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# Bioavailability of Bioactive Compounds

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**ABSTRACT**

Arachidin-1 (Ara-1) and arachidin-3 (Ara-3) are stilbenoids found in hairy peanut roots, which have been shown to possess similar, if not higher, efficacy in biological activities when compared to nonprenylated analogs such as piceatannol and resveratrol. Blackberry essences are concentrated volatiles, which are characterized by low molecular weight, lipophilic properties, and have also been demonstrated to have health benefits. As interest in dietary bioactive compounds functional ingredients increase, more emphasis has been placed on identifying which compounds have high efficacy as well as bioavailability. The ability of a compound to exert its health promoting effects depends on its bioavailable dose, rather than the administered dose. The present study aimed to evaluate the stability of Ara-1 and Ara-3 under cell incubation conditions and attempts to determine bioavailability of Ara-1, Ara-3, and blackberry essences using an *in vitro* Caco-2 transport assay. Caco-2 cells were seeded on semipermeable membranes and allowed to differentiate into a morphology that resembles mature small intestine enterocytes. Sample preparation, extraction methods, and enzymatic treatments were adjusted to enhance recovery of Ara-1 and Ara-3. However, Ara-1 was shown to be more susceptible to the effects of oxidation and degradation. At this time, it is inconclusive whether Ara-1 and Ara-3 possesses higher bioavailability than its analog, resveratrol (Resv), in cell incubation conditions due to little to no recovery during transport. On the other than, individual volatiles from the blackberry essences were tentatively identified and transport rates determined based on the peak area found in the starting material. Results suggests that though esters and monoterpenes make up the two most abundant functional groups in the blackberry essence profile, higher transport rates and recovery were observed in aldehydes.

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**DEDICATION**

This thesis is dedicated to my friends and family – I would not be where I am today without their support. To my younger sister, Cynthia Sze Nga Kar, for inspiring me to take the road less traveled by. That, alone, can make all the difference.

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# CHAPTER 1

## Introduction

Bioactive compounds are essential and non-essential compounds that are usually present in small quantities as natural constituents in food and can be shown to exert a wide range of biological activities in the human body (Biesalski et al. 2009). Previous epidemiological studies have shown that the consumption of plant-derived bioactive compounds play an important role in the protection of humans from various degenerative diseases. For the past 30 years, public interest in foods that provide health benefits beyond basic nutrition has increased dramatically due to several factors, including an increase in healthcare costs and the desire to improve personal health (Kris-Etherton et al. 2002, Luthria 2006). The basis, however, is mainly due to knowledge of the association between protective properties of plant-based diets and a decreased risk of chronic diseases such as cancer and cardiovascular disease.

Berry fruits are a rich source of essences (concentrated volatiles), antioxidants, and polyphenols such as anthocyanins and phenolic acids (Nile and Park 2014, Szajdek and Borowska 2008). Blackberries, in particular, have shown to possess anti-inflammatory properties by inhibiting nitric oxide production as well as protecting against age-related diseases such as neurodegeneration (Skrovankova et al. 2015). Peanut plants (*Arachis hypogaea* L.) contain stilbenoids such as arachidin-1 (Ara-1) and arachidin-3 (Ara-3), which have also been shown to confer antioxidant and cancer-protective properties.

Current research efforts are focused not only on the development of health-promoting foods for health maintenance throughout the life cycle, but also on researching bioactive components to evaluate the necessary dosage for optimum health. Bioavailability, or the rate and extent to which the active ingredient or active moiety is absorbed from food and becomes

available at the site of action, depends on a compound's overall absorption in the body.

The ability of a compound to exert its biological effects depends on its bioavailable dose, rather than the administered dose. To date, there has been no research performed on the bioavailability of berry volatile extracts or Ara-1 and Ara-3. Understanding how these compounds are absorbed and discerning its fate in the body is an important step to determining how readily they may exert biological effects. Therefore, the **goal** of this proposed research is to determine the *in vitro* bioavailability of blackberry volatiles, arachidin-1 (Ara-1) and arachidin-3 (Ara-3) to better understand their health-promoting properties. The **hypotheses** are that 1) Ara-1 and Ara-3 will possess higher absorption potential and subsequent bioavailability than its analog, resveratrol (Resv), due to the presence of prenylated moieties and 2) blackberry volatiles will possess a moderate to high absorption potential and subsequent bioavailability due to the volatiles' low molecular weight and lipophilicity. The **objectives** of this study are to 1) evaluate the stability of Ara-1 and Ara-3 and identify the components of blackberry essences, 2) assess the bioavailability of Ara-1 and Ara-3 by measuring their uptake and transport across the Caco-2 cell monolayer and (3) assess the bioavailability of blackberry volatiles by measuring and identifying compounds transported across the Caco-2 cell monolayer.

# CHAPTER 2

## Literature Review

### Bioactive Compounds in Foods

Bioactive compounds are essential and non-essential compounds (e.g., vitamins or polyphenols) that occur in nature and can be shown to have an effect on human health (Biesalski et al. 2009). The majority are found in foods that predominantly originate from the plant kingdom - in various fruits, vegetables and grains - with some others from animal sources (Rein et al. 2013). Bioactive compounds vary widely in their chemical structures and functions; as such, they are categorized accordingly (Kris-Etherton et al. 2002). Several examples of plant-derived bioactive compounds include polyphenolics, organosulfur compounds, phytosterols, carotenoids and monoterpenes - all of which have subcategories exhibiting a diverse range of chemical and biological properties (Kris-Etherton et al. 2002). They may also differ in attributes regarding sites of action, distribution in nature, and concentrations in foods as well as the human body (Carbonell-Capella et al. 2014).

### Polyphenols

Polyphenols are present in all plants and have been extensively studied for its health effects in a variety of food sources such as legumes, nuts, vegetables, fruits, tea and red wine (Kris-Etherton et al. 2002). They are products of secondary metabolism during normal plant development in addition to acting as a defense mechanism against stressors and contributing to the coloration of plants (Liu 2013). Recent interest in polyphenols are manifold but are largely due in part to its antioxidant properties, ubiquitous abundance throughout the human diet, and their potential role in the prevention of degenerative diseases associated with chronic oxidative stress (Manach et al. 2004).

**Classification**

Polyphenols are extremely diverse, comprising more than 8,000 identified structures that vary from simple molecules to complex polymeric structures (Kris-Etherton et al. 2002). They are further categorized into non-flavonoids and flavonoids on the basis of the number of phenol rings they contain as well as the structural elements that bind them together (Manach et al. 2004). Non-flavonoids consist of phenolic acids, stilbenes (resveratrol, piceatannol, arachidin-1, and arachidin-3), lignans, and tannins (Manach et al. 2004). Flavonoids, on the other hand, can be further subdivided into classes consisting of more than 5,000 compounds, representing the most common group of phenolic compounds found in plant foods (Kris-Etherton et al. 2002). They share a common structure of two aromatic rings (A and B) that are bound by 3 carbon atoms, resulting in the formation of an oxygenated heterocycle (ring C) (Manach et al. 2004). Depending on the function of the heterocycle, flavonoids are classified as flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols, such as catechins and proanthocyanidins (Manach et al. 2004). Recently, a review reported that the intake of dietary flavonoids in Australian, European and US adult populations was between 209 to 1017 mg/day (mean 435 mg/day) (Kent et al. 2018). The variability may be attributed to access to different food supplies and different cultural eating habits (Kent et al. 2018).

The human diet is not only a means of providing sufficient nutrients for metabolic requirements, but can also be capable of providing numerous health benefits (Biesalski et al. 2009). Previously, epidemiological studies have shown that a diet based on plant-derived foods with high amounts of polyphenols may help reduce the risk of chronic diseases such as cardiovascular disease, diabetes and cancer (Liu 2003). In the last three decades, the general population's interest in functional foods, or foods that provide health benefits beyond basic

nutrition, has increased dramatically, thereby reflecting a change in the overall role of nutrition for the future (Luthria 2006). The resulting consumer demand for functional foods can be attributed to several factors, including an increase in healthcare costs, desire to improve personal health, and epidemiological evidence showing the association between protective properties of plant-based diets and a decreased risk of chronic disease (Kris-Etherton et al. 2002, Luthria 2006). Therefore, current research efforts are directed towards the development of health-promoting and functional foods for health maintenance throughout the life cycle (Rein et al. 2013).

**Health Effects of Polyphenols**

Cardiovascular disease (CVD) and cancer are the top 2 leading causes of death in the United States (CDC 2015). Approximately 610,000 men and women die from heart disease each year, while cancer collectively claims the lives of more than half a million Americans in the same time frame (CDC 2015).

Since both CVD and cancer relate to diet, researchers have investigated the role of dietary antioxidants in mitigating oxidative stress. Oxidizing agents may be attributed to intracellular metabolic activities within organisms, or they may be found in food, water, and air (Liu 2003). Though some oxidizing agents are necessary for life, it is important to maintain balance between oxidants and antioxidants for the purpose of optimal physiologic conditions in the body (Liu 2003). Oxidative stress results from the overproduction of oxidants, which can cause damage to biomolecules such as DNA, protein, and lipids, leading to an increased risk of CVD and cancer (Liu 2003). Fruits, vegetables and beverages like tea and wine contain a vast variety of polyphenols that can offset oxidative damage in cellular systems by mechanisms such

as activating signaling pathways that lead to upregulation of the expression of detoxifying enzymes (Liu 2003, World Cancer Research Fund, 2007).

Extensive studies have been undertaken to explore the link between dietary intake of polyphenols and reduced risk of CVD. A cohort study by Knekt and colleagues (1996) studied the association between intake of flavonoids and coronary mortality from 1967-1992 in 5133 Finnish men and women. The subjects ranged from ages 30-69 years and were free from heart disease at baseline. The results indicated that people with very low dietary intakes of flavonoids have a higher risk of coronary heart disease. Apples and onions, rich in the flavonoid quercetin, represented 64% of the subjects' total dietary intake and were inversely associated with total and coronary mortality. Researchers suggest that the mechanism of protection may be attributed to antioxidative effects or the inhibition of thrombogenesis (Knekt et al. 1996). Additionally, a study by Lekakis and colleagues (2005) found that red grape polyphenolics acutely improved endothelial function in 30 male patients with coronary artery disease (Lekakis et al. 2005). Subjects were either administered 600 mg of red grape extract dissolved in 20 mL of water, or 20 mL of water as the placebo. The extract contained 4.32 mg epicatechin, 2.72 mg catechin, 2.07 mg gallic acid, 0.9 mg trans-resveratrol, 0.47 mg rutin, 0.42 mg epsilon-viniferin, 0.28 mg, p-coumaric acid, 0.14 mg ferulic acid and 0.04 mg quercetin per gram (Lekakis et al. 2005).

The American Institute for Cancer Research and World Cancer Research Fund (2007) reported that an estimated 30-40% of all cancers can be prevented from lifestyle habits and diet alone. The relationship between intake of antioxidant flavonoids and risk of cancer was investigated in a prospective study from 1967-1991 amongst 9959 Finnish men and women aged 15-99. Their findings revealed an inverse association between flavonoid consumption and incidence of all sites of cancer combined. More specifically, the consumption of apples shared an

inverse correlation with subsequent incidences of lung cancer (Knekt et al. 1997). In another 8-year prospective cohort study of postmenopausal women, it was found that those who consumed ≥2 cups of non-herbal tea - mainly black tea - had a slightly lower risk for all cancers compared with women who either never consumed or only occasionally consumed tea (Zheng et al. 1996). Tea contains a vast array of antioxidative phenolic compounds such as catechin, epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate (Bhagwat et al. 2003). Depending on whether or not the tea leaves are fermented, other active components can include theaflavins and thearubigins (Bhagwat et al. 2003). The length of fermentation dictates what kind of tea will be produced. For example, green tea is not fermented and contains mostly catechins, while black tea is fully fermented and contains mostly thearubigins and theaflavins (Bhagwat et al. 2003).

**Volatiles**

Volatile compounds are generally lipophilic compounds with a low molecular weight (<300 Da) that vaporize readily at room temperature due to high vapor pressure (Kalua et al. 2007). If the compound is sufficiently volatile, it is perceived as a smell or odor after it reaches the olfactory epithelium, dissolves into the mucus, and bonds with olfactory receptors (Kalua et al. 2007). The most well-known volatile organic compounds (VOCs) are industrial solvents, with many of them being widely use in commercial applications (Pennerman et al. 2015). However, solvents such as benzene, toluene, glycol ethers, chloroform and others have been linked to both long-term and short-term negative effects on human health (Pennerman et al. 2015). On the other hand, little is known about the potential effects on human health – negative or beneficial - of biogenic VOCs (Pennerman et al. 2015). Biogenic VOCs produced by plants are considered secondary metabolites that are not directly involved in the growth process, but serve to help the

plant survive in its environment. More than 100,000 chemical products are known to be produced by plants, and 1,700 of these are categorized as volatiles (Spinelli et al. 2011).

Throughout time, it has been known that both floral and vegetative parts of plants emit unique smells that have been used to enhance the quality of foods. Since then, they have remained commercially important to the food, pharmaceutical, agricultural and chemical industries as flavorants, drugs, pesticides and feedstocks (Schwab et al. 2008). Not only do VOCs possess the ability to attract pollinators, but they also exhibit anti-microbial and/or anti-herbivore activity, modulate acquired resistance to pests, and protect the plants' reproductive parts from external threats (Aprea et al. 2015, Dudareva et al. 2004). Fruits, in particular, produce a wide range of volatile compounds that contribute to the fruits' characteristic aroma and flavor (El Hadi et al. 2013). Such compounds are biosynthesized through metabolic pathways during the growth, harvest, post-harvest, and storage phases, reaching their highest concentrations during ripening (Yang et al. 2009).

**Classification**

Plant-derived VOCs can be categorized based on their chemical structures. They are mainly comprised of terpenoids, esters, aromatics, amines, alcohols, aldehydes, ketones, lactones, thiols, and other miscellaneous compounds. Terpenoids, an important contributor to flavor and aroma in many fruits, are responsible for the smells given off by plants such as pine trees (pinene), ginger (zingiberene), and citrus fruits (limonene) (El Hadi et al. 2013). A single fruit can possess a high number of volatiles that span a wide range of chemical structures. Apples, for example, have been reported to contain over 300 volatiles, with esters being the most abundant volatile compound found within (El Hadi et al. 2013). Volatiles may also be expressed in different concentrations and identities between cultivars. The unique aroma of "Fuji" apples

are largely due in part to ethyl 2-methyl butanoate, 2-methyl butyl acetate, and hexyl acetate, while hexyl acetate, hexyl 2-methyl butanoate, hexyl hexanoate, hexyl butanoate, 2-methylbuutyl acetate, and butyl acetate are the most prominent volatile compounds in "Pink Lady" cultivars (El Hadi et al. 2013). Blackberry volatiles, on the other hand, are mostly comprised of esters and aliphatic alcohols, followd by terpenic and aromatic compounds, aldehydes, and ketones. A total of 74 volatiles were identified an characterized based on GC and MS data in a recent study performed by D'Agostino and colleagues (2015).

**Health Effects of Volatiles**

The attempt to discern the role of plant volatiles beyond its flavor-contributing attributes has gained momentum in recent years. As such, research studies that explore the potential of volatiles to exert biological and pharmacological activities in human health have begun to emerge. In a study by Hannemann and colleagues (2014), it has been shown that aromatic aldehydes such as *o*-vanillin markedly reduces the complications of sickle cell disease by inhibiting polymerization, maintaining red blood cell hydration, and reducing the concentration of sickle hemoglobin (Hannemann et al. 2014). Additionally, phenolic acids and monoterpenes found in Asian medicinal mushrooms have also been found to exhibit antioxidant and antimicrobial properties. For example, cinnamic acid and one of its metabolites have minimum inhibitory concentrations ($IC_{50}$) of 0.7~30 μg/mL against certain bacteria and fungi, while those of monoterpenes alpha-terpineol and linalool against pathogenic and spoilage-forming bacteria range from 0.5%-2% (Pennerman et al. 2015). In another study by Hirota and colleagues (2010), the anti-inflammatory effects of limonene, a cyclic terpene commonly found in citrus fruits, on human eosinophilic leukemia HL-60 clone 15 cells was investigated. Results show that limonene could have potential anti-inflammatory efficacy in the treatment of bronchial asthma by

inhibiting cytokines, reactive oxygen species production, and inactivating eosinophil migration (Hirota et al. 2010).

## Overview of Bioavailability

The ability of a bioactive compound to exert its health-promoting effects upon a living organism depends on its bioavailable dose instead of the administered dose (Holst and Williamson 2008). The bioavailable dose is a more accurate representation of what the body is capable of absorbing, distributing, metabolizing and excreting (ADME) as opposed to what is administered or how much of a compound is present in specific foods. Understanding this concept is integral to optimizing diets and setting appropriate recommendations for human consumption (Caballero et al. 2015). The extent to which a compound will be present in appropriate concentrations at its site of action depends on a variety of factors including dose, formulation, the route of administration, the rate and extent of absorption, distribution through systemic circulation, biotransformation, and excretion (Caldwell et al. 1995). As a result, the term "bioavailability" was introduced to quantitate the fraction of administered compound that is made available to the body.

### Definition of Bioavailability

According to the FDA, bioavailability is defined as "the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action" (FDA, 2016). However, the scope of bioavailability is not just limited to the common oral route of administration for both drugs and bioactive food compounds; in fact, how a dosage form is introduced to the body can have various implications on bioavailability. Routes of administration can either have a systemic effect when delivered enterally (drug placed directly into the gastrointestinal tract) and parenterally (drug bypasses gastrointestinal tract by being placed directly into systemic circulation), or a local effect (such as when a substance is applied

topically where the effect is desired) (Verma et al. 2010). Enteral routes include oral, sublingual, and rectal methods, while parenteral routes include injecting drugs intravascularly, intramuscularly and subcutaneously, or without injections via inhalation (Verma et al. 2010). Each route possesses a different onset of action, or the length of time needed for a drug to exert its effect, and are unique in its advantages and limitations. For example, compounds administered orally are convenient, but must undergo first pass metabolism in which intestinal and hepatic degradation can greatly reduce its concentration before reaching systemic circulation. Oral routes of administration may have average onset of action of 30-90 min , whereas inhalation of gaseous and volatile agents is preferred for drugs that are easily degraded in the gut (Verma et al. 2010). In this way, the compound bypasses first pass metabolism and leads to a more rapid onset of action - usually 2-3 min - due to ease of access into circulation (Verma et al. 2010).

The aforementioned definition of bioavailability is slightly modified to encompass the four stages of ADME from a nutritional standpoint: an emphasis on the oral route of administration through consumption, distribution through systemic circulation to targeted tissues and organs, metabolism through biotransformation, and urinary or fecal excretion (Kussmann et al. 2007).

Compared with pharmaceutical drugs, the bioavailability of polyphenols is difficult to discern due to the complexity of food compounds, factors affecting the digestive process, and different absorptive mechanisms involved with water soluble and lipid soluble molecules (Rein et al. 2013). Additionally, unlike macronutrients, the bioavailability of polyphenols can vary widely after ingestion. Protein, fat and carbohydrates have a higher rates of absorption and utilization - usually more than 90% of the amount ingested - resulting in a higher bioavailability

(Caballero et al. 2015). This is due to many active transport systems in the body for fatty acids, amino acids, small peptide and sugars, which allows macronutrients to be readily absorbed and distributed throughout the body (Caballero et al. 2015). Bioavailability is an essential step in researching functional foods and its associated health claims to evaluate the necessary dosage for optimum health, as the biological properties of bioactive compounds depend on their absorption in the gut and subsequent bioavailability.

A prerequisite to assessing bioavailability is bioaccessibility, or the fraction of a food constituent that, as a result of being released from the food matrix, becomes present in the gut and may pass through the intestinal barrier (Saura-Calixto et al. 2007). A compound must be released from the food matrix, whether by digestive enzymes or colonic microflora, and in the right form to be absorbed before it can become bioaccessible (D'Archivio et al. 2007). The caloric content and makeup of the food matrix can affect the bioaccessibility of digested compounds. For example, an *in vitro* study showed that the bioavailability of isoflavones in food was higher when consumed with fat and protein than compared to isoflavone supplements consumed without food (Walsh et al. 2003). In a human study, researchers determined that carotenoid bioavailability increased in human subjects when salads were consumed with full fat and reduced fat salad dressing, but decreased when compared with consumption of salads with fat free salad dressing (Brown et al. 2004). No absorption of carotenoids was observed when salads with fat-free salad dressing was consumed, as carotenoids are fat-soluble compounds.

Overall, bioaccessibility is a crucial step to consider before assessing a compound's bioavailability. If a bioactive compound cannot be released from the food matrix in which it is bound, it cannot be potentially bioavailable or susceptible to absorption through the gut barrier.

**Factors Affecting Bioavailability**

As mentioned previously, the bioavailability of nutrients can be highly variable and influenced by both exogenous factors (complexity of food matrix, molecular structure of compounds) and endogenous factors (intestinal transit time, metabolism and extent of conjugation) (Holst and Williamson 2008).

One particularly important endogenous factor to consider is the basic structure (molecular size, degree of polymerization, solubility) of the bioactive compound, which determines the rate and extent of intestinal absorption. For example, most polyphenols exist as esters, glycosides, or polymers, but are not directly absorbable in their native forms. All flavonoids except flavanols exist in glycosylated forms, consisting of one or more sugar moiety and an aglycone (Pandey and Rizvi 2009). It is predicted that in order for passive diffusion of aglycones to occur across the small intestine brush border, hydrolysis of the hydrophilic sugar moiety will likely be necessary (Scalbert and Williamson 2000). The fate of glycosides is still unclear, but it is plausible that most glycosides can resist acid hydrolysis in the stomach to arrive intact at the intestine, where only aglycones and very few glycosides can be absorbed (Pandey and Rizvi 2009). Typically, the sugar moieties in flavonoids consist of glucose or rhamnose, but can also be a number of other sugars as well.

Glycoside activities can occur by way of enzymes such as β-glucosidase and lactase-phlorizin hydrolase in the brush border of the small intestine, depending on the linked sugar (Rein et al. 2013). However, if a flavonoid possesses a rhamnose moiety, absorption cannot occur until the colonic microflora cleaves the sugar moiety from the aglycone (Rein et al. 2013). The site of absorption of one polyphenol may be entirely different than another, as some polyphenols are well absorbed in the small intestine, while others may not be absorbed until it

reaches the colon or other parts of the gastrointestinal (GI) tract. Additionally, absorption can depend on molecular weight, as in the case of oligomeric polyphenols and complex lipids. Due to their high molecular weight, these compounds are unlikely to pass through intestinal epithelial cells unless they are broken down first (Rein et al. 2013).

The metabolism and extent of conjugation for bioactive components also play a role in their bioavailability. As the parent compound moves through the course of absorption, it may also undergo extensive metabolism to increase solubility and facilitate excretion from the body. Though the process of conjugation can produce active metabolites - such as those from some dietary polyphenols - it can also decrease the total amount in the blood stream, thus decreasing bioavailability (D'Archivio et al. 2007). In the case of most aglycones after absorption into enterocytes, conjugation reactions create metabolites that are also substrates for transporters such as multi drug resistance-associated protein 2 (MRP2) (Kanazawa 2011). Active excretion by MRP2 effluxes the metabolite back into the digestive lumen. However, other transporters such as MRP1 may play a role in transporting polyphenols into the bloodstream instead (Bohn 2014).

In the large intestine, bacteria may participate in the metabolism of polyphenols or the resulting metabolites that have been re-excreted through enterohepatic circulation. Though microbes may decrease the total amount of a parent compound, it has the potential to produce active metabolites, as in the case of equol (Bohn 2014). Equol is a highly bioavailable metabolite of daidzein, a bioactive compound found in soybeans, and has been shown to exhibit a stronger estrogenic activity than daidzein (Ohtomo et al. 2008).

**Bioavailability Studies of Bioactive Components**

The main goal of bioavailability studies is to determine which bioactive food compounds are the most well absorbed, which are the most active metabolites, and which lead to the formation of active metabolites (D'Archivio et al. 2007). Categories of studies focusing on

bioavailability include *in vitro* methods such as cellular assays and *in vivo* models with animals and human subjects, which are further subcategorized into epidemiological and intervention studies (Biesalski et al. 2009).

*In vitro* models used in the assessment of the bioavailability of bioactive compounds include simulated gastrointestinal digestion, artificial membranes, Caco-2 cell cultures and more. Simulated gastrointestinal digestion, the most widely used *in vitro* method for assessing bioaccessibility and bioavailability, typically consists of homogenization of the sample, lowering the pH and adding pepsin to mirror that of gastric digestion, readjusting the pH and adding pancreatic enzymes for the small intestine phase, followed by centrifugation to isolate desired fractions (Carbonell-Capella et al. 2014). Advantages of this method include the ability to test specific mechanisms of actions as well as the efficiency of each digestion, absorption, or transport mechanism. Additionally, this method is relatively inexpensive and simple to use (Carbonell-Capella et al. 2014). However, disadvantages also present barriers such as the absence of homeostatic mechanisms and the inability to reproduce the dynamic conditions within the GI tract that could otherwise be achieved in cell culture models (Carbonell-Capella et al. 2014).

Caco-2 cell culture assays consist of human colon epithelial cancer cell lines cultured as a monolayer, usually on semipermeable plastic supports that fit into multi-well plates. In this way, Caco-2 cells differentiate to form tight junctions that serve as a model for human intestinal epithelial cells in the gut. Since oral administration is the most common route for the delivery of food, the effectiveness of a compound to reach its intended site of action is dependent on intestinal absorption. To test bioavailability, compounds of interest are added to the apical or basolateral chambers and incubated. After a length of time, aliquots are taken from both sides

and measured for the concentration of the compound (van Breemen and Li 2005). The rates of permeability are then calculated to determine the apparent permeability coefficients ($P_{app}$) that represent how readily a compound is absorbed from the lumen. The Caco-2 model is advantageous for *in vitro* studies mainly due to the cell line's ability to form into a monolayer that closely mimics that of normal human intestinal epithelium, allowing researchers deeper insight into the oral absorption of dietary compounds. However, there are still disadvantages in regards to the Caco-2 model, including the absence of mucin, lack of cellular heterogeneity, and the inability to study regional differences (Ingels and Augustijns 2003).

In animal studies, experiments are typically designed with rat and mice models using a variety of methods, including *in situ* gastric administration, stomach intubation, Using chambers, and the supplementation of bioactive compounds into control diets. Crespy and colleagues (2002) observed that quercetin, but not its glycosides, is absorbed from the rat stomach through an *in situ* gastric administration of quercetin, isoquercitrin (quercetin 3-*O*-glucose), and rutin (quercetin 3-*O*-glucose-rhamnose) (Crespy et al. 2002). The methodology of flavonol administration involved anesthetizing Wister rats before filling the stomach *in situ* with a mix of 6 mL buffer and 15 μmol/L of the selected bioactive compound, then collecting stomach contents, bile, and blood samples for HPLC analysis. In another study, McGhie and colleagues (2003) observed that anthocyanins from berry fruits (boysenberry, black raspberry and blueberry) are absorbed and excreted largely unmetabolized, thus rendering low bioavailability (McGhie et al. 2003). Five male subjects consumed 300 mL of water at 7:00 am on the morning of the experiment, and an additional 300 mL of water was consumed every hr for the next 10 h. At 10:00 am, blackcurrant, boysenberry, or blueberry extract (total volume of 300 mL) was consumed instead. Each subject voided their bladder at every hr starting at 10:00 am until 4:00

pm. The total amount of all anthocyanins excreted as a percentage of the administered dose was less than 0.1%, confirming data from previous studies that the apparent bioavailability of anthocyanins is considerably lower than for catechin or quercetin. Additionally, it was observed that despite the high level of intersubject variation, anthocyanin absorption seems to be influenced by chemical structure of both the sugar moiety and phenolic aglycone. For example, bioavailability was apparently lower for delphinidin 3-*O*-glucoside (0.042%) and cyanidin 3-*O*-glucoside (0.040%) in comparison to delphinidin 3-*O*-rutinoside (0.067%) and cyanidin 3-*O*-rutinoside (0.63%) (McGhie et al. 2003).

In human studies, the administration of bioactive compounds are through oral consumption of a single dose provided as a pure compound, plant extract, whole food, or whole beverage to healthy subjects. Like animal studies, the pharmacokinetics and extent of absorption are typically analyzed by measuring plasma concentrations and/or urinary excretion. In a study by Setchell and colleagues (2003) on the bioavailability, disposition, and dose-response effects of soy isoflavones, 10 healthy women were given, on separate occasions, 10, 20 or 40 g of toasted soy nuts to eat in a single bolus after an overnight fast (Setchell et al. 2003). This dosage reflected increasing amounts of the conjugated forms of daidzein (6.6, 13.2 and 26.4 mg) and genistein (9.8, 19.6 and 39.2 mg). Daidzein and genistein were absorbed quickly, reaching maximum serum concentrations ranging from 2-8 h after ingestion. The bioavailability showed a curvilinear relationship with increasing dosages of isoflavones ingested, showing reduced systemic availability that is likely attributed to reduced absorption of isoflavones (Setchell et al. 2003). In another study on flavanone bioavailability in humans after ingestion of two doses of orange juice, 5 healthy men were recruited to ingest 1 L then 0.5 L of orange juice on two different days after an overnight fast, separated by a wash-out period of 15 days (Manach et al.

2003). The juice contained 444 mg/L of hesperidin and 96 mg/L of narirutin. Researchers have found that flavanone metabolites appeared in plasma 3 h after ingestion, with maximum concentrations appearing between 5-7 h. The peak plasma concentration of hesperetin was 1.28 μmol/L, while naringenin was 0.20 μmol/L after the 1 L dose. Relative urinary excretion was similar for the two compounds and did not depend on the dose: values ranged from 4.1% to 7.9% of the intake. Since urinary elimination was almost complete after 24 h, it was suggested that sustained effect of flavanones depend on a regular consumption of citrus fruits and related products (Manach et al. 2003).

**Berry Volatiles**

The aroma of berries play a major role in its overall quality in fresh or processed fruits. In the case of red raspberries, over 300 volatile compounds have been reported so far. Strawberries, on the other hand, possess one of the most complex fruit aromas. There are approximately 350 volatile compounds present in strawberries, with furaneol and mesifurane as the more dominating aroma compounds (Abbott et al. 2010). Esters make up over 90% of the total number of volatiles in ripe strawberry fruit, while aldehydes and alcohols are responsible for the green, unripe notes in strawberry aroma (El Hadi et al. 2013). Meanwhile, esters and alcohols make up the top two most abundant volatiles in blackberries, with the sweet, fruity and green notes attributed to volatiles such as terpinen-4-ol and 2-heptanol (Meret et al. 2011).

Limited research has been done on the bioavailability of specific purified berry volatiles such as ethyl acetate, hexanal, α -terpinene, and terpinen-4-ol. It may be of interest to explore the prospective health benefits of volatile and semi-volatile terpenic compounds as well as its bioavailability through inhalation (Salvador et al. 2016). The concepts and mechanisms outlined from studies on the bioavailability of volatile aromatic compounds, herbal medicinal products

and essential oils may serve to further research on the bioavailability of berry volatiles. Herbal medicinal products have long been used as a household remedy against colds, muscle aches, and gastrointestinal disturbances. The efficacy of natural volatiles such as those contained in essential oils is well-established for issues such as chronic pulmonary obstruction and acute bronchitis (Kohlert et al. 2000). For example, clinical studies have been undertaken to evaluate the efficacy of peppermint oil for the treatment of irritable bowel syndrome and tension-type headaches (Kohlert et al. 2000). Essential oil components are usually administered through the dermal, pulmonary, or oral routes (Kohlert et al. 2000). Monoterpenoid compounds such as camphor, limonene and 3-carene are readily absorbed through dermal application due to their lipophilic profile (Kohlert et al. 2000). Volatiles, particularly monoterpenes, are well suited to be absorbed into the body via inhalation (Kohlert et al. 2000). The compounds may be absorbed by the lung, in which case systemic availability could be possible (Kohlert et al. 2000). However, factors such as the structure of compound and the breathing mechanics of subjects could influence the degree of absorption during inhalation. Overall, the fate of essential oil components in the body depend on their chemical structure. The major excretion pathway was through the kidneys and lungs, with a minor pathway being fecal excretion (Kohlert et al. 2000). Due to their volatility, essential oil components or their metabolites are likely to be exhaled (Kohlert et al. 2000).

### *In Vitro Studies*

A study by Heinlein and colleagues (2014) investigated the permeability of hop aroma compounds across Caco-2 monolayers, those of which included linalool (terpene alcohol found in strawberries, blackberries, blueberries, red raspberries, black raspberries and cranberries), nerol (monoterpene found in blackberries), and β-myrcene (monoterpene found in black

raspberries) (Heinlein et al. 2014). Stock solutions of compounds in dimethylsulfoxide (DMSO) were prepared, and diluted to a final concentration of 100 μM for each individually tested compound. Nerol exhibited a high initial increase and the beginning of a steady-state after 80 – 100 min, while linalool showed a slower increase and an earlier steady-state after 60 min. Permeability values for β-myrcene remained constant over time. The recovery percentages of nerol, linalool, and β-myrcene were 58.8%, 25.6%, and 12.3%, respectively.

***In Vivo Studies***

Several *in vivo* studies of various berry volatiles have been untaken over the years, including an animal study by Sporstøl and Scheline (1982) that observed the metabolism of raspberry ketone – one of the main volatile compound in raspberries - in rats, guinea pigs and rabbits. Animals were given intragastric dosages of 1 mmol/kg raspberry ketone, and it was found that approximately 90% of the dose was recovered as metabolites in 0-24 h urines in all 3 species (Sporstøl and Scheline 1982). Additionally, urinary metabolite excretion was rapid and nearly complete within 24 h. The most prominent urinary metabolites were raspberry ketone and its corresponding carbinol, both conjugated through glucuronidation and/or sulfonation (Sporstøl and Scheline 1982). Out of the three species, rabbits showed the greatest extent of ketone reduction into carbinol due to the animal's abundance of ketone reductases. Rats excreted the highest number of metabolites, while guinea pigs showed the simplest pattern of metabolism. In another animal study by Igimi and colleagues (1974), the absorption, distribution, and excretion of *d*-limonene – a monoterpene found in many volatile oils as well as black raspberries – was investigated. *D*-limonene was labeled with $^{14}C$ and administered to 21 male Wistar rats (800 mg/kg) orally by stomach tube. *D*-limonene is absorbed rapidly from the GI tract during several

hr, followed by rapid elimination with no significant accumulation in the body (Igimi et al. 1974).

**Arachidin-1 and Arachidin-3**

The prenylated stilbenoids arachidin-1 (Ara-1) and arachidin-3 (Ara-3) are bioactive compounds derived from the root of peanuts (*Arachis hypogaea)*. Both Ara-1 and Ara-3 are derivatives of resveratrol, another stilbenoid compound that has been studied extensively for its biological properties (Huang et al. 2010) (Figure 1). Ara-1 and Ara-3 have attracted the interest of researchers in an effort to further investigate its diverse biological activities, especially its antioxidant and anti-inflammatory effects. Ara-1 and Ara-3 exhibit higher degree of biological activities such as antioxidant, anti-inflammatory, cardioprotective, antiviral and anticancer properties *in vitro* with the potential in improving human health compared to resveratrol and piceatannol, their nonprenylated analogues (Yang et al. 2015). Huang and colleagues (2010) observed that Ara-1 induces programmed cell death in human leukemia HL-60 cells in a manner that shows higher efficacy in comparison to resveratrol (Huang et al. 2010).

Resveratrol, along with quercetin, has been suggested to play a role in lowering the prevalence of coronary heart disease among red wine drinkers, with potential mechanisms including inhibition of LDL oxidation, platelet aggregation, and eicosanoid synthesis (Kaldas et al. 2003). Studies have shown that certain human populations that consume a moderate amount of red wine experience lower incidences of heart disease - this observation has since been labelled “The French Paradox” (Abbott et al. 2010). Although resveratrol is known for its health benefits, it has been reported to have low bioavailability in the body (Meng et al. 2004, Walle et al. 2004). Meng and colleagues (2004) observed that more than 90% of resveratrol was conjugated into other forms after 30 min of ingesting resveratrol. Resveratrol possesses limited

oral bioavailability, consisting of rapid absorption and extensive metabolism, leading to the production of various conjugated metabolites that could otherwise be responsible for its biological effects (Yang et al. 2015).

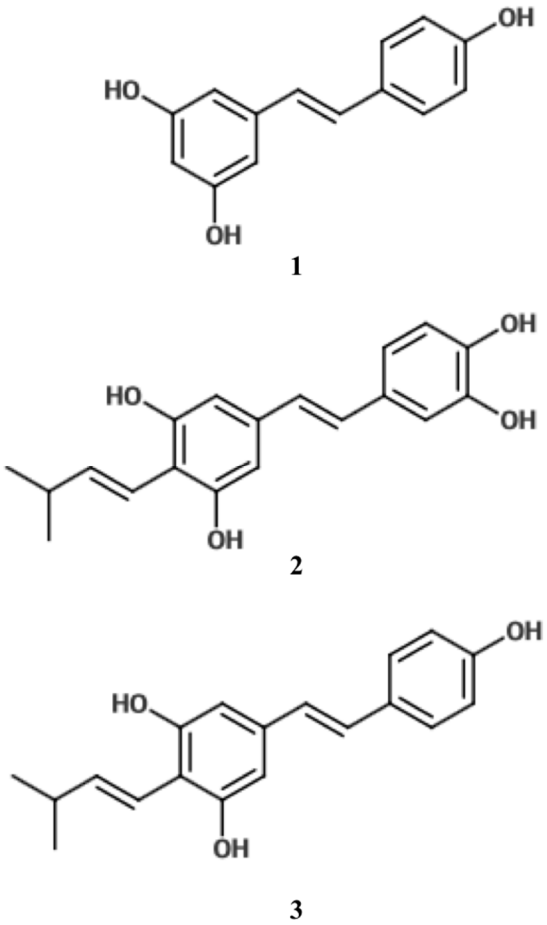


**Figure 1:** Chemical structures of trans-resveratrol (1), trans-arachidin-1 (2), and trans-arachidin-3 (3) (Adapted from Condori et al. 2010).

***In Vitro Studies***

Currently, bioavailability studies on Ara-1 and Ara-3 using the Caco-2 transport assay have not yet been conducted. Most published research on the bioavailability of stilbenoids have primarily focused on resveratrol. Because of resveratrol's related chemical structure to that of Ara-1 and Ara-3, estimations of the bioavailability of these two compounds can be made. A study by Brents and colleagues (2012) found that Ara-1 and Ara-3 are metabolized to a lesser

degree than its non-prenylated parent compounds, piceatannol and resveratrol. This suggests that the lipophilic side chains of Ara-1 and Ara-3 may disrupt the glucuronidation process, thereby enhancing the compounds' bioavailability (Brents et al. 2012). Another study by Brents and colleagues (2012) evaluated the activities of eight human UDP-glucuronosyltransferases (UGTs) towards Ara-1, Ara-3, *trans*-piceatannol, and *trans*-resveratrol. The findings indicated that prenylated stilbenoids may be preferable alternatives to *trans*-resveratrol due to its slower metabolism. Since this occurs in response to *in vitro* metabolism by UGT isoforms found in the liver and intestine, which are the main tissues responsible for metabolism of these compounds *in vivo*, the results may be indicative of the same effects during human metabolism (Brents et al. 2012).

***In Vivo Studies***

Though *in vivo* bioavailability characteristics of Ara-1 and Ara-3 have yet to be elucidated, studies on resveratrol within both animal and human models may serve to shed light on the characteristics of its prenylated analogs. In the past, research has shown that resveratrol is orally absorbed up to about 75% in humans, yet extensive metabolism in the intestine and liver ultimately brings oral availability down to less than 1% (Walle 2011). An increased dosage and repeated dose administration does not seem to alter availability significantly (Walle 2011). In a study by Walle and colleagues (2004), 6 human subjects were administered single doses of resveratrol orally (25 mg) and intravenously (0.2 mg). $C_{max}$ was reached at about 1 h after oral ingestion of 491 ng/mL, with a second peak at 6 h after the dose of 290 ng/mL (Walle et al. 2004). The plasma concentrations then declined rapidly. After the intravenous dose, plasma concentrations rapidly decreased in the first hr without any second peaks, unlike after oral dosage (Walle et al. 2004). Despite this, only trace amounts of unchanged resveratrol (<5 ng/mL)

could be detected in plasma, and most of the oral dose was recovered in urine (Walle et al. 2004). Rapid sulfate conjugation by the intestine and liver appears to be the rate-limiting step in resveratrol's bioavailability, thus leading to resveratrol's low bioavailability (Walle et al. 2004).

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# CHAPTER 3

## Bioavailability of Bioactive Compounds in Peanuts


### Abstract

Arachidin-1 (Ara-1) and arachidin-3 (Ara-3) have been shown to possess similar, if not higher, efficacy in biological activities when compared to nonprenylated analogs such as piceatannol and resveratrol (Resv). Although Resv is highly studied for its diverse biological effects, it possesses extremely low bioavailability. The ability of a compound to exert its health promoting effects depends on its bioavailable dose, rather than the administered dose. Therefore, this study evaluates the stability of Ara-1 and Ara-3 under cell incubation conditions and attempts to determine bioavailability using an *in vitro* Caco-2 transport assay. 100 - 200 ppm of Resv (positive control), Ara-1, and Ara-3 were added separately to Caco-2 cells in semipermeable inserts to evaluate absorption and transport. 0.5 mL aliquots were withdrawn at various time points and analyzed using HPLC to determine rate of transport. Sample preparation, extraction methods, and enzymatic treatments were adjusted to enhance recovery of Ara-1 and Ara-3. However, Ara-1 was shown to be more susceptible to the effects of oxidation and degradation, which may be attributed to its extra hydroxyl group, in comparison to Ara-3. At this time, it is inconclusive whether Ara-1 and Ara-3 possesses higher bioavailability than its analog, Resv, in cell incubation conditions due to little to no recovery during transport.

## Introduction

The prenylated stilbenoids arachidin-1 (Ara-1) and arachidin-3 (Ara-3) are bioactive compounds derived from the root of peanuts (*Arachis hypogaea)*. Both Ara-1 and Ara-3 are analogs of resveratrol (Resv), a well-known stilbenoid that has been studied due to its diverse biological properties (Huang et al. 2010). However, more recently, Ara-1 and Ara-3 have attracted the interest of researchers who are interested in its health-promoting properties, especially its antioxidant and anti-inflammatory effects. Ara-1 and Ara-3 exhibit higher degrees of biological activities such as antioxidant, anti-inflammatory, cardioprotective, antiviral and anticancer properties *in vitro* with the potential in improving human health in comparison to Resv and piceatannol, their nonprenylated analogues (Yang et al. 2015).

Although Resv is widely studied, it has been reported to have low bioavailability in the body due to extensive metabolism and rapid absorption (Meng et al. 2004, Walle et al. 2004, Yang et al. 2015). Although bioavailability studies on Resv have been performed, no work has been done on the bioavailability of Ara-1 and Ara-3 using Caco-2 transport assays. Previous studies have noted that Ara-1 and Ara-3 may be less susceptible to extensive metabolism due to the ability of lipophilic side chains to disrupt the glucuronidation process, thereby enhancing the compounds' bioavailability (Brents et al. 2012). Therefore, the objectives of this study were to 1) evaluate the stability of Ara-1 and Ara-3 under incubation conditions using various sample preparations, transport buffers and enzymatic treatments and 2) assess the bioavailability of Ara-1 and Ara-3 by measuring their uptake and transport across the Caco-2 cell monolayer.

## Materials and Methods

### *Materials*

Ara-1 and Ara-3 compounds were extracted from peanut hairy roots and generously donated by Dr. Medina-Bolivar (Arkansas State University, Jonesboro, AR) following the method of Abbott and colleagues (Abbott et al. 2010). Stock solutions were prepared by solubilizing 1 mg each of dried Ara-1 and Ara-3 in 1 mL of ethanol. The stock solutions were then divided into aliquots at known volumes, dried using nitrogen, and stored in amber glass vials fitted with a cap at -20°C until ready for usage. Resveratrol was obtained from Tokyo Chemical Industry (Philadelphia, PA). β-Glucuronidase from *Helix pomatia,* Type H-2 (≥85,000 units/mL) was purchased from Sigma Aldrich (St. Louis, MO). All media components and reagents were obtained from Gibco® through ThermoFisher (Grand Island, NY).

### *Optimizing Separation of Ara-1, Ara-3 and Resveratrol using HPLC*

Quantitative analysis of Ara-1, Ara-3 and Resv (control) was performed using the modified method of Yang and colleagues (Yang et al. 2016). Samples were analyzed using a System Gold HPLC system (Beckman-Coulter, Fullerton, CA, USA) equipped with a model 126 dual pump, a model 508 autosampler, and a model 168 photodiode array detector with Beckman-Coulter System 32 Karat software (Version 8, 2006). Separation was carried out using a 4.6 mm × 250 mm Aqua 5 μM $C_{18}$ column (Phenomenex) with flow rate at 1 mL/min. The mobile phases consisted of 2% formic acid in water (A) and 100% methanol (B). The gradient system was modified to optimize separation as follows: initial equilibration with 50% B for 2 min , increase from 50-65% B over 30 min , held at 65% B for 2 min , increase from 65-100% B over 5 min before returning to the initial binary gradient of 50% B. The injection volume was 50 μL. UV-visible spectra was monitored at 340 nm for Ara-1, Ara-3 and Resv.

### *Detection Limit of Ara-1 using HPLC*

In addition to using HPLC to quantitate recovery of the compounds throughout the transport assay, the method was also used to determine the detection limit of Ara-1. This parameter was investigated for two reasons: (1) a low response of Ara-1 was observed in comparison to Resv and Ara-3 despite analyzing all three compounds at the same concentration and (2) the detection limit informs the necessary concentrations of Ara-1 required for the treatment in anticipation of multiple dilution factors during the transport assay. To begin, 50 uL of Ara-1 (1 mg/mL) in ethanol was evaporated to dryness with nitrogen and redissolved in 1 uL DMSO in a 13 x 100 mm borosilicate glass culture tube. The mixture sonicated for 1 min and vortexed for 10 sec with 638 uL of working Dulbecco's modified Eagle's Medium (WMEM) to create a standard curve using 1:4 serial dilution in WMEM. Finally, the samples were passed through a 13 mm, 0.45 um PTFE syringe filter into HPLC vials for immediate analysis. Concentrations of Ara-1 and Ara-3 were determined using a standard curve of each compound with concentration ranging from 0.624 ppm to 78 ppm.

### *UPLC Tandem Mass Spectroscopy (UPLC-MS/MS) Analysis*

Liquid chromatography-mass spectrometry qualitative analysis of Ara-1 and Ara-3 was performed using the modified method of Yang and colleagues to confirm characterization after extraction and purification of the compounds from peanut hairy roots (Yang et al. 2016). A Shimadzu 8040 UPLC system was used for the chromatographic separation. The separation method was similar to the conditions described above with the following modification: water was used in mobile phase A and 0.1% formic acid in acetonitrile was used in mobile phase B. UPLC-MS/MS analysis was conducted using a Shimadzu 8040 triple quadrupole liquid chromatograph mass spectrometer with an electrospray ionization source to obtain structural

information of Ara-1 and Ara-3 after initial purification. The separation was performed on a 2.1 × 50 mm 1.9 μM $C_{18}$ column with a flow rate at 0.2 mL/min. A linear gradient was performed from 30-80% B over 8 min. Briefly, all mass spectra were performed in the positive ion mode with an ion spray voltage at 4 kV, sheath gas (high-purity nitrogen) at 45 arbitrary units, auxiliary gas (high-purity nitrogen) at 15 arbitrary units, capillary voltage at 9 V, capillary temperature at 300°C and tube lens offset at 45 V. Full mass scans were recorded in the range *m/z* 100 to 2,000. Ultra-high-purity helium was used as the collision gas, and 35% collision energy was applied in collision-induced dissociation.

***Cell Culture Maintenance***

Caco-2 human colon cancer cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) at passage number 18. Cells were cultured according to the protocol of Salyer and colleagues (Salyer et al. 2013) in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 2% antibiotic-antimicotic, and incubated at 37 °C.

***Preparation of Cell Monolayers for Transport Assay***

At 80-90% confluency, Caco-2 cells were trypsinized and seeded onto Transwell inserts (polycarbonate membrane, 12 mm i.d., 0.4 μm pore size, Corning Inc., Kennebunk, ME) in 12-well plates at a density of $1.0 \times 10^5$ cells/$cm^2$.

The seeded Transwell inserts were treated with 500 uL and 1500 uL of WMEM on the apical and basolateral side, respectively. Cells were allowed to grow and differentiate to form a monolayer for 14-21 days post-seeding. The growing medium were replaced at days 3, 5, and daily thereafter. Transepithelial electrical resistance (TEER) of cells grown in the Transwell were measured using the Millicell ERS-2 voltohmmeter (Millipore Corp., Billerica, MA, USA)

to ensure cell monolayer integrity. Only monolayers with TEER values >400 $\Omega cm^2$ were used for further experimentation. Only passages 25-40 were used in this assay since a defined interval of passages is recommended to ensure a consistent cellular phenotype in all experiments.

***Transport Assay***

On the day of the assay, dried Ara-1 and Ara-3 was first solubilized in DMSO and mixed with a vortex mixer for 1 min. The solution was then diluted in a 15 mL centrifuge tube with WMEM warmed to 37°C to a final concentration of 78 and 74 ppm, respectively, and sonicated for 1 min. The final test solution contained 0.1% DMSO.

Prior to each assay, WMEM in both chambers was aspirated and 500 uL of the treatment were added to the apical chamber in duplicate. Meanwhile, 1 mL of phosphate-buffer solution (PBS) was added to the basolateral chamber.

To start the experiment, the system was incubated at 37 °C for 2 h. 500 uL samples were drawn from the basolateral chamber at 0, 0.5, 1 and 2 h. The samples were mixed with 25 uL of trifluoroacetic acid (TFA) and 25 uL of 70% ethanol and frozen immediately. The basolateral chamber was replenished with 500 uL of PBS at each time point. At 2 h, remaining solutions in both chambers were collected. The apical and basolateral chambers were then washed with 1.5 mL and 1 mL of PBS, respectively, mixed with 25 uL of TFA and 25 uL of 70% ethanol and pooled with the samples drawn at the 2 h time point. The stock solution as well as aliquots drawn from the apical chamber at the end of the experiment were used to calculate mass balance.

The insert membrane was carefully peeled off with a tweezer, placed into a 1.5 mL microcentrifuge tube with 100 uL of cold MQ water, 10 uL of TFA and 10 uL 70% ethanol, then vortexed vigorously for 10 s to solubilize cells from the membrane. All aliquots and cell membrane suspensions were frozen at -20°C until further analysis.

Preliminary HPLC analysis prior to experimentation showed that background peaks from WMEM and PBS – two liquid components in the transport assay system – did not interfere or coelute with peaks from Resv, Ara-1 and Ara-3. Based on this observation, all collected samples were directly injected into the HPLC for analysis using the following procedure: samples were thawed to room temperature, sonicated for 10 min and vortexed for 10 sec. 190 uL was withdrawn and was treated with 10 uL of 20 ppm Resv in DMSO as an internal standard. The mixture was then vortexed once more and passed through a 0.45 μm, 4mm polyethersulfone (PES) filter prior to HPLC injection. PES filters were used to replace PTFE filters used in prior filtration due to low protein binding, hydrophilicity, and faster flow rates.

***Transport Assay with 200ppm Ara-1 and Ara-3***

Under the newly altered experimental conditions, the preparation of samples did not change except for the concentration of Ara-1 and Ara-3. 200 ppm of Ara-1 and Ara-3 were used as treatment solutions.

The samples collected were additionally subjected to solid phase extraction (SPE) in hopes of concentrating Ara-1 and Ara-3 recovery and eliminating other possible interfering compounds. First, Sep-Pak $C_{18}$ classic 260 mg sorbent cartridges were preconditioned with 2 mL of DI water and 2 mL of 100% methanol. Then, 475 uL of thawed samples were spiked with Resv as an internal standard for a final concentration of 1 ppm. Next, 0.5 mL aliquots of the solution were loaded onto the preconditioned cartridges and eluted for cleanup with 1.5 mL of 5% MeOH in 0.5 mL increments. After drying the cartridge, Ara-1 and Ara-3 were eluted with 1 mL of 100% methanol. The eluates were vortexed for 1 min and passed through a 13mm, 0.45 μm PTFE syringe filter for immediate HPLC analysis.

***Extended Transport Assay with 200ppm Ara-1 and Ara-3***

To rule out if Ara-1 and Ara-3 simply took longer than 2 h to transport to the basolateral chamber, the transport assay was extended to 4 h. The concentration was kept constant at 200 ppm for both compounds. Meanwhile, additional modifications were made: the aliquots collected from the basolateral chamber at each time point were mixed with 100 uL of methanol instead of TFA and 70% ethanol. The insert membranes were treated with 0.5 mL of ice-cold MQ water, rinsed with the same volume of water between samples and vortexed vigorously with 0.5 uL 1 N NaOH for 10 s to solubilize cells from the membrane. All aliquots and cell membrane suspensions were frozen at -20°C until further analysis.

All collected samples were thawed to room temperature, sonicated for 10 min and vortexed for 10 sec. 190 uL was withdrawn and was treated with 10 uL of 20 ppm Resv in DMSO as an internal standard. The mixture was then vortexed once more and passed through a 0.45 μm, 4mm polyethersulfone (PES) filter prior to HPLC injection.

To evaluate if Ara-1 and Ara-3 may have been sequestered within the Caco-2 cells during transport, previously frozen cell culture inserts were thawed to room temperature and centrifuged at 13000 rpm for 10 min. The supernatant was transferred into a borosilicate disposable test tube, mixed with 300 uL methanol, vortexed for 1 min, and centrifuged again at 13000 rpm for 10 min. The supernatant was collected in the first test tube. Once again, the cell membrane was mixed with 300 uL methanol, vortexed for 1 min, and centrifuged at 13000 rpm for 15 min. The supernatant was collected, and 2 mL of DI water was added. The solution was loaded into a preconditioned Sep-Pak $C_{18}$ cartridge, cleaned up with 3 mL of 5% methanol, and air dried. Compounds of interested were eluted with 1 mL of methanol. The eluate was dried under

nitrogen, reconstituted with 200 uL 100% methanol and passed through a 0.45 μm PTFE syringe filter for immediate HPLC analysis.

***Time Course Changes in pH of WMEM and PBS under Experimental Conditions***

To gather more information on whether pH influenced the stability of Ara-1 and Ara-3 under experimental conditions, a time course of pH change was performed for both WMEM and PBS. To begin, 4 mL of either PBS or WMEM were warmed at 37°C and divided evenly into two empty wells in a 12-well plate. This was done because the total volume needed for the time course surpassed the volume that each well could hold. The plates were incubated for 4 hr, in accordance to the length of the transport assay, at 37°C with 5% $CO_2$ to resemble experimental conditions. 500 uL aliquots were taken at time points 0, 1, 2, 3 and 4 h and the pH immediately read with a pH meter.

***Time Course Changes in Stability of Ara-1 and Ara-3 in WMEM and PBS under Experimental Conditions***

According to the time course on changes in pH of WMEM under experimental conditions, the experiment was rerun in the same manner except for the following modifications: Ara-1, Ara-3 and Resv was mixed with either WMEM or PBS to a final concentration of 200 ppm and 0.1% DMSO. The duration of the time course was also reduced to 3 h instead of 4 h. Approximately 200 uL was taken as an aliquot and passed through a 0.45 μm, 4 mm PES filter immediately for HPLC analysis.

The purpose of this time course was to compare the stability of the compounds in each aqueous solution to determine whether the transport media needs to be changed. Resv was included because of its role as a positive control in future transport assay.

***Transport Assay with Modified Transport Buffer***

The time course experiments ran previously shows that WMEM may still be a viable transport buffer for Ara-1 and Resv, but not Ara-3. This time, two different transport buffers were used in the assay: 1) WMEM without FBS to prevent the likelihood that the compounds would bind to proteins in the serum and form a complex too large for HPLC detection and 2) PBS.

On the day of the assay, dried Ara-1 and Ara-3 was first solubilized in DMSO and mixed with a vortex mixer for 1 min. The solution was then diluted separately in a 15 mL centrifuge tube with the previously mentioned transport buffers, warmed to 37°C to a final concentration of 100 ppm and sonicated for 1 min. The final test solutions contained 0.1% DMSO.

Prior to each assay, WMEM in both chambers was aspirated and 500 uL of the two separate treatments were added to the apical chamber in duplicate. Meanwhile, 1 mL of PBS was added to the basolateral chamber.

To start the experiment, the system was incubated at 37 °C for 3 h. A 500 uL aliquot was collected from the basolateral chamber at each time point and mixed with 100 uL of methanol. The insert membranes were treated with 0.5 mL of ice-cold MQ water, rinsed with the same volume of water between samples and vortexed vigorously with 0.5 uL 1 N NaOH for 10 s to solubilize cells from the membrane. All aliquots and cell membrane suspensions were frozen at -20°C until further analysis.

At time of HPLC analysis, all samples were thawed to room temperature, sonicated for 10 min and vortexed for 10 sec. 190 uL was withdrawn and was treated with 10 uL of 20 ppm Resv in DMSO as an internal standard. The mixture was then vortexed once more and passed through a 0.45 μm, 4mm PES filter prior to HPLC injection.

### *Transport Assay with HBSS and Enzymatic Treatment*

To further elucidate the behavior of Ara-1 and Ara-3 under experimental conditions, the method was modified to include enzymatic treatment of the samples following collection by β-glucuronidase and sulfatase. If the compounds are undergoing glucuronidation or sulfonated due to metabolic processes in the Caco-2 cells, the enzymes may free the parent compound from its conjugate. Doing so could allow for the HPLC detection of Ara-1 and Ara-3 in its unconjugated form.

The methodology was the same as described in the previous section with the following modifications: Ara-1 and Ara-3 were prepared using Hank's Balanced Salt Solution (HBSS) with a final concentration of 0.1% DMSO. HBSS was chosen because of its role in maintaining pH, osmotic balance, and cell signaling without potential interference from ingredients of WMEM. Prior to each assay, WMEM both chambers was aspirated and 500 uL of the treatment were added to the apical chamber in duplicate. Meanwhile, 1 mL of HBSS was added to the basolateral chamber.

To start the experiment, the system was incubated at 37 °C for 3 h. A 500 uL aliquot was collected from the basolateral chamber from time point 0 to 2 h. At time point 3 h, approximately 400 uL of HBSS was collected from the apical chamber, while approximately 900 uL of HBSS was collected from the basolateral chamber. The apical chamber was rinsed with 0.5 or 0.6 mL of HBSS two times for a final collected volume of 1 mL in both the apical and basolateral chamber. The insert membranes were treated in the same way as described in the previous section. All aliquots and cell membrane suspensions were frozen at -20°C until further analysis.

Enzymatic treatment for time point 0 h aliquots were allowed to thaw to room temperature and mixed with 2 mL of ethyl acetate. The mixture was vigorously vortexed for 1

min, and then centrifuged at 3000×g at room temperature for 10 min. The supernatant was collected in a borosilicate disposable test tube, dried using nitrogen, reconstituted in 250 uL of MeOH, and passed through a 0.45 μm PTFE filter for immediate HPLC analysis.

For the enzymatic treatment of other time points, aliquots were thawed to room temperature, spiked with Resv as an internal standard to a final concentration of 1 ppm, then mixed with 0.5 mL sodium acetate buffer (0.2 M, pH 5.0) and 10 uL of β-glucuronidase sulfatase ($H_2$ type). The samples were incubated for 20 h at 37°C in a water bath and agitated periodically to potentiate enzyme activity. After 20 h, the samples were removed from incubation and mixed with 2 mL of ethyl acetate, vigorously vortexed for 1 min, centrifuged at 3000×g at room temperature for 10 min. The supernatant was collected in a borosilicate disposable test tube, dried using nitrogen, reconstituted in 250 uL of MeOH, and passed through a 0.45 μm PTFE filter for immediate HPLC analysis.

Enzymatic treatment of insert membranes was as follows: 1.5 mL of sodium acetate buffer (0.2 M, pH 5.0) and 30 uL of β-glucuronidase sulfatase ($H_2$ type) were added to thawed insert membrane samples. Resv was added for a final concentration of 1 ppm as an internal standard. The samples were incubated for 20 h at 37°C in a water bath and agitated periodically to potentiate enzyme activity. After 20 h, they were removed and transferred to a 15 mL centrifuge tube, mixed with 4 mL of ethyl acetate, and subjected to the same protocol as time points 1 and 2 as described above.

Enzymatic treatment of HBSS was as follows: approximately 0.4 mL of sodium acetate buffer (0.2 M, pH 5.0) and 8 uL of β-glucuronidase sulfatase ($H_2$ type) were added to thawed solutions. Resv was added for a final concentration of 1 ppm as an internal standard. Incubation times and subsequent steps were the same as described above.

### *Apical Time course Experiment of Ara-1 and Ara-3*

In a final attempt to examine the fate of Ara-1 and Ara-3 in the apical layer in real time under experimental conditions with Caco-2 cells, the method was modified to include alkali treatment either with or without enzymatic treatment of only apical samples following collection by β-glucuronidase and sulfatase as previously described above. Alkali treatment was attempted to elicit cleavage of potential dimers or polymers of Ara-1 and Ara-3 that may have formed in the apical chamber.

The methodology was the same as described in the previous section in terms of preparation for the assay with the following modification: 100 ppm of Ara-1 was solubilized in HBSS and 0.1% DMSO, while DMEM was used as the transport buffer for Ara-3 (since solubility was problematic in PBS) with the same concentration of DMSO. 4 wells each in a 12 well plate were used to track Ara-1 and Ara-3 separately throughout the time course experiment. The contents in the apical chamber of the first wells were collected for time point 1 h, as it was assumed that nothing transported at time point 0 h. The second wells were for time point 2 h, third wells for time point 3 h to be subjected to alkali treatment without enzymatic treatment, and the fourth wells for time point 3 h to be subjected to both alkali and enzymatic treatment.

The original treatment solutions as well as time point 1 h and 2 h were directly injected for HPLC analysis after being passed through a 0.45 μm, 4 mm PES filter.

Time point 3 h samples without enzymatic treatment were prepared in the following manner: 1 mL of alkali treatment (2 N NaOH, pH 6-7) was mixed with the thawed samples and heated at 60°C for 15 min. After cooling to room temperature, 1.5 mL of ethyl acetate was added, vortexed vigorously for 1-2 min, and centrifuged at 3000 rpm for 10 min. The mixture

was dried under nitrogen, reconstituted in 250 uL of MeOH, and filtered through a 0.45 μm PTFE filter for immediate HPLC analysis.

Time point 3 h samples with enzymatic treatment were prepared in the same manner, except with 20 uL of β-glucuronidase sulfatase ($H_2$ type) added after pH of the alkali-treated sample was adjusted to pH 5 with HCl. The samples were incubated at 37°C for 20 h. After incubation, the samples were mixed with 2 mL of ethyl acetate, vigorously vortexed for 2 min, dried under nitrogen, reconstituted in 250 uL of methanol, and filtered through a 0.45 μm PTFE filter for immediate HPLC analysis.

### *Statistical Analysis*

All statistical analyses will be performed using Statistical Analysis Software (System Version 9.4; SAS Institute Inc., Cary, NC) using a one-way analysis of variance test (ANOVA). Data will be expressed as means $\pm$ standard error of mean (SEM). A difference of $p<0.05$ will be considered significant.

## Results

### *Optimizing Separation of Ara-1, Ara-3 and Resveratrol using HPLC*

The chromatogram of Ara-1, Ara-3 and Resv in WMEM is shown in Figure 1. The compounds were solubilized in WMEM with a final concentration of 0.1% DMSO because it was important to determine if separation was possible if samples from the transport assay were to be directly injected into the HPLC, since WMEM is the transport buffer. The figure shows that the background peaks stemming from WMEM are close to, but do not interfere with the elution of Resv at 5.2 min. Ara-1 elutes second around 10.6 min, and Ara-3 elutes last at 13.6 min due to its nonpolar structure.

### *UPLC Tandem Mass Spectroscopy (UPLC-MS/MS) Analysis*

The UPLC-MS/MS spectra of Ara-1 and Ara-3 is shown in Figure 2, which was performed to verify the structure of the compounds after extraction. According to Yang and colleagues, the MS/MS fragments for Ara-1 were listed as *m/z* 313 --> 257, 239, 229, 211 and Ara-1 as *m/z* 297 --> 241, 223, 213, 195, which is consistent with the results shown (2016).

### *Transport Assay*

Samples collected from time point 0 h were screened using HPLC to determine if Ara-1 and Ara-3 were transporting immediately after being applied to the cells, which may be indicative of a faulty monolayer and/or incomplete tight junctions between Caco-2 cells. No compounds were detected in the basolateral chamber at this time point.

At time point 0.5 and 1 h, neither Ara-1 nor Ara-3 were detected in the basolateral chamber. This is unusual, as a previous transport study of Resv by intestinal Caco-2 cells performed showed that the highest concentration (40 μM of Resv) yielded a detectable response of 2000 pmol $mL^{-1}$ in the basolateral chamber at time point 1 h (Kaldas et al. 2003). Granted, Resv is slightly different in structure by the addition of a prenylated group and extra hydroxyl group in Ara-1 and an extra hydroxyl group in Ara-3. There was no basis or prior literature to compare transport data to for Ara-1 and Ara-3, so it was assumed that perhaps a higher concentration was needed to increase peak response, given that the transport system works but the concentration of the transported compounds was still below detection level.

Another noteworthy observation is that the standard curve made from the same stock solution of Ara-1 and Ara-3 in WMEM became increasingly unreliable due to decreased peak response and possible deterioration of the compounds in refrigerated storage. A difference of the stock solution before and after 7 days in storage is shown in for Ara-1 in Figure 3 and Ara-3 in

Figure 4. Ara-3 showed a decrease of 35.2% in peak area after 7 days, while Ara-1 showed a much sharper decrease of 64.7%.

Additionally, the presence of minor peaks was also evident before and after storage – several increased, while others decreased. The numbers in the chromatogram correspond to notable increases or decreases in possible oxidation products or other unidentified byproducts. For example, Peak 2 area (figure 3) increased by 12.1% while lambda max changed from 267 nm to 266 nm. Peak 3 area, on the other hand, decreased by 49.4% while the lambda max remained the same. Minor peak formation was not as prominent in Ara-3 before and after storage (figure 4); however, a stark increase was shown in Peak 2 with an increase by 251.9% in area. The lambda max interestingly changed from 279 nm to 325 nm, indicating either an inaccurate lambda max due to low response, instrumental variability, co-elution, or the formation of a different compound altogether.

Overall, altered experimental conditions were implemented to elucidate whether the lack of transport seen in Ara-1 and Ara-3 were due to: instability of the compounds under experimental conditions (e.g. effects of pH, possible oxidation, complexation in WMEM), extended transport time beyond 2 h of experiment, accumulation of Ara-1 and Ara-3 within Caco-2 cells, flaw in the transport system itself, and/or lack of equipment sensitivity.

***Transport Assay with 200ppm Ara-1 and Ara-3***

Increasing the concentration of Ara-1 and Ara-3 from 74 ppm and 78 ppm, respectively, to 200 ppm of each did not considerably increase peak response as previously thought. There were also issues regarding the standard curves of Ara-1 in WMEM. HPLC analysis showed that responses ranging 0.5 ppm to 200 ppm fluctuated and therefore did not yield a linear response. However, Ara-3 maintained linearity and did not seem to have issues. Ara-3 showed peaks with

the same retention time (RT) as Ara-1 in several wells at time points throughout the transport assay, but the lambda max was 263 nm instead of 340 nm. This variability could be due to factors such as instrument variability and too low of a response; nonetheless, the compound could not be confirmed given the information available. Even if the compound in question was Ara-1, the standard curve cannot be used to quantitate the amount transported due to its unreliability.

Incorporating SPE as a final step to extract Ara-1 and Ara-3 did not seem to have an effect. No peaks were detected except that of Resv as the internal standard, indicating that the extraction method was effective at recovering similarly nonpolar compounds. In this regard, it is assumed that the absence of Ara-1 and Ara-3 after SPE indicates that little to none of the compounds transported to the basolateral chamber.

***Extended Transport Assay with 200ppm Ara-1 and Ara-3***

The length of the experiment was modified from 2 h to 4 h in the event that Ara-1 and Ara-3 took longer than 2 h to transport across the monolayer. However, neither Ara-1 nor Ara-3 were detected in the basolateral chamber throughout all time points, evidencing that their absence was likely not due to extensive transport rates. The compounds are being affected somehow either before they managed to cross into the basolateral chamber, or they were accumulating inside the Caco-2 cells.

Additionally, the treatment solutions of Ara-1 and Ara-3, prepared immediately prior to experimentation, were analyzed on the HPLC immediately after preparation for mass balance purposes. In comparison to the apical layer at the end of the 4 h time point, both compounds' areas had diminished by approximately 99.99% ± 0.00% and 96.45% ± 0.25%, respectively.

To confirm whether the compounds were sequestered inside the cells, insert membranes were lysed and the contents repeatedly extracted to elute the Ara-1 and Ara-3 through SPE. Once again, Resv as an internal standard consistently appeared in all the samples, indicating that the extraction method was successful. However, no responses from the corresponding peaks associated with Ara-1 and Ara-3 were observed, which further suggests that little to none of the compounds were caught in the monolayer. Since the recovery of Ara-1 and Ara-3 in the apical layer was extremely low, this suggests that the compounds have either degraded in the solution they were solubilized in (WMEM) before transport occurred, or they have undergone structural changes that eludes HPLC detection (perhaps high molecular weight products of dimerization or degradation products).

Perhaps the most noteworthy finding was observed after removing the original treatment solution used for this modified experiment from the refrigerator 7 days later. A distinct color change between Ara-1 and Ara-3 in WMEM occurred in storage after unwrapping the foil used to protect the light-sensitive compounds, as shown in Figure 5. On the left (Fig.5-A), Ara-1 changed from a bright pink color to that of a dark red, while Ara-3 (Fig. 5-B) remained unchanged. The right (Fig. 5-C) is plain WMEM stored at 5 °C for the same amount of time. Typically, polyphenols experience browning due to oxidation and/or polymerization, which could explain why Ara-1 displayed inconsistent peak responses and had decreased in area after refrigerated storage – even before experimentation or contact with Caco-2 cells. It may be undergoing oxidation, serum binding, biotransformation, polymerization or another reaction that causes deterioration of the parent compound and the production of new compounds, which could also explain the prevalence of minor peaks when compared to Ara-3.

### *Time Course Changes in pH of WMEM under Experimental Conditions*

Figure 6 shows that PBS, with an expected pH of 7.4, decreased at 1 h to approximately pH 6.5. On the other hand, WMEM was higher than expected at approximately pH 8. The two time courses were performed separately, but perhaps the two systems work together at physiological pH to buffer changes during the transport assay. Nonetheless, the high pH of WMEM alone is worrisome given that Resv was previously shown to be unstable in a 50 mM phosphate buffer (pH 8.0) at 37°C, leading to degradation within a matter of hr (Zupančič et al. 2015).

### *Time Course Changes in Stability of Ara-1, Ara-3 and Resv in WMEM and PBS under Experimental Conditions*

Data regarding the stability of Ara-1 and Resv in both WMEM and PBS could be gathered, but the recovery of Ara-3 in PBS proved to be extremely difficult. The area of Ara-3 in WMEM did not seem to diminish; in fact, recovery was about 97.0% ± 0.2% at 3 h. However, when the same experiment was conducted with PBS, Ara-3 was barely detected at time point 0 h and beyond despite multiple attempts, close attention to pipetting technique, and carefully solubilizing the compound in PBS. To determine if the issue had to do with the instrument itself, other samples with known concentrations were analyzed with no issues. To determine if PBS was the issue, a 0.1 mg of Ara-3 was dissolved in 1 mL of 80% methanol. Ara-3 showed a strong response in this corresponding chromatogram – suggesting that the strange results observed previously was not an instrumental error. Normally, one would assume that Ara-3 simply is not solubilized well in PBS. However, dissolving Ara-3 in DMSO and PBS was performed in the past and analyzed using HPLC with no issues. The peak response was not as high as WMEM, but detectable nonetheless.

The end of the 3 h time course showed that Ara-1 had 77% ± 4.11% and 96.1% ± 11.56% recovery in WMEM and PBS, respectively (Figure 7). On the other hand, Resv had 109.6% ± 8.0% in WMEM and 114.8% ± 13.5% in PBS at the end of 3 h (Figure 8). The higher recovery in Resv could be attributed to inaccurate pipetting or insufficient solubility in the beginning of the experiment. There were no significant differences between WMEM and PBS as transport buffers in the recovery of Ara-1 or Resv at each time point.

Overall, it appears that even though there was no significant difference, Ara-1 may be more susceptible to degradation in WMEM compared to PBS. The recovery of Resv in WMEM and PBS were very comparable. A comparison of Ara-3 in WMEM and PBS was not possible; however, data has shown that WMEM could still be a viable transport buffer.

***Transport Assay with Modified Transport Buffer***

Despite eliminating FBS (fetal bovine serum) in the WMEM transport buffer, HPLC analysis shows that neither Ara-1 nor Ara-3 were detectable throughout the experiment in the basolateral chamber, but Resv (control) was detected in both the apical and basolateral chamber. This suggests that the issue of recovery regarding Ara-1 and Ara-3 was likely not due to protein binding in the presence of FBS.

PBS was used as an alternate transport buffer to simplify the transport assay system and to prevent the complex ingredient profile from interfering with the recovery of Ara-1 and Ara-3. The attempt was also another chance to verify whether the stock of Ara-3 used previously in the time course experiment was indeed contaminated or problematic. Once again, neither Ara-1 nor Ara-3 were detectable in the basolateral chamber. Additionally, only 0.13% ± 0.03% of Ara-1 remained in the apical chamber at time point 3 h out of the amount originally applied at time point 0 h. Ara-3 was not detected at time point 0 h or 3 h at all. On the other hand, 10.23% ±

0.49% of Resv remained in the apical chamber, while none was recovered in the basolateral chamber, likely due to solubility issues in during preparation.

***Transport Assay with HBSS and Enzymatic Treatment***

To further elucidate the behavior of Ara-1 and Ara-3 under experimental conditions, the method was modified to include enzymatic treatment of the samples following collection by β-glucuronidase and sulfatase. Unfortunately, the compounds did not seem to be undergoing glucuronidation or sulfonation due to metabolic processes in the Caco-2 cells because the parent compounds of Ara-1 or Ara-3 were not detected in the basolateral chamber using HPLC analysis throughout the duration of the transport assay. Another reason could be that theyw ere polymerized or complexed similarly as the parent compound.

The insert membranes of Ara-1 and Ara-3 were subjected to enzymatic treatment to yield potential parent compounds that may have been metabolized within the cell. After analysis, it is observed that Resv consistently appeared as an internal standard, but responses from Ara-1 and Ara-3 could not be observed according to inconsistent retention time and lambda max.

***Apical Time course Experiment of Ara-1 and Ara-3***

Finally, the fate of Ara-1 and Ara-3 in the apical layer was observed in real time under experimental conditions with Caco-2 cells using alkali treatment of apical samples either with or without enzymatic treatment of. Subjecting samples to alkaline conditions were performed to elicit cleavage of potential dimers or polymers of Ara-1 and Ara-3 that may have formed in the apical chamber. This was previously difficult to test because withdrawing aliquots during the transport assay in the presence of cells meant that the well could no longer be used - this is because the volume of aliquot necessary for analysis meant that the treatment solution (approximately 0.5 mL) in the apical chamber must be completely withdrawn. Even if this were

not the case, any volume taken from the apical chamber would interfere with mass balance at the end of the transport assay. Lastly, the preservation of limited quantities of Ara-1 and Ara-3 as well the length of time needed for monolayer formation were other reasons why this time course experiment was not attempted previously.

At time point 1 h, Ara-1 in HBSS decreased to 37.9% of the original area after HPLC analysis. At time point 2 h, no Ara-1 was detected at all. Ara-1 was not detected at time point 3 h after alkali treatment with or without enzymatic treatment. The results strongly suggest that the stability of Ara-1 is related to the presence of Caco-2 cells, as the previous time course experiment of Ara-1 in WMEM and PBS without Caco-2 cells yielded 104.1% ± 4.8% and 100.8% ± 9.6% recovery at time point 1 h. Though the transport buffers used were different, one can predict that the responses of PBS and HBSS would be similar.

The original area of Ara-3 could not be determined because HPLC analysis showed no response at all. As a result, a comparison could not be made between all other time points, regardless of alkali treatment or enzymatic treatment, since the original treatment did not contain Ara-3 for an unknown reason. The treatment solution could have very well contained Ara-3, but the amount remained undetected by HPLC. Originally, it was thought that Ara-3 was not soluble in PBS, but it appears that Ara-3 has been affected somehow during storage or preparation in such a way that prompts little to no response even in DMEM during HPLC analysis.

**Discussion**

For a long time, Resv has been known to possess a wide range of purported health benefits, including anti-inflammatory, antioxidant, and anti-cancer activities. Despite the diverse biological activities of Resv, the compound possesses limited oral bioavailability due to rapid absorption and extensive metabolism, leading to metabolites such as glucuronidated and sulfonated conjugates (Yang et al. 2015).

Therefore, recent research has been shifted to similar activities exhibited by Ara-1 and Ara-3, the prenylated analogs of Resv. In some cases, these biological activities surpass the potency of their nonprenylated analogs. For example, Ara-1 had the highest efficacy in inducing cell death in human leukemia HL-60 cells through the intrinsic apoptotic pathway (Huang et al. 2010). It was much more cytotoxic than Ara-3 and Resv with an $EC_{50}$ of 4.2 uM compared to 17.6 uM, and its potency was likely attributed to the 4-isopentenyl moiety and the additional 3'-hydroxyl moiety (Huang et al. 2010). According to the researchers, the isopentenyl group may increase the hydrophobicity of the compound, thereby increasing compound interaction with the cell membrane and increase cell update (Huang et al. 2010).

Bioavailability studies on Ara-1 and Ara-3 using the Caco-2 transport assay have not yet been conducted until now. Most published research on the bioavailability of stilbenoids have primarily focused on Resv. A study by Brents and colleagues (2012) found that Ara-1 and Ara-3 are metabolized to a lesser degree than its non-prenylated parent compounds, piceatannol and Resv (Brents et al. 2012). Researchers suggested that the lipophilic side chains of Ara-1 and Ara-3 may disrupt the glucuronidation process, thereby enhancing the compounds' bioavailability (Brents et al. 2012). However, results from the study differ than that of the present study. Though data gathered from the present study do not confirm whether Ara-1 or Ara-3 are truly being metabolized and to what extent, one can assume that the cells are interacting with the compounds somehow to modify the structure of the parent compound. This is support by the observation that the parent compounds are not recovered at any time point in the basolateral chamber, but are rapidly decreasing as observed in the time course experiment in the apical chamber when in contact with cells.

Furthermore, another study by Brents and colleagues (2012) evaluated the activities of eight human UDP-glucuronosyltransferases (UGTs) towards Ara-1, Ara-3, *trans*-piceatannol, and *trans*-resveratrol. The findings indicated that prenylated stilbenoids may be preferable alternatives to *trans*-resveratrol due to its slower metabolism. Whether Resv is metabolized more in comparison to Ara-1 and Ara-3 cannot be confirmed in the present study, as the time course stability of Resv with Caco-2 cells was not performed. However, one can infer that based on previous results of Resv stability in PBS and WMEM in cell culture conditions in addition to consistent transport to the basolateral chamber as positive control, that Resv may be metabolized to a lesser degree compared to Ara-1 and Ara-3. Future studies are needed to elucidate if this is the case.

The very same structural advantage that provides Ara-1 with higher efficacy of biological activities may be the same feature that decreases its bioavailability. In a review by Chen and colleagues, it is proposed that though the presence of a prenyl-moiety in a flavonoid makes the backbone compound more lipophilic and enhances biological activity, it may also decrease its bioavailability and plasma absorption (2014). Another important study by Xiao and Högger highlighted the impact of structure on the stability of stilbenoids in cell culture conditions. It was found that the glycosylation and methoxylation of stilbenoids enhanced the stability, and the hydroxylation of stilbenoids decreased the stability (2015). Ara-3 shares the same structure as Resv, except for the presence of a prenylated moiety, while Ara-1 possesses the prenylated moiety in addition to an extra hydroxyl group (2015). This is supported by the low recovery of Ara-1 from the apical chamber in the present study as well as the color change observed after 7 days in refrigerated storage. Specifically, Ara-1 in WMEM took on a dark brownish color compared to Ara-3 in WMEM and WMEM alone. Ara-1 has an extra hydroxyl group on the B

ring at the ortho position compared to Ara-3 or resveratrol. The more hydroxyl groups are present, the more likely they are to be affected by oxidation, which could lead to quinone formation. Quinones may bind to other quinones or proteins and other compounds, which in turn leads to polymerization and subsequent color change.

Overall, more work needs to be done to elucidate the exact mechanism of what is occurring between Caco-2 cells and Ara-1 and Ara-3 in the apical layer. A potential direction that this research could take would be performing LC-MS and NMR on degradation products observed during HPLC analysis. Additionally, if Ara-1 and Ara-3 are being oxidized, adding an antioxidant to the apical chamber of the transport assay may potentially protect the compounds long enough to be transported to the basolateral chamber.

**Conclusion**

In conclusion, Ara-1 and Ara-3 are not detected by HPLC in the basolateral chamber or insert membranes and possess low recovery in the apical chamber after performing transport assays with various transport buffers, preparation methods, and sample treatments over the course of 3 to 4 h. This research demonstrates that Ara-1 and Ara-3 are not stable possibly due to polymerization or degradation from the effects of oxidation, pH, and/or cellular interactions between the compounds and Caco-2 cells during incubation.

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# APPENDIX

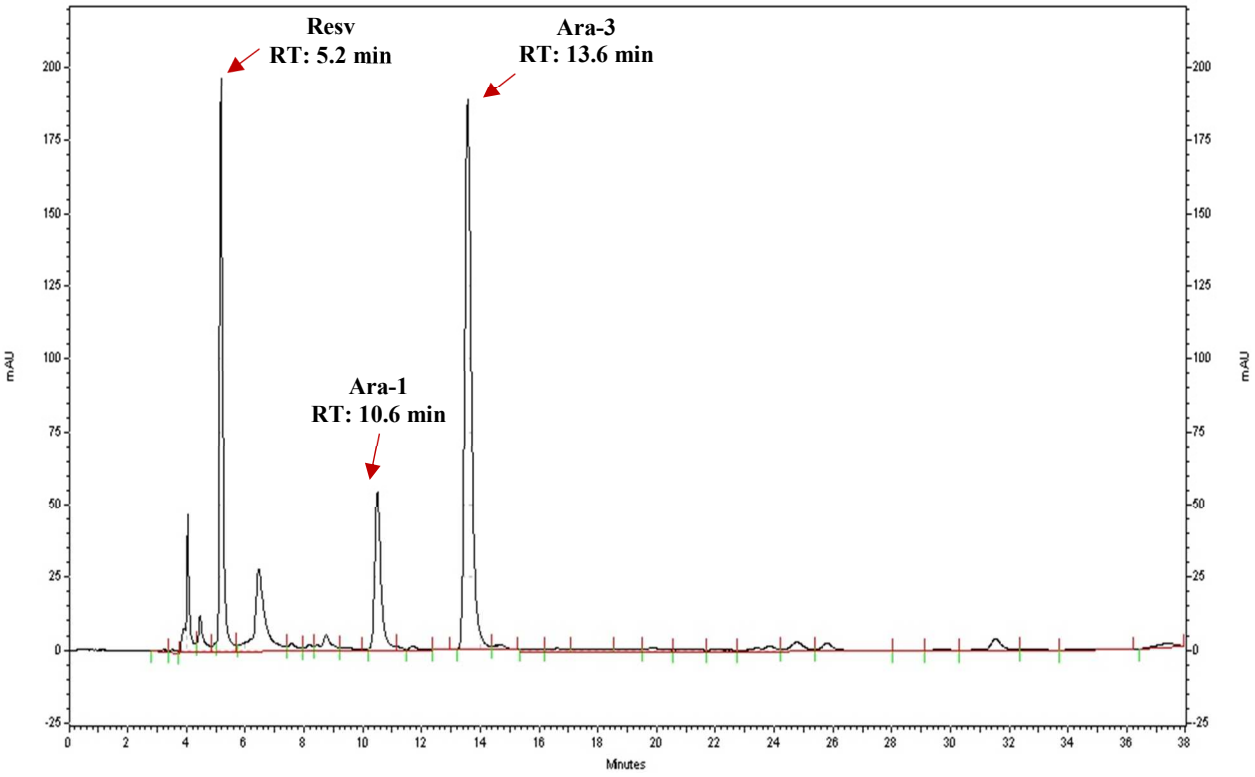


**Figure 1:** HPLC chromatogram of arachidin-1, arachidin-3 and resveratrol as control in WMEM at 340 nm.

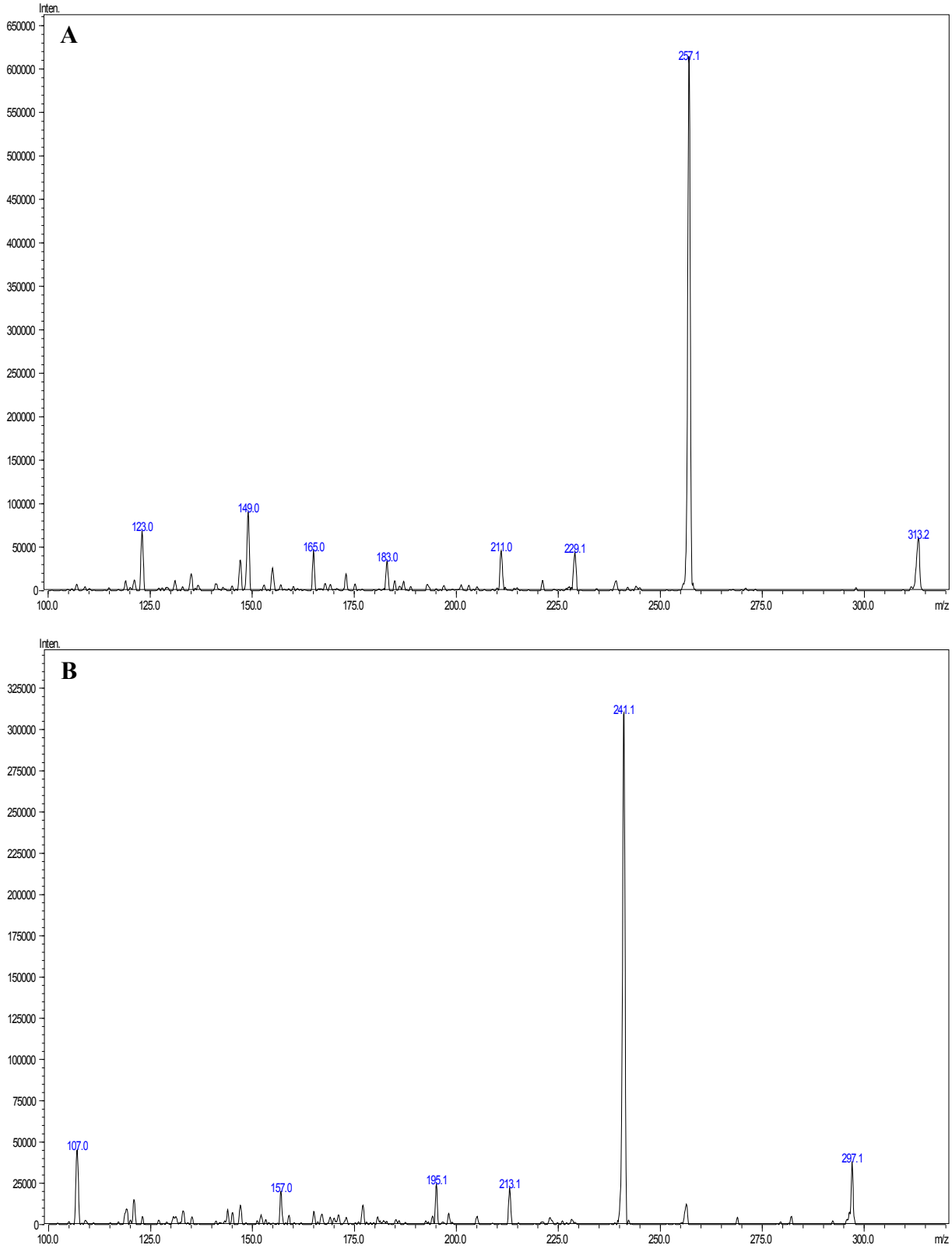


**Figure 2:** UPLC-MS/MS spectra of Ara-1 (A) and Ara-3 (B).

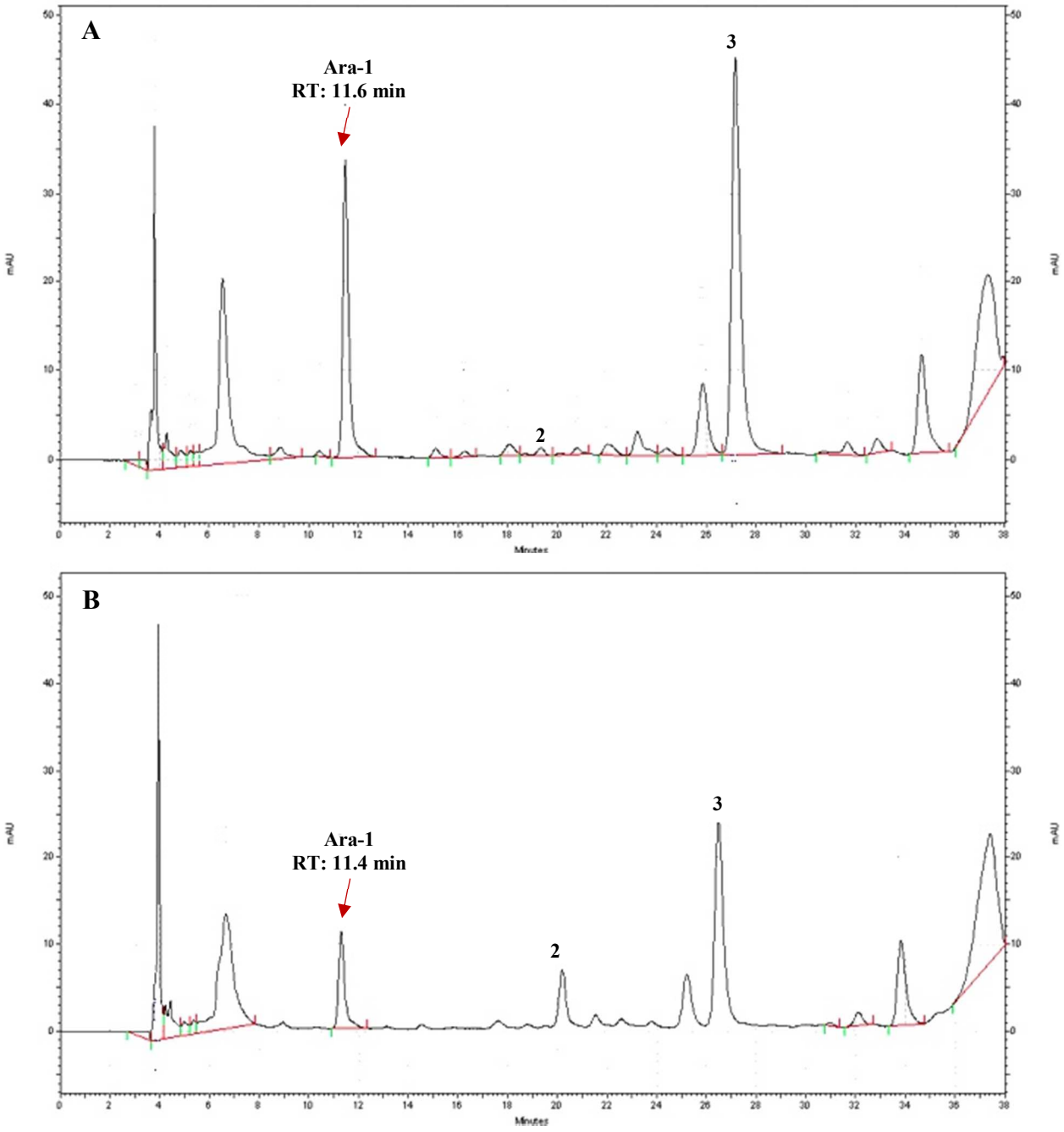


**Figure 3:** HPLC chromatograms of Ara-1 from stock solution in WMEM at 78 ppm before (A) and after 7 days of 5°C storage (B) at 340 nm.

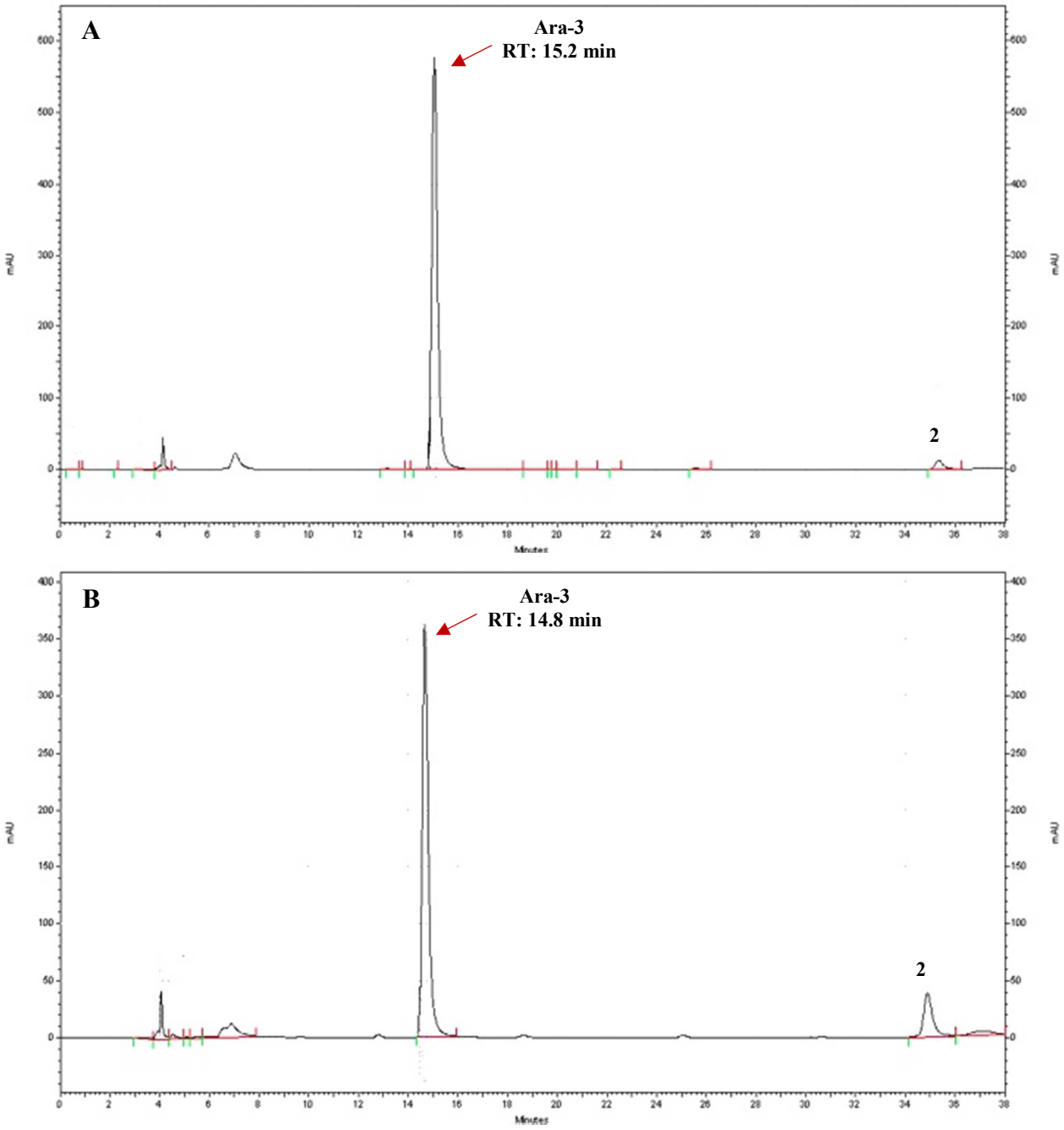


**Figure 4:** HPLC chromatograms of Ara-3 from stock solution in WMEM at 78 ppm before (A) and after 7 days of 5°C storage (B) at 340 nm.

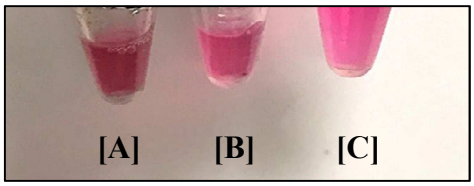


**Figure 5:** Original treatment solution made with 200 ppm Ara-1 in WMEM (A), 200 ppm Ara-3 in WMEM (B), and WMEM (C) stored after 7 days at 5°C.

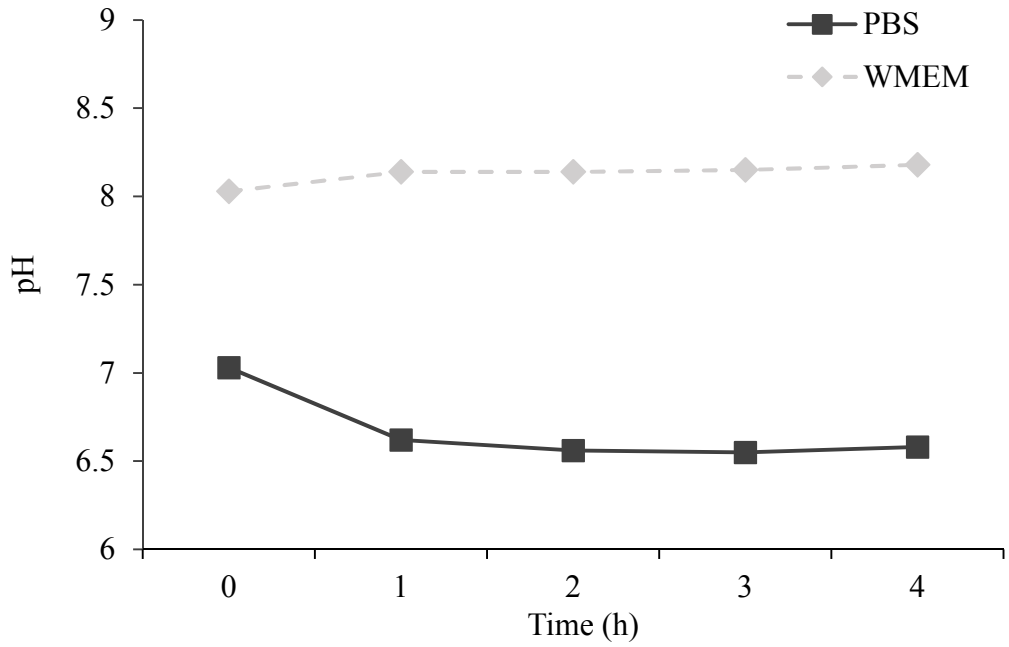


**Figure 6:** Change in pH of PBS and WMEM over 4 h in transport assay conditions at 37°C, 5% $CO_2$.

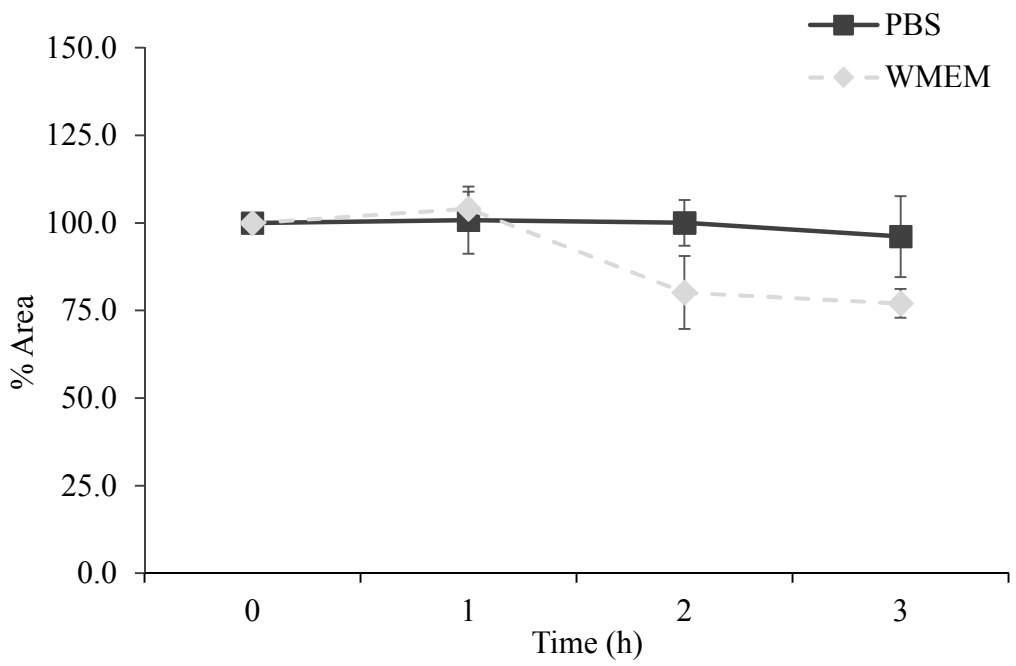


**Figure 7:** Percent change in area of Ara-1 (n = 2) in PBS and WMEM at transport assay conditions with standard error of mean bars (SEM). $p < 0.05$.

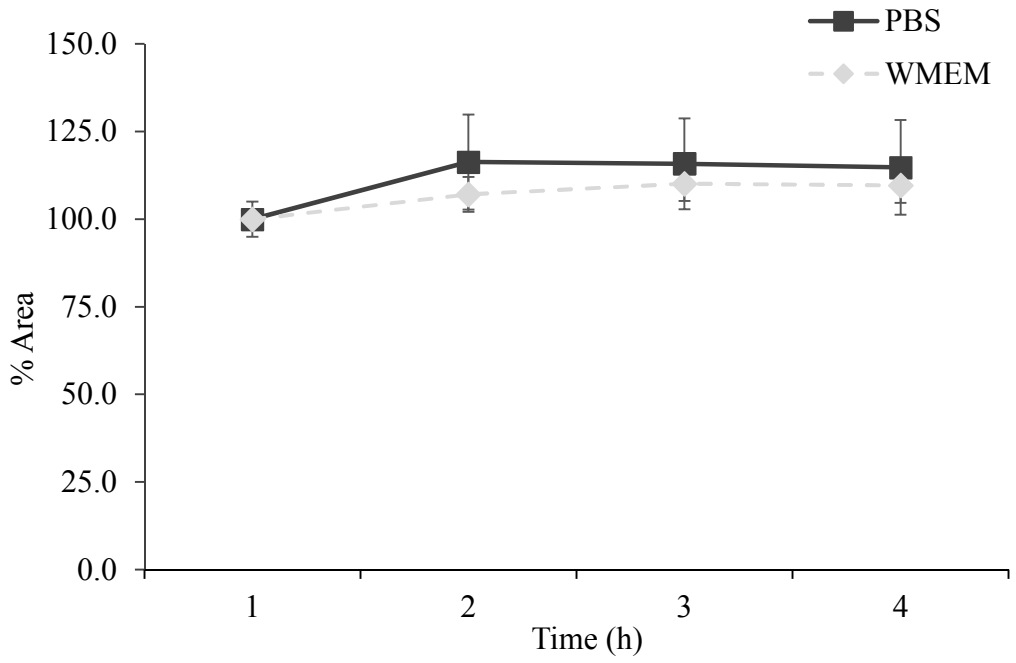


**Figure 8:** Percent change in area of Resv (n = 2) in PBS and WMEM at transport assay conditions with standard error of mean bars (SEM). $p < 0.05$.

# CHAPTER 4

## Bioavailability of Bioactive Compounds in Blackberry Volatiles


### Abstract

The use of berries and its components as functional ingredients have been on the rise, especially due to antioxidant activities found in polyphenols. However, the bioavailability of polyphenols tends to be low. Other plant metabolites such as volatiles tend to possess lower molecular weight and lipophilic chemical structures, which may enhance bioavailability. An *in vitro* Caco-2 model was used to determine the bioavailability of volatiles found in blackberry essences. Blackberry essences were extracted from locally purchased blackberries, and a 1:1 dilution of blackberry essences combined with working cell culture media (WMEM) was applied Caco-2 cells in semipermeable inserts to evaluate absorption and transport over a period of 0 – 4 h. 0.5 mL aliquots were taken from the basolateral chamber every hr and analyzed using headspace solid phase microextraction gas chromatography (HS-SPME-GC). Individual volatiles were tentatively identified and transport rates determined based on the peak area found in the starting material. Results suggests that though esters and monoterpenes make up the two most abundant functional groups in the blackberry essence profile, higher transport rates and recovery were observed in aldehydes.

## Introduction

The latest research efforts have been focused on identifying dietary bioactive compounds found in foods that have been shown to exert biological activities. Fruits, especially berries, can be a rich source of antioxidants and polyphenols such as anthocyanins and phenolic acids (Nile and Park 2014, Szajdek and Borowska 2008). Despite the well-known health effects of anthocyanins and polyphenols, these compounds tend to possess poor bioavailability and their stability can be affect by a variety of factors (Lila et al. 2016, Parisi et al. 2014). Other plant metabolites such as volatiles show biological effects as well (Ayseli and İpek Ayseli 2016, Pennerman et al. 2015). The attempt to discern the role of plant volatiles beyond its flavor-contributing attributes has gained momentum in recent years. As such, research studies that explore the potential of volatiles to exert biological and pharmacological activities in human health have begun to emerge.

In general, volatile compounds tend to be lipophilic in nature and possess molecular weights lower than 300 Da and vaporize readily at room temperature (Kalua et al. 2007). If the compound is sufficiently volatile, it is regarded as a smell or odor after it binds with olfactory receptors in the nose (Kalua et al. 2007). Volatiles produced by plants are considered secondary metabolites that help the plant survive in its environment. More than 100,000 chemical products are known to be produced by plants, and 1,700 of these are categorized as volatiles (Spinelli et al. 2011).

For much of history, the floral and vegetative parts of plants have been used to enhance food quality and add to its organoleptic properties. Since then, the use of volatiles have expanded to pharmaceutical, agricultural and chemical industries as flavorants, drugs, pesticides and feedstocks (Schwab et al. 2008). Fruits, in particular, produce many volatile compounds that contribute to their characteristic aroma and flavor (El Hadi et al. 2013). Plant-derived volatiles

can be categorized according to their chemical structures, which include terpenoids, esters, aromatics, amines, alcohols, aldehydes, ketones, all of which vary in their biological activities and subsequent absorption once it has entered the body. Blackberries, in particular, are dominated by esters and alcohols but also possess a wide variety of other volatiles that contribute to its aroma and biological activities (D'Agostino et al. 2015, Meret et al. 2011). For example, blackberry volatiles have been shown in a previous *in vitro* study to show significant modulation of production of NO, interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) in a RAW264.7 macrophage cell model in comparison to six other berries (Gu 2018).

Perhaps the most important aspect of researching dietary bioactive compounds is to determine the compounds' bioavailability, or the extent to which a compound is absorbed and distributed. The ability of a compound to exert its biological effects depends on its bioavailable dose, rather than the administered dose. Limited research has been done on the bioavailability of specific berry essences, or concentrated volatiles. Therefore, it is of interest to explore the prospective health benefits of volatile and semi-volatile compounds found in blackberry essences. The objective of this study is to assess the bioavailability of blackberry essences by measuring and identifying individual volatile compounds transported across the Caco-2 cell monolayer.

## Materials and Methods

### *Materials*

Blackberry essences were obtained from Dr. Luke Howard's lab (University of Arkansas, Fayetteville, AR). All materials were capped, tightly sealed and stored in amber glass bottles at -20°C until use.

***Headspace-Solid Phase Microextraction-Gas Chromatography- Mass Spectrometry (HS-SPME-GC-MS) Profile of Blackberry Volatiles***

HS-SPME-GC-MS analysis was conducted to determine the profile of blackberry essences prior to experimentation. Frozen blackberry essences were first allowed to thaw to room temperature. An 85 μm carboxen/polydimethylsiloxane, Stableflex, 24 Ga, manual SPME fiber was then inserted into 20 mL glass headspace vial with 3 mL of blackberry incubated for 30 min at 65°C with magnetic stirrer. Volatiles adsorbed to the SPME fiber were then desorbed at 270°C for 2 min in the injection port of a Varian 3800 GC (Agilent Technologies, Santa Clara, CA, USA) with a HP-5 (5% phenyl-methylpolysiloxane) column (30 m $_X$ 250 μm $_X$ 1 μm) (Agilent Technologies, Santa Clara, CA, USA) connected to a Bruker (Billerica, MA, USA) model Esquire-LC/MS ion trap mass spectrometer. For gas chromatography, the conditions were as follows: flame ionization detector (FID) temperature kept at 280°C, total run time at 29 min, and the fiber kept in the injection port for 10 min after each run. The injection port operated in splitless mode with a constant helium flow rate at 25 psi. The initial temperature gradient was 25°C, held for 4 min, ramped at 12°C/min to 289°C, and held at 3 min. The mass spectrometry analysis was performed in positive ion mode under the following conditions: capillary voltage at 4 kV with polarity [-] for positive ion 56 mode analysis, nebulizer gas pressure 30.0 psi, dry gas flow of 9.0 mL/min, and a temperature of 300°C. Data were collected in full scan mode over a mass range of m/z 50-1000 at 1.0 s per cycle.

***Headspace Solid Phase Microextraction Gas Chromatography (HS-SPME-GC) of Blackberry Volatiles in Transport Assay***

The identification of individual blackberry volatiles collected during the transport assay were subject to the same method as described above with the following modifications: an 85 μm

carboxen/polydimethylsiloxane, Stableflex, 24 Ga, manual SPME fiber was inserted into 4 mL glass headspace vial with 0.25 mL of samples from all time points as well as the apical and basolateral layer, and incubated for 30 min at 65°C with magnetic stirrer. Blackberry volatiles were identified by GC-MS and further confirmed by retention indices using data of a n-alkane mixture (C7-C20).

***Cell Culture Maintenance***

Caco-2 human colon cancer cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) at passage number 18. Cells were cultured according to the protocol of Salyer and colleagues (Salyer et al. 2013) in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 2% antibiotic-antimicotic, and incubated at 37 °C in a humidified 5% CO2 atmosphere. Cells were subcultured at 80-90% confluency.

***Preparation of Cell Monolayers for Transport Assay***

At 80-90% confluency, Caco-2 cells were trypsinized and seeded onto Transwell inserts (polycarbonate membrane, 12 mm i.d., 0.4 μm pore size, Corning Inc., Kennebunk, ME) in 12-well plates at a density of $1.0 \times 10^5$ cells/cm$^2$.

The seeded Transwell inserts were treated with 500 uL and 1500 uL of WMEM on the apical and basolateral side, respectively. Cells were allowed to grow and differentiate to form a monolayer for 14-21 days post-seeding. The growing medium were replaced at days 3, 5, and daily thereafter. Transepithelial electrical resistance (TEER) of cells grown in the Transwell were measured using the Millicell ERS-2 voltohmmeter (Millipore Corp., Billerica, MA, USA) to ensure cell monolayer integrity. Only monolayers with TEER values >400 Ωcm$^2$ were used

for further experimentation. Only passages 25-40 were used in this assay since a defined interval of passages is recommended to ensure a consistent cellular phenotype in all experiments.

***Transport Assay***

In preparation for the transport assay, frozen blackberry volatiles were first allowed to thaw to room temperature from -20°C. The stock solution was prepared by creating a 1:1 dilution of WMEM warmed to 37°C and thawed blackberry essences immediately prior to experimentation.

Prior to each assay, WMEM in both chambers was aspirated and 500 uL of the treatment were added to the apical chamber in duplicate. Meanwhile, 1 mL of phosphate-buffer solution (PBS) was added to the basolateral chamber.

To start the experiment, the system was incubated at 37 °C for 4 h. 500 uL samples were drawn from the basolateral chamber every hr for up to 4 hr. The basolateral chamber was replenished with 500 uL of PBS at each time point. At 4 h, remaining solutions in both chambers were collected. The apical and basolateral chambers were then washed with 2000 uL and 1000 uL of PBS, respectively, pooled with the samples drawn at the 2 h time point and vortexed for 10 s. The stock solution as well as aliquots drawn from the apical chamber at the end of the experiment were used to calculate mass balance.

The insert membrane was carefully peeled off with a tweezer and suspended with 0.5 mL of ice-cold MQ water. The cell membrane was rinsed once more with 0.5 mL of ice-cold MQ water. Then, 500 uL 1 N NaOH was added and the mixture was vortexed for 10 s to solubilize cells from the membrane. All aliquots and cell membrane suspensions were frozen at -20°C until further analysis.

### *Statistical Analysis*

All statistical analyses was performed using Statistical Analysis Software (System Version 9.4; SAS Institute Inc., Cary, NC) using a one-way analysis of variance test (ANOVA). Data was expressed as means ± standard error of mean (SEM). A difference of $p<0.05$ was considered significant.

## Results

### *Headspace Solid Phase Microextraction Gas Chromatography- Mass Spectrometry (HS-SPME-GC-MS) Profile of Blackberry Essences*

Figure 1 is a GC-MS spectrum highlighting the numerous volatiles that make up the complex profile of blackberry essence. Figure 2 categorizes the various functional groups that are found in the blackberry essence profile. Abundance is as follows: esters (32%), monoterpenes (25%), alcohols (13%), acids (11%), aldehydes (7%), ketones (7%) and other unknown compounds (5%).

The tentative identification of individual volatiles found in the blackberry essence using HS-SPME-GC-MS are listed in Table 1. The volatiles are arranged by retention time and characterized by percent area of all peaks found in the essence profile. The observed retention index (RI) was matched as closely as possible to the reported RI of the volatile as calculated using data of a n-alkane mixture ($C_7$-$C_{20}$).

### *Headspace Solid Phase Microextraction Gas Chromatography (HS-SPME-GC) of Blackberry Essences in Transport Assay*

While the quantitation of each volatile recovered during the transport assay could not be performed due to limited time and an incomplete range of standards for all blackberry volatiles found in the essence, identification of the volatiles was performed by GC and further confirmed by retention indices as previously shown. Figure 3 is an example of a GC chromatogram of

blackberry volatiles at time point 0 h, 1 h, and 4 h from both the apical and basolateral chamber during the transport assay. The changes in peak areas give a visual representation in the transport of each compound as time progresses.

Table 2 is a summarized version individual berry volatiles during transport assays (n=19) at time points 0, 1, 2, 3, and 4 h in both the apical chamber and basolateral chamber performed using the same methodology as previously described. Since quantitation was not completed for the individual volatiles, the percent area of each volatile found in the starting material was used as a reference to determine transport instead. Each tentatively identified volatile in Table 2 is arranged by retention time and observed RI after performing GC analysis. The column "percent transported at each time point" refers to each volatile's observed peak area at each time point during the assay divided by the peak area in the starting material. The value is then multiplied by 100 to yield a percentage of how much of the volatile was transported and recovered at each time point in comparison to the starting material. The column "percent area of starting material in berry essence profile" refers to the peak area of that volatile divided by all other volatile peak areas in the starting material and multiplied by 100 to yield a percent value. "% sum of area recovered in basolateral chamber" refers to the percentage of how much of the starting material was transported to the basolateral chamber, whereas "percent sum of total area recovered" indicates the percentage of how much of the starting material was recovered during the transport assay. Ideally, the values should add up to yield 100%; however, a percent mass balance is simply impossible. In this case, numbers above or below 100% may be attributed to the formation of byproducts. For example, a value over 100% could be that the breakdown product of another volatile present in the blackberry essence turned into the volatile of interest. On the other hand, a value under 100% could mean that the volatile of interest has been modified

structurally or produced other breakdown products, therefore reducing the area of the total parent compound. The volatiles with blank rows in the "percent transported at each time point" column are those with peak interference from background noise, whether due to components of PBS or WMEM co-eluting at the same time. Therefore, the values were omitted due to false responses.

Several volatiles were recovered at time point 0 h, which could be possible due to faulty monolayer formation or incomplete tight junctions between the Caco-2 cells. Another explanation is that the volatiles were low in molecular weight and/or highly lipophilic, expediting passive transport almost immediately across the cell monolayer. Several volatiles high in recovery at this time point include 1,3-benzenediol, 4-ethyl- or perilla alcohol, decanal, 2-heptenal, α-terpineol and/or dodecane, and linalool (Table 2). 1,3-benzenediol, 4-ethyl is a benzenediol with a low molecular weight of 124.139 g/mol, whereas decanal is a ten-carbon aldehyde that occurs naturally and is used in fragrances and flavoring (Kim et al. 2016). 2-heptenal is considered a fatty aldehyde lipid molecule, which may explain its affinity to cross the cell membrane (Kim et al. 2016). Linalool, a terpene alcohol, was shown to possess Papp values in the range of $2.6 \times 10^{-6}$ to $1.8 \times 10{-}4$ cm $s^{-1}$, indicating a bioavailability of more than 90% (Heinlein et al. 2014).

At time point 1 h, 1,3-benzenediol,4-ethyl or perill alcohol, 2-propyl-1-pentanol and several other volatiles found abundantly were detected. 2-Propyl-1-pentanol is a primary alcohol with a molecular weight of 130.23 g/mol that peaks in transport at time point 1 h but tends to decrease over time. The transport of 1,3-benzenediol, 4-ethyl is also highest at time point 1 h but steadily decreases over time. Interestingly, linalool and decanal maintained somewhat consistent transport over time. Several occurrences are observed at time point 2 h. The volatiles with the highest percent of transport included 2-heptanone and 2-hexenol, which are not readily detected

at other time points. They were also only observed in a small number of wells throughout the transport assay; therefore, the data for these volatiles may be inconsistent. On the other hand, aldehydes such as 2-heptenal, pentanals and the terpene alcohol linalool continue to transport across the monolayer. Results from time point 3 h show that out of the top four most abundant volatiles, three were aldehydes, specifically 2-hexenal, 2-heptenal and pentanal. 2-hexenal, is an aldehyde that occurs in many plants and is responsible for the "green" smell and taste (Kim et al. 2016). Finally, the basolateral chamber at time point 4 had an usually high amount of pinocarveol, which may be another example of inconsistent data due to its absence at every other time point as well as the apical chamber. The next most abundant volatile was decanal, followed by 2-heptenal and gamma-ionone – a high molecular weight sesquiterpenoid (192.3 gm/mol) found in fruits and used readily in the fragrance and food industries (Kim et al. 2016). Interestingly, gamma-ionone was detected at later time points compared to other abundant volatiles, which could be due to its bulky structure and high molecular weight.

Overall, it appears that though monoterpenes and esters constitute the major groups of compounds found in black berry essences, compounds belonging to aldehydes and alcohols tended to yield the highest percentage of transport throughout the time points in the transport assay. For example, decanal and 2-heptenal had the top two highest sum of percentage areas recovered, followed by 1,3-beneznediol, 4-ethyl, linalool, heptanal and pentanal. However, the sum of % areas for these volatiles were more than 100%, which may mean that other volatiles may have had breakdown products that contributed to final yield.

**Discussion**

The aroma of berries play a major role in its overall quality in fresh or processed fruits. In addition to its pleasant aromatic profile, berries also produce volatiles with functional properties that help the plant survive in its environment. Research on the biological activities of plant

metabolites have mostly been focused on polyphenols, especially antioxidants found within berries. However, polyphenols are typically characterized by low oral bioavailability as demonstrated by *in vitro* and *in vivo* studies. As of recent, biological activities exhibited by volatiles (the concentrated form also known as essences) found in products such as essential oils have garnered research interest. Volatiles found within blackberry essences, including compounds such as linalool and nerol, have been shown to have antimicrobial and antioxidant effects (Heinlein et al. 2014, Ladeira Coêlho et al. 2016). Structurally, volatiles tend to be lipophilic and low in molecular weight, which could result in rapid absorption and transport across the small intestine due to the affinity towards cell membranes. These characteristics could give volatiles an advantage over compounds such as polyphenols and therefore having a higher chance of eliciting biological activities, as a compound must be absorbed and distributed to have an effect in the body. Currently, studies regarding the bioavailability of berry essences do not exist, although the bioavailability of several volatile components have been elucidated.

In a study by Heinlein and colleagues (2014) investigating the permeability of hop aroma compounds across Caco-2 monolayers, it was noted that nerol exhibited a high initial increase and the beginning of a steady-state after 80 – 100 min, while linalool showed a slower increase and an earlier steady-state after 60 min. The recovery percentages of nerol and linalool were 58.8% and 25.6%, respectively. In the current study, nerol was not found in the blackberry essence profile, which could be due to many factors. This may include differences in cultivars, environmental conditions during plant growth, soil composition, external stressors, and the like. Linalool, on the other hand, was found in the blackberry essence profile. Although quantitation of each volatile was not calculated due to limitations in the present study, an alternative was to use the percentage of starting material as a reference when tracking the amount of volatile

transported over time. Linalool has shown a relatively steady rate of transport throughout the assay, which corresponded with the data from Heinlein and colleagues.

Other volatiles that exhibited high rates of transport tended to be structurally similar to each other – specifically aldehydes such as decanal, 2-heptenal, heptanal, and pentanal. Heptanal tended to decrease over time, indicating rapid transport initially but slowing down as the transport assay continued. 2-heptenal fluctuated throughout the 4 time points, whereas decanal and pentanal maintained a steady rate of transport. 1,3-benzenediol, 4-ethyl appeared in abundance during the first two hr and slowed down as time went on.

In the future, more research needs to be done on the quantitation of individual volatiles during transport assays. The mechanism behind volatile transport across the Caco-2 cell monolayer will provide insight on how the compounds are absorbed in addition to determining their permeability and potential metabolites responsible for biological effects.

**Conclusion**

In conclusion, even though monoterpenes and esters are readily found in the blackberry essence profile, compounds categorized as aldehydes and alcohols tended to yield the highest percentage of transport throughout the time points in the transport assay. This research demonstrates that blackberry essence possesses a complex mixture of different volatiles, each differing in their structure, biological activity, and absorption.

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# APPENDIX

**Table 1.** Identification of individual volatiles and percentage area of total peaks found in blackberry essence using HS-SPME-GC-MS.

| retention time (min) | % area | observed RI | reported RI | tentative identification | retention time (min) | % area | observed RI | reported RI | tentative identification |
|---|---|---|---|---|---|---|---|---|---|
| 5.68 | 0.01 | 710 | 711 | 2-pentanone (methyl propyl ketone) | 15.39 | 0.17 | 1048 | 1048 | ocimene |
| 6.02 | 0.07 | 719 | 718 | acetoin (3-hydroxy-2-butanone) | 15.45 | 0.08 | 1051 | 1049 | (z)-2-octenal |
| 7.32 | 0.17 | 754 | 753 | 2-methyl-2-butenal or pentanal | 15.65 | 2.02 | 1060 | 1057 | mesifuran |
| 7.44 | 0.2 | 757 | 756 | ethyl methylpropanoate | 15.9 | 4.99 | 1072 | 1072 | 1-octanol |
| 7.78 | 0.02 | 766 | 767 | methyl 2 methylbutanoate | 16.16 | 0.06 | 1084 | 1084 | m-cresol, 3-methyl-phenol |
| 7.91 | 0.06 | 769 | 768 | 2-methyl-2-pentanol | 16.28 | 0.11 | 1090 | 1089 | guaiacol |
| 8.09 | 0.01 | 774 | 773 | toluene | 16.38 | 3.51 | 1094 | 1093 | 2-nonanone |
| 8.28 | 0.06 | 779 | 779 | 3-methyl-2-buten-1-ol | 16.53 | 3.53 | 1102 | 1100 | linalool |
| 8.5 | 0.18 | 785 | 785 | dimethyl disulfide | 16.6 | 2.15 | 1105 | 1104 | nonanal |
| 8.63 | 0.12 | 789 | 789 | 1-hexen-3-ol | 16.79 | 0.26 | 1115 | 1115 | 1,3,8-p-menthatriene |
| 9.02 | 0.02 | 799 | 798 | 4-methyl-3-penten-2-one or 3-hexanal | 16.85 | 0.22 | 1118 | 1118 | p-cymenene or 2-phenylethyl alcohol |
| 9.2 | 0.02 | 805 | 804 | ethyl butanoate | 16.92 | 0.13 | 1121 | 1122 | trans-p-mentha-2,8-dienol |
| 10.05 | 0.1 | 834 | 833 | 3-ethoxy-1-propanol | 17.01 | 0.3 | 1126 | 1126 | menthone |
| 10.24 | 0.06 | 841 | 843 | 1-(methylthio)ethanethiol | 17.08 | 0.12 | 1130 | 1131 | nerol oxide |
| 10.35 | 0.01 | 845 | 844 | (e)-2-hexenal or ethyl 2-methylbutanoate | 17.16 | 0.23 | 1134 | 1132 | limonene oxide |
| 10.41 | 0.02 | 847 | 848 | ethyl-2-methylbutanoate | 17.3 | 0.28 | 1141 | 1142 | (e)-limonene oxide or α-fenchyl alcohol |
| 10.57 | 0.19 | 852 | 854 | (e) 2-hexanal | 17.45 | 0.56 | 1149 | 1149 | 2-octyl acetate |
| 10.8 | 0.74 | 860 | 858 | 3-hexenol (z) | 17.53 | 0.8 | 1153 | 1154 | nonanol |
| 10.92 | 0.45 | 865 | 866 | 3-methyl-2-butanone | 17.7 | 0.76 | 1161 | 1162 | benzyl acetate or borneol |
| 11.3 | 10.86 | 878 | 877 | isovaleric acid or 2,4-dimethylthiazole | 17.8 | 0.2 | 1166 | 1166 | p-cymenol or (e,z)-2,6-nonadienol |
| 11.79 | 1.37 | 895 | 895 | 2-heptanone | 17.92 | 1.08 | 1173 | 1172 | (e)-linalool oxide |
| 12.13 | 25.64 | 908 | 907 | formic acid, hexyl ester - hexyl formate | 18.06 | 0.48 | 1180 | 1179 | terpinen-4-ol |
| 12.23 | 1.29 | 912 | 911 | 4-methyl-1-hexanol | 18.18 | 2.08 | 1186 | 1187 | 1-methyl-1-octanol, nonan-2-ol |
| 12.65 | 0.06 | 929 | 929 | ethyl 3-hydroxy-3-methylbutanoate | 18.27 | 3.52 | 1190 | 1190 | dihydrocarveol |

**Table 1. (Cont.)**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 12.75 | 0.05 | 934 | 935 | ethyl 3-hydroxybutanoate | 18.41 | 1.11 | 1198 | 1195 | α-terpineol |
| 12.87 | 0.09 | 939 | 939 | α-pinene | 18.55 | 2.38 | 1205 | 1203 | myrtenol |
| 13.05 | 0.15 | 946 | 946 | 1-isothiocyanato-butane or pseudocumene | 18.78 | 1.6 | 1218 | 1217 | (e)-carveol or (e,e)-2,4-nonadienal |
| 13.17 | 0.12 | 951 | 953 | (+)-camphene | 18.88 | 0.47 | 1224 | 1223 | linalyl formate |
| 13.3 | 0.05 | 956 | 960 | benzaldehyde | 18.98 | 0.49 | 1229 | 1229 | (z)-carveol |
| 13.4 | 1.03 | 960 | 963 | benzaldehyde | 19.12 | 0.19 | 1237 | 1239 | hexyl 2-methylbutyrate or benzothiazole |
| 13.52 | 0.25 | 965 | 965 | 3-octanone | 19.32 | 1.76 | 1249 | 1250 | (z)-2-decenal |
| 13.6 | 0.16 | 969 | 968 | ethyl isohexanoate, 4-methyl pentanoic acid ethyl ester | 19.4 | 0.9 | 1253 | 1254 | carvone |
| 13.71 | 1.24 | 973 | 972 | sabinene or trans-2-pentenol | 19.47 | 0.79 | 1257 | 1256 | bornyl formate or p-anisyl alcohol |
| 13.93 | 1.07 | 982 | 981 | octanol | 19.77 | 0.41 | 1274 | 1275 | perilla aldehyde |
| 14.09 | 0.25 | 989 | 985 | sulcatone | 19.99 | 0.1 | 1286 | 1287 | 4-ethylguaiacol |
| 14.23 | 0.55 | 995 | 992 | β-myrcene | 20.21 | 0.46 | 1299 | 1299 | gamma butyrolactone |
| 14.31 | 0.65 | 998 | 998 | 4-carene | 20.31 | 0.15 | 1305 | 1302 | nonyl acetate |
| 14.46 | 0.65 | 1005 | 1006 | octanal | 21.35 | 0.96 | 1364 | 1364 | eugenol, 4-allyl-2-methoxyphenol |
| 14.56 | 0.32 | 1009 | 1009 | 3-carene | 21.67 | 1.11 | 1383 | 1382 | geranyl acetate |
| 14.65 | 2.74 | 1014 | 1014 | hexyl acetate | 23.23 | 0.96 | 1478 | 1477 | r-δ-decenolactone or linalyl isovalerate |
| 14.88 | 0.55 | 1024 | 1027 | p-cymene | 25.15 | 1.1 | 1603 | 1604 | cedr-8(15)-en-9-ol |
| 15.02 | 2.23 | 1031 | 1033 | limonene | 25.3 | 0.16 | 1613 | 1614 | (e)-2-hexenoic acid |
| 15.23 | 0.72 | 1041 | 1041 | acetophenone | 26.32 | 0.15 | 1685 | 1685 | γ-dodecalactone |

RI: retention index

**Table 2.** Summary of individual berry volatiles recovered during transport assay at time points 0, 1, 2, 3, and 4 h.

| | | | % transported at each time point | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **retention time (min)** | **observed RI** | **tentative identification** | **T0** | **T1** | **T2** | **T3** | **T4 Baso** | **T4 Api** | **% area of starting material in berry essence profile** | **% sum of area recovered in basolateral chamber** | **% sum of total area recovered** |
| 3.5 | 656 | acetic acid | | | | | | | | | |
| 4.3 | 706 | pentanal | 23.1 | 17.8 | 29.9 | 26.8 | 15.8 | 8.7 | 2.7 | 90.3 | 122.1 |
| 4.9 | 723 | butanoic acid, methyl ester | 3.1 | 3.7 | 3.6 | 4.1 | 3.1 | 0.0 | 14.6 | 14.5 | 17.6 |
| 6.6 | 771 | toluene | 0.0 | 13.2 | 0.0 | 29.6 | 14.0 | 32.2 | 2.4 | 56.8 | 89.0 |
| 7.3 | 791 | hexanal | 4.9 | 8.5 | 10.4 | 8.7 | 0.0 | 11.8 | 6.2 | 27.7 | 44.4 |
| 8.3 | 822 | pentanoic acid, methyl ester | 0.0 | 0.0 | 0.0 | 0.0 | | 73.2 | 0.5 | 0.0 | 73.2 |
| 9.1 | 846 | 2,3-dimethyl-1-pentanol or 3-hexanol, 5-methyl- | | | | | | | | | |
| 9.4 | 855 | 2-hexenal | 27.7 | 0.0 | 0.0 | 32.9 | 0.0 | 35.6 | 1.1 | 32.9 | 96.2 |
| 10.1 | 877 | 2-hexenol | 0.0 | 0.0 | 72.8 | 0.0 | | 37.1 | 0.7 | 72.8 | 109.9 |
| 10.6 | 892 | 2-heptanone | | | | | | | | | |
| 10.9 | 902 | heptanal | 17.3 | 14.0 | 18.5 | 15.0 | 0.0 | 58.8 | 2.6 | 47.5 | 123.6 |
| 11.5 | 923 | 2-heptanol | | | | | | | | | |
| 11.9 | 937 | 4-methylheptan-2-one | | | | | | | | | |
| 12.2 | 947 | 2-heptenal | 37.8 | 0.0 | 34.7 | 29.9 | 25.6 | 26.6 | 1.1 | 90.3 | 154.7 |
| 12.3 | 951 | 2-heptanone, 4-methyl | 0.0 | 0.0 | 89.1 | 0.0 | 0.0 | 0.0 | 0.6 | 89.1 | 89.1 |
| 12.6 | 961 | benzaldehyde | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 0.0 | 0.0 |
| 12.9 | 972 | 1-octen-3-ol | 0.0 | 10.5 | 0.0 | 0.0 | 0.0 | 0.0 | 2.7 | 10.5 | 10.5 |
| 13.4 | 989 | unknown | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.9 | 0.0 | 0.0 |
| 13.6 | 996 | 5-hepten-2-ol, 6-methyl- (sulcatol) | 8.0 | 9.7 | 8.7 | 6.6 | 3.8 | 3.4 | 10.4 | 28.7 | 40.1 |
| 14.7 | 1040 | 2-propyl-1-pentanol | 0.0 | 31.0 | 28.1 | 14.2 | 17.0 | 9.1 | 3.3 | 90.4 | 99.5 |
| 15.2 | 1060 | 2-nonenal (e) | 0.0 | 0.0 | 0.0 | 16.0 | 0.0 | 11.6 | 2.5 | 16.0 | 27.6 |
| 16 | 1092 | 4-methyl-benzaldehyde | | | | | | | | | |
| 16.2 | 1100 | linalool | 30.2 | 17.2 | 25.2 | 21.8 | 15.3 | 31.7 | 1.8 | 79.4 | 141.4 |
| 16.6 | 1117 | nonanal | | | | | | | | | |
| 17 | 1135 | hexanoic acid, 2-ethyl- | | | | | | | | | |
| 17.3 | 1148 | pinocarveol | 0.0 | 0.0 | 0.0 | 0.0 | 98.8 | 0.0 | 1.1 | 98.8 | 98.8 |
| 17.6 | 1161 | borneol | | | | | | | | | |
| 18.1 | 1183 | terpinen-4-ol | | | | | | | | | |
| 18.5 | 1200 | alpha terpineol and/or dodecane | 33.8 | 14.5 | 11.6 | 10.0 | 11.0 | 17.0 | 3.8 | 47.1 | 98.0 |
| 18.7 | 1209 | decanal | 38.3 | 30.9 | 22.2 | 15.6 | 40.7 | 33.4 | 2.5 | 109.5 | 181.1 |
| 18.9 | 1218 | benzaldehyde, 2,4-dimethyl- | | | | | | | | | |
| 19.2 | 1232 | myrtenal | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 77.8 | 0.6 | 0.0 | 77.8 |
| 19.5 | 1245 | nonanoic acid | | | | | | | | | |
| 20 | 1268 | butanoic acid, heptyl ester | 4.0 | 6.2 | 5.6 | 5.6 | 0.0 | 0.0 | 6.7 | 17.4 | 21.4 |
| 20.3 | 1282 | butanoic acid, heptyl ester | | | | | | | | | |
| 20.6 | 1295 | 1,3-benzenediol, 4-ethyl- or perilla alcohol | 45.1 | 60.5 | 14.0 | 10.8 | 0.0 | 21.1 | 4.0 | 85.3 | 151.5 |
| 22.5 | 1347 | gamma-ionone | 8.5 | 0.0 | 11.3 | 8.5 | 17.8 | 13.3 | 3.6 | 37.6 | 59.4 |
| 24.5 | 1400 | tetradecane | | | | | | | | | |
| 26.6 | 1507 | 2,4-di-tert-butyl phenol | | | | | | | | | |
| 29.5 | 1700 | 2-hexadecanol | | | | | | | | | |

RI: retention index (n=19).

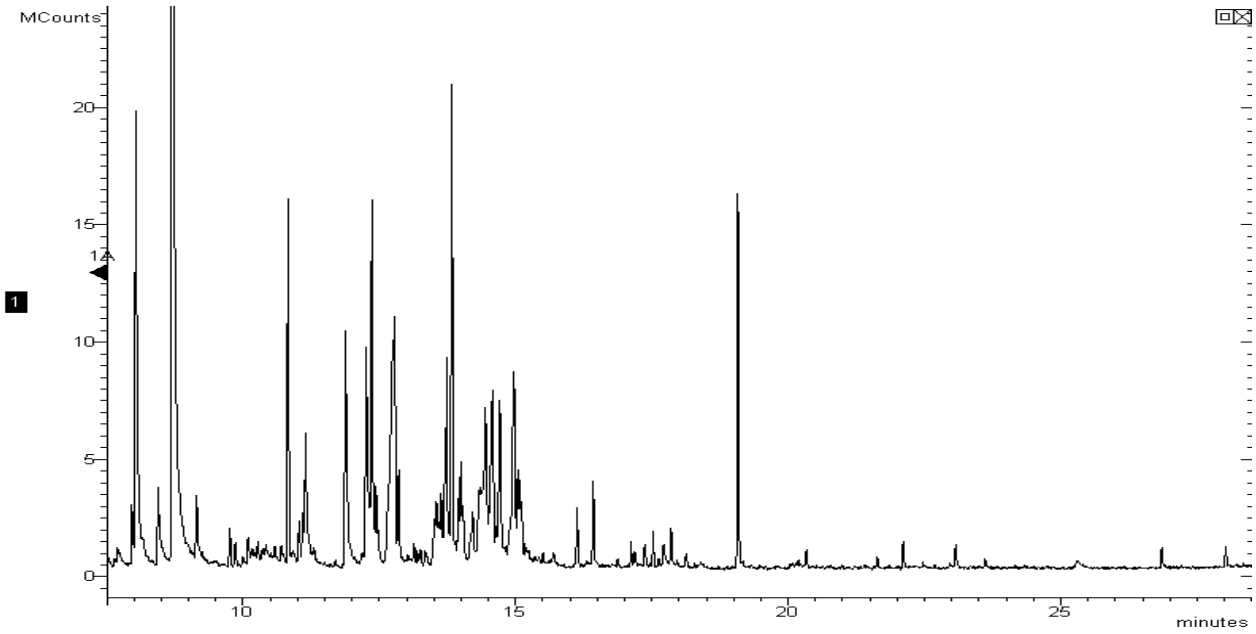


**Figure 1.** GC-MS chromatogram of blackberry essence profile.

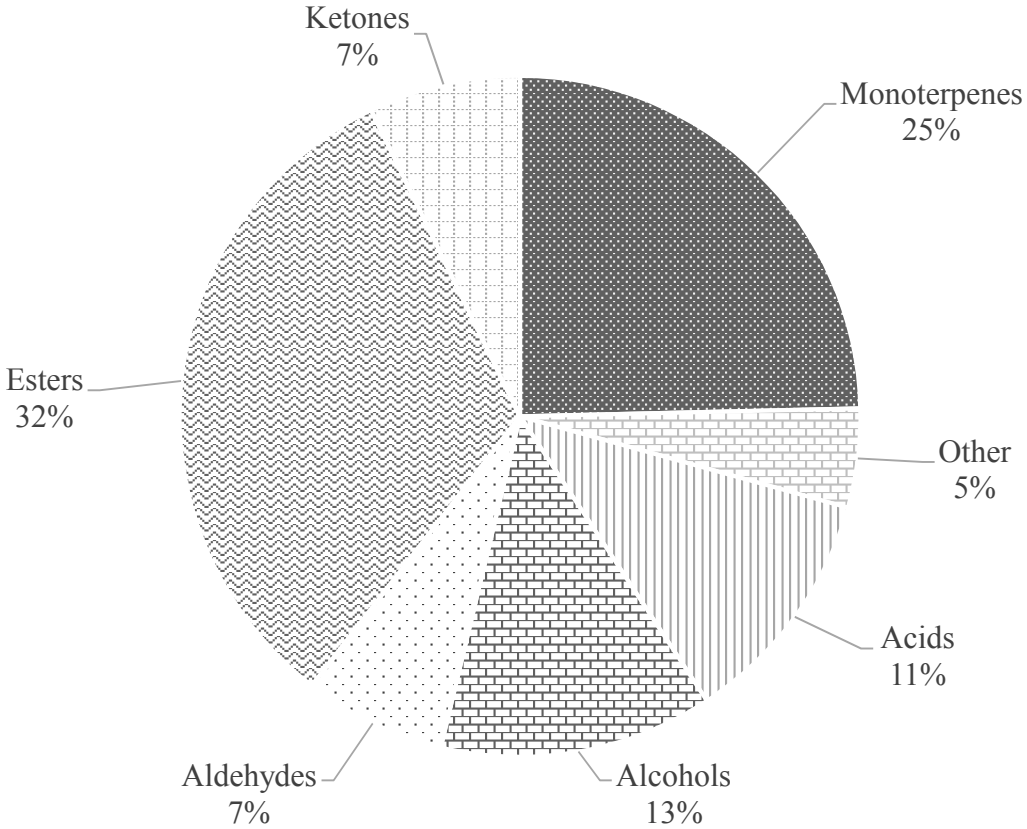


**Figure 2.** Distribution of functional groups found in blackberry essence profile.

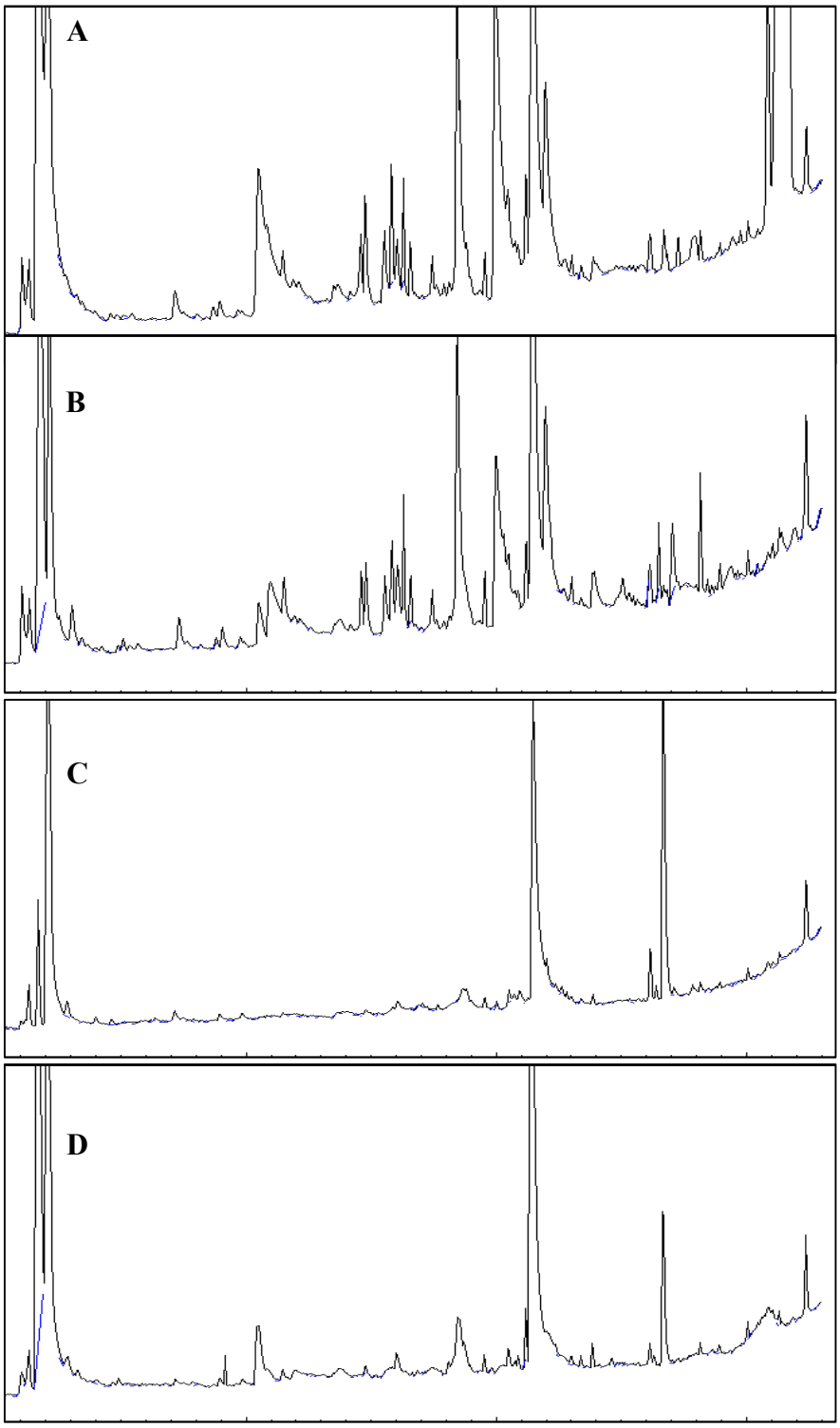


**Figure 3.** GC chromatogram of blackberry volatiles at various time points during the transport assay. (A) 0 h from basolateral chamber; (B) 1 h from basolateral chamber; (C) 4 h from apical chamber; (D) 4 h from basolateral chamber.

# CHAPTER 5

## Overall Conclusion

This study demonstrated that Ara-1 and Ara-3 are not detected by HPLC in the basolateral chamber after performing transport assays with various transport buffers, preparation methods, and sample treatments over the course of 3 to 4 h. Overall, the stability of both compounds may be decreased due to polymerization or degradation from the effects of oxidation, pH, and/or cellular interactions between the compounds and cells during incubation conditions. Further research is necessary to ascertain why the use of Ara-1 and Ara-3 during cell culture assays may pose issues with stability and recovery. Additionally, the exact mechanism of the interaction between Caco-2 cells and Ara-1 and Ara-3 in the apical layer should be elucidated. Regarding the bioavailability of blackberry essences, compounds categorized as aldehydes and alcohols tended to yield the highest percentage of transport throughout the time points in the transport assay. Though monoterpenes and esters comprise the two largest functional groups in the blackberry essence profile, compounds that are less bulky and lower in molecular weight tended to transport at higher rates in comparison. This study demonstrates that blackberry essence possesses a complex mixture of different volatiles, each differing in their structure, biological activity, and absorption. Therefore, more research in this area needs to be conducted for a clearer understand on the apparent permeability of each volatile.

# APPENDIX


December 7, 2016

MEMORANDUM

TO: Dr. Sun-Ok Lee

FROM: Ines Pinto, Biosafety Committee Chair

RE: Protocol Modification

PROTOCOL #: 16011

PROTOCOL TITLE: Testing bioactive components (arachidin-1 and 3, conjugated linoleic acid, saponins, berry volatiles) for proliferation and inflammatory responses

APPROVED PROJECT PERIOD: Start Date September 10, 2015 Expiration Date September 9, 2018

The Institutional Biosafety Committee (IBC) has approved your request, dated December 5, 2016, to modify Protocol # 16011, "Testing bioactive components (arachidin-1 and 3, conjugated linoleic acid, saponins, berry volatiles) for proliferation and inflammatory responses".

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

*Form 1: GENERAL INFORMATION, contd*

## PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:

List all personnel (including PI and Co-PI) to be involved in this project:

| Name: (first and last) - POSITION (Title, academic degrees, certifications, and material field ofexpertise) | QUALIFICATIONS/TRAINING/RELEVANT EXPERIENCE Describe previous work or training with biohazardous and/or recombinant DNA and include Biosafety Levels) |
|---|---|
| Example: Bob Biohazard - Associate Professor, PhD Microbiology | 14 yrs working with E. coli at BL1, Salmonella enterica at BL2, 8 yrs working with transgenic mice. |
| Sun-Ok Lee - Assistant Professor | 16 years of experience in conducting research involving human cell and animal tissue cultures |
| Cindi Brownmiller - Research Associate | 12 months of experience in cell cultures |
| Lacy Nelson-Program Technician II | No experience. Get a training from Dr. Lee |
| Inah Gu- M.S. student | 5 months of experience in cell cultures |
| Macy Shirley - Undergraduate student | No experience. Get a training from Dr. Lee |
| Danielle Ashley- M.S. student | No experience. Get a training from Dr. Lee |
| Wing Shun Lam- M.S. student | No experience. Get a training from Dr. Lee |
| Brittany Frederick- M.S. student | No experience. Get a training from Dr. Lee |
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Additional Personnel Information (if needed):

All personnel took the EH&S Online training including bloodborn pathogens, hazardous waste, biosafety, fire safety, and autoclave safety.
All personnel are provided with the hepatitis A & B immunizations. The use of departmental cost center is allowable. It will keep a log as whom have received it and whom have declined.