exhibit a maximum oxidation rate (current) at a certain potential.

1.3.6 Objectives of this study

More information can be gleaned from an oxidation if the potential used in oxidation is known. The voltage may indicate whether a chemical readily oxidizes (as evidenced by a strong signal at a low potential), or not. The electric signal in the bimodal sensing technique is essentially the charge transferred from a solution over the course of an oxidation under an applied potential. The signal is expressed in units of C/m^2 is generally interpreted as a reflection of the total oxidation that occurs within the testing solution. With respect to the potential (V) pushing into the solution, the magnitude of the charge transfer may be interpreted to convey information about the the ease with which the sample is oxidizing as well as the concentration of phenolics in the sample.

As mentioned, the measurement of the electrons passing into the working electrode is not necessarily indicative of an H^+ being lost from a hydroxyl moiety, although a positive relation does exist (Hotta, et al., 2001). Hotta and others surmised that other chemical reactions such as

the loss of H^+ from second hydroxyl groups or even the polymerization of the chemical may be occurring when larger than stoichiometrically predicted numbers arise, and their group looked for insight into the exact chemistry of the situation. Other researchers (Zielinska et al., 2008, Brenes et al. 2000) have found an electron count provided by an electrochemical analyzer useful even without an exacting knowledge of the polymerization or multi-step oxidation of a chemical in solution.

The two signals of the bimodal sensing apparatus yield two tracks of measure that might provide more information about a sample than a single-signal apparatus might. Additionally, the simplicity of the electrochemical apparatus and its use, due to a straightforward oxidation and minimal reagents (none other than the same) may make this apparatus particularly suited for the development of an analytical method.

The purpose of our work was to investigate the possibility of developing an electrochemical method to quantitatively measure samples containing phenolic substances by better establishing quantification of the signals, working parameters for the apparatus, and an understanding of parameters of influence on the electrochemical technique.

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CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

Chitosan

A 1% solution (w/v) of chitosan was prepared by dissolving chitosan powder in distilled, deionized water with enough hydrochloric acid to keep the solution pH < 6 and the chitosan dissolved. Chitosan produced from hydrolyzed chitin is the only natural polysaccharide that may hold a positive charge; most polysaccharides are negatively charged. The slide formed the electrode portion of the bimodal sensing apparatus and was prepared by coating a transparent indium-tin oxide.

Other chemical materials

Caffeic acid, ferulic acid, vanillic acid, chlorogenic acid, protocatechuic acid, ascorbic acid, p-hydroxycoumaric acid, p-hydroxybenzoic acid, salicin, saligenin, sodium carbonate monohydrate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were purchased from Sigma-Aldrich (St. Louis, MO). Chitosan (85% deacetylation), transparent indium tin oxide (ITO)-coated glass slides (surface resistivity 8-12 Ω/sq). Platinum and silver wires (Surepure Chemetals Inc., NJ). Ultrapure water (Cayman Chemical Company, Ann Arbor, MI). All other materials were of analytical lab grade unless otherwise specified and were used without further purification.

2.2 Instrumentation

CHI627C electrochemical analyzer (CH Instruments, Inc., Austin, TX) spectrophotometer (Sptectronic Genesys), Genesys 20 spectrophotometer (Thermo, Spectronic, MA), rotary evaporator (Buchi, Switzerland).

2.3 Methods

2.3.1 Sample preparation.

Chitosan solutions

85 % deacetylated, 200,000 kDa chitosan from crab shells (Sgima-Aldrich, St. Louis, MO) was dissolved in acidic solution (pH 5.5-6) as described by Wu, Yi, Li, et al., (2003).

Transparent Electrode Glass Slides

To prevent any confusion between slides and to also prevent any mis-orientation of the electrodes, 1-inch, transparent, indium-tin oxide-coated glass slides (surface resistivity $8 - 12\ \Omega$, Fisher Scientific) were etched with a small distinguishing mark in one corner of the glass and were cleaned between uses by ultrasonicating in 0.1 M HCl for 3 min and rinsing with copious amounts of water.

Casting of Chitosan film

A 100 µL aliquot of 1% (wt/wt) chitosan solution (1% wt/v) was pipetted and spread over the ITO slide or at ambient temperature over night. The chitosan was spread over the slide as evenly as possible, leaving a narrow perimeter of uncoated indium-tin oxide around the perimenter (~1.5 mm) of the electrode. The coated slides were dried at ambient temperature overnight or in an oven set to ~39 °C for ninety minutes. The chitosan-coated ITO was

neutralized by NaOH (0.1 M) for 15 min.to raise the pH above 5.5 and then rinsed with copious amounts of water. This precipitated the polymer onto the slide to prevent the dissolution of chitosan in test solutions. Following neutralization, the slides were rinsed under a stream of distilled water for two (2) minutes to wash off any residual sodium hydroxide. The slides were again dried either at ambient temperature or in a 39 °C-oven at which point they were considered ready for use.

Preparation of phenolic solutions

Pure phenolic of nonphenolic substances were weighed out and dissolved in a small amount of acetone. Solubilized chemicals were diluted to a concentration of 10.0 mM in 0.1 M potassium phosphate buffer at a pH of 7 which had been prepared using standard grade salts (Sigma-Aldrich, St. Louis, MO) and ultrapure water (Cayman Chemical, Ann Arbor, MI). The acetone was removed using vacuum distillation (Buchi, Switzerland) for 8-15 minutes and the volume corrected using ultrapure water. Dilutions of the 10.0 mM solutions as well as the initial solutions were kept under refrigeration when not in use and discarded after 14 days.

2.3.2 Analysis of electrical signal

A 0.525 mL of sample solution was placed in a rubber chamber clamped over a transparent indium tin oxide slide that had been coated with a ~1uM thick film of chiotsan. A three-electrode system including a Ag/AgCl quasi-reference electrode and Pt counter electrode was prepared and set up using platinum/silver wires (99.95% pure) from Surepure Chemetals Inc. (Florham, Park, NJ) as decribed by Liu, Gaskell, Cheng, Yu and Payne (2008). Experimental conditions of voltammetry and coulometry were controlled by a CHI627C electrochemical analyzer (CH Instruments, Inc., Austin, TX).

Coulometry

Aliquots (0.525 mL) of sample solutions were oxidized under a 0.5 V potential and the charge conducted was measured at a sensitivity of 10^{-6}. Samples were oxidized at ambient temperature for 1000 seconds and the charge recorded was converted to and reported in units of Coulombs / meter^2.

2.3.3 Analysis of optical signal

The oxidized sample was pipeted out of the cell and the rubber cylinder unclamped and carefully removed from the chitosan-coated ITO slide. The slide was gently rinsed with distilled water and allowed to dry at ambient temperature. The slide was then taped to the outside of a cuvette holder in a spectrophotometer (Spectronic Genesys) so that the region of interest was in the path of the spectrophotometer light and normal to it. The absorbance spectrum from a second slide over which buffer had been oxidized subtracted from the sample's absorbance signal measurement. The absorbance spectrum between 530 and 560 nm was measured and the values summed to integrate in the fashion of a Riemann sum with unit 2 nm to yield an AUC value. Results were reported as area under the curve (unitless).

2.3.4 Folin Ciocalteu method

The procedure put forth by Singleton and Rossi (1965) was followed with a total reaction volume of 10.00 mL. The final composition of the reaction mixture contained 100 uL of sample, 500 uL of Folin-Ciocalteu reagent, 1.5 mL of 20% sodium carbonate, and 7.9 mL of ultra-pure water. After 2 hours' reaction at ambient temperature, the absorbance was measured at 765 nm. Phenolic content was calculated against a standard curve of gallic acid (100 to 500 μg / mL) and reported in the gallic acid equivalents (GAE) per milliliter of solution.

2.4 Statistical Analysis

Data were analyzed using SPSS (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL). Data were reported as mean measured values with the standard deviation denoted using positive t-bars above graphical values (n = 3 unless otherwise specified). Differences between means were determined by analysis of variance (ANOVA) with Tukey's HSD post hoc test. Significance was declared at $p > 0.05$.

CHAPTER 3: RESULTS

To develop an analytical method, the selection, evaluation, and validation, of parameters and conditions are needed. In this chapter, the results from experiments to select the working conditions and understand some of the influences on responses of polyphenols in the spectroelectrochemical apparatus are presented.

Two signals of the electrochemical apparatus, an electrical and an optical response, were studied in a series of experiments. Before each signal's response to the oxidation of polyphenolic compounds could be studied, working parameters for the apparatus needed to be selected. Amongst all of the working parameters that could be established, the operating conditions of voltage and duration were expected to exert the greatest influence on the oxidative response of a given sample. Thus, initial experiments were conducted to select a voltage and duration. Following the establishment of oxidative parameters, the response of pure phenolic chemicals was studied to establish the range of concentration that yielded a linear response in either signal. The next objects of study were the influences of ion strength and solvent composition. Thirdly, evaluations of interference by monophenols and ascorbic acid on the polyphenolic response were conducted and compared to the effect on the measurement of polyphenols by the conventional Folin Ciocalteu method. The effects of a given parameter on the electrical and optical signals were considered individually and the results from each condition guided further experiments that ultimately led to a proposal of conditions for the development of a determination of polyphenolic content using an electrochemical apparatus with spectroelectrochemical responses.

Operating parameters of voltage and duration were chosen using cyclic voltammograms of pure phenolic/nonphenolic compounds and by choosing a point at which the accumulation of charge could be differentiated from that of a buffer. The quantification of the optical signal by

the area under the curve from 530 to 560 nm was selected using spectra from several polyphenolic and monophenlic solutions, and an evaluation of the effect of ion strength and solvent composition was assessed by the magnitutde of the baseline and bimodal signals of polyphenolic solutions (respectively). Lastly, a measurement of interference by monophenolic compounds and ascorbic acid on the bimodal signals and a comparison to the effect of these chemicals in the FC method was performed.

3.1 Electrochemical Determination

3.1.1 Selection of operating parameters for the electrochemical assay

In any study, many parameters need to be controlled. In the electrochemical oxidation apparatus of this project, the voltage and duration were the two primary operating parameters. To choose the operating voltage, cyclic voltammograms (-0.4, 1.2 V) of pure phenolic compounds (10 mM, pH 7) were recorded. The cyclic voltammetry provided insight into the magtitude of response at a given voltage, while the duration confirmed the total magnitude to be expected. Upon selection of the operating voltage, experiments of duration were conducted to select a length of time that increased the sensitivity of the signal but did not last unreasonably long; the duration of the assay was chosen in consideration of brevity and signal magnitude.

The working concentration range corresponding to a linear range was identified based on the limit of quantitation of the electric signal and an upper limit was to maximize the working concentration domain while maintaining a linear relation between the signal and concentration. These analyses were used to select the operating and working parameters for a new technique to detect polyphenolic substances using the bimodal sensing apparatus.

3.1.1.1 Selection of voltage

A constant voltage was used during the operation of the eletrochemical oxidation apparatus. The operating voltage strongly influences the rate and degree of oxidation within an electrochemical cell. For the development of an assay to detect and quantify polyphenolic substances, a voltage needed to be selected that would a) induce a polyphenolic sample to oxidize and b) do so at a rate resulting in a signal easily discerned from that of buffer.

To choose a voltage for the operation of the bimodal sensing technique, cyclic voltammograms were performed on selected pure phenolic acids (gallic acid, p-hydroxybenzoic acid, vanillic acid, protocatechuic acid, p-hydroxycoumaric acid, caffeic acid, ferulic acid, saligenin, chlorogenic acid) and nonphenolics (salicin, trolox) and the current was studied in relation to the voltage to select an operating voltage for the assay. Structures of the compounds used are shown below.

Figure 3.0: Phenolic and non-phenolic chemicals.

Polyphenolics
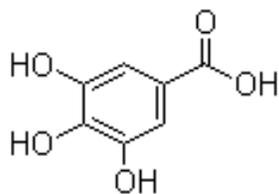
Gallic acid
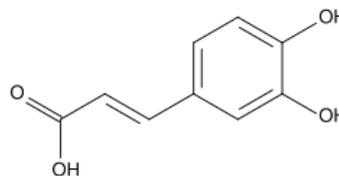
Caffeic acid
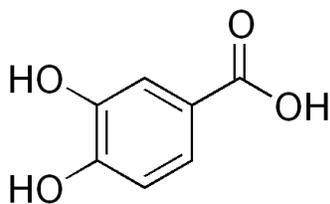
Protocatechuic acid
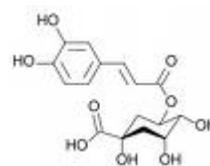
Chlorogenic acid

Monophenolic Compounds

p-Hydroxybenzoic acid

Ferulic acid
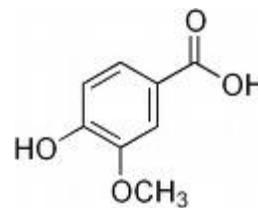
Vanillic acid

p-Hydroxycoumaric acid
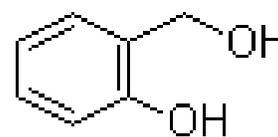
Saligenin
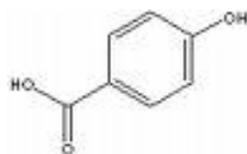
Non-phenolics
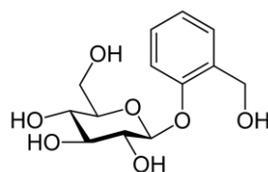
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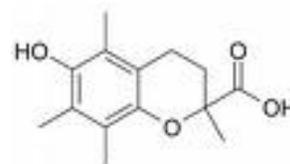
Trolox

Cyclic voltammograms may offer insight into the oxidizing maximum of a chemical. Voltammograms plot the current conducted by a sample in relation to an applied current. The selection of the operating potential was done using cyclic voltammograms on 10 mM pure phenolic solutions. The cyclic voltammogram (CV) measured the current conducted by a sample in response to an increasing potential applied to a sample, followed by a decreasing potential that

was applied in a continuous cycle with under a steady rate of change. A CV allows an experimenter to see differences in oxidizing potential among chemicals.

Of the pure phenolics used for the cyclic voltammetry analysis to choose an operating voltage for the electrochemical apparatus, the caffeic, p-hydroxycoumaric, vanillic, ferulic, and protocatechuic acids were chosen for their near-ubiquity and distribution in plants (Robbins, 2003, Shahidi, 1992). Gallic, p-hydroxybenzoic, and chlorogenic acids were chosen for their widespread availability (although less so than the caffeic, p-hydroxycoumaric, vanillic, etc.) while trolox, salicin, and saligenin were chosen because of their non-phenolic acid-based antioxidant activity.

In our selection of 11 chemicals (4 polyphenolic, 5 monophenolic, and 2 non-phenolic chemicals) there appeared to be two major kinds of responses from the solutions: a strong response and a weak response in current, indicating an abundant or minimal amount of oxidation permitted by the chemical solution. The stronger responses were all on the order of 0.4 mA or greater while the weaker responses conducted somewhat less than 0.4 MA over most of the range of voltage (as tested between -0.4 and 1.2 V). Interestingly, the strong response was associated with polyphenolics and the minimal response was associated with monophenolics. The voltage was chosen based on the discrepancy the currents of the monophenolic and polyphenolic chemicals exhibited and with attention to the amplitude of the polyphenols' electric signal. As examples of these two kinds of responses, the cyclic voltammograms of gallic acid (a polyphenolic chemical) and p-hydroxycoumaric acid (a monophenolic chemical) are shown in Figures 3.1 and 3.2.
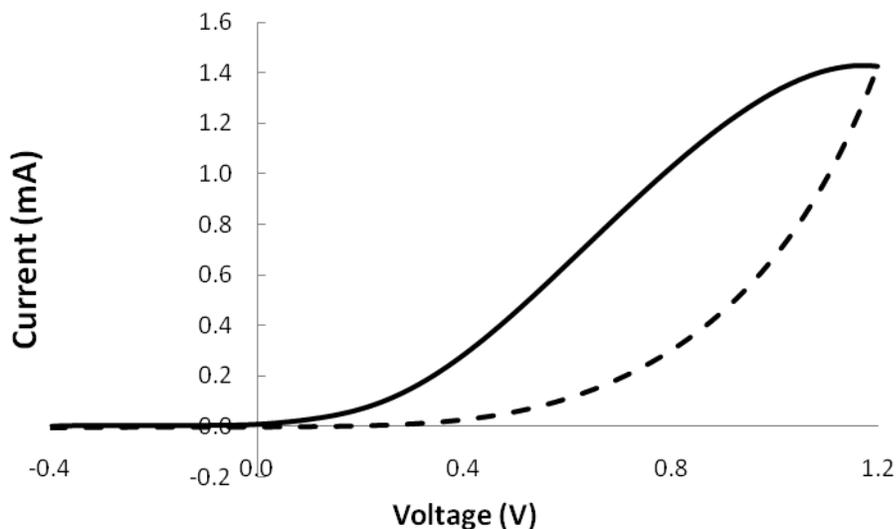
Figure 3.1. Cyclic voltammogram of gallic acid. 10 mM gallic acid in 0.1 M pH 7 potassium phosphate buffer used in cyclic voltammetry ranging between -0.4 and 1.2 V. The response to the increasing voltage is portrayed by a solid black line while the descending current is portrayed using a dashed black line.

Figure 3.1: A cyclic voltammogram of 10 mM gallic acid was conducted between -0.4 V and 1.2 V. The current conducted in response to the increasing voltage is portrayed with a solid black line and the decreasing current is portrayed with a dashed black line. The gallic acid began to conduct electricity greater than one tenth of a milliamp at approximately 0.2 V and the current increased with the applied potential until reaching a maximum current of 1.4 mA when a voltage of 1.2 V was applied. The current response to the decreasing voltage (dashed line) was much weaker than the current in response to the increasing voltage: as the applied potential decreased from 1.2 V to 0.8 V, the current quickly fell from 1.4 mA to 0.3 mA (as opposed to a current of 1.0 mA upon an increasing voltage). The current generally increased or decreased with increasing or decreasing potential.
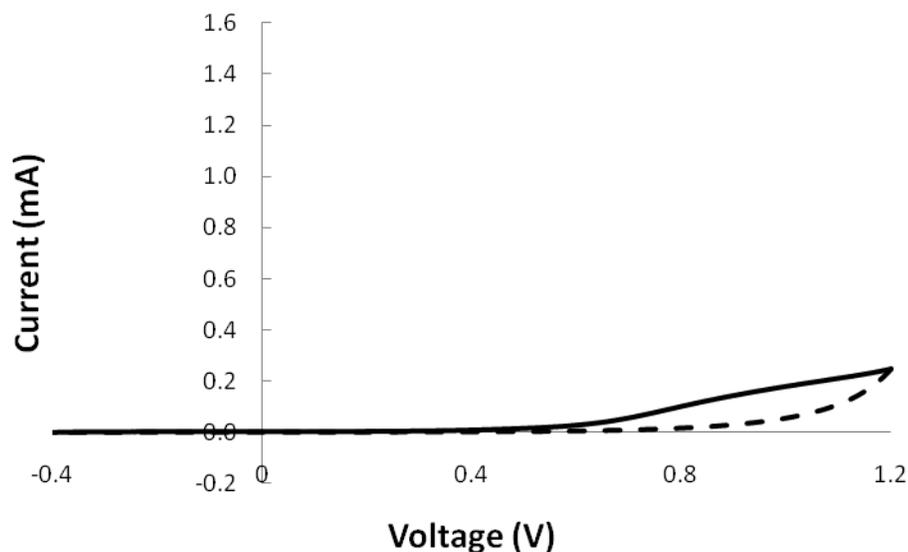
Figure 3.2. Cyclic voltammogram of p-hydroxycoumaric acid. 10 mM p-hydroxycoumaric acid in 0.1 M pH 7 phosphate buffer used in cyclic voltammetry ranging between -0.4 and 1.2 V. The response to the increasing voltage is portrayed by a solid line while the descending current is portrayed by a dashed line.

Figure 3.2: Another example, a cyclic voltammogram (from -0.4 V to 1.2 V to -0.4 V) was conducted on the monophenolic p-hydroxycoumaric acid (Figure 3.2). Over the increasing segment of voltage the p-hydroxycoumaric acid began to conduct electricity (on a scale of tenths of a milliamp) at approximately 0.6 V. The current conducted increased with respect to the voltage appliedand a maximum voltage (within the testing range) of 0.25 mA was measured under an applied potential reached 1.2 V.

In comparison to the current conducted by the polyphenolic gallic acid, the current conducted by the monophenolic p-hydroxycoumaric acid was about one-half that of the polyphenolic. This general relation was observed amongst the test monophenolics and polyphenolics overall.

The CVs were used to select an operating voltage that could possibly help to differentiate between polyphenolic and monophenolic compounds. The first goal was to select the voltage that enabled or improved the oxidation (and sensing) of polyphenolics without a contributory charge from the oxidation of monophenolic compounds. A second goal was to select a voltage that

maximized the charge-based electric response from a given sample. To achieve both goals, the choice was made to consider the region of voltages where the current conducted by monophenolics could be lumped together with background noise, and the polyphenols exhibited some degree of measurable current. Thus, the voltages over which the monophenolic compounds negligibly conducted while polyphenolc conducted a "decent" amount of current were to be considered. This necessitated the judgement of what would (effectively) constitute a "zero" value and what would constitute a non-zero value. The cyclic voltammograms of the selected 10 mM polyphenolic and monophenolic acids that had been subjected to cyclic voltammetry between -0.4 V and 1.2 V were studied for differences in the response between polyphenolics and monophenolics (Figure 3.3). A current of 0.2 mA was chosen as a limit between background noise and measurable signal.
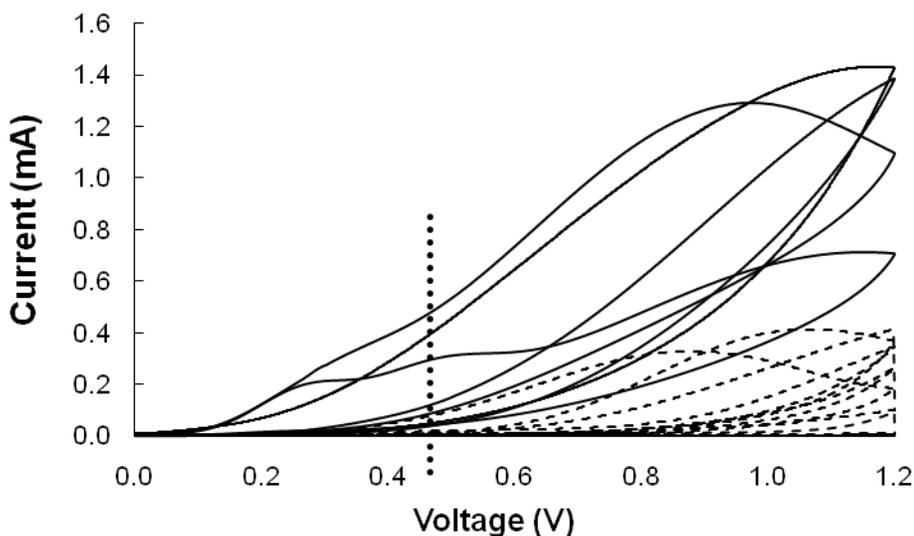
Figure 3.3. Voltammograms of selected phenolics and nonphenolic solutions. Cyclic voltammetries of 10 mM solutions of phenolics and nonphenolics were performed from -0.4 to 1.2 V. Polyphenolics are represented with solid black lines while monohydryoxyphenolics and non-phenolic solutions are represented with dashed gray lines. Polyphenolic solutions tested: caffeic acid; protocatechuic acid; gallic acid; and chlorogenic acid. Monophenolic solutions and non-phenolic reducing agents tested: saligenin; p-hydroxybenzoic acid; p-hydroxycoumaric acid; vanillic acid; ferulic acid; salicylic acid; and the water-soluble vitamin E analog trolox.

Figure 3.3: In general, most of the selected monophenolic and non-phenolic samples conducted little or no measurable current between a voltage of 0.2 V and 0.5 V (<0.2 mA conducte while the polyphenolic compounds had begun to conduct current at a rate greater than 0.2 mA by 0.2 V. It was surmised that the limited charge transferred by monophenols (that oxidized to a small degree) at a voltage between 0.2 V and 0.5 V would not yield a significant amount of noise overall. A 0.5 V potential was the highest voltage at which a monophenolic exhibited a current less than 0.2 mA and that the polyphenolic substances were conducting greater than 0.2 mA.

A different rate of the conduction by oxidizing chemicals indicated a possibility to determine polyphenols in the presence of monophenolic substances at an applied voltage of 0.5 V. Assuming little or no oxidative interaction, this opened up the possibility of determining polyphenols in the presence of monophenolics. It was hoped that a long-enough duration would help to increase the total charge transferred by the polyphenols, create a larger discrepancy between polyphenol and monophenol signals, and enable the distinguishment of signal due to polyphenols over that of monophenols.

Many other chemicals are known to oxidize in the (0.2, 0.5) V range, and could potentially alter the polyphenolic examination. It was desired to identify and minimize these potential interferences with improved operating technique.

The current conducted by polyphenolics was observed to be greater than that of monophenolics under an applied potential of 0.5 V. This voltage did not yield the fastest oxidation of polyphenolic chemicals, but was theorized to generate a measurable signal over time and allow oxidation from polyphenolic compounds to be distinguished from that of monophenolic compounds. Because of this differentiation noted at 0.5 V, 0.5 V was chosen as the operating voltage for further assay development.

3.1.1.2 Selection of the duration

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The duration of oxidation was chosen by examining the time by which the electrical signal had grown large enough to be distinguished from a buffer. At this point in the project, the only other constraint on the operating parameters was the 0.5 V voltage that induced polyphenolic chemicals to conduct current (oxidize) at a faster rate than monophenolics. The duration of oxidation was the second parameter of operation to be chosen. It would influence the electrical signal by limiting the sum total charge from an oxidizing chemical during the test phase. An appropriate duration for the assay would be long enough to accumulate enough charge to resolve a polyphenolic compound from a buffer. The longer the duration, the greater the proportion of sample that would be oxidized and the less chemical required in a sample volume to oxidize enough to create a measurable signal.

In terms of eliciting a signal from this electrochemical apparatus: the longer the duration, the greater the signal and the lower the detectable concentration, effectively increasing the sensitivy of the instrument. Such amplification was expected to affect all concentrations of chemicals. However, the duration was limited by the feasibility of the run-time. A duration of sufficient length to increase the sensitivity of the instrument was desired, but not one that would require longer than ~1 hour to oxidize the sample, disassemble the electrochemical cell, dry the chitosan film, and take an optical measurement (see sections 3.2). The longest programmable running time for the potentiostat was 1000 seconds (~17 minutes). By this time, decent differentiation and sensitivity was observed, so 1000 seconds was chosen as the duration.

3.1.1.3 Estimation of the working concentration range

Having selected the voltage and duration, it was next necessary to know whether a linear relationship existed between the electrical signal intensity and the concentration of the polyphenolics. A linear relation is essential for the development of a quantitative assay. Concentrations of pure phenolics were tested and their electric signals plotted, as shown in Figure 3.4. A linear relationship between the electrochemical signal intensity and the concentration

seemed to exist over a portion of the concentration range selected. It was necessary to choose a

concentration range using a limit of quantitation and a linear regression.
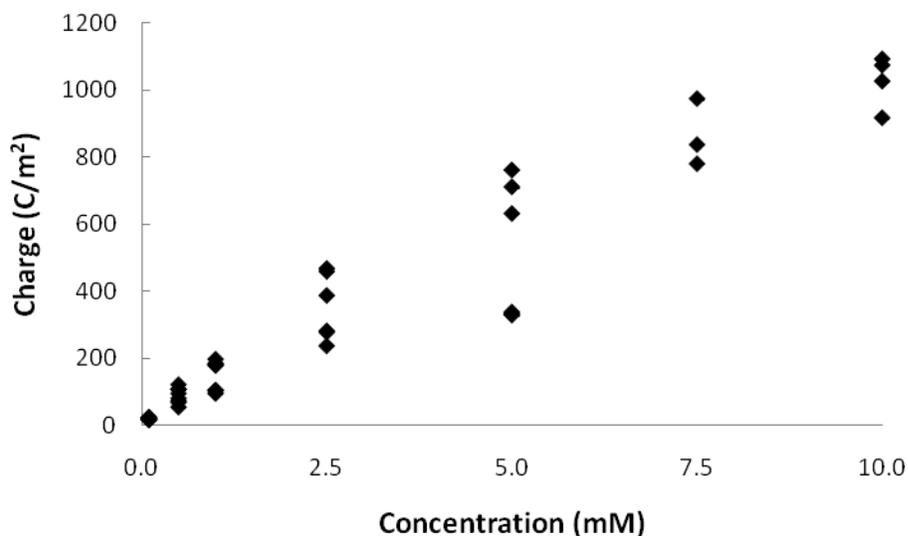
Figure 3.4. Plot of the electric signal (charge) vs. concentration for protocatechuic acid. The
charge transferred during 1000 seconds of oxidation at 0.5 V. Data from protocatechuic acid
solutions ranging from 0.1 mM to 10.0 mM (0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0 mM) are shown.

Figure 3.4: Protocatechuic acid in 0.1 M phosphate buffer (pH 7) was oxidized (0.5 V,

1000 seconds) at concentrations of 0.1, 0.5, 1.0, 2.5, 5.0, 7.5 and 10 mM. The electric signal was

not linear over the entire range tested, and appeared to saturate beyond a concentration of 5.0

mM. For this reason, concentrations greater than 5 mM were considered to be out of range for

these working parameters.

Determination of the quantitation limit

The quantitation limit was derived in reflection of the detection limit. As pulled from

recommendations on the statistically significant limit of quantitation (Armbruster, Tillman,

Hubbs, 1994, Breaux, Jones, Boulas, 2003), the threshold to the quantitation limit was chosen to

be a concentration three times that of the concentration at the detection limit. The detection limit

expressed the lowest concentration at which the signal of a sample was distinguishable from the baseline electric signal of a buffer (Eksperiandova, Belikov, Khimchenko, Blank, 2010) based on a confidence interval of three standard deviations. Several polyphenolic acid solutions were diluted in series to determine the detection limit. The results for protocatechuic acid are expressed in Figure 3.5.
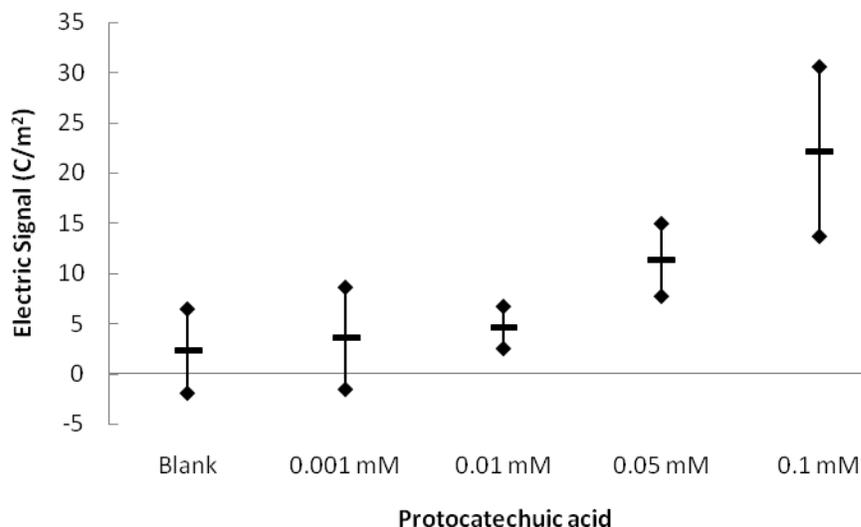
Figure 3.5. Limit of detection of the electric signal in protocatechuic acid. Protocatechuic acid solutions at concentrations of 0.001, 0.01, 0.05, and 0.1 mM were oxidized at 0.5 V for 1000 seconds. Data are expressed as the mean value +/- 3 standard deviations.

Figure 3.5: The detection limit of the electrochemical system was determined by the lowest concentration found to be statistically distinguishable from the buffer at a confidence interval of three standard deviations. A 1000-second oxidation at 0.5 V, a 0.1 mM pH 7 of potassium phosphate buffer solution transferred 2.3 ± 1.4 C/m^2 across the anode (3 trials). As limited by 3σ, the upper limit to noise was $2.3 + 3*1.4$ C/m^2 or 6.5 C/m^2. The data from the oxidations of a series of dilutions (0.001, 0.01, 0.05, and 0.1 mM) showed that lowest concentration significantly different from the buffer was 0.05 mM. The mean electric signal values for the dilutions of protocatechuic acid were: 3.5 C/m^2 (0.001 mM); 4.6 C/m^2 (0.01 mM); 11.3 C/m^2 (0.05 mM); and 22.1 C/m^2 (0.1 mM). Other polyphenolic solutions also showed a significant difference from the

buffer at a concentration of 0.05 mM (22.8 C/m^2 for 0.05 mM gallic acid, and 21.8 C/m^2 for 0.05 mM caffeic acid). As such, the 0.05 mM concentration was established as the detection limit of the electrochemical apparatus.

The limit of detection was then used to determine the limit of quantitation. A qualification of 3 times the concentration of that at the detection limit was used to find the quantitation limit. Hence, with the detection limit of 0.05 mM, the limit of quantitation was calculated to be 0.15 mM. The validation of 0.15 mM polypenolic acid as a limit to the linear range of the electric signal was not performed because the 0.15 mM concentration was not a dilution routinely prepared during other concurrent experiments in this study. Instead, most of the experiments were conducted with polyphenolic solutions of 0.5, 1.0, 2.5, and 5.0 mM. As such, this thesis can only assert linearity to a concentration of 0.5 mM for this electrochemical determination.

Selection of the upper limit of the working domain

To select the upper limit of the working range, a plot of the electric signal vs. concentration was examined between 0 and 10.0 mM. Along this range, the relationship between the signal and the concentration appeared to saturate beyond a concentration of 5.0 mM. A linear relation between the charge transferred (following a 1,000 second oxidation at 0.5 V) and the concentration of a polyphenolic seemed to exist up to a concentration of 5.0 mM. The upper limit for the concentration range was tentatively chosen to be 5.0 mM.

Linearity of the relation between electrochemical signal and the concentration

In order to be used for a quantitative assay, the linearity of the signal-concentration curve needed to be evaluated. To accomplish this analysis, linear regressions were fit to the electric data from selected polyphenolic solutions. The coefficient of determination was calculated and

used as an indicator of linearity of the data for each polyphenolic solution. The results from the

assessment of linearity are portrayed in Table 3.1.

Table 3.1. The linearity of electric signal. R^2 values for the linear trendlines of several polyphenol over the concentration domain 0.05 – 5 mM. Concentrations tested were 0.5, 1.0, 2.5, and 5.0 mM . Solutions were dissolved in a pH 7, 0.1 M phosphate buffer. Three samples of each data point were oxidized.

Chemical	R^2 value
Gallic acid	0.93
Protocatechuic acid	0.94
Caffeic acid	0.96

Using a potential of 0.5 V for 1000 seconds and testing concentrations of 0.5, 1.0, 2.5,

and 5.0 mM, the electric signal vs. concentration relation was evaluated for linearity based on the

R^2 values of a linear trendline (Table 3.1). The R^2 values of trendlines for gallic acid,

protocatechuic acid, and caffeic acid were 0.93, 0.94 and 0.96, respectively.

The linearity of the electric signals of the selected polyphenolic acids under oxidation

conditions of 0.5 V for 1000 seconds were relatively close to 1.00. This indicated that the chosen

working range of 0.5 mM to 5.0 mM might be adequate for use in an electrochemical quantitative

assay for polyphenolic acids.

3.1.1.4 Summary of operating parameters selected

The parameters of 0.5 V, 1000 seconds and working range of 0.5 to 5.0 mM were chosen

as the working values for polyphenolic compounds in the further development of an

electrochemical assay for polyphenolic quantification. The parameters selected might offer

selective oxidation of polyphenolic compounds when in mixture with monophenolic chemicals

(the voltage), to increase the sensitivity and magnitude of the electric signal (the duration) and generate signals in linear relation to the concentration tested.

3.1.2 Evaluation of parameters on the electrochemical signal

Experiments were next conducted to investigate any effect certain experimental condition might effect on the signal. Because of the liquid nature of the sample, the ion strength or solvent composition were considered to be potential influences of the electrical signal from this electrochemical apparatus.

3.1.2.1 Effect of ion strength on the electrical signal

The electric signal was due to an anodic oxidation-reduction reaction. The oxidation of phenolic antioxidants is known to be influenced by the pH of their environment (its one of the reasons behind the pH adjustment in the FC method). For this reason, a stable pH (provided through a buffer) in the bimodal sensing assay was a necessary testing condition to ensure uniformity and reproducibility from sample to sample. Additionally, part of the electrochemical apparatus was in contact with a chitosan film at all times. Chitosan film is soluble in acidic solutions so the used of an acidic buffer would dissolve the chitosan during its oxidation and make the optical signal disappear. On the other hand, an alkaline pH might influence the activity of the phenolics being tested – and possibly the measured values of the electrochemical assay. It was for these reasons that the buffer was adjusted to pH 7 in all solutions. A buffer at pH 7 was used throughout all experiments to keep the pH high enough (by a unit pH) to prevent solubilizing of the chitosan film during contact with the sample but maintain a non-alkaline pH that might have an effect on the reactivity of the sample.

It was important to know if the ion strength from a buffer would affect the electric signal. Salt concentrations have been shown to interfere with the conventional FC method but would be important for adjusting and maintaining the pH of a potentially acidic food sample. An

experiment to test for interference of ion strength on the baseline of the electric signal of the bimodal sensing apparatus was conducted by oxidizing a buffer at three different strengths (0.1, 1.0, and 2.0 M). Interference on the baseline signal was interpreted by considerations of the variation of the magnitude of the baseline signal and evaluated in comparison to the magnitude of the electrochemical signal from a 0.5 mM polyphenolic solution (the lowest in the working range). The results of this experiment are portrayed in Figure 3.6.
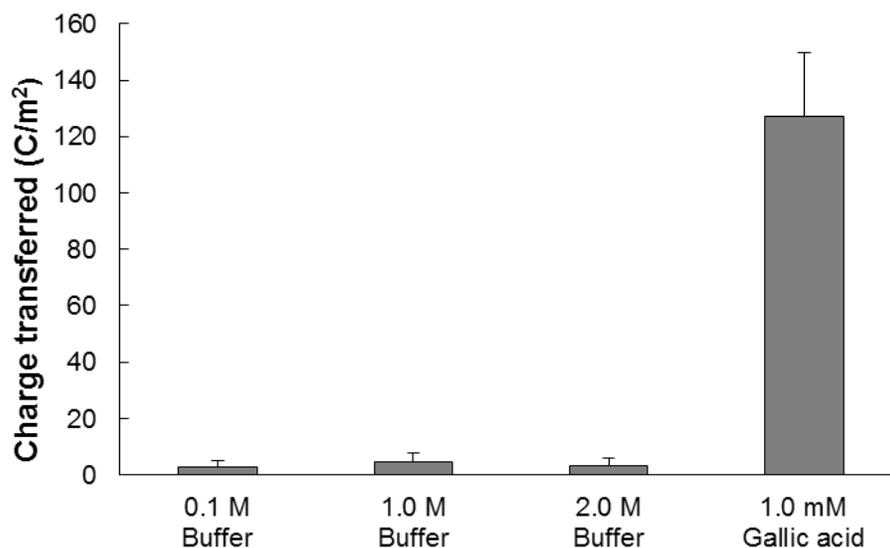
Figure 3.6. Effect of ion strength on electrochemical signal. Average chronocoulometric charges for 0.1 M, 1.0 M, and 2.0 M solutions of potassium phosphate buffer (pH 7) shown in comparison to the electrical signal of a polyphenol (0.5 mM gallic acid in 0.1 M pH 7 phosphate buffer). All solutions were oxidized at 0.5 V for 1000 seconds.

Figure 3.6: The electric signals (charge) of three different ion strengths (0.1 M, 1.0 M, and 2.0M) were compared to each other and contrasted against the electric signal of a polyphenolic acid (Figure 3.6). The contrast of the polyphenolic chemical was used to put the magnitude of influence by the ion strength in proportion with electric signals that would be 'typically' seen in the assay. The value of the electric signal of 1.0 mM gallic acid was used as a reference to which the magnitude of the baseline signal variation could be compared.

The 0.1 M buffer transferred a total of 3.6 C/m^2 after 1000 seconds at 0.5 V, while the 1.0 M buffer transferred 4.6 C/m^2 and the 2.0 M buffer transferred a total of 3.3 C/m^2. The baseline

electrochemical measurements corresponding to different ion strengths were not detectably influenced by the ion strength. In comparison to the magnitude of the electrochemical signal measurement of 0.5 mM gallic acid (127 C/m^2), the variation of the measured baseline signals (standard deviation squared of the mean baseline values) was approximately 0.6 % of the magnitude of the polyphenol. It was concluded that the ion strength did not significantly affect the optical signal of the blank.

These observations were interpreted to mean that the ion strength of a buffer did not affect the baseline signal of the bimodal sensing assay.

3.1.2.2 Effect of solvent on the signal

An experimenter may use a variety of solvents to extract and prepare food samples. In this electrochemical apparatus, the electric signal is a measure of the oxidation occurring in the solution and is dependent on the conduction of electricity in the sample. The conduction of electricity through a solution can be influenced by the composition of the solution. In turn, anything that affected the conduction of electricity would also affect the measured signal from the oxidation of a sample. An experiment was designed and performed to investigate the influence of solution composition on the electrical signal, the results of which are displayed in figure 3.7.

Figure 3.7. Solvent interference on the electric signal. (next page) Electric signals for the oxidation of 2.5 mM phenolic acids (A: 3,4-dihydroxybenzoic acid; B: 3,4,5-trihydroxybenzoic acid; C: 3,4-dihydroxycinnamic acid) at 0.5 V for 1000 seconds in four solvent solutions: 100% buffer; 10% dimethylsulfoxide and 90% buffer; 10% acetone and 90% buffer; or 50 % acetone and 50% buffer. The ion strength of the buffer was adjusted to maintain a final testing concentration of 0.1 M potassium phosphate. Values shown are the median and standard deviation of at least three oxidations
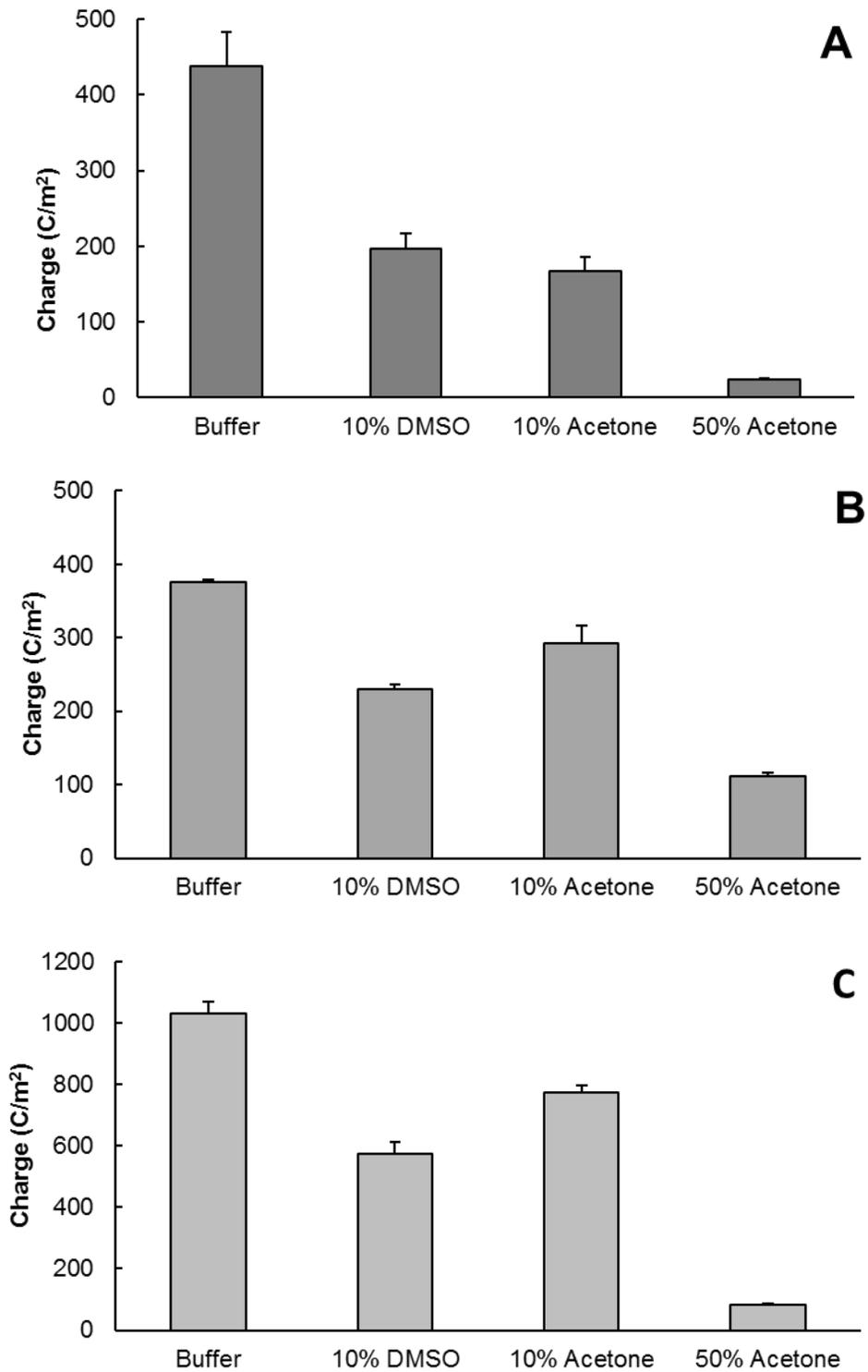
Figure 3.7 The electrical signals of several phenolic acids in solutions of different compositions were measured to answer the question of solvent influence. The effect a solvent

had on the electrical signal was assessed by comparing the coulombs transferred over an oxidation of the phenolic acids in different solvents. The charge transferred by polyphenolic acids in a 100% of an aqueous buffer was used a baseline reference value of oxidation. Figure 3.8 shows the results of this experiment using 2.5 mM protocatechuic acid (3,4-dihyrdroxybenzoic acid), 2.5 mM gallic acid (3,4,5-trihydroxybenzoic acid), and 2.5 mM caffeic acid (3,4-dihydroxycinnamic acid) in solutions of either 100% 0.1 M potassium phosphate buffer at a pH7 of 7 (buffer) , 90% buffer with 10% dimethylsulfoxide (DMSO), 90% buffer with 10% acetone, or 50% buffer and 50% acetone.

The electric signals of several phenolic acids in solutions containing 10% DMSO, 10% acetone, and 50% acetone were measured (Figure 3.7) and compared to a reference of the electric signal from the oxidation of the phenolic acid in 100% aqueous buffer. The buffer used (0.1 M potassium phosphate, pH 7) was prepared so that the final ion concentration would be 0.1 M in all solutions.

Electric signals for the 2.5 mM protocatechuic acid in different solvent compositions were: 439 C/m^2 (buffer alone); 197 C/m^2 (10% DMSO); 167 C/m^2 (10% acetone); 24 C/m^2 (50% acetone). The signals of the second phenolic acid tested, 2.5 mM gallic acid, were: 375 C/m^2 (buffer alone); 230 C/m^2 (10% DMSO); 290 C/m^2 (10% acetone); 111 C/m^2 (50% acetone). The signals of the final phenolic acid tested, 2.5 mM caffeic acid, were: 1033 C/m^2 (buffer); 575 C/m^2 (10% DMSO); 773 C/m^2 (10% acetone); and 81 C/m^2 (50% acetone).

For the 2.5 mM protocatechuic acid, a comparision between the sample in 100% buffer and the 10% acetone showed the oxidation measured was smaller for the 10% acetone solution. At the 10% level, the acetone exerted a greater dampening effect on the electric signal than did the 10% DMSO solution. In comparison to the effect of the acetone and DMSO on the electric value of the 2.5 mM protocatechuic, the 10% DMSO influenced the electric signal to a greater degree than the acetone did for gallic and caffeic acid solutions.

For all phenolic acids tested, the 50% acetone solvent solutions diminished the charge transferred during oxidation to a greater degree than the 10% acetone or the 10% DMSO did. From this experiment it was surmised that the amount of a solvent may dose-dependently influence the electric oxidation of a phenolic acid, and that the degree to which a solvent affected the signal might vary.

3.1.2.3 Selectivity for polyphenols by the electric signal

Operating conditions had been chosen with the intent to improve the selectivity of the oxidative apparatus' response for polyhydroxyphenolics by oxidizing at a voltage of 0.5 V and choosing a domain of the electromagnetic spectrum for the slight selectivity of polyhydroxyphenolic acids over monohydroxyphenolic acids. To validate a lack of minimized monohydroxyphenolic aicd response, several polyhydroxy- and monohydroxyphenolic acids were oxidized at 0.5 V for 1000 seconds and their signals were compared. The results from these oxidations are shown in Figure 3.8.
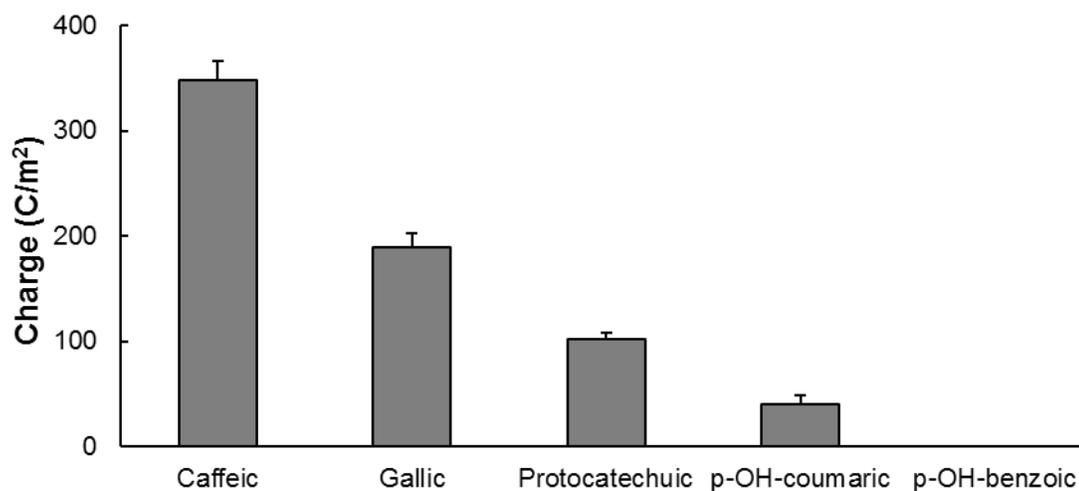

Figure 3.8. Electrical signal of several phenolic acids following 1000-second, 0.5 V anodic oxidation. The signal is in units of Coulombs per square meter. 1.0 mM caffeic, gallic, and protocatechuic acid were compared to the electric signal generated by 1.0 mM p-hydroxybenzoic acid, 1.0 mM p-hydroxycoumaric acid, and the buffer.

Figure 3.8 The values of the electric signal of 1.0 mM phenolic acids following anodic oxidation at 0.5 V for 1000 seconds are shown in Figure 3.8 and are as follows: 1.0 mM caffeic acid, 348 C/m^2; 1.0 mM gallic acid, 189 C/m^2; 1.0 mM protocatechuic acid, 102 C/m^2; 1.0 mM p-hydroxycoumaric acid, 40 C/m^2; 1.0 mM p-hydroxybenzoic acid, 0 C/m^2; and buffer 0 C/m^2. The electric signals of the polyhydroxy- and monohydroxyphenolic acids solutions were compared in and as groups (of polyhydroxyphenolic acids or monohydroxyphenolic acids) to investigate the use of oxidative parameters (0.5 V, 1000 sec) in this electrochemical setup for the oxidation of polyphenolic compounds.

A variation of electric signal intensity was observed within the polyhydroxyphenolic acids. The caffeic acid (3,4-dihydroxycinnamic acid) was observed to have an electric signal roughly 1.5 and 3.5 times as strong as the gallic or protocatechuic acid tested (348 C/m^2 vs. 189 and 102 C/m^2 for 1.0 mM solutions). This variation of signal intensity is similar to the variation seen in other phenolic-sensing assays, many of which use a standard chemical and compare measured activity to the activity of a known standard. It was speculated whether this electrochemical apparatus could benefit from the selection and use of a standard chemical in further analyses.

The electric signals of pure phenolic acid solutions were examined to evaluate the selectivity of oxidation of polyhydroxyphenolic compounds in this electrochemical apparatus at the prescribed working parameters (0.5 V and 1000 sec). The monophenolics displayed electric activity due to oxidation, but not as extensive as the polyhydroxyphenolic acids. One of the monohydroxyphenolic acids tested, p-hydroxybenzoic acid (p-OH-benzoic), exhibited an electric signal much smaller than the polyhydroxyphenolic acids (effectively 0 C/m^2 vs. 100-300 C/m^2). This seemed to indicate that there was some selectivity of oxidation for polyhydroxyphenolic acids under the working parameters of 0.5 V and 1000 seconds. A second monohydrophenolic acid, p-hydroxycoumaric acid (p-OH-coumaric), transferred 40 C/m^2 which was a charge on the

order of that transferred by the polyhydroxyphenolic acids (measured values range from 102 to 348 C/m^2).

While this similar intensity of charge transferred by the p-hydroxycoumaric acid was close to the oxidation-related values of the polyphenolic compounds, the 40 C/m^2 could be too low to be considered 'in range' if this apparatus was used for quantification. Depending on the relation between an electric signal and the concentration and the limits of the measurable range, a concentrated solution of a p-hydroxycoumaric acid could transfer enough charge to fall be erroneously measured as a polyphenolic compound. This left open the possibility of calculating a monophenolic sample to contain polyphenol and would be an instance of a false-positive. However, when the signal of the 1.0 mM p-hydroxycoumaric acid was compared to recorded values for 0.5 mM polyphenol (the limit of quantitation for polyphenols) it was found to be too low for calculation of a (false) polyphenolic content. The minimum value recorded for a 0.5 mM polyphenol was 50 C/m^2, but most values ranged between 70 and 130 C/m^2. This weaker activity was considered to be an instance of a "functional polyphenol" and it was concluded that some monohydroxyphenolic acids could oxidize at 0.5 V. It was noted that the working conditions of 0.5 V and 1000 seconds did not create selective conditions for the oxidation of polyhydroxyphenolic compounds over monohydroxphenolic compounds, but did somewhat increase the propensity of polyphenolics to oxidize (over that of monophenolics).

3.1.2.4 Effect of monophenolic acid on the electrical signal

Having established that the signal of the polyphenolics was stronger than most of the monophenolics under the operating conditions of 0.5 V and 1000 seconds, our attention turned to investigations of interference of monophenolics on polyphenolics. Interference experiments of monophenolics on polyphenolics were conducted much like the previous interference experiments: by varying the factor of potential interference while holding other factors constant. A control solution of a 1.0 mM polyphenolic compound was oxidized and four other solutions

with increasing concentrations of a monophenolic (0.1, 0.5, 1.0, and 2.0 mM) were oxidized as well. The concentration levels of monophenols were chosen to effect ratios between the concentration of polyphenols and monohydroxyphenolic acids measured in common fruits, based on data seen in the literature (Antolovich, 2000, Moore et al., 2005, Zadernowski, 2005, Hernanz, 2007). Variance from the magnitude of the control's electric response was interpreted as influence and interference by the monophenolic compound. Two polyphenols, gallic acid and caffeic acid, were tested in combination with two monophenols, p-hydroxycoumaric acid or p-hydroxybenzoic acid, for a total of four classes of chemical combination at 5 levels of monophenols (when including the control). Three, 1000-second oxidations were conducted on each solution and the results are featured in Figures 3.9 and 3.10.
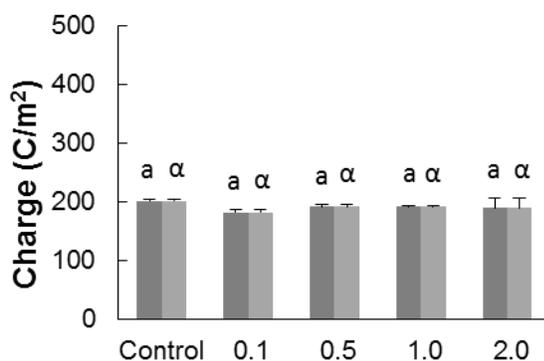
Figure 3.9. Effect of monophenolic acid on electric signal of gallic acid. 1 mM gallic acid with increasing amounts of p-hydroxycoumaric acid or p-hydroxybenzoic acid at five different levels. 1 mM gallic acid with: no added monophenol (control); 0.1 mM monophenolic acid; 0.5 mM monophenolic acid; 1.0 mM monophenolic acid; or 2.0 mM monophenolic acid. All combinations of polyphenolic and monophenolic acid tested with three samples.

Figure 3.9 Increasing concentrations of p-hydroxycoumaric and p-hydroxybenzoic acid in combination with 1.0 mM gallic acid were oxidized as part of an experiment to test for monophenolic interference on the electric response of a polyphenol in the electrochemical apparatus. Concentrations of 0 mM (control), 0.1 mM, 0.5 mM, 1.0 mM, and 2.0 mM were used. The chart of the results was formatted to portray the electric signals of solutions with p-hydroxycoumaric acid (pC) or p-hydroxybenzoic (pB) acid on gallic acid in the same diagram.

Unbounded dark gray bars denoted pC + gallic acid while the effect of p-hydroxybenzoic acid (pB) on gallic acid was portrayed using unbounded light bray bars.

Mean measured charge transferred over the oxidations for the 0("control"), 0.1, 0.5, 1.0, and 2.0 mM treatment levels of p-hydroxycoumaric acid on gallic acid had electric signal values of 201.1 C/m^2, 196.5 26.8 C/m^2, 189.0 C/m^2, 189.0 C/m^2, and 181.2 C/m^2, respectively. The mean electric signals for gallic acid and p-hydroxybenzoic acid had values of 181.4 C/m^2 (0.1 mM pB), 191.4 C/m^2 (0.5 mM pB), 191.6 C/m^2 (1.0 mM pB), and 189.7 C/m^2 (2.0 mM pB). The non-zero treatment levels exhibited similar means while the control had a more unique mean (201.1 C/m^2).

Tukey's Honestly Significant Different (HSD) test showed no statistical difference between treatment levels in the signals of the bimodal sensing assay. This was conveyed in figure 3.12 by the letters above each group. All of the p-hydroxycoumaric acid treatment levels with gallic acid were statistically a single group so a small "a" was placed above each column. All of the p-hydroxybenzoic treatment levels on gallic acid were of a single group by Tukey's HSD. This was denoted by placing an 'α' over each column. The analysis of variance (ANOVA) showed the electrochemical determination of gallic acid oxidation was not affected by the addition of the monophenolic compounds tested. ANOVA did not prove the addition of increasing amounts of monohydroxyphenolic acid to be completely without effect; it showed the absence of an effect by the monophenolic compounds on the gallic acid's electrical response. A test for equivalence among different levels of treatment failed. A second combination of mono- and poly-hydroxyphenolic acids using caffeic acid with p-hydroxycoumaric or p-hydroxybenzoic acidswas also conducted, the results of which are shown in figure 3.10.
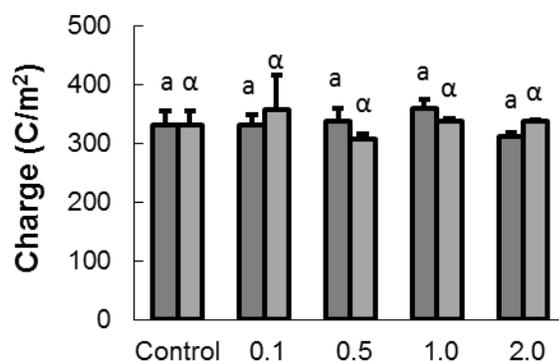
Figure 3.10. The interference of monophenolics on the electrical signal of the bimodal sensing assay. 1 mM caffeic acid with increasing amounts of p-hydroxycoumaric acid (dark gray bars with thick black borders) or p-hydroxybenzoic acid (light gray columns with thick black borders) at five different levels. 1 mM caffeic acid with: no added monophenol (control); 0.1 mM; 0.5 mM; 1.0 mM; or 2.0 mM monophenolic acid. All combinations of polyphenolic and monophenolic acid tested with three samples. Columns topped by the same letter were not statistically different from one another by Tukey's Honestly Significant Difference post hoc test in an analysis of variance.

Figure 3.10 The second half of the experiment to test for interference of monohydroxyphenolics on the electric signal of polyhydroxyphenolics used caffeic acid (3,4-dihydroxycinnamic acid) in combination with p-hydroxycoumaric and p-hydroxybenzoic acids (pC, pB). The concentration of the caffeic acid was held constant while the monophenolic concentrations increased over the treatment levels of 0 mM ("control"), 0.1 mM, 0.5 mM, 1.0 mM, and 2.0 mM concentrations. Mean measured values of the p-hydroxycoumaric acid on caffeic acid were portrayed using dark gray bars with thick black borders and had mean electrical response values of 331.3 C/m^2, 331.0 C/m^2, 337.1 C/m^2, 358.8 C/m^2, and 312.0 C/m^2, respectively (Figure 3.10). The electrical signals for caffeic acid treated with increasing amounts of p-hydroxybenzoic acid transferred charge in the amounts of 357.8 C/m^2, 308.1 C/m^2, 338.5 C/m^2, and 337.2 C/m^2 for the non-zero treatment levels of 0.1 mM, 0.5 mM, 1.0 mM, and 2.0 mM, respsectively.

The intra-composition solutions of caffeic acid with either p-hydroxybenzoic (pB) or p-hydroxcoumaric (pC) acid were evaluated in comparison to the electric signal of the 1.0 mM control caffeic acid. An analysis of variance (ANOVA) was conducted to statistically assess

whether a given treatment level of caffeic acid with pB or pC yielded an electric signal that was statistically different from the control. Tukey's Honestly Significant Difference (HSD) post hoc test at a significance level of $p > 0.05$ was used as part of the ANOVA and became the indicator of statistical difference (of a treatment, from the control). The HSD ANOVA values were portrayed in Figure 3.10 by superscripts above each column. Columns topped by the same letter were not statistically different from one another. A lack of difference (shared letter) was interpreted to mean that the treatments of pC or pB exerted no interference on the caffeic acid and its electric signal at (the treatment's) concentration. I

For the solutions of caffeic acid with pC or pB, the ANOVA with Tukey's HSD calculated no intra-level effect by a single monophenolic compound on the electric response. Neither p-hydroxybenzoic acid nor p-hydroxycoumaric acid was found to exert an effect on the electric signal of the caffeic acid such that the measured electric signal was significantly different from that of the control. Under an ANOVA using Tukey's HSD, a lack of variation does not indicate equivalence of all treatment levels. The ANOVA indicates the absence of a significant change due to a different treatment level.

It was concluded that the presence of a monophenol in solution with a polyphenol did not interfere with the electrochemical determination of the polyphenol using the specified electrochemical apparatus.

Even though the operating parameters chosen improved the selectivity of the oxidation for that of poyphenolic compounds over monophenolic compounds, the electrical signal itself could not offer concrete information that any oxidized substance was a polyphenol; any compound that oxidized in the sample could contribute to the electrical signal. Hence, the electric signal alone was not sufficient for the detection of polyhenolic compounds. This was controlled by the optical signal in the chitosan film. To use the optical signal in the film as part of a quantitative assay, a method of measurement for the signal needed to be established.

3.2 The Optical Signal

The electrical signal and the optical signal each played a critical part in the electrochemical determination, but neither response could be used on its own to electrochemically determine polyphenolic content in a sample. The electric signal was not specific to a given oxidizing chemical while the chitosan's post-electrode reaction was specific for phenolic substances. For this reason a consideration of both signals would be necessary when evaluating potential working parameters or other variables of influence. In this section of the results, the parameters of influence on the optical signal are presented.

3.2.1 Development of the quantification of the optical response

Chitosan films were known to show color in response to any phenolic oxidized during the test (Liu et al., 2008). A chitosan film laid across the indium-tin oxide electrode underwent a post-electrode, phenolic-specific reaction to yield a film with a modified absorbance spectrum following the oxidation of a phenolic compound. Thus, the chitosan film contained the information that was selective for phenolics. In contrast, the measured value of the electric signal represented the oxidization of all chemicals in solution, phenolic or not. So while the 0.5 V operating parameter encouraged the oxidation of polyphenols over that of monophenols and the measurement of the charge transfer indicated how much total oxidation occurred in a sample, the optical response in the chitosan was essential for the determinations of a phenolic oxidation). This formed the basis of the optical signal for the electrochemical apparatus.

It was necessary to put a number to the optical response. Spectra of chitosan films following oxidations of several pure phenolic compounds were examined to this end. Following the oxidation of a sample, the chitosan-coated transparent electrode was rinsed and set to dry. Slides were taped to the side of a cuvette holder in a spectrophotometer and the absorbance between 300 and 700 nm was recorded. The absorbance of a chitosan-coated slide was subtracted

from all spectra. The area under the absorbance curve between 530 and 560 nm was selected as the measurement of the optical response for the determination of polyphenolic compounds.

Having selected a quantification parameter, the optical response was measured with respect to polyphenolic and monophenolic compounds and comparisons of selectivity were made, along with an evaluation of the linearity of the optical signal with respect to the concentration of the polyphenolic compound oxidized.

3.2.1.1 Selection of the quantifier of the optical response

A major concern was the selection of a wavelength for use in quantification of polyphenolics (at which a monohpenolic compound did not show significant absorbance). As shown in Figure 3.11 polyphenolic and monophenolic acids exhibited absorbance over much of the 300-700nm range used to derive this quantifier.
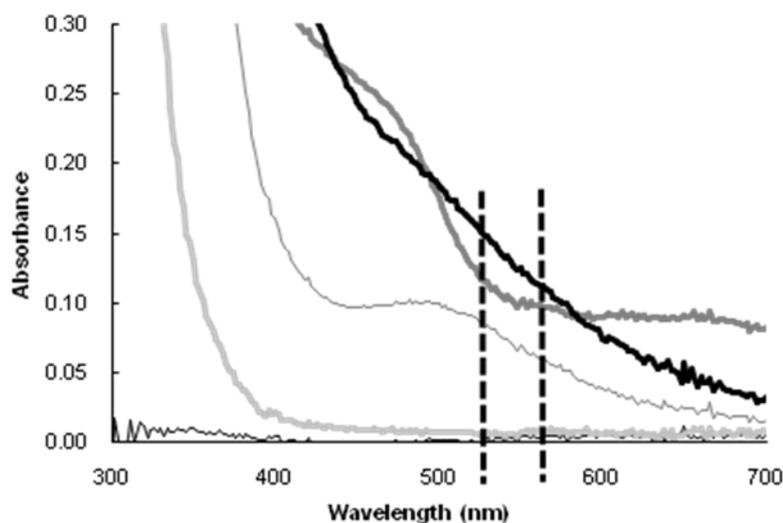
Figure 3.11. Absorbance spectra of dried chitosan films. Following oxidationf of selected pure phenolic acid solutions for 1000 seconds at 0.5 V. The solid black line represents the absorbance spectrum of 2.5 mM caffeic acid; the thick dark gray line represents the absorbance of 2.5 mM gallic acid; the thin dark gray line of 2.5 mM protocatechuic acid; the thick light gray line represents 5.0 mM p-hydroxycoumaric acid; and the thin black line represents the absorbance of the film over which 2.5 mM p-hydroxybenzoic acid was oxidized. All lines are averages of spectra from 3 separate oxidations. Vertical dotted lines are drawn at approximately the $\lambda = 530$ nm and $\lambda = 560$ nm values to indicate the area of interest for use as quantifier of the optical response of polyphenolic compounds in the electrochemical apparatus.

Absorbance spectra of the domain 300 to 700 nm were recorded from individual dried chitosan films following oxidations of phenolic acids. Film spectra were compared to select a quantifier for the optical response. It was desired to use a measurement that would be shared only by the polyphenolic spectra and not by all of the phenolic spectra. The choice used in this project was an area of the curve where there seemed to be a slight difference between the absorbance spectra of polyphenols versus the spectra of monophenols. Several spectra used to select this area for measurement are shown in Figure 3.11 and a report of the considerations of the sample spectra follows.

Figure 3.11: As a quantifier for the optical response, a single wavelength corresponding to a peak of absorbance shared by polyphenolic compounds was the first choice. This hinged on the existence of a signle peak of absorbance common to all phenolic or (better yet) polyphenolic samples. Due to the existence of numerous absorption maxima exhibited by the different phenolic solutions it was not feasible to use a single wavelength for quantification. Nor was a composite of several wavelengths specific to polyphenolic substances apparent. It was concluded that the use of a single or several wavelengths for the quantification of the optical signal would be insufficient.

A second option for measurement was that of the area under the curve for a portion of the absorbance range. As shown in Figure 3.11, chitosan films following oxidations of a monophenolic compound exhibited absorbance in the region between 300 and 400 nm. A polyphenolic-specific optical measurement was not practical using the area over the 300 – 400 nm spectrum due to the absorbance of monophenolics in this domain; there was a possibility of false-positives from monophenolics using this area of the curve for an optical signal. A concentrated monophenolic compound could potentially oxidize enough to modify the chitosan absorbance spectrum and erroneously be interpreted as polyphenolic content. The spectra were examined over other regions for a potential quantifier of the optical response.

A comparison of absorbance spectra showed a modest polyphenolic-specific asborption (over that of monophenolic compounds) might exist between 530 and 560 nm. Between 530 and 560 nm the slope of the polyphenols was gently negative and the absorbance of the monophenolic acids (those tested) was negligible. The chitosan films from all polyphenolics tested showed absorption over this range. The major disadvantage of this range was that the absorbance spectrum between 530 and 560 nm did not span an absorbance peak but a side slope of the absorbance curve. The area under the absorbance spectrum curve between 530 and 560 nm was tentatively chosen as the quantifier for the optical response.

3.2.1.2 Optical signal of polyphenolic versus monophenolic compounds

The optical signal was known to be selective for the post-anodic reaction of all phenolics (Liu et al., 2008); when monophenolic chemicals oxidized, they created an optical signal. It was the operating potential that encouraged the oxidation of polyphenolics more than that of monophenolics. Further insight into the oxidation of polyphenolics versus that of monophenolics (under the conditions of 0.5 V lasting 1000 seconds) could be ascertained from a comparison of the optical signals of each other. An experiment to gauge the degree of oxidation of each kind of phenolic using the optical signal as an indicator of oxidation was performed. Monophenolics and polyphenolics were oxidized and the results, as presented in figure 3.12, compared.
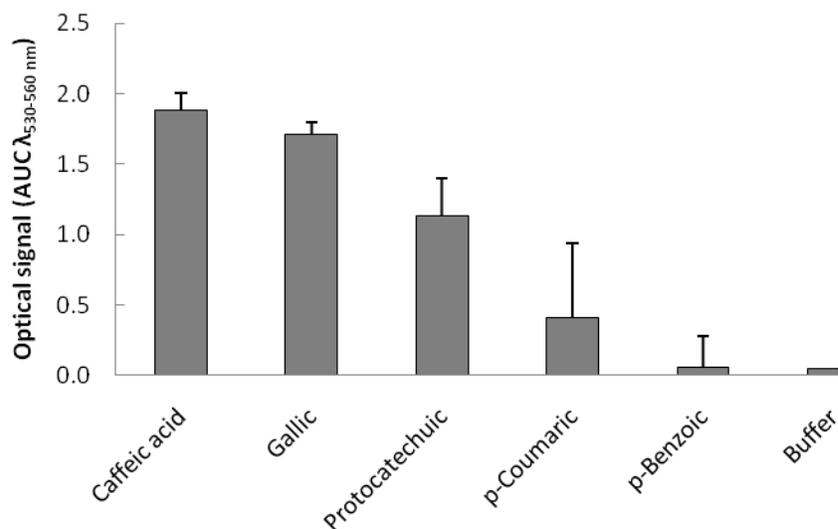
Figure 3.12. Optical signal of selected pure phenolic compounds. The optical signal of several 1.0 mM mono- or poly-phenolic acid solutions (p-hydroxybenzoic acid, caffeic acid, gallic acid, protocatechuic acid) and one 5.0 mM monophenolic acid (p-hydroxycoumaric acid) following a 1000-second oxidation at 0.5 V is featured. Values presented are the mean + standard deviation of three oxidations. The signal was calculated from area under the curve in the absorbance spectrum between 530 and 560 nm in the dried chitosan film over which the phenolic solutions were oxidized.

Figure 3.12 The optical signals (area under the curve between 530 and 560 nm) of several pure polyphenolic and monophenolic acid solutions were measured and compared (Figure 3.12). 1.0 mM caffeic acid, 1.88 AUC; 1.0 mM gallic acid, 1.71 AUC; 1.0 mM protocatechuic acid, 1.1 AUC; 5.0 mM p-hydroxycoumaric acid, 0.41 AUC 1.0 mM p-hydroxybenzoic acid, 0.05 AUC were found to be the values of the optical signal analysis to validate the selectivity of the bimodal sensing assay for polyphenolic compounds.

The polyphenolic acid solutions created an optical signal on the order of 1-2 AUC and the monophenolic compounds generated optical signals ranging from 0.01 to 0.5 AUC. The monophenolic acids created an optical signal, but only did so in more concentrated solutions. It took a concentration of 5.0 mM of p-hydroxycoumaric acid to be mistaken as a polyphenolic chemical of approximately 0.3 mM.

These results were interpreted to confirm that the optical signal was specific for phenolics and could not be used in the capacity of a response selective for the oxidation of polyphenolics alone. The applied voltage of the oxidation improved operating conditions to oxidize polyphenolics to a greater degree than monophenolics. The experiment to test whether the optical response – as measured by $AUC_{530-560nm}$ – was selective for polyphenolic compounds showed no selectivity. Certain monophenolics like p-hydroxycoumaric acid could potentially be in solutions at concentrations so high they could generate enough optical response to be mistaken as a low-concentration polyphenolic solution. Presumably, this activity was not unique to a single monophenol. In comparison with the experiment to asses the electric response due to polyphnenols (vs. that of monophenols, section 3.1.2.3), the results were much the same: if a monophenolic sample oxidized at all, it showed in the total measured oxidation (from the electric response) and in the measured phenolic oxidation (the optical response). It was concluded that it was not possible to distinguish the oxidation of polyphenols from that of monophenols using the optical quantification selected and indicated a need to reconsider the quantification of the optical signal to something stronger (like the absorbance peaks in the 300-400 nm range).

3.2.1.3 The linearity of the optical signal

With the selection of a quantification of the optical response, its performance as a linear signal (with respect to concentration) needed to be evaluated. Several concentrations (0.5, 1.0, 2.5, and 5.0 mM) of three polyphenolic acids were oxidized and their optical signals measured and charted. Plots of AUCs versus the concentrations were fitted with lines of best fit. Linearity of signal-concentration relationships was assessed by coefficients of determination for the linear regressions of each polyphenol. The values of the coefficients of regression are featured in Table 3.2.

Table 3.2. The linearity of the optical signal. R^2 values for the linear trendlines between the optical response and concentration over the domain (0.5 mM, 5.0 mM) for three polyphenolic acids (gallic acid, protocatechuic acid, caffeic acid). Concentrations used to generate the trendlines were 0.5, 1.0, 2.5, and 5.0 mM.

Chemical	R^2 value
Gallic acid	0.92
Protocatechuic acid	0.94
Caffeic acid	0.96

The linearity of the optical signal (area under the curve from 530 to 560 nm) was evaluated by the R^2 values of linear regression lines for the polyphenolic acids tested: gallic acid; protocatechuic acid; and caffeic acid.

The optical signal of gallic acid was fit with a linear trendline with an R^2 value of 0.92, while the trendline R^2 value for protocatechuic acid was 0.94 and for caffeic acid was 0.96 (Table 3.2).

The coefficients of regression of the optical signal as it regressed to a linear line of best fit for polyphenolic acids in the bimodal sensing apparatus were interpreted as indicators of decent linearity between the optical signal and the concentration of a polyphenolic compound. It was concluded that the measurement of 530-560 nm AUC would function acceptably when used as part of a quantitative technique for polyphenolic acids with the bimodal sensing apparatus.

3.2.2 Evaluation of parameters on the optical signal

As shown by Liu et al. (2008), the optical signal was dependent on a post-electrode reaction between an oxidized phenolic and the chitosan film coating the electrode. Influences on the oxidation of a sample would be expected to influence the optical signal. For this reason,

influence by ion strength, solvent composition, and the presence of monphenolics were investigated.

3.2.2.1 Effect of ion strength on the optical signal

The optical signal is known to be due to a post-oxidative interaction between the chitosan film over the workin electrode and the oxidized phenolic. The electrochemical oxidation of a chemical is known to be influenced by the conductivity of its environment and this is related to the ion strength of a solution.

It was important to know whether the ion strength of a buffer would affect the optical signal. An experiment to test for interference of ion strength on the baseline of the optical signal of the electrochemical apparatus was conducted by oxidizing three different strengths of potassium phosphate buffer (0.1, 1.0, and 2.0 M, pH 7). The ion strength was first considered on the baseline signals measured, and the final evaluation of the evaluation of an effect of ion strength was interpreted in comparison to the magnitude of an optical signal from the oxidation of a polyphenolic acid solution (gallic acid, 1.0 mM in 0.1M buffer). The results of this experiment are portrayed in Figure 3.13.

Figure 3.13. Effect of ion strength on optical signal. Mean optical signals (area under the curve (AUC $\lambda_{530:560nm}$)) for 0.1 M, 1.0 M, and 2.0 M solutions of potassium phosphate buffer (pH 7) and of 0.5 mM gallic acid (in 0.1 M phosphate buffer, pH 7). All solutions were oxidized at 0.5 V for 1000 seconds.
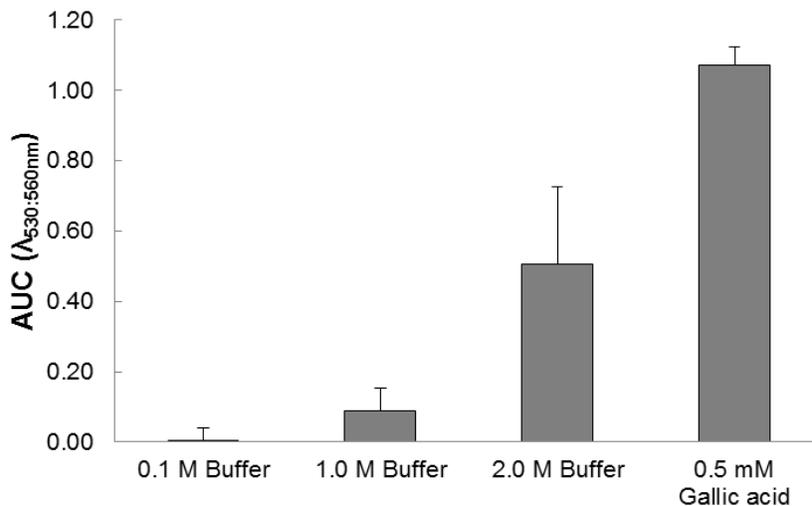

Figure 3.13 Buffers three ion concentrations (0.1 M, 1.0 M, and 2.0M) were oxidized in an electrochemical apparatus. For each sample (3 oxidations per ion strength), the electrochemical cell was disassembled and the chitosan film on the electrode rinsed with distilled water. Films were dried and then the chitosan-coated electrodes (transparent indium tin oxide on a glass slide) were taped to the outside of a cuvette holder in a spectrophotometer. The absorbance between 530 nm and 560 nm was measured and the area under the absorbance curve (AUC) summed to yield the optical signal measurement. The mean measured values different ion strengths of buffer were compared to each other and contrasted against the mean optical response value of a 0.5 mM polyphenolic acid, as shown in Figure 3.13.

The reason for the contrast of the optical baselines (from different ion strengths of buffer) to the optical signal of a polyphenolic chemical was used to better evaluate variation due to the effect of ion strength on the optical baseline. The value of the optical signal of 0.5 mM gallic acid was used as a reference to which the magnitude of the baseline signal variation could be compared and the concentration of that polyphenolic solution (0.5 mM) was chosen because it

would be expected to produce the lowest optical signal encountered as it was the concentration of a polyphenol at the lower limit of the working range of the technique (0.5-5.0 mM).

The optical signals of the three buffers featured AUC values of 0.0 (0.1 M buffer), 0.1 (1.0 M buffer), and 0.5 (2.0 M buffer). The film from the 0.5 mM gallic acid solution was measured to have a mean optical signal value of 1.1.

The variation of the measured optical signal with respect to the ion strength of the buffer was significant between the 2.0 M buffer and the other, weaker buffers (0.1 M, 1.0 M). The optical signals of all three of the ion strengths (as a gropu) did not vary significantly from the measured value of the 0.5 mM gallic acid signal. However, when the two lower-concentrations of buffers were evaluated as a group in comparison to the 0.5 mM gallic acid, the difference between measure optical signals was significant.

It was concluded that ion strengths of 2.0 M exerted an effect on the blank optical signal of the blank, but that lower ion strengths did not. It was further concluded that buffers of ion strength less than or equal to 1.0 M would facilitate the discrimination of the baseline from optical signals of polyphenolic compounds.

3.2.2.2 Solvent effect on the optical signal

The effect of the 10% DMSO, 10% acetone and 50% acetone solvents on the optical signal of 2.5 mM protocatechuic acid were measured. The mean measured optical signals of protocatechuic, gallic, and caffeic acids in solutions of 100% buffer, 10% dimethylsulfoxide (DMSO), 10% acetone, or 50% acetone are shown in Figure 3.14.
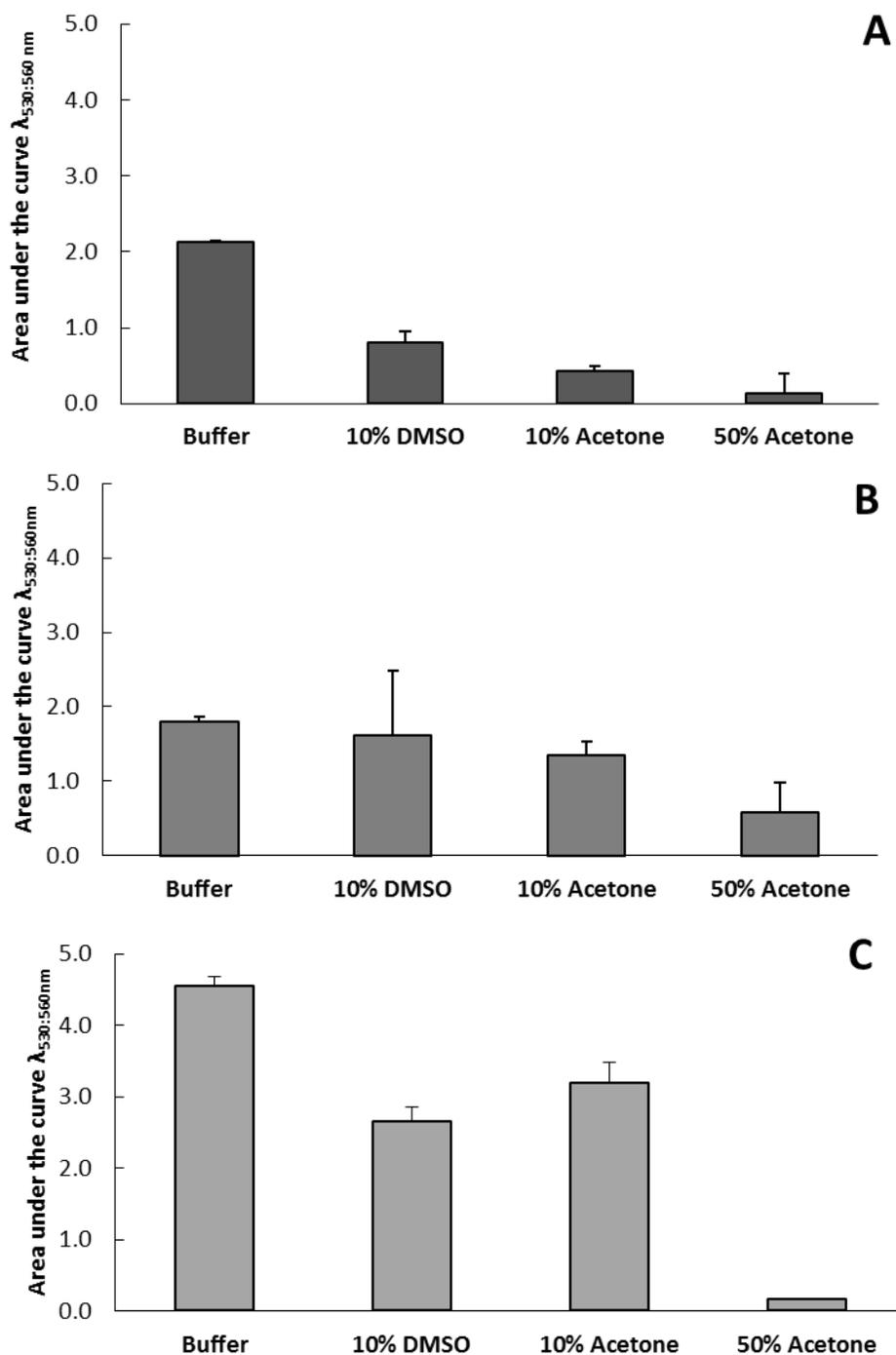
Figure 3.14. Solvent effect on the optical signal of a polyphenol. $AUC_{(530\text{-}560nm)}$ for 1000-second oxidations at 0.5 V of A: protocatechuic acid; B: gallic acid; C: caffeic acid; when dissolved in solutions of either 100% 0.1 M potassium phosphate buffer, 10% DMSO and 90% buffer, 10% acetone and 90% buffer, or 50 % acetone and 50% buffer. The ion strength of the buffer was adjusted to maintain a testing concentration of 0.1 M phosphate in each solution. Values and error bars shown are the average and standard deviation values of three oxidations.

Figure 3.14 The same trend seen with the electrical signal (3.1.2.2) was observed with the optical. When compared to the signal of the solution in 100% 0.1 M phosphate buffer the signal diminished with increasing amounts of organic solvent. The 2.5 mM protocatechuic acid in 100% buffer had an area under the curve of 2.1 AUC, 10% DMSO solvent diminished the optical signal of the 2.5 mM protocatechuic acid to 0.8 AUC, the 10% acetone diminished the signal to 0.4 AUC, and the mean optical signal of the protocatechuic acid in the 50% acetone solution was 0.15 AUC. For gallic acid (3,4,5-trihydroxybenzoic acid) the effect of the solvents resulted in optical signals of 1.8 AUC (buffer), 1.6 AUC (10% DMSO), 1.4 AUC (10% acetone), and 0.6 AUC (50% acetone). The mean measured values of caffeic acid (3,4-dihydroxycinnamic acid) in various solvents were 4.5 AUC (buffer), 2.7 AUC (10% DMSO), 3.2 AUC (10% acetone), and 0.2 AUC (50% acetone).

From these results it was concluded that the solvent used in a solution exerted an effect on the optical signal of a polyphenolic compound. From this conclusion it was inferred that the measured signals of samples in different solvents would not be directly comparable. However, the calculation of conversion factors between solvents would require the re-establishment of a linear portion of a working range between the signal and concentration for polyphenolics in different solvents; this was beyond the scope of this project. Ultimately it was apparent that a signal needed to be evaluated on terms specific to the solution composition because the solvent was shown to exert an influence on the measured optical signal of a polyphenolic compound in the bimodal sensing assay.

It was conjectured that the electrochemical technique might benefit from the use of a standard given the variance of the optical signal of the same compound in different compositions of solvents. Additionally, because both the optical and electric signal were diminished, it would be interesting to see if they were diminished to the same degree. This could be done by charting the ratio of the diminished signals to see if a constant relation existed and might reveal a relation between the optical and electric signals that heretofore had not been noted. If such a relation was

conserved across different solvents, this two-part variable could be introduced into an equation for quantification of polyphenolics that could enable the quantification across solvents, sort of like a translator. Such a chart was plotted and is shown in the appendix in figure A.21.

3.2.2.3 Effect of monophenolic acids on the optical signal

Thus far, all tests had been performed on solutions of a single phenolic in solution with no other chemicals save solvent or buffer. To begin to evaluate the bimodal sensing assay in use with more complex solutions, experiments to test for the interference of monophenolics and ascorbic acid were conducted. The same solutions were also tested by the FC method for use in a comparison to the conventional method (Section 3.4).

To test for interference of monophenolic acids (on the polyphenolic response), a series of solutions was oxidized in the bimodal sensing assay. The concentration of the polyphenolic compounds (gallic acid or caffeic acid) in solution were kept constant while the monophenolic compounds (p-hydroxycoumaric or p-hydroxybenzoic acid) were increased.

The results were analyzed for interference within a given treatment (intra-level) by conducting an analysis of variance. Interference by the monophenolic compounds was detected by a changed electric or optical signal in the presence of increasing amounts of monophenolic. Any interference due to monophenolics was expected to manifest in an altered signal. To assess the interference by a monophenolic chemical, an analysis of variance was conducted. Significance was declared at a level of $p > 0.05$ using Tukey's Honestly Significant Different (HSD) post hoc test in an analysis of variance on the intra-treatment level signals.

The phenolic acids used in this experiment were chosen because of their prevalence in plants and fruits, as gathered from several studies of polyphenolic compounds in fruits and plants (Hernanz et al., 2007; Zadernowski, 2005; Antolovich, 2000). As calculated from these papers the ratio of polyphenolic to monophenoic acid ranged from 20:1 to 1:3. These ratios were

mimicked were in this experiment around a constant polyphenolic concentration of 1.0 mM through concentrations of 0.1:1, 0.5:1, 1:1, and 2:1.

Table 3.3. Diagram of concentrations used in the monophenol interference experiment. 1.0 mM gallic acid (GA) or 1.0 mM caffeic acid (CA) used in combination with para-coumaric acid (pC) or para-hydroxybenzoic acid (pB) at concentrations of 0.0, 0.1, 0.5, 1.0 or 2.0 mM.

		Monophenolic acid (mM)				
		0.0	0.1	0.5	1.0	2.0
1 mM GA	pC					
	pB					
1 mM CA	pC					
	pB					

Gallic acid and caffeic acid were tested in mixture with p-hydroxycoumaric and p-hydroxybenzoic acids (a total of 4 mixtures/treatments) and each treatment was tested at 4 levels, (0.1, 0.5, 1.0 and 2.0 mM of monophenolic acid) while maintaining a constant concentration of the polyphenolic acid (1.0 mM). The concentration array is physically depicted in Table 3.3.

Intra-treatment results were statistically analyzed for an effect by a monophenolic chemical using an analysis of variance (ANOVA) with Tukey's Honestly Significant Difference post hoc test. Each non-zero treatment level was compared to a control solution (of 1.0 mM polyphenolic acid, no monophenolic acid) to evaluate whether the presence of a monophenol interfered with the polyphenolic response.
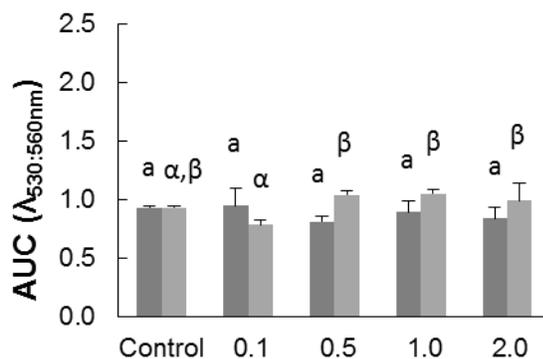
Figure 3.15. The interference of monophenolics on the optical signal. 1 mM gallic acid with increasing amounts of p-hydroxycoumaric acid or p-hydroxybenzoic acid at five different levels. 1 mM gallic acid with: no added monophenol (control); 0.1 mM monophenolic acid; 0.5 mM monophenolic acid; 1.0 mM monophenolic acid; or 2.0 mM monophenolic acid. All combinations of polyphenolic and monophenolic acid tested with three samples. Different letters above treatment levels indicate significant difference within the type of treatment.

Figure 3.14 The treatment levels and representation designations of the monophenolic concentration on the 1.0 mM polyphenolic acid were 0 (control), 0.1 mM, 0.5 mM, 1.0 mM, and 2.0 mM, and were represented by borderless dark gray columns. The mean optical signal from 3 oxidations of gallic acid with p-hydroxycoumaric acid were 0.93 AUC, 0.95 AUC, 0.81 AUC, 0.90 AUC, 0.84 AUC, for the control, a, b, c, and d levels respectively (Figure 3.15) and were portrayed using borderless light gray columns. The mean measured optical signals for gallic acid in solution with increasing amounts of p-hydroxybenzoic acid were 0.79 AUC, 1.04 AUC, 1.05 AUC, and 0.99 AUC for the non-zero treatment levels a,b,c, and d of monophenol (respectively).

An analysis of variance (ANOVA) using Tukey's Honestly Significant Difference (HSD) at a significance level of $p > 0.05$ showed that the addition of p-hydroxycoumaric acid to gallic acid did significantly change the measured optical response at any of the treatment levels. For treatments of gallic acid with p-hydroxybenzoic acid, the measured optical signals broke into two groups. Neither group varied significantly from the control, but the two groups varied significantly from one another. The two overlapping groups of values were A: the control and the

0.1 mM treatment level of p-hydroxybenzoic acid and B: the control, 0.5 mM, 1.0 mM, and 2.0 mM treatment levels.

The minimal variation with respect to the monophenolic concentration was interpreted to mean that the monophenolics had no statistically significant effect on the optical signal of the gallic acid.

A second polyphenolic, caffeic acid, was oxidized in combination with p-hydroxycoumaric acid and p-hydroxybenzoic acid. The measured optical signals from these oxidations are shown in Figure 3.16 with bars with thick black borders. Caffeic acid with p-hydroxycoumaric acid are portrayed using dark gray columns and caffic acid with p-hydroxybenzoic acid are shown as light gray columns.
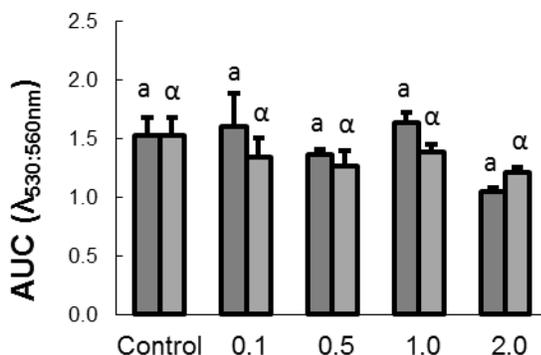

Figure 3.16. The interference of monophenolics on the optical signal. 1 mM caffeic acid with increasing amounts of p-hydroxycoumaric acid or p-hydroxybenzoic acid at five different levels. 1 mM caffeic acid with: no added monophenol (control); 0.1 mM monophenolic acid; 0.5 mM monophenolic acid; 1.0 mM monophenolic acid; or 2.0 mM monophenolic acid. All combinations of polyphenolic and monophenolic acid tested in three trials. Different letters above treatment levels indicate significant difference within the type of treatment by Tukey's HSD post hoc test.

Figure 3.16 The optical signal ($AUC_{530-560 \, nm}$) for 1.0 mM caffeic acid with increasing amounts of p-hydroxycoumaric acid (pC) was measured. The mean optical response values of three oxidations of caffeic acid with p-hydroxycoumaric acid were: 1.52 AUC (control) 1.61 AUC (0.1 mM pC) 1.36 AUC (0.5 mM pC); 1.63 AUC (1.0 mM pC); and 1.05 AUC (2.0 mM pC), respectively (Figure 3.16). Mean measured optical responses for solutions of caffeic acid with

increasing amounts of p-hydroxybenzoic acid were: 1.34 AUC; 1.26 AUC; 1.38 AUC; and 1.21 AUC for the 0.1 mM, 0.5 mM, 1.0 mM and 2.0 mM p-hydroxybenzoic acid treatment levels, respectively.

Following an ANOVA using Tukey's HSD, no statistically significant variation in the optical signal was calculated for the addition of p-hydroxycoumaric nor p-hydroxybenzoic acid to caffeic acid at a significance of 5 percent.

The lack of variation was encouraging in the development of a method specific for the detection of polyphenolic chemicals and unaffected by monophenolic species present in solution and was a glad finding to one of the chief aims in the development of the parameters.

3.3 The Selection of a Standard Chemical

All quantitative assays are based on the conversion of a measured signal to the amount of substance of inquiry. The conversion from a measured attribute to a quantified substance is often calculated using the activity of a standard chemical. A standard must satisfy certain attributes: the magnitude and variation of activity of the standard should be representative of the chemicals assayed; the activity of the standard should be a mechanism common to all of the chemicals for which the assay tests.

It was thought necessary to have an idea of a broader set of polyphenolic compunds before a standard could be proposed.

3.4 Interference on the Folin Ciocalteu Method and Spectroelectrochemical Apparatus

The results from the interference studies of the polyphenolic activity were presented in sections 3.1.2.4 and 3.2.2.4. In parallel to those experiments of monophenol interference on the polyphenolic signal, the same solutions were tested by the FC method to be able to compare the

electrochemical determination apparatus to the conventional method. This section presents the activity of these solutions in the FC method and then compares the acitivity of all three measured signals of the solutions (the electrical and optical response using the electrochemical apparatus, and the activity of the solutions in the FC method).

3.4.1 Influence of monophenolic compounds

As the conventional method for quantifying polyphenolic compounds, the Folin Ciocalteu (FC) method is widely used. The method is a robust and rugged method that quantifies the phenolic content of a sample in reference to a standard. The FC method measures total phenolic content, and is not specific for polyphenolic compounds. Hence, in comparison to the FC method, the electrochemical apparatus presented in this assay could be slightly more selective for polyphenols, which may exert a greater positive effect on human health than monophenols (Stevenson and Hurst, 2007). A comparison of the polyphenol determination by an electrochemical method and the FC method was conducted. Using solutions of increasing concentration of monohphenolic compound in solution with a constant concentration of polyphenol, the values measured by the FC method were compared with those measured by the spectroelectrochemical apparatus (section 3.1.2.4 and 3.2.2.3).

3.4.1.1 Interference by monophenolic compounds on the FC determination of polyphenols

The results presented in Figure 3.17 are those from the Folin-Ciocalteu method using solutions of 1.0 mM gallic or caffeic acid in combination with 0 (control), 0.1 mM, 0.5 mM, 1.0 mM, or 2.0 mM selected monophenol (p-hydroxycoumaric or p-hydroxybenzoic acid). The activity of a chemical in the FC method is scaled to the activity of a standard chemical, gallic acid, and is reported in units of µg/mL gallic acid equivalents.

Figure 3.19 shows a compilation of the electric, optical and FC results for the effect of a monophenol on the measured activity of a polyphenolic compound using solutions of either 1.0

mM gallic or caffeic acid with increasing concentrations of p-hydroxybenzoic acid or p-hydroxycoumaric acid.
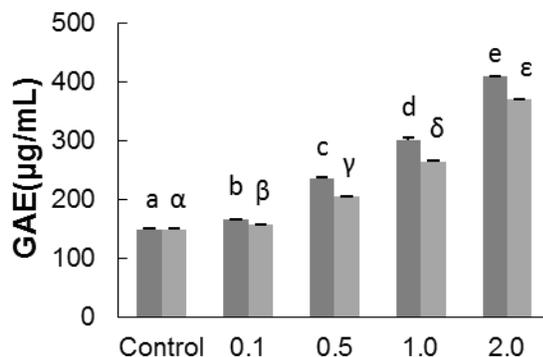
Figure 3.17. Interference of monophenols on FC determination of polyphenolic compounds. Results are espressed as gallic acid equivalents (GAE) in units of μg GAE /mL sample. 1.0 mM gallic acid with increasing amounts of p-hydroxycoumaric acid or p-hydroxybenzoic acid at five different levels are portrayed. 1 mM gallic acid with: no added monophenol (control); 0.1 mM monophenolic acid; 0.5 mM monophenolic acid; 1.0 mM monophenolic acid; or 2.0 mM monophenolic acid. The bar heights are the mean measured values from the FC method of 3 trials and the error bars represent the standard deviation of the values, while the letters above each treatment level are the Tukey's Honestly Significant Difference variables and signify treatments of significant different by an analysis of variance.

Figure 3.17 Gallic acid with p-hydroxycoumaric acid was portrayed using borderless dark gray columns and gallic acid with increasing amounts of p-hydroxybenzoic acid was depicted using borderless light gray columns.

The treatment levels of 0 mM (control), 0.1, 0.5, 1.0, and 2.0 mM p-hydroxycoumaric acid on 1.0 mM gallic acid had GAE signal values of 149.3 μg/mL GAE, 166.6 μg/mL GAE, 235.7 μg/mL GAE, 301.4 μg/mL GAE (n = 5), and 409.3 μg/mL GAE. The values from the FC method of 1.0 mM gallic acid treated with increasing amounts of p-hydroxybenzoic acid were 157.0 μg/mL GAE, 205.7 μg/mL GAE, 264.7 μg/mL GAE, and 371.3 μg/mL GAE for the 0.1, 0.5, 1.0, and 2.0 mM p-hydroxybenzoic acid treatment levels (Figure 3.17).

Each treatment of monophenolic acid on the GAE signal proved to be statistically different from the others. Each statistically different treatment level was assigned a letter which was placed above the column. Bars with the same letter above them were found to be statistically

without difference. As shown in Figures 3.17 and 3.18, each treatment level of monophenolic acid on the polyphenolic signal as measured by the FC method was distinct from the control, and further each was significantly different from each other, indicating that monophenols interfered with the signal of polyphenols in the FC method assessment. This was interpreted to mean that the monophenolic acid exerted a dose-dependent influence on the GAE signal of the FC method.

Combinations of caffeic acid with p-hydroxycoumaric or p-hydroxybenzoic acid were also tested by the FC method. The results from these solutions are presented in Figures 3.17 and 3.18.
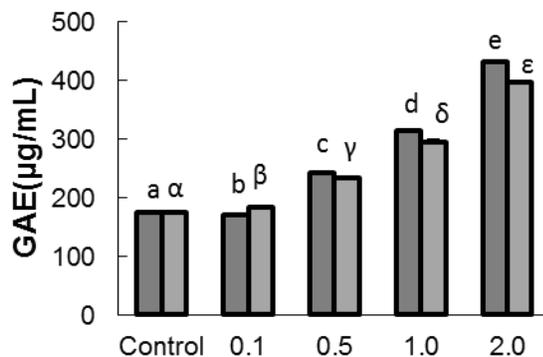

Figure 3.18. The interference of monophenolics on the GAE signal of caffeic acid. 1 mM caffeic acid with increasing amounts of p-hydroxycoumaric acid or p-hydroxybenzoic acid at five different levels. 1 mM caffeic acid with: no added monophenol (control); 0.1 mM monophenolic acid; 0.5 mM monophenolic acid; 1.0 mM monophenolic acid; or 2.0 mM monophenolic acid. All combinations of polyphenolic and monophenolic acid tested with three samples. The bar heights represented the mean values of 3 trials and the error bars represented the standard deviation of the values, while the letters above each treatment level were meant to indicate whether a treatment was statistically different from another treatment by the analysis of variance (ANOVA) with Tukey's Honestly Significant Difference test.

Figure 3.18: The treatment levels of "control", 0.1 (mM), 0.5 (mM), 1.0 (mM), and 2.0 (mM) of the monophenolic p-hydroxycoumaric acid on 1.0 mM caffeic acid had GAE values of 174.6 µg/mL GAE, 171.6 µg/mL GAE, 241.7 µg/mL GAE, 314.3 µg/mL GAE, and 432.0 µg/mL GAE (Figure 3.18).

The values from the FC method of 1.0 mM caffeic acid treated with increasing amounts of p-hydroxybenzoic acid were 183.6 µg/mL GAE, 233.6 µg/mL GAE, 294.3 µg/mL GAE, and 396.6 µg/mL GAE for the non-zero treatment levels of 0.1, 0.5, 1.0, and 2.0 mM.

When tested by the FC method, each successive treatment level of monophenolic acid was statistically different from all previous signals. This was interpreted to mean that the monophenolic acid exerted a dose-dependent influence on the signal in the FC method.

3.4.1.2 Comparison of monophenolic interference in the spectroelectrochemical and FC methods

A compilation of the optical (section 3.2.2.3), electrochemical (section 3.1.2.4), and FC (section 3.4.1.1), results of monophenolic interference experiments appears in Figure 3.19.
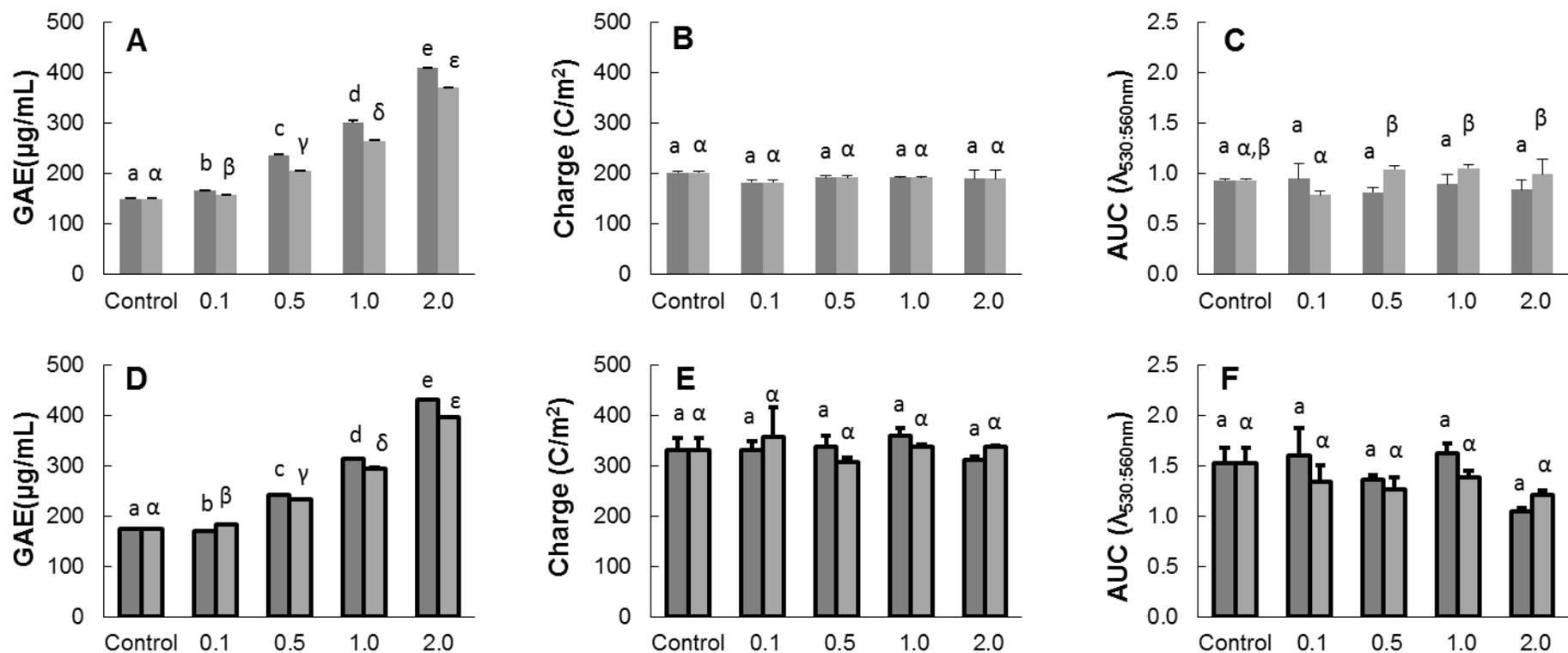
Figure 3.19. Effect of monophenolic acid on polyphenolic determinations by electrochemical and FC methods. P-hydroxycoumaric acid in dark gray, p-hydroxybenzoic acid in light gray, gallic acid in unbordered columns, caffeic acid in columns with black borders. Concentration of polyphenolic acid (gallic or caffeic) kept constant at 1.0 mM throughout. Concentration of monophenolic acid at 0 mM (control), 0.1 mM, 0.5 mM, 1.0 mM, or 2.0 mM. Chart A: GAE gallic acid and monophenolic acid. Chart B: electrical response of gallic acid and monophenolic acid. Chart C: optical response of gallic acid and monophenolic acid. Chart D: GAE of caffeic acid and monophenolic acid. Chart E: electrical response of caffeic acid and monophenolic acid. Chart F: optical response of caffeic acid and monophenolic acid. Values of a given chart that are marked by the same letter are not significantly different from one another by Tukey's Honestly Significant Difference post hoc test in an analysis of variance.

Figure 3.19 An interference experiment was conducted using polyphenols (1.0 mM gallic acid or 1.0 mM caffeic acid) in solution with increasing concentrations of a monphenolic compound (0, 0.1, 0.5, 1.0, or 2.0 mM of either p-hydroxycoumaric or p-hydroxybenzoic acid). The polyphenolic content of solutions was measured according to the electrochemical technique presented in this project and by the FC method.

Neither signal of the bimodal sensing apparatus was consistently affected by the addition of a monophenolic acid (the exception to this trend was the optical signal of gallic acid with p-hydroxybenzoic acid), while data from the FC method assay showed dose-dependent signal interference by a monohpenolic compound.

The FC method was clearly subject to interference by monophenolic acids. In comparison, the bimodal sensing assay did not yield nearly as much to the dosage of monophenolic acids. Due to constraints on the sample size and resulting power of this experiment, an analysis of equivalence could not be reliably conducted. Using the data for the 3 trials conducted a t-test analysis of equivalence among the different treatment levels of a combination of two chemicals failed. The variation of the bimodal sensing results were too great to conduct an equivalence analysis – in order to achieve the desired power for a t-test based equivalence analysis, the number of trials would have needed to be substantial. Thus, the bimodal sensing assay was interpreted to exhibit little or no interference from the addition of a monophenoic acid while the polyphenolic signal in the FC method was shown to display interference from monophenols. It was concluded that the bimodal sensing assay offered better selectivity for polyphenolic species than the FC method.

3.5 Effect of Ascorbic Acid

A second test for interference in the electrochemical determination of polyphenolic compounds was conducted using increasing concentrations of ascorbic acid. Following

evaluation for interference by ascorbic acid on a given signal or within a certain test, the responses of the electric, optical, and FC measurements were compared.

3.5.1.1 Interference by ascorbic acid on phenolic compound determination in the FC method

Ascorbic acid is known to interfere with the quantification of phenolic compounds when using the FC method. To measure this effect, solutions with 1.0 mM polyphenolic acid and increasing ascorbic acid concentrations were oxidized at 0.5 V for 1000 seconds. The ascorbic acid was tested at four levels: 0.1 mM; 0.5 mM; 1.0 mM; and 2.0 mM. The range of treatment levels of ascorbic acid between 0 and 2.0 mM was chosen to mimic the amount that would be found in natural products; 2.0 mM is about the concentration of ascorbic acid in a product offering 100 % RDA of vitamin C in 8 oz fluid, a common level of ascorbic acid in juices.

GALLIC + ASCORBIC ACIDS   **CAFFEIC + ASCORBIC ACIDS**
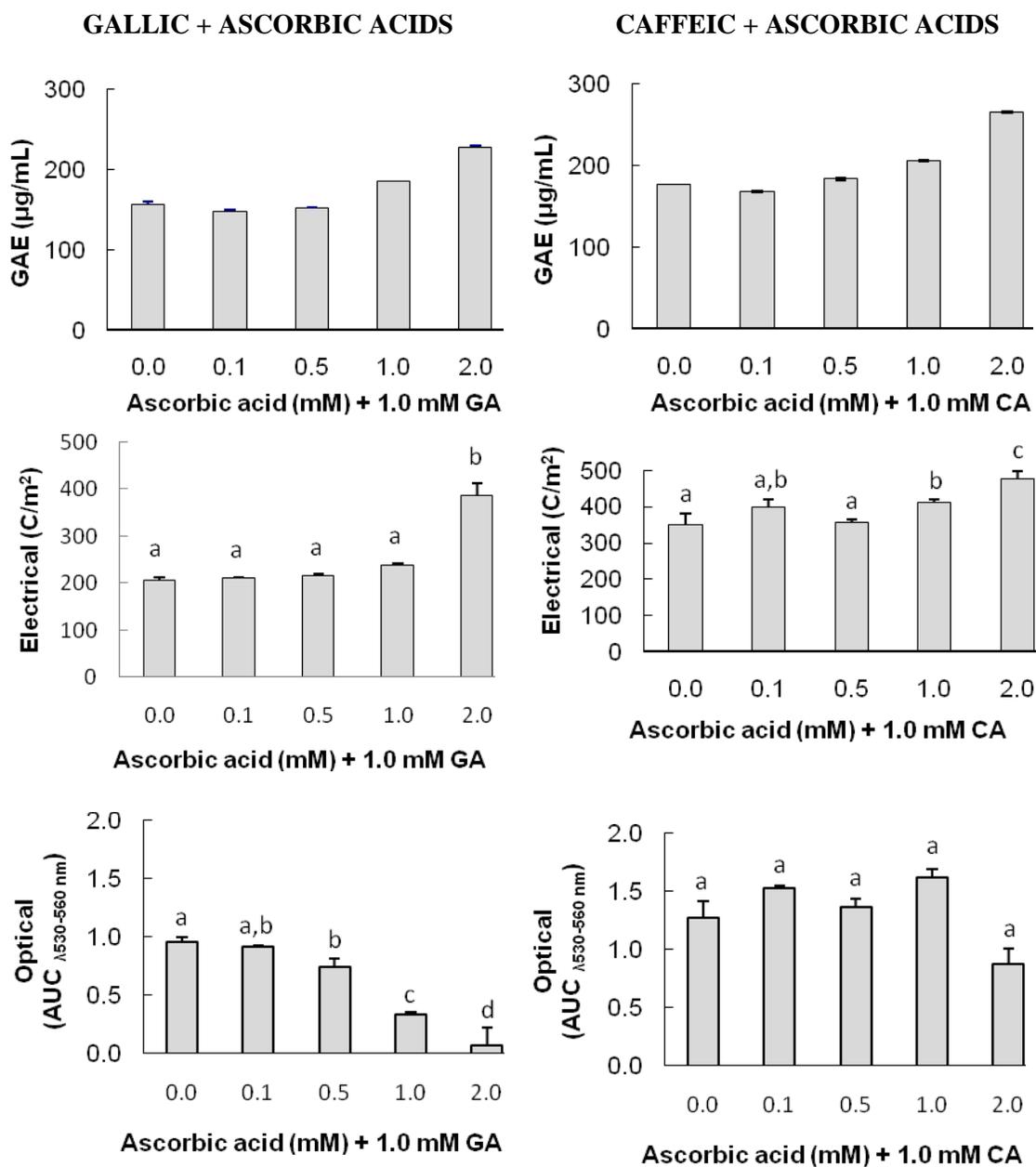
Figure 3.20. Effect of ascorbic acid on FC and electrochemical determinations of polyphenolic compounds. Solutions of 1.0 mM gallic acid (left hand column) or 1.0 mM caffeic acid (right column of charts) with increasing amounts of ascorbic acid (0, 0.1, 0.5, 1.0, and 2.0 mM) were tested using the bimodal sensing apparatus and the FC method. Column heights represent the mean values of 3 trials and the error bars represent the standard deviation of the values, while the letters above each treatment level indicate statistical difference from another treatment using Tukey's Honestly Significant Difference post hoc test in an analysis of variance (ANOVA).

Figure 3.20

FC method The treatment levels of 0, 0.1, 0.5, 1.0, and 2.0 mM ascorbic acid on 1.0 mM gallic acid had values of 157 GAE, 148 GAE, 152 GAE, 186 GAE, and 229 GAE, respectively (Figure 3.20). The electrical signals for 1.0 mM caffeic acid treated with 0.0, 0.1, 0.5, 1.0, and 2.0 mM ascorbic acid transferred charge in the amount of 177 GAE, 169 GAE, 184 GAE, 207 GAE, and 266 GAE respectively (Figure 3.20).

The dose-dependent response to ascorbic acid in the FC method had been expected. One of the known limitations of the FC method is the interference of ascorbic acid. The analysis next turned to an assessment of resistance by the electrochemical method to interference from ascorbic acid.

Electric signal: The mean values measured of treatment levels of 0, 0.1, 0.5, 1.0, and 2.0 mM ascorbic acid on 1.0 mM gallic acid were 207 C/m^2, 211 C/m^2, 215 C/m^2, 237 C/m^2, and 385 C/m^2, respectively, over the course of the oxidations.

There was a dose-dependent trend to the electric signals of gallic or caffeic acid with increasing amounts of ascorbic acid that became pronounced at 1.0 mM and higher concentrations. Gallic acid solutions with ascorbic acid at concentrations of 0, 0.1, 0.5, and 1.0 mM were not significantly different. The anodic oxidation measured from 1.0 mM gallic acid with 2.0 mM ascorbic acid was greater than lower treatment levels. The electrical signals for 1.0 mM caffeic acid treated with 0.0, 0.1, 0.5, 1.0, and 2.0 mM ascorbic acid transferred charge in the amount of 348 C/m^2, 399 C/m^2, 357 C/m^2, 412 C/m^2, and 475 C/m^2 respectively. Statistically, the measured signal of the gallic acid solutions with ascorbic acid broke into 2 groups: one group (denoted by a small 'a' over the columns of the control, the 0.1, 0.5, and 1.0 mM treatment levels of ascorbic acid and a second group (denoted with a small 'b' over the column) of the solution of gallic acid with 2.0 mM ascorbic acid.

As for the mixtures of caffeic acid with ascorbic acid, signals from the different treatment levels broke into three partially overlapping groups. The first group, denoted with a small 'a'

above the columns, included the control (1.0 mM gallic acid, 0.0 mM ascorbic acid), the 0.1 mM level of ascorbic acid (GA 1.0 mM) and the 0.5 mM level of ascorbic acid. The second group included the 0.1 mM and the 1.0 mM levels of ascorbic acid, and the third statistically distinct group included the 2.0 mM level of ascorbic acid treatment alone. The charge transferred by the control and the two least-concentrated ascorbic acid solutions (0.1, 0.5 mM) were not statistically distinct by Tukey's HSD. The mixture of 1.0 mM caffeic acid and 1.0 mM ascorbic acid showed a greater amount of oxidation than the first three mixtures, and was statistically different from the control and the 0.5 mM ascorbic acid treatment (the 0.1 mM ascorbic acid treatment was high enough that it could not be declared significantly different from either the 0 and 0.5 mM treatments or the 1.0 mM treatment).

The most concentrated treatment (with 2.0 mM ascorbic acid) responded electrically with a higher charge value and was found to be statistically different from all of the other treatments by the ANOVA.

Ascorbic acid at treatment levels of 2.0 mM interfered with the (polyphenolic) charge when tested with gallic and caffeic acid. The purpose of this experiment was to determine whether or not ascorbic acid interfered with the determination of polyphenolic content. It was shown that ascorbic acid did interfere with the measured electric signal of the bimodal sensing apparatus. However, the interference from ascorbic acid was not shown to interfere with the electric response when present in concentrations up to 0.5 mM. Experimentation to ascertain the limit of interference was beyond the scope of this project. The interference of the ascorbic acid on the electric signal was not entirely surprising. Ascorbic acid was known to have an oxidation-reduction potential of ~0.3 V, depending on the medium. At an operating potential of 0.5 V, it had been expected that some ascorbic acid would also oxidize. From the electric signal alone, it could not be determined whether the ascorbic acid oxidized in addition to, in place of, or in proportion to the amount of polyphenol oxidizing. The phenolic-specific signal in the chitosan film was expected to offer more insight into this matter.

Optical signal: The treatment levels of 0, 0.1, 0.5, 1.0, and 2.0 mM ascorbic acid on 1.0 mM gallic acid had values of 0.96 AUC, 0.92 AUC, 0.74 AUC, 0.34 AUC, and 0.07 AUC, respectively. The optical signals for 1.0 mM caffeic acid treated with 0.0, 0.1, 0.5, 1.0, and 2.0 mM ascorbic acid absorbed light in the amount of 1.27 AUC, 1.53 AUC, 1.36 AUC, 1.62 AUC, and 0.88 AUC respectively.

Interference of ascorbic acid on the bimodal sensing assay did not make the assay inferior to the FC method; simply they are both susceptible to interference from ascorbic acid. Because of the interference of ascorbic acid, at this time it would be recommended to eliminate ascorbic acid from a sample before measuring the polypehenolic content with the bimodal sensing assay.

The optical signal decreased with respect to the dosage of ascorbic acid in mixtures of gallic acid and ascorbic acid, but erratically varied in the mixtures of caffeic acid and ascorbic acid. A lack of a shared trend between the two polyphenolic compound mixtures made it impossible to typify an effect from the ascorbic acid on the optical signal. It could be that these two polyphenolic substances interacted differently with ascorbic acid and thus provided varying results, but the overall effect of interference was noted.

CHAPTER 4: CONCLUSIONS AND FUTURE RESEARCH

This study indicated that it may be possible to establish operating conditions (0.5 V for 1000 seconds over a domain from 0.5 to 5.0 mM polyphenolic substance) for the electrochemical detection of polyphenolic compounds with an optical response in a chitosan film cast across the working electrode. Two responses from the oxidation of polyphenolic samples were detected and quantified, an electrical and an optical. Linearity of the electrochemical response was verified for the working range; the ion strength on the baseline electric value was determined to be inconsequential at concentrations up to 1.0 M; and the composition of the solvent was found to influence both the electric and optical signals. Interference by monophenolic compounds on the detection of polyphenolic chemicals was not found up to 2.0 mM concentrations of monophenol, and ascorbic acid was found to interfere with both the electric and optical signals at concentrations of 1.0 mM or more (electric) and 0.5 mM or more (optical). Interference by monophenolic and ascorbic acid in the conventional Folin Ciocalteu (FC) method (for the quantification of phenolics) was also evaluated and the results compared to the results from the electrochemical analyses. Monophenols were found to interfere with polyphenolic detection by the FC method but not by the electrochemical technique presented in this thesis. Ascorbic acid was found to interfere with this electrochemical technique to a greater extent than it did with the FC method.

The use of this reagentless electrochemical technology for the investigation of polyphenolic compounds takes less time than the conventional method. However, the quantification of polyphenolic compounds using the proposed conditions is more subject to interference from ascorbic acid and influence by organic solvents than the FC assay is. Sample

refinement may be necessary in order to develop this electrochemical technique into a quantitative assay.

To complete the development of a novel assay, an equation that relates the electric and optical measurements discretely to the concentration of a polyphenolic sample is needed. Additional avenues of research may include a critical review of the parameters of optical quantification, investigation of the nature of the interference from ascorbic acid and organic solvents, and expanded testing of the apparatus' signals using a greater variety of polyphenolic samples and, ultimately, food-based samples.

Figure A.1. Comparison of trends of solvent effect on electrical and optical signals. The electrical and optical signals of A: protocatechuic acid; B: gallic acid; C: caffeic acid are superimposed. Mean values of electric and optical signals in 100% aqueous buffer are normalized to compare the relative effect a different solvent has on each signal for a given 2.5 mM concentration of the chemical.
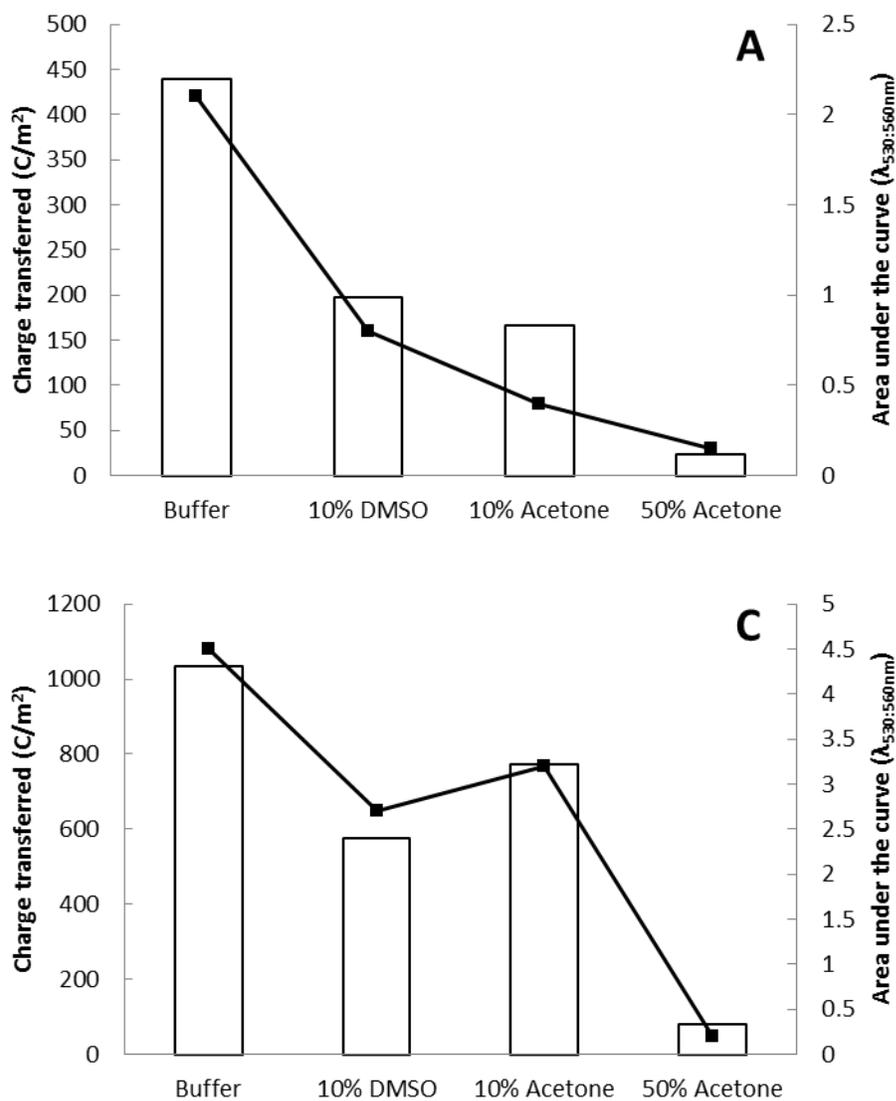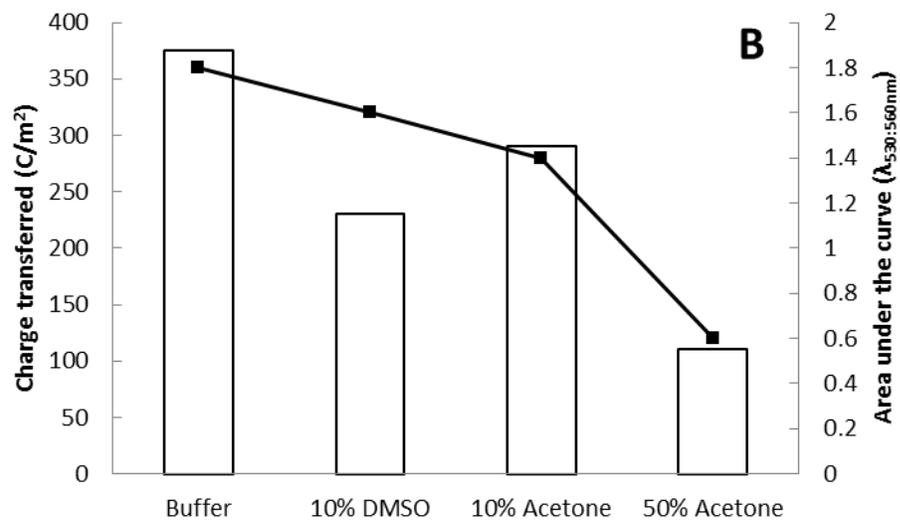
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