Biotransformation and Antioxidant Capacity of Polyphenol-Rich Potato Extracts after Digestion in a Computer Controlled Dynamic Human Gastrointestinal Model

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This thesis is dedicated to my beloved family.

My parents Georges and Marlene,

my brother Halim, and my sister Carelle

who supported and encouraged me with their endless love and understanding.

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ABSTRACT

In vitro digestions mimicking gastric and intestinal enzymatic action in a chemical milieu have been used to examine the effects of digestion on polyphenol structure and function. However, most these studies have not accounted for the effects of human colonic bacterial action on polyphenols. The Computer Controlled Dynamic Human Gastrointestinal Model (GI model) is composed of five vessels representing different compartments of the gastrointestinal (GI) tract, the stomach, the small intestine (SI), the ascending colon, transverse colon, and descending colon. The aim of this thesis was to assess the change in polyphenolic profiles and antioxidant capacities of polyphenol-rich 'Onaway' potato extracts (PE) following their enzymatic and microfloral digestion in the vessels of the GI model. The predominant components in the PE were chlorogenic acid (CGA), caffeic acid (CA), ferulic acid (FA), and the flavonoid rutin (RU). A major change in the polyphenolic profile was detected in the colonic vessels after 24 h digestion of the PE as assessed by liquid chromatography mass spectrometry (LC-MS) and high performance liquid chromatography (HPLC). Major biotransformation was seen in the ascending colon vessel, where there was first contact of polyphenols with human colonic microflora showing a mixture of parent compounds and microbial polyphenol metabolites. At 24 h the parent polyphenols, CGA, CA, FA, and RU, were absent in the last 2 colonic vessels coinciding with the appearance of microbial metabolites. Different phenolic profiles were detected among the three colonic vessels with the most abundant microbial metabolites including derivatives of propionic acid, acetic acid, and benzoic acid. Antioxidant capacity assays; ferric reducing antioxidant power (FRAP) and 2,2′-azino-bis-(3-ethylbenzthiazoline sulfonic acid) (ABTS), increased in all vessels of the GI model upon the addition of the PE. A significant increase in FRAP activity ($P < 0.05$) was detected in colonic vessels after 16 h of fermentation which is likely the time required to produce the microbial metabolites. The ascending colon showed the greatest ($P < 0.05$) increase in ABTS at 24 h compared to baseline as it contained both the parent compounds and their microbial metabolites. The current study shows that the digestive process involving human colonic bacteria causes major changes in the polyphenolic profile that differ according the colonic region. Microflora play an important role in biotransformation of polyphenols by producing new metabolites that have a relatively high antioxidant activity.

RÉSUMÉ

Afin d'étudier l'effet de la digestion sur la structure et la fonction des polyphenols, les études in vitro qui reproduisent les conditions enzymatiques et chimiques de l'estomac et de l'intestin grêle sont utilisées. Malheureusement, la majorité de ces études, ne prennent pas en compte l'effet de la microflore intestinale. Le Modèle gastro-intestinal humain dynamique contrôlé par ordinateur (modèle GI) est composé de cinq récipients/bioréacteurs qui représentent respectivement les compartiments du système digestif humain: l'estomac, l'intestin grêle, le côlon ascendant, le côlon transverse, et le côlon descendant. Le bute de cette thèse était d'évaluer le changement du profil polyphénolique et de la capacité antioxydante des extraits de pommes de terre 'Onaway' riches en polyphénols après leur digestion enzymatique et microbactérienne par la microflore intestinale en utilisant le modèle GI. Les composants prédominants dans les extraits de pommes de terre étaient l'acide chlorogénique (CGA), l'acide caféique (CA), l'acide férulique (FA) et la flavonoïde rutine (RU). Un changement majeur dans le profil polyphénolique a été détecté par chromatographie en phase liquide couplée à la spectrométrie de masse (LC-MS) et chromatographie liquide haute performance (HPLC) dans les récipients/bioréacteurs du côlon après 24 h de digestion. Les biotransformations majeures ont été détectées dans le récipient/bioréacteur qui représente le côlon ascendant où les polyphénols sont fraichement exposés avec la microflore du colon. Ce récipient est composé d'un mélange de polyphénols parents et ses métabolites microbiens. Apres 24 h, les polyphenol parents, CGA, CA, FA, et RU étaient absents dans les deux dernier récipients/bioréacteurs coloniques. Des profils phénoliques différents ont été détectés parmi les trois récipients du côlon. Les métabolites microbiens les plus abondants étaient des dérivés de l'acide propionique, l'acide acétique et l'acide benzoïque. Le pouvoir de réduction des ions ferriques (FRAP) et l'activité antioxydante par 2, 2'-azino-bis [3-éthylbenzothiazoline-6-sulfonique] (ABTS) ont été augmentés après l'addition des extraits des pommes de terre. Une augmentation significative de l'activité FRAP ($P < 0.05$) a été détectée dans les récipients coloniques après 16 h de fermentation. Ceci pourrait correspondre au temps requis pour produire les métabolites microbiens. Le côlon ascendant a subi la plus grande ($P < 0.05$) augmentation en ABTS par rapport au scénario de référence avant l'addition des extraits des pommes de terre car il contenait à la fois les composés parents et de leurs métabolites microbiens. Cette étude montre que lors du processus digestif humain, la microflore intestinale entraîne des changements majeurs dans le profil polyphénolique qui diffèrent selon la région du côlon. La microflore joue un rôle important dans la biotransformation des polyphénols en produisant de nouveaux métabolites qui ont une activité antioxydante élevée.

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CONTRIBUTION OF AUTHORS

Joelle Khairallah (Candidate) was primarily responsible for developing the protocol of the study and conducting all experiments (digestion using the GI model, the antioxidant assays, preparation of samples for HPLC and LC-MS injection) in addition to organizing and analyzing the data. The candidate also wrote all the chapters of this thesis, and the mentioned manuscript, along with preparing the figures, tables, and pathway of biotransformation.

Dr. Stan Kubow (Supervisor of Candidate, Associate Professor, School of Dietetics and Human Nutrition, McGill University): Provided the original idea and impetus for the study, ongoing guidance and feedback in all aspects of the project; including protocol and methodology development. Dr.Kubow also edited and critiqued every chapter in this thesis. The input of Dr. Kubow was also in meeting with the candidate frequently to discuss and guide the progress of the work being done.

Ms. Shima Sadeghi Ekbatan (Doctoral Student; School of Dietetics and Human Nutrition): Participated in the all the lab work related to the GI model and part of the antioxidant assays. Gave suggestions for the study design development and analysis of results.

Dr. Kebba Sabally (Postdoctoral Fellow; School of Dietetics and Human Nutrition): Provided training with the HPLC and carried out the LC-MS detection of microbial metabolites.

Dr. Atef Nassar (Postdoctoral Fellow; Plant Science Department): Guided the statistical analysis.

Dr. Lekha Sleno (Collaborator; Chemistry Department, UQAM): Supervised the LC-MS assessment of polyphenols.

Ms. Laetitia Rodes (Doctoral Student; Department of Biomedical Engineering): Explained the aspects of the gut model, assisted with lab work on the model, and gave suggestions for the protocol.

Dr. Danielle Donnelly (Committee member; Plant Science Department): Guided the development of the protocol development and study design, and helped edit the thesis.

Dr. Satya Prakash (Associate Professor; Department of Biomedical Engineering): Shared the Computer Controlled Dynamic Human Gastrointestinal model and was available for guidance during the study.

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LIST OF ABBREVIATIONS

ABTS	2,2′-azino-bis-(3-ethylbenzothiazoline sulphonic acid)
CA	Caffeic acid
CGA	Chlorogenic acid
cryptoCGA	Cryptochlorogenic acid
CVD	Cardiovascular diseases
CQA	Caffeoylquinic acid
DPPH	1,1-diphenyl-2-picryl-hydrazyl
FA	Ferulic acid
FAA	5-O-feruloyl-L-arabinofuranose
FAX	Feruloyl-arabinoxylan
FEP	Fresh edible portion
FRAP	Ferric reducing antioxidant power
GI	Gastrointestinal
GI Model	Computer Controlled Dynamic Human Gastrointestinal Model
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LI	Large intestine
neoCGA	Neochlorogenic acid
ORAC	Oxygen radical absorbance capacity
PA	Phenolic acid
PE	Potato extract
RU	Rutin
SHIME	Simulated Human Intestinal Microbial Ecosystem
SI	Small intestine
V1	Stomach
V2	Small intestine
V3	Ascending colon
V4	Transverse colon
V5	Descending colon

Chapter 1

Introduction, Objectives, and Hypotheses

INTRODUCTION

1.1.Rationale and Statement of Purpose

Polyphenols are phytochemicals found in abundant concentrations in plants (Crozier et al., 2009). They can be divided into five groups based on the number of rings and different structures attached to them: flavonoids, phenolic acids, phenolic alcohols, stilbenes, and lignans (D' Archivio et al., 2007). Each group can be further subdivided (D' Archivio et al., 2007). Phenolic acids constitute one-third of the polyphenol intake in the diet and they include the hydroxycinnamic acids: caffeic acid (CA), chlorogenic acid (CGA); the ester of CA with quinic acid, in addition to ferulic acid (FA) and coumaric acid (Scalbert and Williamson 2000; Han et al., 2007). Rutin (RU) is a flavone; a subcategory of the flavonoid group (Nijveldt et al., 2001).

Polyphenols are a major source of antioxidants and several health benefits have been linked to their powerful antioxidant effects such as preventing low-density lipoprotein oxidation that may reduce the risk of cardiovascular diseases (CVD) (Yang et al., 2011). Epidemiological studies have associated the consumption of fruits, vegetables, and whole grains with a decreased risk of chronic diseases such as CVD, cancer, and diabetes, specifically attributed to their polyphenolic content (Verlangieri et al., 1985; Liu 2003; Arts and Hollman 2005; Mellen et al., 2008; Annema et al., 2011). A synergistic effect resulting from the presence of different polyphenols in fruits and vegetables is suggested to increase the antioxidant and antiproliferative activities (Chu et al., 2002; Sun et al., 2002). Additionally, in vivo and in vitro studies have shown a potential effect of polyphenols in attenuating inflammation in general and inflammatory bowel diseases in particular by different mechanisms that include inhibition of the nuclear factor (NF)-kB signaling pathways, as well as altering mRNA and protein expressions of inflammatory mediators induced with inflammation (Romier et al., 2009).

Polyphenols are supplied in large quantities in the human diet mainly from fruits, vegetables, whole grains, and beverages such as red wine, tea, and coffee (D' Archivio et al., 2007). Typically, the most commonly consumed food

commodities are the major contributors of polyphenols in the diet (Crozier et al., 2009). Potatoes, for instance, despite their relatively low polyphenolic content are ranked third after oranges and apples, and first among vegetables in contributing to the total phenolic content because they are widely consumed as a staple food by many populations worldwide (Chun et al., 2005; Camire et al., 2009). Potatoes contain phenolic acids, mainly CGA, in addition to CA, FA, and gallic acid (Reddivari et al., 2007).

Upon ingestion, polyphenols are digested by the intestinal enzymes and colonic microflora (Manach et al., 2004; Saura-Calixto et al., 2007). The digestive process is the first step, before absorption and metabolism, in modulating the bioavailability of polyphenols by affecting their stability and bioaccessibility (Saura-Calixto et al., 2007). A large amount of polyphenols reach the colon for later digestion by the microflora (Jenner et al., 2005). Results from animal studies and clinical trials comparing individuals with an intact colon to healthy ileostomy participants suggest that a large amount of CGA, CA, FA and RU reach the large intestine (LI) to get metabolized by the microflora (Hollman et al., 1995; Olthof et al., 2001; Lafay et al., 2006; Stalmach et al., 2009; Stalmach et al., 2010). This latter phenomenon is evident by the increase in the plasma levels of some microbial metabolites like derivatives of phenylpropionic acid, hippuric acid, benzoic acid, and phenylacetic acid, after more than 6-8 h from ingesting polyphenol-rich food, or supplements of pure polyphenol compounds (Hollman et al., 1997; Renouf et al., 2010). Colonic metabolism yields polyphenol metabolites with structures and functions that differ from the parent compound (Han et al., 2007). Microfloral action increases the health benefits of the polyphenols (Crozier et al., 2009). For instance, after the consumption of a meal containing onions, plasma levels of quercetin, a microbial metabolite of RU, were increased. This was accompanied by a decrease in urinary markers of oxidative stress and an increase in resistance of lymphocyte DNA to damage (Boyle et al., 2000).

In vitro digestions, mimicking gastric and intestinal enzymatic action and chemical milieu, are a tool to examine the effects of digestion on polyphenol structure and function. However, the majority of studies have not examined the

microbial action on these substrates. Basic in vitro fermentors and human fecal incubations have been used to assess the effect of bacteria on the generation of polyphenol metabolites (Kroon et al., 1997; Gonthier et al., 2006; Gumienna et al., 2011). Recently, polyphenol metabolism has been studied using novel complex continuous in vitro gut models that account for a different range of factors like pH, anaerobic conditions, and temperature (van Duynhoven et al., 2011). Continuous models consisting of several connected vessels representing different parts of the colon and are used for long term experiments as the microflora in the vessels are allowed a period of days to weeks for adaptation, unlike static models which are more limited (van Duynhoven et al., 2011).

The Computer Controlled Dynamic Human Gastrointestinal Model (GI model) (Fig. 3.1) is a computerized continuous model reproduced as an analogue of a previously described model, Simulated Human Intestinal Microbial Ecosystem (SHIME), by Molly et al. (1993). It is a continuous model that aims to demonstrate more physiological relevance by mimicking the different stages of the human gastrointestinal (GI) tract and accounting for the human microbial ecosystem. Briefly, the model is composed of 5 double jacketed vessels each representing part of the human intestinal tract: the stomach, the small intestine, the ascending colon, the transverse colon, and the descending colon. It is fully computer controlled. For example, the pH is controlled differently in each vessel and automatically adjusted according to the GI portion it represents. The temperature is kept at 37^{o}C. The anaerobic milieu is maintained in the vessels by flushing nitrogen daily through the air space.

The GI model was used in this thesis as a model to study the interactions among gastric and intestinal digestive processes, human microflora and the polyphenols. However, a limitation is that absorption is not simulated in this model. Hence, the production of post-absorptive metabolites generated via hepatic and intestinal cell metabolism, such as hippuric acid that is produced post-absorptively from benzoic acid, cannot be measured. Also, gut epithelial cells can have minimal esterase activity that is involved in the polyphenol breakdown (Andreasen et al., 2001). Such intestinal cellular metabolism can alter the

presentation of gut metabolites in the lumen including new or methylated metabolites (Kemperman et al., 2010). Likewise, peristalsis that affects the movement of luminal contents is not truly simulated despite the use of magnetic stirrers in the vessels to keep the solution and their constituents in motion. Despite these limitations, the GI model is a useful tool that simulates in vitro enzymatic and microbial digestion of polyphenols; it includes the gastric and pancreatic enzymatic components of the digestive process, which is more accurate and controlled compared to the classical pepsin-HCl, pancreatin-NaOH GI in vitro digestion used extensively to study polyphenol metabolism (Vallejo et al., 2004; Bermúdez-Soto et al., 2007; Saura-Calixto et al., 2007; Tagliazucchi et al., 2010). Anaerobic conditions are maintained in the GI model; unlike some in vitro digestions that did not account for the confounding aerobic effects on polyphenol breakdown (Vallejo et al., 2004). Additionally, the GI model is relatively better suited for assessment of microbial metabolism as compared to the use of one basic fermentor as the digestate moves from one colonic vessel to another simulating the transit of food. Importantly, the GI model compares relatively well to the microfloral component in the human GI tract as evidenced by studies of colostomy patients (Stalmach et al., 2009; Stalmach et al., 2010) and by the similarity of the contents of a three-stage culture system to the colonic contents of sudden death victims (Macfarlane et al., 1998).

Although in vitro studies have assessed the effects of digestion on stability and antioxidant capacity of polyphenols and the different metabolites produced, there is scant information regarding microfloral action on polyphenols or polyphenol biotransformation throughout digestive tract, especially in different segments of the colon. The aim of this study was to assess the change in polyphenolic profiles and antioxidant capacities of polyphenol-rich potato extracts following their enzymatic and microfloral digestion using the GI model.

1.2 Thesis objectives

To determine the HPLC and LC-MS profiles of polyphenols and their metabolites and to assess the antioxidant capacities of digests of polyohenol-rich potato extracts after enzymatic and microbial digestion in the GI model.

1.3 Hypothesis

The processes of digestion and microbial fermentation/metabolism of polyphenolic-rich potato extracts will alter the polyphenolic structures and antioxidant capacities of the content of digests in the GI model.

Chapter 2

Literature Review

2.1 Polyphenols - Antioxidant Activity and Classification

Reactive oxygen species and reactive nitrogen species, which include free radicals, are constantly produced as a by-product of the various biological reactions taking place within the human body (Fernandez-Panchon et al., 2008). Oxidative stress occurs when free radicals overwhelm the endogenous antioxidant capacity causing damage to cellular constituents (Boots et al., 2008). Therefore, the body requires an exogenous source of antioxidants, mainly supplied by the diet, to help protect from oxidative stress (Han et al., 2007; Fernandez-Panchon et al., 2008). Polyphenols are the major source of antioxidants as they are supplied in large quantities through the diet (D' Archivio et al., 2007).

Polyphenols are phytochemicals also known as "phenolic secondary metabolites" found in abundant concentrations in plants (Crozier et al., 2009). They are characterized by having one or more hydroxyl groups on aromatic rings (Manach et al., 2004). Thousands of polyphenolic structures have been identified, and those are categorized based on the number of phenol rings and the different structures attached to them (D' Archivio et al., 2007). They can be divided into 5 main groups: flavonoids, phenolic acids, phenolic alcohols, stilbenes, and lignans; each of which is also subdivided (D' Archivio et al., 2007).

2.1.1 Phenolic Acids

Phenolic acids (PA) constitute a group of polyphenols that are divided into two groups: i) the hydroxycinnamic acids which are the derivatives of cinnamic acid, such as caffeic acid (CA), ferulic acid (FA), p-coumaric acid and sinapic acid and ii) the hydroxybenzoic acids such as gallic acid and protocatechuic acid that are derivatives of benzoic acid (D' Archivio et al., 2007). Hydroxycinnamic acids have been studied extensively since they are more common in the human diet (Manach et al., 2004). Hydroxycinnamic acids are found as monomers, dimers, or in their bound form as esters (Zhao and Moghadasian 2010). In grains, hydroxycinnamic acids are primarily found in the form of FA, which is an insoluble bound form ester, while soluble hydroxycinnamates in a free form are found in fruits and vegetables (Zhao and Moghadasian 2010). Some of the fruits

and vegetables that contain hydroxycinnamic acids are apple, potato, kiwi, plum, cherry, artichoke, and blueberry (Manach et al., 2004).

2.1.1.1 Chlorogenic Acid and Caffeic Acid

Caffeic acid (CA) is the most abundant PA, representing between 75-100% of the hydroxycinnamic acid content in most fruits (D' Archivio et al., 2007). CA rarely occurs in its free form, and is mainly found bound to quinic acid by an ester bond, forming chlorogenic acid (CGA) (Manach et al., 2004; Zhao and Moghadasian 2010). CGA, also known as 5-caffeoylquinic acid (CQA), can also be found as isomers of neochlorogenic acid (3-CQA) (neoCGA) or cryptochlorogenic acid (4-CQA) (cryptoCGA) (Shakya and Navarre 2006). The concentration of both isomers in potato makes up approximately 15-20 % of the CGA content (Shakya and Navarre 2006).

Caffeic acid inhibited peroxynitrite-induced dopamine oxidation (Kerry and Rice-Evans 1999) and CGA was able to protect granulocyte oxidation in vitro (Bouayed et al., 2007). Moreover, CGA was found to act as an anxiolytic agent; it reduced anxiety in mice exposed to light/dark conditioning (Bouayed et al., 2007).

2.1.1.2 Ferulic Acid

Ferulic acid (FA, trans-4-hydroxy-3-methoxycinnamic acid) (Liu 2007) is the major phenolic acid in cereal grains (D' Archivio et al., 2007), and is found mostly in whole grains and whole grain products with minimal amounts seen in some fruits and vegetables (Liu 2007). FA is primarily found covalently bound to different structural components such as cellulose or lignin, or to sugars like monosaccharides and disaccharides (Liu 2007). For instance, the bound form of FA has been reported to constitute 62-85 % of the different grains: corn, wheat, rice, oat (Adom and Liu 2002). In potato, 40 % of the phenolic content is in the bound form, and most of this is FA that is ester linked to cell wall components (Chu et al., 2002). The antioxidant capacity of FA was reported to attenuate lipid peroxidation and the oxidative action of free radicals associated with diabetes in a

model of rats given streptozotocin to induce diabetes (Balasubashini et al., 2004) and.

2.1.2 Rutin

Rutin (RU, quercetin-3-rhamnosyl glucoside or quercetin-3-rutinoside) (Olthof et al., 2003; Yang et al., 2008), previously known as Vitamin P, is a flavone, which belongs to a subcategory in the flavonoid group and is one of the phenolic groups with the most powerful antioxidant activity (Nijveldt et al., 2001). Flavonoids mainly occur as glycosides in commonly consumed foods such as onion, tomato, and citrus fruits (Heim et al., 2002; Scalbert et al., 2002). RU is the glycoside of the aglycone quercetin (Manach et al., 1997; Yang et al., 2005). RU is formed when quercetin binds to the rutinoside molecule, which is a disaccharide composed of glucose and rhamnose (Hollman et al., 1995).

Both quercetin and RU are sold as antioxidant supplements in the United States (Graefe et al., 2001). RU and vitamin C, at similar concentrations, showed a strong radical scavenging activity with the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay (Yang et al., 2008). RU also inhibited lipid peroxidation and reduced the oxygen free radicals induced by Fe^{2+} in a concentration-dependent manner exhibiting a relatively high reducing power and potent antioxidant activity (Yang et al., 2008). Additionally, RU reduced DNA damage induced by menadione in human colon cells (Caco-2), implying its possible contribution to decreasing the oxidative damage associated with lower intestinal inflammation (Schaefer et al., 2006).

2.2 Daily Intake and Dietary Sources Polyphenols- Chlorogenic acid, Caffeic Acid, Ferulic Acid, and Rutin

Polyphenols are a major dietary source of antioxidants and are supplied mainly from grains, fruits, and vegetables, but also from certain beverages, primarily red wine, tea, and coffee (D' Archivio et al., 2007). Those food commodities consist of a mixture of diverse polyphenols (Manach et al., 2004). The average daily intake of polyphenols in North America is estimated to be 1 g/day (Scalbert and Williamson 2000), which varies with individual daily food

choices and differs among populations according to the food commodities consumed (Manach et al., 2004). Diet can play a very important role in determining the phenolic intake; where for instance the intake of total polyphenols could reach up to 2.6-3.0 g/person/day in the Spanish diet (Saura-Calixto et al., 2007).

PA make up one-third of the total polyphenolic intake (Scalbert and Williamson 2000). CGA, which is the ester of CA, is also present in high concentrations in coffee, as one cup of coffee could contain 70-350 mg of CGA (Clifford 1999). Some fruits like apples and pears contain approx. 62-385 mg and 60-28 mg CGA/kg, respectively (Clifford 1999). Blueberries contain 500-2000 mg CGA/kg (Clifford 1999). FA, mainly found in whole grain products, is believed to be the "second contributor to total hydroxycinnamic acids in people who consume whole grains" (Zhao and Moghadasian 2010). The consumption of FA from whole grains complements the intake of the other phenolic compounds found in fruits and vegetables (Liu 2007).

Individuals who drink coffee, eat bran-rich foods and citrus fruits could consume up to 500-800 mg of cinnamates per day (Clifford 1999). The dietary intake of cinnamates could also reach 1 g/day in people who drink a lot of coffee (Clifford 1999). Flavonoids make up the other two-thirds of the polyphenolic intake (Scalbert and Williamson 2000). In general, flavones are found in different fruits and vegetables like onion, celery, parsley, cranberry, and fruit peel (Nijveldt et al., 2001). Buckwheat and green asparagus are reported to be the major sources of the flavone RU (Yang et al., 2008). RU is also found in high concentrations in tea (Olthof et al., 2003).

It is important to note that despite the polyphenolic content of different food commodities, the most important commodities contributing the largest amounts of dietary polyphenols are foods that are consumed the most (Crozier et al., 2009). For instance, cereals are the principal dietary source of polyphenols in the Spanish diet despite their low polyphenolic content, whereas fruits and beverages high in polyphenols ranked next (Saura-Calixto et al., 2007). In the Greek diet, tomatoes, oranges, and potatoes were the main source of total phenolic

intake and antioxidant capacity (Dilis and Trichopoulou 2010). Apples, oranges and potatoes were the most consumed fruits and vegetables in the American and French diets and hence the major food sources of polyphenols (Chun et al., 2005; Brat et al., 2006).

2.3 Potatoes as a Source of Polyphenols

Potatoes (*Solanum tuberosum L.)* are widely consumed as a staple food worldwide by many populations (Camire et al., 2009). In addition to being viewed as a starchy food, potatoes are also a good source of vitamins and minerals including potassium, phosphorous, vitamin B6, thiamin, and folate. They also provide significant amounts of vitamin C as one medium sized potato can provide up to 45 % of the recommended daily value (Agriculture and Agri-Food Canada 2007; Camire et al., 2009). Additionally, potatoes provide a major source of polyphenols; specifically PA. In particular, CGA is the most abundant phenolic acid in potatoes, along with significant amounts of CA, FA, protocatechuic, and p-coumaric acids (Reddivari et al., 2007). Potatoes also contain flavonoids like RU and some cultivars contain carotenoids such as beta-carotene and lutein (Reddivari et al., 2007; Camire et al., 2009).

In Canada, potatoes constituted 44 % of the total vegetable intake in 2008 (Statistics Canada. Agriculture Division 2009). There has been an apparent recent increase in potato consumption as potatoes constituted 30 % of vegetable intake in 2003 and 37 % in 2005 (McLaughlin 2005; Agriculture and Agri-Food Canada 2007). In the French diet, potatoes along with lettuce and onions were the most important vegetable sources of phenolics. Potatoes had a high average consumption by both men and women (i.e., 63.48 g fresh edible portion (FEP)/day consumed by women and 90.04 g FEP/day consumed by men) and potatoes were thus indicated to be the principal vegetable source of polyphenols (Brat et al., 2006). In the French diet, artichokes, parsley, and brussels sprouts, which were the vegetables with the highest polyphenolic content, were not considered as important contributors to polyphenolic intake in the French population because of their lower consumption (Brat et al., 2006). Likewise,

strawberries and plums did not contribute significant amounts of polyphenols to the American diet, despite their relatively high phenolic content, due to relatively low intake (Chun et al., 2005). Therefore, the high consumption of potatoes makes them an important source of polyphenols and antioxidants, with the potential for consideration as a functional food (Thompson et al., 2009).

2.4 Bioaccessibility of Polyphenols

Polyphenols are primarily found in the bound form in food as esters, glycosides, or polymers, and are rarely consumed in the free form (Manach et al., 2004) . Once ingested, polyphenols are subject to digestion by intestinal enzymes and colonic microflora (Saura-Calixto et al., 2007). The health effects attributed to polyphenols depend partly on the dietary intake of polyphenol-rich foods, and more importantly on the amount of polyphenols that is bioaccessible.

Bioaccessibility refers to the "amount of food constituent" found in the digestive tract upon digestion, that is available for absorption through the intestinal walls (Saura-Calixto et al., 2007). Bioaccessibility is considered an aspect of bioavailability, which is defined as "the proportion of the nutrient that is digested, absorbed, and metabolized through normal pathways" (D' Archivio et al., 2007).

Digestion is the first step in modulating the bioavailability of the polyphenols by affecting their stability and bioaccessibility (Bermúdez-Soto et al., 2007; Tagliazucchi et al., 2010). Polyphenols are subject to digestion in the upper GI tract and a large amount reaches the colon for later digestion by the microflora (Jenner et al., 2005). Absorption takes place after digestion, and it is affected by the structure of polyphenols whereby esterified or glycosylated forms are less available for absorption (Scalbert et al., 2002). Polyphenols with low molecular weight are more readily absorbed as compared to those with a high molecular weight (Scalbert et al., 2002). Metabolism occurs during absorption in the small intestine (SI) and by the liver during first pass metabolism leading to the formation of sulfated, methylated and glucuronated polyphenolic structures in the plasma and urine (Scalbert et al., 2002; D' Archivio et al., 2007).

2.5 Gastrointestinal Digestion of Polyphenols- Chlorogenic Acid, Caffeic Acid, Ferulic Acid, and Rutin

Generally, to assess the changes of polyphenol structures upon gastrointestinal (GI) digestion, the in vitro dual pepsin-pancreatin digestion procedure is applied. This involves pH adjustment to 2.0 and 7.5 in the gastric and pancreatic digestions, respectively, during incubations at 37^{o}C in a shaking water bath over a 2-3 h time-frame (Bermúdez-Soto et al., 2007; Tagliazucchi et al., 2010; Kahle et al., 2011). Such studies have shown that PA including CGA and CA are stable under simulated gastric conditions, but some losses occur upon pancreatic digestion (Bermúdez-Soto et al., 2007; Tagliazucchi et al., 2010; Gumienna et al., 2011; Kahle et al., 2011). For example, no change in profiles of CGA and the neochlorogenic acid (neoCGA) isomer were noted after either purified CGA or chokeberry concentrate containing high concentrations of CGA or neoCGA were subjected to gastric digestion (Bermúdez-Soto et al., 2007). Additionally, the original amounts of CGA and neoCGA in chokeberry juice samples and pure CGA subject to gastric conditions were completely recovered (Bermúdez-Soto et al., 2007). Similarly, pure CA was completely recovered following exposure to in vitro gastric digestive conditions of pepsin-HCl action at pH 2 (Tagliazucchi et al., 2010). However, after pancreatic-simulated digestion of synthetic CGA, lower amounts of CGA were detected (Bermúdez-Soto et al., 2007). The loss of pure CGA resulted from its isomerization into neoCGA during pancreatic digestion since a simultaneous peak of neoCGA replaced the CGA (Bermúdez-Soto et al., 2007). Similarly, upon in vitro pancreatic digestion of synthetic CGA with simulated pancreatic juice for 24 h, 37 % of CGA was degraded and this was accompanied by the formation of CGA isomers; 3-CQA and 4-CQA (Kahle et al., 2011). A partial degradation of CGA into CA and quinic acid was also reported in the SI (Kahle et al., 2011). This observation was suggested to be caused by a minimal esterase enzymatic activity possibly present in the pancreatin used to simulate the pancreatic digestions (Gumienna et al., 2011; Kahle et al., 2011). This study did not support the earlier observations of

Bermúdez-Soto et al. (2007) after pancreatic-simulated digestion of chokeberry juice.

Little esterase activity was detected in vivo in the SI mucosa and lumen of rats (5-10 %), while the majority of esterase was detected in the large intestine (LI) lumen (50-95 %) (Andreasen et al., 2001). Minimal esterase activity in the SI possibly caused the intestinal release of trace amounts of FA esterified to spinach cell wall polysaccharides when rats were gavaged dietary spinach cell walls with ^{14}C labelled phenolic groups (Buchanan et al., 1996). The breakdown of the spinach cell wall polysaccharide upon enzymatic activity is important in releasing FA hence making a small portion available for absorption in the upper GI despite the larger amounts of FA released from microbial action (Buchanan et al., 1996).

Only 2.6 % of total FA in wheat bran was released after simulated gastric and intestinal digestion as compared to a larger release in FA, up to 95 %, after fermentation by microflora (Kroon et al., 1997). Food items containing FA in different forms including different wheat fractions, freeze-dried bread, bread, and flour enriched with pure FA were digested in the TIM system simulating upper GI digestion and absorption (Mateo Anson et al., 2009). FA from the flour enriched with pure FA, in the free form, was mostly collected in the dialysate and hence was the most bioaccessible (60 % bioaccessible) since unbound pure FA did not require further digestion. On the other hand, less than 1 % of FA from wheat and breads was bioaccessible as the majority of FA was in the bound form. This latter study results are in accordance to a rat feeding done with free FA or one of the two FA sugar esters; 5-O-feruloyl-L-arabinofuranose (FAA) or feruloyl-arabinoxylan (FAX) (Zhao et al., 2003). The free FA was efficiently and almost completely absorbed before reaching the cecum since no digestion was needed to release FA from a food matrix (Zhao et al., 2003). On the other hand, around 67 % of the FAA and almost all of the FAX were detected in the cecum since FA was in a more complex bound form. The digestion of FA thus depends on the form in which it is ingested.

After simulated gastro-intestinal digestion of tomatoes, Toor et al. (2009) reported the recovery of 71-77 % of total flavonoids of which RU constituted a

major part. This was in line with the 70-80 % recovery of flavonoids after the gastric and intestinal simulated in vitro digestion of chokeberry juice concentrate (Bermúdez-Soto et al., 2007). Both RU and its aglycone quercetin from chokeberry juice concentrate and the synthetic form of RU were completely recovered and unaffected by simulated gastric digestions (Hollman et al., 1995; Bermúdez-Soto et al., 2007). Quercetin was more stable at a lower pH as 74.8 % of quercetin was recovered after gastric digestion of onions at a low pH compared to 46.5 % recovery at intestinal pH (Boyer et al., 2005). Moreover, less quercetin was recovered when compared to its glycoside (74.3 % vs. 86.4 %, respectively), possibly because of the presence of the glucoside molecule that stabilizes quercetin and makes it less susceptible to enzymatic and chemical degradation (Boyer et al., 2005).

2.6 Absorption of Polyphenols - Chlorogenic Acid, Caffeic Acid, Ferulic Acid, and Rutin

Clinical trials with healthy ileostomy participants were done to assess the amount of phenolics absorbed in the upper GI. The phenolics excreted in the ileostomy effluent represented the amount likely to reach the colon under normal conditions. The ileostomy effluent was collected 13 h after participants consumed supplements of either fried onions rich in quercetin glucoside, pure RU (100 mg), or pure quercetin (100 mg) (Hollman et al., 1995). Results revealed that 52 %, 24%, and 17 % of quercetin glucosides from onions, pure quercetin, and RU were absorbed, respectively (Hollman et al., 1995). The lower absorption of RU was thought to be caused by the size of rutinoside disaccharide molecule. Only one-third of the ingested CGA supplement dose (1000 mg) was absorbed in the upper GI of ileostomy participants (Olthof et al., 2001).

Similarly, 71 % of ingested CGA and sulphated CA and FA were collected in the ileostomy effluent of participants 24 h after the consumption of coffee rich in CGA (0.345 mg) (Stalmach et al., 2010). Some studies have reported the absorption of some undegraded CGA in the upper GI of rats and humans (Lafay et al., 2006; Lafay et al., 2006; Farah et al., 2008). CA is more readily absorbed in

the upper GI as only 5 % was collected in the ileostomy effluent after CA supplement consumption (Olthof et al., 2001).

Likewise, FA in the free form is more readily available for absorption, as free FA given at different dietary doses (10, 50, or 250 μmol/day) was efficiently absorbed with approximately 50 % of the ingested dose appearing in the urine 18 h after feeding (Adam et al., 2002). On the contrary, when rats were fed their standard diet supplemented with different sources of cereal matrices like whole wheat or bran, FA absorption was reduced and urinary excretion was 90-95 % less (Adam et al., 2002). Therefore, the absorption of FA depends on its digestion, which itself is affected by the form in which FA is found (bound or free). Data from human studies also showed that FA from tomatoes (Bourne and Rice-Evans 1998) and beer (Bourne et al., 2000) was more bioavailable (11-25% and 19-98% respectively), compared to 3 % from bran (Kern et al., 2003). The lower FA biodegradability in wheat bran is likely due to the poor digestibility of the bran cell wall polysaccharides leading to a minimal release of FA for later absorption (Adam et al., 2002).

When healthy individuals with an intact colon consumed CGA-rich coffee, analysis of plasma showed a peak of CGA conjugates, sulfated CA, and FA, within 1 h of ingestion (Stalmach et al., 2009). However, plasma levels of metabolites such as dihydroferulic acid, dihydroferulic acid-4-*O*-sulfate and dihydrocaffeic acid-3-*O*-sulfate peaked after more than 4 h post-ingestion pointing out further degradation and absorption in the large intestine (LI) (Stalmach et al., 2009). Renouf et al. (2010) and Farah et al. (2008) also reported the appearance of two plasma levels whereby firstly, FA, CA, and conjugated forms of CGA plasma levels were detected 0.5-1 h after the consumption of coffee or CGA-rich green tea extracts while the dihydrocaffeic and dihydrofeulic acid metabolites appeared 6-8 h after ingestion. Likewise, quercetin plasma levels of participants who ingested pure RU peaked 9 h after ingestion as compared to a peak that appeared after less than 0.7 h or 2.5 h after ingestion of quercetin from onions or apples, respectively (Hollman et al., 1997).

2.7 Microfloral Metabolism of Polyphenols

2.7.1. Microflora in the Human Body

The GI tract harbours between 10 and 100 trillion microbes mainly found in the distal part of the GI tract; an amount that is around 10 times the number of cells in the human body (Williamson and Clifford 2010). The LI contains an immense amount of microflora estimated to encompass 200 g of living cells, found as 10^{12} microorganism/g of gut content (Possemiers et al., 2011). Therefore, this huge microbial ecosystem "should be considered as a separate organ", that possesses a high metabolic capacity (Possemiers et al., 2011).

Polyphenols reach the colon through two pathways; directly through the GI tract after ingestion or through bile (as glucuronides and/or sulfates) via the enterohepatic circulation (Gao et al., 2006; Possemiers et al., 2011). The amount of polyphenols excreted by the latter route is minimal given that the amount of conjugated metabolites detected in the intestine of rats from bile accounted for approximately 0.4 % of a gavaged dose of CA (Azuma et al., 2000) and 6 % of a perfused dose of FA (Adam et al., 2002).

The polyphenols that reach the colon will either get absorbed or acted upon by the microbial species, causing either a decrease or increase in their biological activity (Possemiers et al., 2011), as a result of the further degradation and transformation of the parent polyphenols (Selma et al., 2009; Kemperman et al., 2010). Microflora cause biotransformation by decarboxylation, demethylation, dehydroxylation reactions, or upon the hydrolysis of esters, glycosides, or glucuronides (Selma et al., 2009). The composition of the intestinal microbial community is affected by age, diet, and the environment (Possemiers et al., 2011). This causes a large inter-individual variability in the production rate and concentration of the polyphenolic metabolites when polyphenols are metabolized by intestinal microflora (Renouf et al., 2010).

2.7.2 Microfloral Metabolism of Chlorogenic Acid, Caffeic acid, Ferulic acid, and Rutin

Only 2-15 % of the ingested flavonoids and one-third of the ingested CGA are absorbed in the upper GI tract (Olthof et al., 2001; Gao et al., 2006). Moreover, the primary site of degradation of the FA esters is the colon (Kroon et al., 1997). Microfloral species are mainly responsible for releasing the bound phenolic acids that reach the LI because of their esterase activity (Chesson et al., 1999; Andreasen et al., 2001). Bacterial strains *Escherichia coli*, *Bifidobacterium lactis and Lactobacillus gasseriin* degraded CGA into CA and quinic acid thus making them bioaccessible (Couteau et al., 2001). Cinnamoyl esterase enzymatic activity in the same strains of bacteria was also responsible for the release of FA from ethyl ferulate (Andreasen et al., 2001; Couteau et al., 2001). Approximately 50-95 % of cinnamoyl esterase activity was found in the luminal microflora of the LI (10 times higher than the SI mucosa) (Andreasen et al., 2001). Up to 95 % of FA release takes place in the colon upon the fermentation of FA-containing food items like wheat bran with human pooled fecal material (Kroon et al., 1997). The β-glycosidic linkage of glycosides in flavonoids resists hydrolysis by the pancreatic enzymes (Heim et al., 2002). RU needs to be deglycosylated to release the aglycone quercetin by the action of the enzyme β-glucosidase (Bokkenheuser et al., 1987; Schneider et al., 1999). Enzymes detected in fecal inoculums that fermented RU consisted of high levels of β-glucosidase, α-rhamnosidase, and β-galactosidase (Aura et al., 2002).

After the cleavage of the ester bond of CGA in the colon, the released CA is then converted into dihydrocaffeic acid and other microbial metabolites with CA disappearing upon fermentation (Gonthier et al., 2006; Farah et al., 2008). Microbial metabolites (m-coumaric acid, hippuric acid, and derivatives of phenylpropionic, hippuric, and benzoic acid) made up the majority of metabolites in the plasma and urine of rats fed a diet supplemented with CGA, CA, or quinic acid (Gonthier et al., 2003). In urine, these metabolites made up 57.4 % of ingested CGA and 28 % of CA while the originally ingested compounds (CGA and CA) ,FA, and iso-ferulic acid made up only 1.3 % of the CGA intake and 12.8

% of CA intake (Gonthier et al., 2003). Similarly, after the consumption of RU supplements, the urine of participants with an intact colon contained microbial metabolites of different phenylacetic acid derivatives (Olthof et al., 2003). In total, the microbial metabolites accounted for about half of the ingested amount of RU (Olthof et al., 2003). None of the metabolites were detected in the urine of participants with an ileostomy indicating their production upon microbial action (Olthof et al., 2003). Metabolites also appeared in the glucuronated and sulfated form, which shows that further metabolism occurs in the liver after absorption (Rechner et al., 2002; Stalmach et al., 2009).

Fermentation of methyl ferulate with human and ruminant microflora first caused its demethylation into FA followed by several transformations including hydrogenation, demethylation, and dehydroxylation (Russell et al., 2008). The production of metabolites (hydrogenated FA and CA, and hydrogenated 3-Hydroxycinnamic acid) increased with time and was accompanied by a simultaneous decrease in FA and methylated FA (original compound) which disappeared after 24-72 h (Russell et al., 2008). A similar decrease in FA was observed by Kroon et al. (1997) after FA was released upon the fermentation of wheat bran with microflora. This is thought to be caused by either the metabolism of FA by the microflora or the transformation of FA into other metabolites (Kroon et al., 1997).

2.8 Effect of Digestion on Antioxidant Capacity

Few studies looked at changes in antioxidant capacity in food products with a complex food matrix, or juices, or the synthetic form of the compounds when subject to simulated gastric and pancreatic digestion. After in vitro gastric digestion of red grapes, ferric reducing antioxidant power assay (FRAP) and 2,2′-azino-bis-(3-ethylbenzothiazoline sulphonic acid) assay (ABTS) showed a significant increase in antioxidant activity (Tagliazucchi et al., 2010). The FRAP assay showed a slight but significant reduction in antioxidant capacity upon the transfer of grape digestates from gastric to intestinal milieu; at time zero of intestinal digestion (Tagliazucchi et al., 2010). The decrease in FRAP was

correlated with a 15 % decrease in total polyphenol as the anthocyanins in the red grape samples were highly affected by the change in pH when transferred into the basic milieu (Tagliazucchi et al., 2010). On the other hand, the ABTS assay showed a significant increase at time zero of intestinal digestion, which suggests that ATBS-related antioxidant activity increases with an increase in pH (Tagliazucchi et al., 2010). This latter observation is thought to be caused by the deprotonation of the hydroxyl moiety on the aromatic ring accompanying the pH change (Tagliazucchi et al., 2010). Upon pancreatic digestion, both antioxidant assays showed that a significant increase in antioxidant capacity was accompanied by the release of polyphenols from the food matrix of grapes making them more bioaccessible (Tagliazucchi et al., 2010). Similarly, the polyphenolic content of apples increased upon gastric and pancreatic simulated digestion as did the antioxidant capacity measured by ABTS (Tarko et al., 2008). Despite no increase in the polyphenol quantities after gastric and pancreatic digestion of apple peel rich in quercetin glycosides, the radical scavenging activity increased significantly (Tarko et al., 2008). This could be caused by digestive enzymes (and low pH) that changed polyphenol-structure resulting in greater antioxidant capacity rather than increased phenolic content (Tarko et al., 2008). Examples could include the degradation of polymers into monomers that could be more reactive, the production of molecules with more hydroxyl groups (Tarko et al., 2008), or the deprotonation of hydroxyl groups (Tagliazucchi et al., 2010).

The health effects of polyphenols are both systemic and at the level of the gut. The unabsorbed CGA, CA, FA, or RU protect against oxidative stress locally and the metabolites produced upon microbial action may have beneficial effects both at the gut level and systemically. For instance, the antioxidant capacity of the microbial metabolite dihydroferulic acid was higher than that of CGA and CA when tested with ABTS and oxygen radical absorbance capacity (ORAC) assays (Gómez-Ruiz et al., 2007). Additionally, the antioxidant activity of the synthetic compounds like quercetin, the metabolite of RU, was increased upon in vitro digestion when assessed with the ABTS assay (Tagliazucchi et al., 2010). Quercetin has also shown potent peroxynitrite- and reactive oxygen species-

scavenging action, in addition to potent anti-inflammatory effects (Boots et al., 2008).

CONNECTING STATEMENT

Polyphenols have been attributed with several health benefits related to powerful antioxidant effects such as prevention of low-density lipoprotein oxidation that may reduce the risk of developing cardiovascular diseases (Yang et al., 2011). Polyphenols are supplied dietarily, mostly by some commonly consumed grains, fruits and vegetables, which include potato (D' Archivio et al., 2007; Crozier et al., 2009). Most polyphenols exist as esters, polymers, or glycosides in foods and therefore require further digestion into simpler forms to be available for absorption (Manach et al., 2004). The digestive process is the first step, before absorption and metabolism, in modulating polyphenol bioavailability by affecting their stability and bioaccessibility (Saura-Calixto et al., 2007). In vitro models of digestion that include gastric and pancreatic digestive enzymes are considered as a good alternative to in vivo studies to assess the effect of digestion on polyphenol profiles (Saura-Calixto et al., 2000; Tagliazucchi et al., 2010). However, animal and clinical studies have shown that a large portion of ingested polyphenols reach the colon for further metabolism by colonic microflora (Olthof et al., 2001; Gonthier et al., 2003; Jenner et al., 2005; Lafay et al., 2006; Renouf et al., 2010). Microfloral species in the colon play a major role in determining the bioaccessibility and bioavailability of polyphenols (Gonthier et al., 2003; Kemperman et al., 2010). Microbial metabolism yields secondary metabolites with structures and antioxidant functions that differ from the parent compound (Han et al., 2007). However, only few studies have accounted for the microbial metabolism when examining the effect of digestion on polyphenol structures and functions (Saura-Calixto et al., 2007; Gumienna et al., 2011). The current study examined the polyphenolic biotransformation of three phenolic acids chlorogenic acid, caffeic acid, ferulic acid, and the flavonoid rutin present in potato extracts upon human enzymatic and microbial digestion using the Computer Controlled Dynamic Human Gastrointestinal Model (Martoni et al., 2007). This model is an in vitro system with physiological relevance to the different stages of the human gastrointestinal tract as it accounts for enzymatic action and the microbial ecosystem. To assess the change in bioactivity, the in vitro antioxidant activity of

polyphenolic rich potato extracts was assessed at different stages of digestion via antioxidant capacity assays.

Chapter 3

Manuscript

Biotransformation and Antioxidant Capacity of Polyphenol-Rich Potato Extracts after Digestion in a Computer Controlled Dynamic Human Gastrointestinal Model

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3.1 Introduction

Polyphenols have been extensively studied for their antioxidant capacity and are gaining importance because of various health benefits including anticarcinogenic, cardioprotective, anti-inflammatory, and neuro-protective activities (Kondratyuk and Pezzuto 2004). Polyphenols are the major antioxidants in the human diet as they are widespread in various foods (D' Archivio et al., 2007). Grains, fruits, vegetables, and beverages, mainly red wine, tea and coffee, are the major dietary sources of polyphenols (D' Archivio et al., 2007). The amount of polyphenols differs from one food item to another. However, staple foods contribute the most to the phenolic intake and are the most important polyphenolic dietary sources (Crozier et al., 2009).

Potatoes are consumed as a staple food by many populations worldwide (Camire et al., 2009). Despite their relatively low phenolic content, potatoes have been listed as the major source of polyphenols along with oranges and apples in the French, American, and Greek diets given their significant consumption (Chun et al., 2005; Brat et al., 2006; Dilis and Trichopoulou 2010). In addition to their starch content, potatoes provide polyphenols including phenolic acids (PA) such as chlorogenic acid (CGA), caffeic acid (CA), and ferulic acid (FA) as well as the flavonoid rutin (RU) and micronutrients including vitamin C, potassium, and phosphorous (Reddivari et al., 2007; Camire et al., 2009). PA and flavonoids are widely consumed as they make up one-third and two-thirds of the total dietary polyphenolic intake respectively (Scalbert and Williamson 2000).

In food, polyphenols are found in complex structures such as esters or glycosides that require digestion in order to become bioaccessible for absorption (Manach et al., 2004). For instance, CGA is the ester of CA with quinic acid and FA is primarily found bound to plant cell walls (Zhao and Moghadasian 2010). Similarly, RU is a quercetin glycoside attached to rutinoside, a large disaccharide molecule (Hollman et al., 1995). In order to assess the health benefits of polyphenols in vivo, it is important to assess their fate once ingested.

Upon ingestion, polyphenols, including CGA, CA, FA and RU, are subject to digestion by intestinal enzymes and colonic microflora (Saura-Calixto et al.,

2007). Digestion modulates the bioaccessibility of polyphenols in the gastrointestinal (GI) tract thus determining how much is available to be used by the body (Saura-Calixto et al., 2007). Absorption and metabolism follow digestion in determining the final bioavailability of the polyphenols in vivo (Manach et al., 2004). Evidence from *in situ* intestinal perfusion of polyphenols in animal studies (Lafay et al., 2006) as well as clinical trials measuring polyphenols excreted in the ileostomy effluent of participants after polyphenol supplementation report low absorption of polyphenols in the upper GI tract (Hollman et al., 1995; Olthof et al., 2001; Stalmach et al., 2010). The size of the molecule plays a role in hindering absorption. For example, CA and the aglycone quercetin are both absorbed more efficiently than CGA, the ester of CA, and RU, the glycoside of quercetin (Hollman et al., 1995; Azuma et al., 2000; Olthof et al., 2001). Additionally, studies of healthy volunteers consuming FA-rich foods have reported that FA bound in a complex format in the form of bran is much less bioavailable (3%) than the free form found in tomato (11-25%) and beer (19-98%) (Bourne and Rice-Evans 1998; Bourne et al., 2000; Kern et al., 2003). The binding of FA to cell wall polysaccharides with a low biodegradability in whole grain products decreases the release of FA for absorption (Adam et al., 2002).

Only one-third of ingested of CGA and 2-15% of the ingested flavonoids are absorbed in the upper GI tract (Olthof et al., 2001; Gao et al., 2006; Stalmach et al., 2010). Therefore, substantial amounts of these polyphenols reach the large intestine (LI) for later metabolism and digestion by the microflora, which possess a high metabolic capacity (Possemiers et al., 2011). For instance, the LI is an essential organ in releasing FA from the food matrix for further absorption (Chesson et al., 1999). Additionally, microbial action causes further degradation and transformation of polyphenols contributing to their absorption and subsequent putative health benefits (Selma et al., 2009; Kemperman et al., 2010). This phenomenon is evident by the late appearance of microbial metabolites in the plasma of healthy participants with an intact colon after approximately 6-12 h or more following ingestion of polyphenolic supplements of CA, CGA, or RU (Graefe et al., 2001; Farah et al., 2008; Renouf et al., 2010). The contribution of

microflora in the breakdown of polyphenols is supported by the fact that heat inactivated microflora caused no change to the phenolic composition (Plumb et al., 1999; Gonthier et al., 2006). Additionally, no transformation of ingested phenolics or production of metabolites was detected in germ free animals (Scheline and Midtvedt 1970; Peppercorn and Goldman 1972).

To date, most in vitro studies testing the effects of digestion on polyphenol profiles have only accounted for the upper GI portion involving enzymatic and chemical digestive conditions (Bermúdez-Soto et al., 2007; Tagliazucchi et al., 2010). The few studies that have accounted for the microbial action on polyphenols have used a basic fermentor involving human or rat fecal incubations (Kroon et al., 1997; Gonthier et al., 2006; Saura-Calixto et al., 2007; Gumienna et al., 2011). Additionally, the change in antioxidant capacity upon enzymatic and microbial digestion has been sparsely assessed. The aim of this study was to assess the impact of enzymatic and microbial digestion of the polyphenols CGA, CA, FA and RU present in polyphenol-rich PE on antioxidant capacity and phenolic profiles using the Computer Controlled Dynamic Human Gastrointestinal Model (GI model). The GI model is a continuous model used as a modern tool to assess the effect of a digestion of polyphenols, instead of performing incubations in inoculated basic fermentors.

3.2 Materials and Methods

3.2.1 Polyphenolic Extraction of the 'Onaway' Potato

Twelve Potato cultivars grown in Canada were previously analyzed for their polyphenolic content of CGA, CA, FA, and RU (Vunnam 2010). The polyphenolic content varied among cultivars which also had season-to-season variability (Vunnam 2010). 'Onaway' had a relatively high and consistent content of these four polyphenols and ascorbic acid and was selected to generate the polyphenol-rich extracts.

The PE was generated by POS Bio-Sciences (POS Bio-Sciences, SK, Canada). Briefly, 20 kg of 'Onaway' potato were diced, freeze-dried and extracted by agitating them with 200 L of a 90 % (v/v) aqueous ethanol solution

(SDAG-13) for 1 h at room temperature. The ratio of powder: aqueous ethanol was 1:10 (w/v). After extraction, the extract was separated from the solids by centrifugation at 1,076 x g for 10 min. The extract was then concentrated under vacuum at 40-50°C until approx. 15 L and the ethanol percentage, measured using a hydrometer, was less than 10 %. Water was added back during evaporation in order to attain low ethanol content. Afterwards, the concentrate was freeze-dried to generate powdered 'Onaway' PE and analyzed (Table 3.1). To quantify the polyphenolic content of the freeze-dried powder, 0.01 g were re-solubilised and extracted in 3 ml of 95 % methanol. The polyphenolic content of the 'Onaway' extracts in mg per g dry matter (mg/g) basis was 8.9 CGA, 0.6 CA, 0.2 FA, and 1.2 RU. The polyphenolic-rich powder was stored at -80 ^{o}C until utilization for the simulated artificial GI digestion.

3.2.2 'Onaway' Potato Extract Digestion in the Computer Controlled Dynamic Human Gastrointestinal Model

Similar to the SHIME model by Molly et al. (1993), the GI model (Fig. 3.1) is composed of 5 double jacketed vessels each representing part of the human intestinal tract: the stomach (vessel 1; V1), small intestine (SI) (vessel 2; V2), ascending colon (vessel 3; V3), transverse colon (vessel 4; V4), and the descending colon (vessel 5; V5) (Martoni et al., 2007). It is computer controlled by the Labview® software. The system was validated by different tests such as the production of short chain fatty acids, gas production, and enzymatic activity (Molly et al., 1994).

The last 3 vessels, also called the fermentation vessels, were inoculated with a 15 % solution of pooled human fecal suspension prepared form freshly collected fecal samples of 5 healthy individuals. Fecal donors did not follow any restricted diet prior to the study, had no GI problems, and had no antibiotic treatment in the past 6 mo. The microbial ecosystem was kept sustained by continuous feeding referred to as GI food, which is set at pH 2 and kept at 4^{o}C to prevent contamination. The GI food was composed of (g/L) 1 arabinolactan, 2 pectin, 1 xylan, 3 starch,0.4 glucose, 3 yeast extract, 1 peptone, 4 mucin, and 0.5 cystein powder as described by Molly et al. (1993).

Prior to the treatment, GI food was administered into the system for a 2 wk period in order to ensure the growth and stabilization of the microflora. After this stabilization period, 130 g of 'Onaway' PE containing 1.4 g of total polyphenols was administered into the gut model system. This amount is slightly above the average daily total polyphenol intake of 1 g (Scalbert and Williamson 2000) and could be consumed daily through food or supplement intake. The extracts were dissolved using 25 ml of methanol and solubilized into the GI food as described in other studies involving the feeding of polyphenol solutions to microflora (Rechner et al., 2004; Gao et al., 2006).

Throughout the simulated gut digestion period, temperature was kept at 37^{o} C by the flow of hot water through the double-jacketed vessels. The pH in the vessels was measured by pH probes and automatically adjusted using 0.2 M HCl solution or 0.5 M NaOH solutions. When the digestates reached the SI vessel (V2), simulated pancreatic juice was added composed of (g/L) 12 $NaHCO_3$ (S7277-1kg, Sigma), 6 Oxgall (DF0128-17-8, Fisher Scientific), and 0.9 pancreatin (P1750-100g, Sigma). A flow of 0.5 M NaOH adjusted the pH of the stomach vessel (V1) into a range of 6.5 - 7.5. Pepsin (P7125, Sigma) was freshly prepared according to Gumienna et al. (2011) and added once food was pumped into vessel 1 and pH was adjusted to 2. The pH range in vessels 3, 4, and 5 was maintained at 5.60 - 5.80, 6.20 - 6.40, and 6.60 - 6.80, respectively. Conditions in all the vessels of the simulated gut were kept strictly anaerobic by flushing nitrogen into the system to keep the head-space oxygen free. The fluid in the vessels was continuously stirred using a magnetic stirrer and the vessels were wrapped with aluminum foil to prevent polyphenol photodecomposition. Digestion lasted 24 h and samples were taken from all the vessels before polyphenol addition (T=0) and every 8 h during the 24 h digestion. Treatments were followed by a 72 h washout period in which only GI food was administered into the gut model system. To ensure the stability of the polyphenols and inactivate digestive enzymes after the removal of digests, 10 ml of a 0.5 M HCl solution was added to decrease the pH of samples ($pH \leq 2$) of samples withdrawn from vessels 2 to 5 (Bermúdez-Soto et al., 2007; Tagliazucchi et al., 2010). The

aliquots were centrifuged at 1000 x g for 20 min and stored at -80^{o} C for later analysis.

3.2.3 High Performance Liquid Chromatography (HPLC) and Liquid Chromatography Mass Spectrometry (LC-MS) Identification of Phenolic Compounds-

Samples were thawed in the lab with UV filtered light systems, vortexed, and filtered with 25 mm Syringe Filters (0.45 µm, MCE, sterile) (Fisher Scientific Ottawa, ON) into 1 ml glass vials before HPLC injection (High Performance Liquid Chromatography). Samples were kept chilled at all times and shielded from bright light. The HPLC identification of a compound was based on the retention time as compared to that of purchased pure standards (CGA C3878, CA C0625, FA 12,870 and RU R5143) (Sigma-Aldrich, St. Louis, MO) and verified by LC-MS (Liquid Chromatography-Mass Spectrometry). A Varian HPLC system equipped with a Varian 9012 tertiary pump module, refrigerated auto-sampler model 410 and single variable wavelength detector module 9050 operated by a Varian Star 5.3 software was used for sample analysis. Phenolic compounds were separated based on a modified method (Shakya and Navarre 2006), using a reverse phase HPLC Gemini-NX (5 µm, 100 mm × 4.6 mm) column (Phenomenex, Torrance, CA, USA) and a 4.6 mm × 2.0 mm guard column. Two buffers, buffer A and buffer B, were used as mobile phases. Buffer A was a 10 mM formic acid solution prepared by dissolving 0.4603 g of formic acid in 1 L distilled water. The pH was adjusted 3.5 using 1 M NH_4OH solution. Buffer B was a 5 mM ammonium formate solution prepared by dissolving 0.3153 g of ammonium formate in 1 L 100 % methanol and stirred on a magnetic stirrer. The solvent gradient was as follows: 0-1 min 100 % buffer A, 1-5 min 0-30 % buffer B, 5-8.5 min 30-70 % buffer B, 8.5-12 min 70-100 % buffer B. UV detection was conducted at 320 nm. A flow rate of 1.0 ml/min was used and 20 µl of sample were injected. Samples were analyzed in duplicate. The same column and elution conditions were used for LC-MS analysis using a 6210 LC-MS Time of Flight system (Agilent Technologies, Santa Clara, CA, USA) in negative electrospray

ionization mode with internal calibration using calibrant ions at *m/z* 119.0363 and 966.0007 at a scanning speed of 1 spectrum/s. Source conditions were as follows: capillary voltage of -4000 V, gas temperature of 350°C, drying gas flow of 12 l/min, nebulizer gas at 50 psi and fragmentor and skimmer voltages at 100 V and 60 V, respectively.

3.2.4 Assessment of Scavenging Capacity- ABTS Procedure

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay was used to estimate the total antioxidant scavenging activity (Gao et al., 2000). This assay is a colorimetric assay in which the ABTS radical ($ABTS^{\bullet+}$) is generated in a stock solution (dark blue-green color) after mixing 5 ml of 7 mM ABTS with 88 µl of 140 mM potassium persulfate ($K_2S_2O_8$). An ABTS working solution was prepared by diluting the stock solution with 95 % ethanol to give an absorbance of 0.7 ± 0.05 at 734 nm. An aliquot of 100 µl from each of the filtered samples was added to 1200 µl ABTS working solution, vortexed, and the reduction of the ABTS radical by the samples was detected by recording the absorbance at 734 nm after 2 min of mixing. All samples were analyzed in duplicate. Trolox (238813-5G- Sigma-Aldrich), an analogue of Vitamin E, was used as a standard. A standard curve was prepared from the spectrophotometer readings of different concentrations of Trolox that ranged between 2.5 -785 µM. The standard curve was used to calculate the antioxidants activity in the digested and undigested samples based on Trolox equivalents.

3.2.5 Ferric Reducing Antioxidant Power Assay- FRAP

The Ferric Reducing Antioxidant Power Assay (FRAP) is a colorimetric assay used to determine the total antioxidant capacity of the sample through the reduction of the ferric tripyridyltriazine complex to a ferrous complex (Benzie and Strain 1996). The intensity of the blue color developed when the pale yellow coloured ferric tripyridyltriazine complex is reduced into the ferrous form, is measured spectrophotometrically at 593 nm in order to assess the total antioxidant capacity. The FRAP reagent was prepared by mixing 300 mM acetate buffer at

pH 3.6, 10 mM TPTZ solution (2, 4, 6-tripyridyl-s-triazine), and 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$) in a 10:1:1 ratio followed by its incubation for 10 min at 37°C. Acetate acid buffer was prepared by mixing 16 ml of glacial acetic acid and 3.1 g of sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) in 1 L distilled water. TPTZ solution was prepared by mixing 0.3123 g of TPTZ (2, 4, 6-tripyridyl-s-triazine), and 0.33 ml HCl (1 M) in 100 ml distilled water. Ferric chloride solution (0.2 M) was prepared by dissolving 0.54 g of ferric chloride in 100 ml distilled water. An aqueous mixture of ferrous sulfate ($FeSO_4.7H_2O$) was used as a standard. A stock solution of 1 mM was prepared by dissolving 0.278 g $FeSO_4.7H_2O$ in 1 L distilled water and thereafter used to prepare different dilutions ranging from 0.1 to 1.0 mM. A standard curve was used to calculate the antioxidant capacity in the samples as ferrous sulfate equivalents. The reaction was carried out using a 96-well plate upon the addition of 30 µl H_2O, 10 µl standards or samples, and 200 µl FRAP solution. Samples were left to react at room temperature for 20 min, and then absorbance was read at 593 nm in a microplate reader (Infinite PRO 200 series, Tecan Group, San Jose, CA, USA). All readings were performed in duplicate.

3.2.6 Statistical Analysis

The experiment was designed as a repeated measures model where treatment with PE and vessels (1 to 5) (main factors) were studied at different time periods (0, 8, 16, and 24 h). Data of antioxidant scavenging activity (ABTS and FRAP) were tested for normality using the UNIVARIATE procedure of the Statistical Analysis System (version 9.2 of the SAS system for windows. SAS Institute Inc., Cary. NC, USA) then were statistically analyzed using the MIXED procedure of SAS. Significant least square means (LSMeans) were compared using Tukey's HSD (honestly significant difference) *post-hoc* test at 0.05 probability level ($P < 0.05$). Vessel effect was examined as the mean differences of T=0 and T=24. This effect was statistically studied using the GLM (general linear model) procedure of SAS and the significant LSMeans were compared using Tukey's HSD *post-hoc*

test. Pearson correlation coefficient between ABTS and FRAP was calculated using the CORR procedure of SAS.

3.3 Results and Discussion

3.3.1 Identification of Polyphenols by HPLC and LC-MS

Table 3.2 shows LC-MS results from the assessment of the colonic vessels for the presence of microbial metabolites at baseline; before the addition of the 'Onaway' PE (T=0), and 24 h after the addition of the extracts in the system (T=24). The microbial metabolites dihydrocaffeic acid, 3-Phenylpropionic acid, 3-Hydroxybenzoic acid, cinnamic acid**,** and trace amounts of 3-Hydroxyphenylacetic acid were detected in vessel 4 (the transverse colon) at T=0. Dihydrocaffeic acid, cinnamic acid, 3-Hydroxyphenylacetic acid, and trace amounts of 3-Hydroxybenzoic acid and 3-Phenylpropionic acid were also detected in V5 (the descending colon) in addition to trace amounts of benzoic acid. No metabolites were detected in V3 (the ascending colon).

The detection of microbial metabolites at T=0 can be explained by the fact that volunteers were not given a polyphenol-restricted diet prior to the study; therefore, their diet was reflected in the fecal water. The same metabolites have been detected in the fecal water of healthy free living individuals subject to no dietary restrictions (Jenner et al., 2005; Gill et al., 2010). The amount of microbial metabolites in fecal water decreased with a low polyphenolic intake (Jenner et al., 2005) and increased with polyphenol supplementation, portraying the effect of dietary intake (Gill et al., 2010).

In Figure 3.3, chromatogram 3.3A is a representative HPLC chromatogram showing generally the absence of phenolic compounds with very small peaks at T=0, before the addition of polyphenols. Fifteen microbial metabolites listed in Table 3.2 were detected at T=24 using LC-MS. The different pH range in the colonic vessels likely determines the bacterial species present in each vessel and thus can be responsible for the observed difference in profiles and metabolite production among the 3 colonic compartments. The microbial metabolites 3-Phenylpropionic acid and cinnamic acid were detected in V4 and

V5 but not in V3. In addition, the dihydroferulic acid and 3-Hydroxybenzoic acid metabolites were not detected in V4 but in V3 and V5. The metabolite coumaric acid was not detected in V5 but in V4 and V3.

Figure 3.2 is a representation of the extracted ion chromatogram of the LC-MS polyphenolic profiles of the last 3 vessels of the GI model showing the colonic segments; the ascending colon (V3) (Fig. 3.2A), the transverse colon (V4) (Fig. 3.2B), and the descending colon (V5) (Fig.3.2C) at T=24. The profiles show the parent compounds (CGA, CA, FA, and RU) and the detected microbial metabolites generated by the microbial species. In V3 (Fig. 3.2A), 2 large peaks appear around the almost undetectable CGA peak, which was originally the most abundant polyphenol in the PE samples. The 2 peaks represent the isomers, neoCGA (neochlorogenic acid) and cryptoCGA (cryptochlorogenic acid). The same isomerization of GCA into neoCGA and cryptoCGA was reported after 0.1 h of CGA fermentation in a basic fermentor (Rechner et al., 2004). However, in the chromatograms representing V4 and V5 (Fig 3.2B and 3.2C), the peaks of CGA and its isomers were absent. Likewise, the other identified parent compounds (CA, FA, and RU) were still detectable in V3 but not in V4 and V5 (Fig. 3.2; Table 3.2). Only trace amounts of FA and CA were detected in V4 and V5 respectively while CGA, its isomers, and RU were completely absent (Table 3.2). None of the parent compounds were detected in the colonic vessels at T=0, prior to polyphenol addition. The disappearance of FA, CA, CGA, or RU was also reported after 2-24 h of fermentation with fecal slurry (Kroon et al., 1997; Aura et al., 2002; Gonthier et al., 2006). CA was listed as one of the FA metabolites produced upon in vitro fermentation of FA in a basic fermentor; before CA itself underwent additional biotransformation (Braune et al., 2009). The production of CA from FA may explain the presence of trace amounts of FA first in V4 followed the detection of trace amounts of CA in V5.

HPLC profiles of the colonic vessels V3, V4, and V5 at T=24 are shown in Figures 3.3B, 3.3C, and 3.3D respectively. Similar to the results from LC-MS, all parent compounds including the CGA peak were still detectable in V3 by HPLC, but not in V4 and V5 (Fig. 3.3). Moreover, and in line with the LC-MS

profiling in Fig. 3.2, these chromatograms demonstrate different profiles among the 3 colonic vessels showing different biotransformation patterns in different segments of the colon. New peaks were detected in all colonic vessels at T=24, pointing out the biotransformation of parent compounds by the microflora. The most abundant microbial metabolites in the colonic vessels were 3-Hydroxyphenylpropionic acid, dihydrocaffeic acid, and quinic acid (Figure 3.2; Table 3.2). The identification of these 3 microbial metabolites by HPLC (Figures 3.3B, 3.3C 3.3D) was based on their appearance and retention time detected with LC-MS. In V3, CA and quinic acid were the most abundant metabolites followed by 3-Hydroxyphenylpropionic acid and dihydrocaffeic acid (Fig.3.2; Table 3.2). The amount of 3-Phenylpropionic acid appeared to increase when in the comparison of V3 to V5. The 3-Hydroxybenzoic acid and dihydroferulic acid metabolites were absent in V4 but re-appeared in V5. The quinic acid metabolite, although still detectable in V4 and V5, appeared to substantially decrease from V3. Similarly, coumaric acid disappeared upon reaching V5. Based on the data presented above, it appears that V3 that represents the ascending colon shows the most abundant metabolic profiles in the simulated GI model, which might be attributed to its location as the first segment where the parent polyphenols undergo biotransformation via the microflora. It appears that polyphenol biotransformation continues in the transverse colon and descending colon, V4 and V5 respectively, to generate additional newly formed metabolites.

A proposed pathway, based on compiling information from the literature and the results portrayed here, was developed and is presented in Figure 3.5. It shows the possible pathways of biotransformation of the parent polyphenolic compounds in the PE sample; CGA, CA, FA, and RU. In the ascending colon (V3), in addition to isomerization of CGA, a large peak of CA was produced with the simultaneous decrease in CGA (Figure 3.3B and Table 3.3). The production of abundant levels of CA and quinic acid, the major metabolites in V3, explain the appearance of 2 large peaks in V3 only and indicate the initiation of CGA biotransformation as it comes into contact with the microflora. The hydrolysis of the ester bond between CA and quinic acid is possible by the enzymatic esterase

activity of the microflora (Andreasen et al., 2001; Couteau et al., 2001). The CA and quinic acid moieties produced from CGA and the CA were, in turn, biotransformed into other metabolites as verified by LC-MS (Table 3.2). Similar microbial metabolites, namely m-coumaric acid, hippuric acid, and derivatives of phenylpropionic acid, hippuric acid, and benzoic acids have been detected in the plasma and urine of rats fed CGA, CA, and quinic acid supplemented diets (Gonthier et al., 2003).

As described above, quinic acid, 3-Hydroxyphenylpropionic acid and dihydrocaffeic acid were the most abundant microbial metabolites in all 3 colonic vessels. Also, 3-Phenylpropionic acid increased from V3 to V5 and was among the most abundant in V5. The microbial metabolite, 3-Hydroxyphenylpropionic acid, is a common metabolite of all parent compounds (Fig. 3.5). CA and FA are substrates for enzymatic action in the colon leading to the formation of 3-Hydroxyphenylpropionic acid as a common product (Rechner et al., 2004; Braune et al., 2009). Additionally, 3-Hydroxyphenylpropionic acid is one of the products from the biotransformation of RU (Rechner et al., 2004). All these substrates (CGA, CA, FA, and RU) decreased upon microbial fermentation, and were not detected in V4 and V5, while 3-Hydroxyphenylpropionic acid increased in all 3 vessels (Table 3.2). The metabolite of 3-Hydroxyphenylpropionic acid, 3-Phenylpropionic acid, was not detected in V3, found in trace amounts in V4, and detected again in V5. Reactions that take place in the colon leading to the formation of microbial metabolites, aside from the degradation of the ester bond, include hydrogenation, demethylation, dehydroxylation, and decarboxylation (Russell et al., 2008; Selma et al., 2009). Dihydroferulic acid was also detected in the colonic vessels and was most abundant in V5. Both dihydrocaffeic acid and dihydroferulic acid were detected in the plasma levels of participants 6-12 h after the consumption of CGA food sources (Stalmach et al., 2009; Renouf et al., 2010). The colon was reported to be the site of converting CA and FA into dihydrocaffeic acid and dihydroferulic acid, respectively (Russell et al., 2008; Braune et al., 2009; Stalmach et al., 2010)

When CGA and CA were incubated in a fermentor in vitro for 48 h, benzoic acid was also one of the most abundant microbial metabolites along with dihydrocaffeic acid and 3-Hydroxyphenylpropionic acid (Gonthier et al., 2006). Benzoic acid was among the most abundant metabolites in V4 as its amount increased from V3, but decreased again in V5. The decrease in benzoic acid might be due to its transformation into 3-Hydroxybenzoic acid which was absent in V4 but detected in V5 (Table 3.2). This latter observation is in line with the observations by Gonthier et al. (2003) who did not detect benzoic acid in the urine of rats supplemented with CGA, CA or quinic acid but did detect its metabolite 3-Hydroxybenzoic acid. Therefore, the present study findings indicate that the transformation of benzoic acid into 3-Hydroxybenzoic acid seems to take place in the colon before it is absorbed. No benzoic acid was detected by Rechner et al. (2004) after the in vitro basic fermentation of CGA possibly because it got transformed after 48 h. Hippuric acid was not detected in any of the vessels in our study (Fig. 3.2; Table 3.2). It was the most abundant metabolite in the urinary excretions and plasma levels of the rats fed CGA, CA, or quinic acid supplemented diets and the only metabolite detected in urine and plasma of rats in the quinic acid fed group (Gonthier et al., 2003). The lack of detection of hippuric acid in the present study supports the concept that hippuric acid is formed after absorption and not by microbial activity as suggested by Rechner et al. (2004).

The disappearance of RU in V4 and V5 (Figs. 3.2B, 3.2C, 3.3B, and 3.3C) is in concert with a complete transformation into quercetin that, in turn, is transformed into other phenylacetic acid derivatives. The beta-glycosidic linkage of glycosides in flavonoids resists hydrolysis by the pancreatic enzymes (Heim et al., 2002). RU needs to be deglycosylated first to release the quercetin molecule from the rutinoside disaccharide by the action of beta-glucosidase enzymes (Bokkenheuser et al., 1987; Schneider et al., 1999). The beta-glucosidase enzymatic activity has been detected in the fecal slurry used to ferment RU along with other enzymes like alpha-rhamnosidase, and beta- glucuronidase (Aura et al., 2002). Quercetin was greatly decreased to almost trace amounts as it got to V5 (Table 3.2). Similarly, 3, 4- Dihydrophenylacetic acid decreased as it reached V5.

This decrease was accompanied by an increase in 3-Hydroxyphenylacetic acid and protocatechuic acid in V5, pointing out to the biotransformation these products from RU in the transverse and descending colon. The formation of 3-Hydroxyphenylacetic acid from 3,4-Dihydroxyphenylacetic acid was reported to occur at around 8 h of fermentation (Aura et al., 2002). Phenylacetic acid derivatives accounted for about half of the amount of RU supplements consumed by volunteers with an intact colon (Olthof et al., 2003). None of the metabolites were detected in the urine of ileostomy participants signifying the importance of the microfloral action in the production of these metabolites (Olthof et al., 2003). Rechner et al. (2004) also detected hydroxyphenylacetic acid derivatives when RU was fermented for 48 hours with fecal slurry. Similarly, the fermentation of citrus fruit extracts with RU in an in vitro dynamic model of the LI containing microflora from fecal material of 10 healthy volunteers produced mainly hydroxyphenylacetic acid and hydroxyphenylpropionic acid derivatives (Gao et al., 2006). Protocatechuic acid was found in trace amounts in V3 at T=24 but was detected in higher concentrations in V4 and V5. Protocatechuic acid is the metabolite formed from the quercetin moiety once RU was broken down by microflora in a basic fermentor (Rechner et al., 2004).

Figure 3.4 shows the HPLC polyphenolic profiles of the undigested 'Onaway' PE (Fig. 3.4A), the stomach vessel (V1) (Fig. 3.3B), and the SI vessel (V2) (Fig. 3.4C) at T=24, with the identified parent compounds CGA, CA, FA, RU, the CGA isomer neoCGA (neochlorogenic acid), and the amino acid tryptophan. Polyphenols in the PE underwent minimal changes upon their digestion in the stomach (Fig. 3.4B) and SI (Fig. 3.4C) whereby a peak of cryptoCGA (cryptochlorogenic acid) appeared upon gastric and pancreatic digestion as compared to the absence of cryptoCGA in the undigested PE in chromatogram 3.4A. The neoCGA peak is still showing in the chromatograms Fig.3.4B and 3.4C. Bermúdez-Soto et al. (2007) reported the isomerization of CGA into neoCGA after simulated pancreatic digestion of pure CGA because of an increase in pH, but not after simulated gastric in vitro digestion. Similarly, Kahle et al. (2011) and Bouayed et al. (2011) also reported the isomerization of

CGA into neoCGA and cryptoCGA upon pancreatic digestion and no isomerization of CGA in apples after simulated gastric digestion. In the PE samples, isomerization of CGA seems to appear after both, gastric and pancreatic digestions. Almost no difference in the HPLC profiles is detectable between chromatograms 3.4B and 3.4C representing the upper GI, which suggests minimal change in polyphenolic profile at the level of the upper GI. All parent compounds were still detectable in V1 and V2 with no change except for the isomerization of CGA into cryptoCGA and possibly neoCGA. This latter observation is in concert with the complete recovery of PA without significant losses upon the in vitro dual pepsin-pancreatin digestion of grapes or chokeberry juice (Bermúdez-Soto et al., 2007; Tagliazucchi et al., 2010). In the colonic vessels (Fig. 3.3), major differences in the polyphenolic profiles were observed as compared to profile of the undigested PE (Fig. 3.4A). Moreover, the polyphenolic profiles in the colonic segments are very different from the profiles of the vessels representing the upper GI, unlike the minimal change in the HPLC profiles detected between the stomach and SI vessels. This difference between the colonic vessels and those representing the upper GI in addition to the production of new metabolites described earlier, is owed to the action of the microfloral species present in the latter vessels.

3.3.2 Changes in Antioxidant Capacity with ABTS and FRAP Assays

The addition of polyphenols caused an increase in the antioxidant capacity as measured with both the FRAP and ATBS assays (Fig 3.6 and 3.7). Antioxidant activity was measured in the 5 vessels of the GI model before the addition of any polyphenols (T=0) and in samples withdrawn every 8 h. Upon the addition of polyphenol-rich PE, both ABTS and FRAP assays showed an increase in antioxidant capacity with time from T=0 to T=24 h. Figure 3.6 shows the increase in FRAP activity measured in the stomach, V1 (Fig. 3.6A), and the SI, V2 (Fig. 3.6B); representing the upper GI vessels, and in the colonic vessels V3 (Fig. 3.6C), V4 (Fig. 3.6D), and V5 (Fig. 3.6E). Figure 3.6A and 3.6B show a significant increase in FRAP activity in both, the stomach and SI vessel respectively, after 8, 16, and 24 h compared to baseline. The FRAP values in the

ascending vessel compartment (V3) (Fig. 3.6C) were significantly different from the baseline value after 16 h of fermentation. The FRAP values in the transverse (V4) (Fig. 3.6D) and descending (V5) (Fig. 3.6E) colonic vessels were significantly different from the baseline value after 24 h of fermentation.

ABTS results are shown in Figure 3.7. Fig. 3.7A shows the ABTS measured in V1 and 3.7B shows the ABTS measured in the V2, while Fig. 3.7C, 3.7D, and 3.7E show the results for the colonic vessels V3, V4, and V5 respectively. A similar trend was observed in all vessels of the system whereby a significant difference ($P < 0.05$) in ABTS compared to baseline values was detected 8 h after the addition of the extracts in vessels 1 to 4 (Fig. 3.7). After 8 h, the scavenging capacity in the vessels increase significantly at T=8, T=16, and T=24 h relative to T=0 h. In V5, there was a small insignificant decrease in ABTS at T=8 h, followed by a significant increase at T=16 h and T=24 h; similar to that in the other vessels of the GI model (Fig. 3.7E).

Parent compounds were not detected by LC-MS and HPLC in vessels V4 and V5 at 24 h of bacterial fermentation (Figs. 3.2B, 3.2C, 3.3C, and 3.3D; Tables 3.2 and 3.3). LC-MS detected only trace amounts of FA in V4 and trace amounts of CA in V5 (Table 3.2). Therefore, the antioxidant capacity measured with both assays in these vessels may be attributed to the metabolites generated upon digestion and biotransformation of parent compounds by microbial action. It appears that microbial-mediated biotransformation in the colonic compartments leads to disappearance of the parent compounds in the transverse and descending colon segments coupled with a simultaneous production of various new microbial metabolites (Table 3.2). In that regard, FRAP activity was shown to increase significantly in the colonic vessels only at 16 and 24 h, which coincides with the time demonstrated to be associated with the generation of the microbial metabolites including 3-Hydroxyphenylpropionic acid, 3-Hydroxyphenylacetic acid, and protocatechuic acid (Table 3.2; Fig 3.2). Thus, metabolic microbial breakdown of polyphenols over a 16 h period appears to be needed for the generation of microbial metabolites with antioxidant capacity. Hence, in the colonic vessels, the parent compounds contribute only sparsely, if any, to the

antioxidant capacity. After healthy participants ingested FA-rich whole wheat bread, the parent compound FA accounted for only about 6 % of the increase in antioxidant capacity of plasma as it was extensively biotransformed into other newly formed metabolites responsible for the increase the antioxidant capacity (Anson et al., 2011). Similarly, only microbial metabolites and none of the parent/original polyphenols in pomegranate juice were detected in the plasma or urine of healthy volunteers after they consumed pomegranate juice daily for 5 days (Cerdá et al., 2004).

The plateau observed by ABTS after 8 h of the addition of polyphenols in all vessels (colonic and upper GI) (Fig. 3.7) is similar to the trend observed with FRAP in the upper GI vessels (Fig. 3.6A and 3.6B). Interestingly, a similar significant increase in ABTS values after 8 h from baseline was observed in both the SI, for instance, that contained only parent phenolic compounds, and the transverse colon vessel (V4) in which only microbial-generated polyphenolic metabolites were present (Fig. 3.7B and Fig. 3.7D). This latter observation may signify that some of the microbial metabolites have a similar scavenging capacity as parent compounds.

The increase in ABTS and FRAP values between T=0 and T=24 shown in Fig. 3.8 indicates the increase in antioxidant capacity within each vessel upon the addition of the polyphenol-rich PE. A tendency ($P = 0.05$) was noted for a greater increase in FRAP activity from baseline in the upper GI vessels (V1 and V2) as compared to colonic vessels V3, V4, and V5 (Figure 3.8A) over 24 h. The previously observed trend, where the colonic vessels required more time to produce microbial metabolites and to show an increase in FRAP activity (Fig. 3.6) could contribute to the smaller increase. Previous investigations have shown that the in vitro generation of microbial metabolites from CGA or RU is time dependent as different metabolites were generated and detected when fermentation time was extended (Rechner et al, 2004). For instance, in the same fermentor, the degradation of CGA produced CA initially after 5 h of fermentation followed by the production of dihydroCA and then 3-Hydroxyphenylpropionic acid after 8 and 24 h respectively (Rechner et al., 2004).

Since CGA and RU make up a significant portion of the PE polyphenolic content, the metabolites generated may have required time to get produced in order to contribute to antioxidant activity. In addition to the time required to generate the metabolites, the different metabolite composition in the different vessels (Table 3.2) may have caused this difference. The rate of microbial conversion also influences the bioavailability of polyphenols in vivo as this determines types of polyphenolic metabolites generated that have different bioavailability (Kemperman et al., 2010), and may also have different antioxidant capacities.

The ascending colon vessel (V3) showed the presence of parent polyphenolic compounds together with the first appearance of microbial polyphenolic metabolites (Fig 3.2A and 3.3B; Table 3.2), while HPLC and LC-MS results showed the absence or trace concentrations of parent polyphenolic compounds in the transverse (V4) and descending (V5) colonic vessels (Tables 3.2 and 3.3). The absence of parent compounds is in contrast to their presence in the stomach and SI vessels (V1 and V2 respectively) as shown by HPLC (Table 3.3). Opposite to the significant increase in FRAP antioxidant activity seen after 8 h in the stomach and SI vessel (Fig. 3.6A and Fig. 3.6B), 16 and 24 h were required to significantly increase the FRAP antioxidant capacity in the ascending and transverse colon, respectively (Fig. 3.6C and Fig. 3.6D) which might be related to: (a) disappearance of the parent polyphenolic compounds; and (b) time-mediated accumulation of microbial polyphenolic metabolites with antioxidant activities. No significant difference in FRAP activity, however, was noted between vessels (Fig 3.8A) which could be related to the relatively wide variation in FRAP activity among vessels, the small sample sizes as well as the contribution of accumulated microbial polyphenolic metabolites to FRAP antioxidant activity in the colonic vessels.

Results of the difference in the ABTS assay measured in all vessels between T=0 and T=24 are shown in Figure 3.8B. Results show that V3, the ascending colon, had the largest increase in ABTS activity from baseline values, which may be related to the appearance of the greatest abundance of both parent polyphenols together with polyphenolic microbial metabolites in this vessel

(Table 3.2, Fig 3.2A and 3.3B). The increase in ABTS was significantly ($P < 0.05$) greater in vessel 3 than in vessels 1, 4, and 5. The ascending colon vessel represents the colonic segment where the initial contact between the polyphenols and microflora takes place leading to microbial polyphenolic biotransformation whereas the polyphenolic content of the other vessels in the system was restricted to either parent compounds in V1 and V2 or microbial metabolites in V4 and V5. For this reason, the largest increase in ABTS activity in this vessel may be attributed to the presence of both parent compounds and their microbial metabolites, which are shown to react similarly with the ABTS radical (Fig. 3.7).

The above results thus suggest that ingested polyphenols together with their generated microbial metabolites can contribute to antioxidant capacity of human fecal fluid. This latter observation is in line with in vitro studies that reported high antioxidant capacity of microbial metabolites in feces. An assessment of the total antioxidant activity of feces obtained from 14 health volunteers using the ABTS assay showed a high radical scavenging antioxidant capacity that was almost 20-fold greater than that detected in the plasma (Garsetti et al., 2000). The microbial metabolites 3-Hydroxyphenylpropionic acid and m-coumaric acid were both characterized by having a high antioxidant capacity in vitro that was only slightly less than CGA as assessed by the ABTS assay (Gómez-Ruiz et al., 2007). Dihydroferulic acid, another microbial metabolite, had a high antioxidant capacity that was greater than both CGA and CA when assessed via the ABTS and ORAC (oxygen radical absorbance capacity) assays (Gómez-Ruiz et al., 2007). Likewise, the antioxidant capacity of dihydroferulic acid was similar to CA when tested with the FRAP assay in phosphate-buffered saline and human plasma (Lekse et al., 2001). Dihydrocaffeic acid showed a radical scavenging capacity greater than α-tocopherol when tested using the DPPH assay (Silva et al., 2000). When incorporated into the human enterocytes, dihydrocaffeic acid significantly increased the erythrocyte ability to reduce extracellular ferricyanide, hence contributing to an intracellular and extracellular antioxidant protection (Lekse et al., 2001). Quercetin the metabolite of RU

showed the higher ABTS and FRAP activity when tested in vitro in comparison to FA, RU, CA, and CGA (Nilsson et al., 2005).

The increase in ATBS antioxidant capacity was significantly ($P < 0.05$) lower in V5, the last colonic vessel, in comparison to the V2 and V3 in the GI model apart from the stomach vessel (Fig. 3.8B). The relatively lower antioxidant capacity might be related to the lack of presence of parent polyphenolic compounds together with further breakdown of microbial polyphenolic metabolites that could exert antioxidant activities including 3-Phenylpropionic acid, benzoic acid, and 3-Hydroxyphenylacetic acid, as structural differences of polyphenols influence their antioxidant activities (Rice-Evans et al., 1996). The number and arrangement of the hydroxyl groups on the aromatic rings affects the antioxidant capacity of polyphenols where polyphenols with two hydroxyl groups on an aromatic ring have a better radical scavenging capacity than those with one hydroxyl group (Rice-Evans et al., 1996; Cao et al., 1997). Extensive microbial biotransformation causes change in structure of the molecules by which end product microbial metabolites have less hydroxyl groups (Olthof et al., 2003). Hippuric acid, for instance, which is formed from benzoic acid upon absorption was reported to have no antioxidant capacity because it has no hydroxyl groups (Olthof et al., 2003).

Some studies have reported a significant increase of 7–25 % in plasma antioxidant capacity after the consumption of polyphenol rich food like strawberries or spinach, or grape seed extract by healthy volunteers (Cao et al., 1998; Vinson et al., 2001). Other studies, however, have reported no effect on plasma antioxidant activity in human and rat studies after the consumption of tea catechin, ellagitannins in pomegranate juice, and quercetin consumption due to the decreased antioxidant activity of the microbial metabolites circulating in the plasma (Kimura et al., 2002; Cerdá et al., 2004; Santos et al., 2008). The lack of plasma antioxidant effect of some polyphenolic metabolites may be due to the excessive structural changes that the parent compounds undergo upon digestion (Olthof et al., 2003) or methylation of metabolites in the liver before they circulate into the plasma (Santos et al., 2008). The conflicting results regarding

plasma antioxidant capacities reported by different studies may also be caused by the use of different analytical methods to measure antioxidant capacity in plasma (Fernandez-Panchon et al., 2008). The differing mechanisms of antioxidant assessment of the ABTS and FRAP assays could have caused the different trend observed in samples with higher abundance of microbial metabolites (colonic vessels) with the two assays. A plateau was observed with ABTS after 8 hours (Fig. 3.7) compared to a gradual increase in antioxidant capacity with FRAP (Fig. 3.6). It is possible that the phenolic structures of some microbial metabolites generated in the colonic vessels might be more reactive with the ABTS radical as compared to the Fe^{3+} ion generated in FRAP assay. Water insoluble extracts of strawberries and other fruit and vegetable extracts were also found to react better with the ABTS assay compared to the FRAP assay because they contain phenolic structures that are more reactive when tested with ABTS (Nilsson et al., 2005). Pearson correlation coefficient showed moderate positive correlation 0.42 ($P<0.0001$) between ABTS and FRAP.

In summary, the present results suggest that polyphenols from PE undergo major changes in their profiles by colonic microbial action leading to the production of several microbial metabolites that can contribute towards increasing the antioxidant capacity of fecal fluid. Biotransformation is initiated in the ascending colon (V3) which is the colonic segment in which the polyphenols first get into contact with the colonic microflora. Biotransformation continues as polyphenols are passed into the subsequent colonic segments, the transverse colon and descending colon, where the parent compounds disappear and new metabolites are generated. The vessels representing different segments of the colon showed different polyphenolic profiles where some metabolites were produced in some colonic vessels while others disappeared. This latter observation is caused by the different pH range in the colonic vessels thus creating a different microbial composition in the 3 different segments. Compared to the changes in the colonic segments, minimal change occurs in the upper GI vessels and almost no change in polyphenol profile occurs between the stomach and the SI. The main microbial metabolites were derivatives of phenylpropionic

acid, phenylacetic acid and benzoic acid. Upon the addition of the polyphenol-rich PE, an increase in antioxidant activity was detected in all vessels of the system with FRAP and ABTS. Time is required for the generation of microbial metabolites, therefore, the FRAP antioxidant capacity in the colonic segments increased with time and started to become significantly greater from the baseline values after 16 h of fermentation. The largest increase was detected in the ascending colon (V3) when assessed with ABTS as this vessel contains both; the parent polyphenols and their metabolites. Structural changes that occur upon biotransformation, and the disappearance of metabolites and parent compounds upon further microbial action in the descending colon (V5), can cause a lower antioxidant capacity detected with ABTS. The present study illustrates the importance of tracking biotransformation of commonly ingested polyphenols in the 3 major colonic segments as microbial metabolism led to differing patterns of polyphenolic metabolite profiles in each of these compartments. The above finding could indicate that polyphenols may exert altered physiological and antioxidant effects depending on the colonic segment due to differences in microbial metabolite profiles.

Table 3.1 Proximate analysis of 'Onaway' extract

Component of Potato Extract	Amount in mg/g of 'Onaway' extract
Carbohydrate	0.732
Ash	0.068
Moisture	0.013
Protein	0.412

Table 3.2 LC-MS results of microbial metabolites detected in the colonic vessels at baseline (T0) and after 24 hours of fermentation.

Phenolic Compound	Molecular Weight	m/z	V3		V4		V5	
			T0	T24	T0	T24	T0	T24
Chlorogenic acid	354	353	-	+	-	-	-	-
Caffeic acid	180	179	-	+	-	-	-	T
Ferulic acid	194	193	-	+	-	T	-	-
Rutin	610	609	-	+	-	-	-	-
Quercetin	302	301	-	T	-	+	-	+
Dihydrocaffeic acid	182	181	-	+	+	+	+	+
Dihydroferulic acid	196	195	-	T	-	-	-	+
3- Hydroxyphenyl-propionic acid	166	165	-	+	-	+	-	+
3-Phenylpropionic acid	150	149	-	-	+	+	T	+
3- Hydroxybenzoic acid	138	137	-	+	+	-	T	+
Benzoic acid	122	121	-	+	-	+	T	T
Protocatechuic acid	154	153	-	T	-	+	-	+
3-Hydroxyphenylacetic acid	152	151	-	+	T	+	+	T
Vanillic acid	168	167	-	+	-	+	-	+
Hippuric acid	179	178	-	-	-	-	-	-
Cinnamic acid	148	147	-	-	+	+	+	+
Coumaric acid	164	163	-	+	-	+	-	-
Quinic acid	192	191	-	+	-	+	-	+
3,4-Dihydroxyphenylacetic acid	168	167	-	+	-	+	-	+
3-Hydroxyphenylacetic acid	152	151	-	+	T	+	+	+

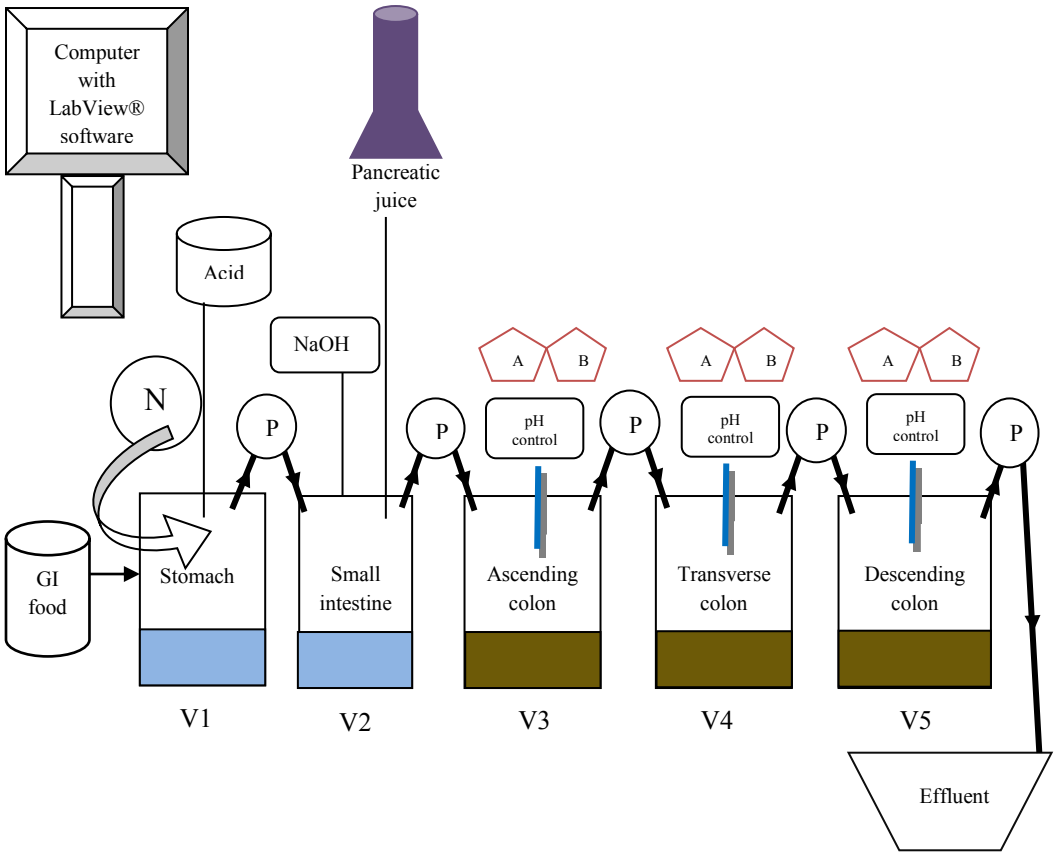
(V3) ascending colon, (V4) transverse colon, and (V5) descending colon, (-) absent; (+) present; (T) trace amounts

Table 3.3 HPLC concentration of parent phenolics of 'Onaway' potato extract in different vessels of Computer Controlled Dynamic Human Gastrointestinal Model after 24 hours of fermentation

Vessel number	CGA (μg/ml)	CA (μg/ml)	FA (μg/ml)	RU (μg/ml)
1	479.62 ± 71.8	20.85 ± 5.1	2.00 ± 1.1	20.95 ± 1.6
2	193.26 ± 71.8	4.59 ± 5.1	1.00 ± 1.1	11.50 ± 1.6
3	9.37 ± 71.8	34.47 ± 5.1	2.51 ± 1.1	5.34 ± 1.6
4	ND	5.25 ± 5.1	0.45 ± 1.1	1.46 ± 1.6
5	0.40 ± 71.8	2.33 ± 5.1	ND	1.26 ± 1.6

Data are represented as LSmeans ± SE. (CGA) chlorogenic acid, (CA) caffeic acid, (FA) ferulic acid, (RU) rutin. ND= not detected

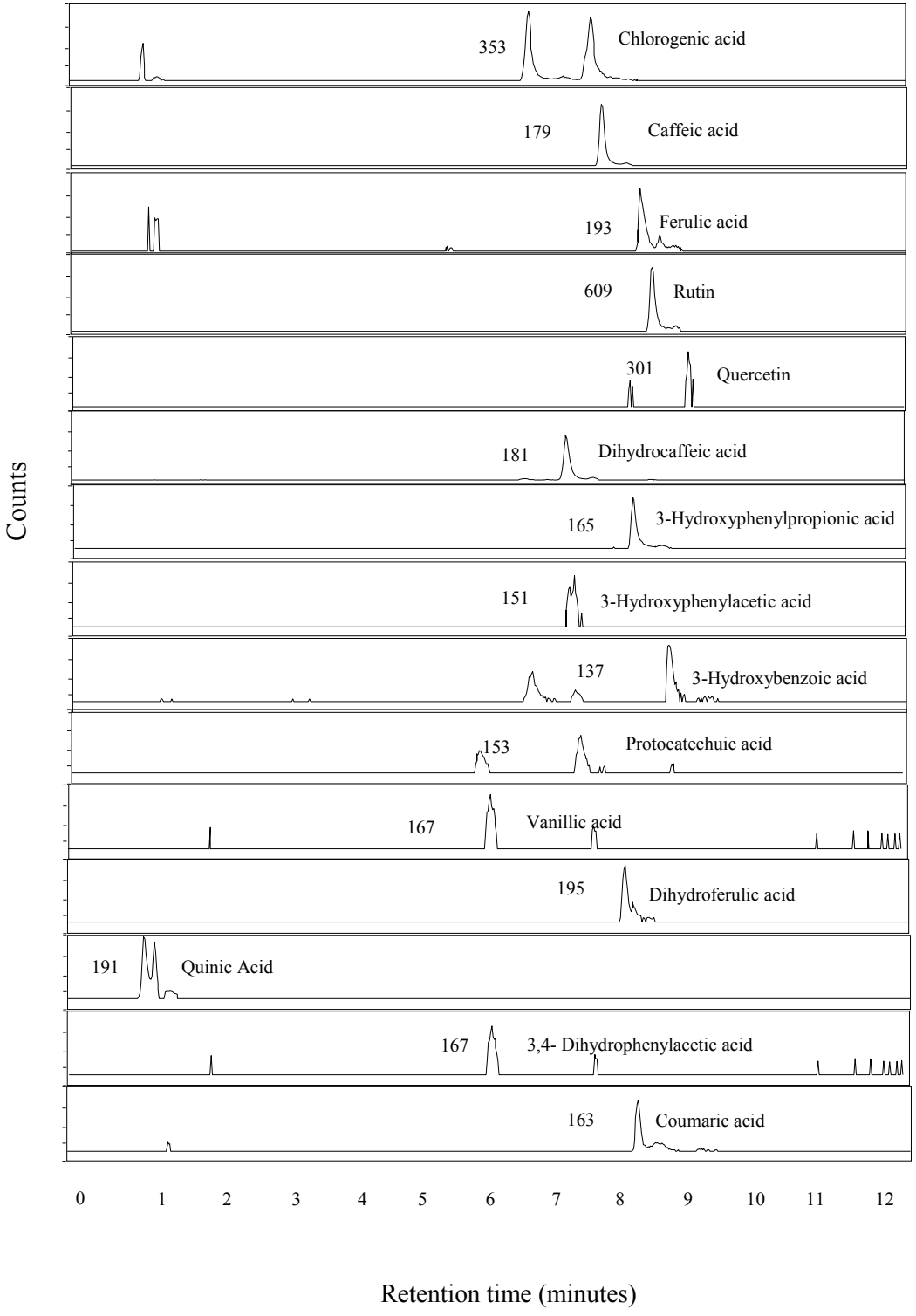
Figure 3.1 Schematic representation of the Computer Controlled Dynamic Human Gastrointestinal Model.



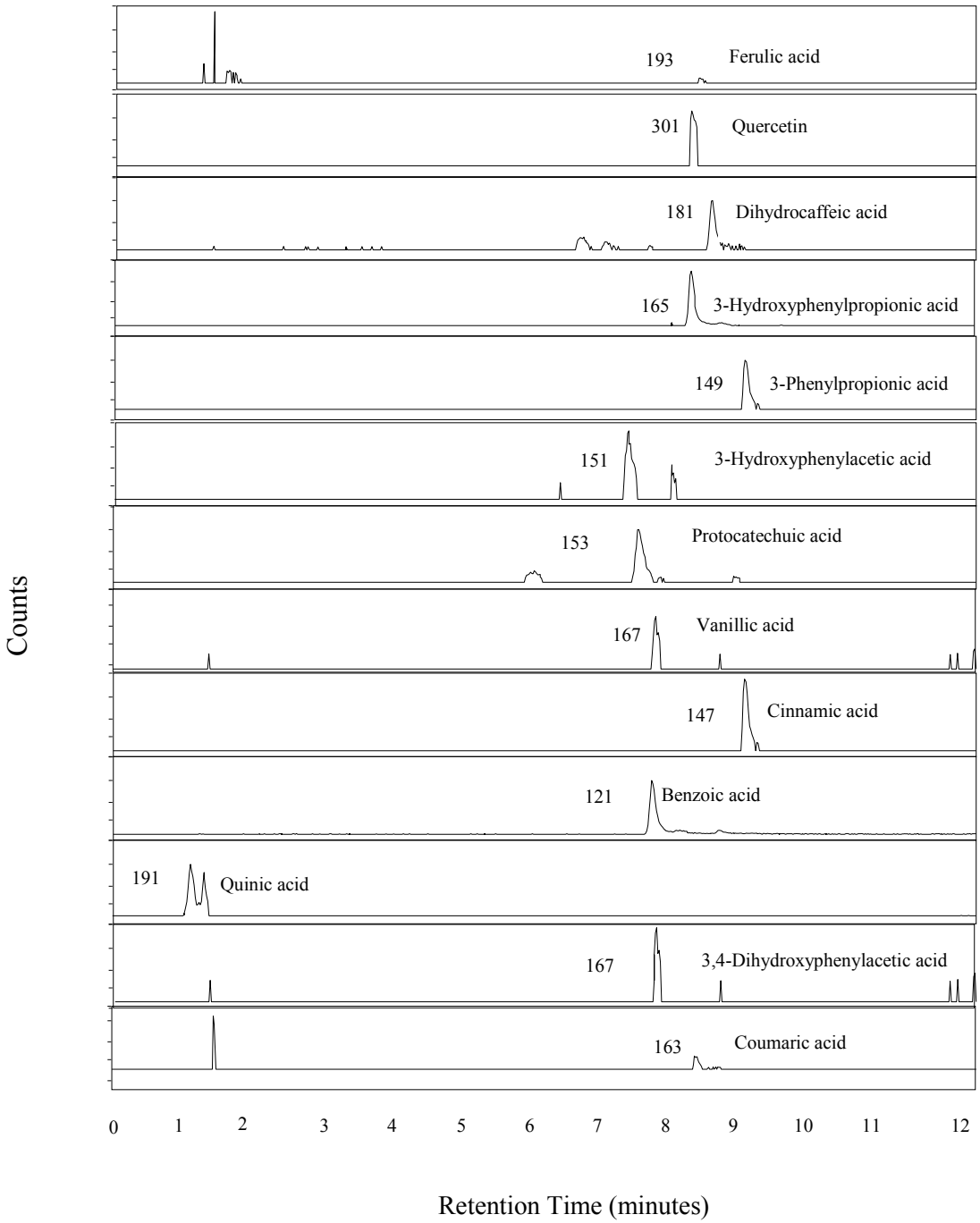
Adapted from Martoni et al, 2007. The schematic representation shows the 5 vessels (V1 to V5) that simulate the different compartments of the gastrointestinal tract. Vessels are joined by pumps. (P) pump; (A) acidic HCl solution to control pH; (B) basic NaOH solution to control pH; (N) nitrogen gas; (GI food) the food mixture given to the bacterial species to ensure their survival. The polyphenol-rich 'Onaway' extracts were dissolved in the GI food in order to allow the administration of the polyphenols into the system to simulate their digestion.

Figure 3.2 Extracted ion chromatogram from colonic vessels after 24 h of fermentation.

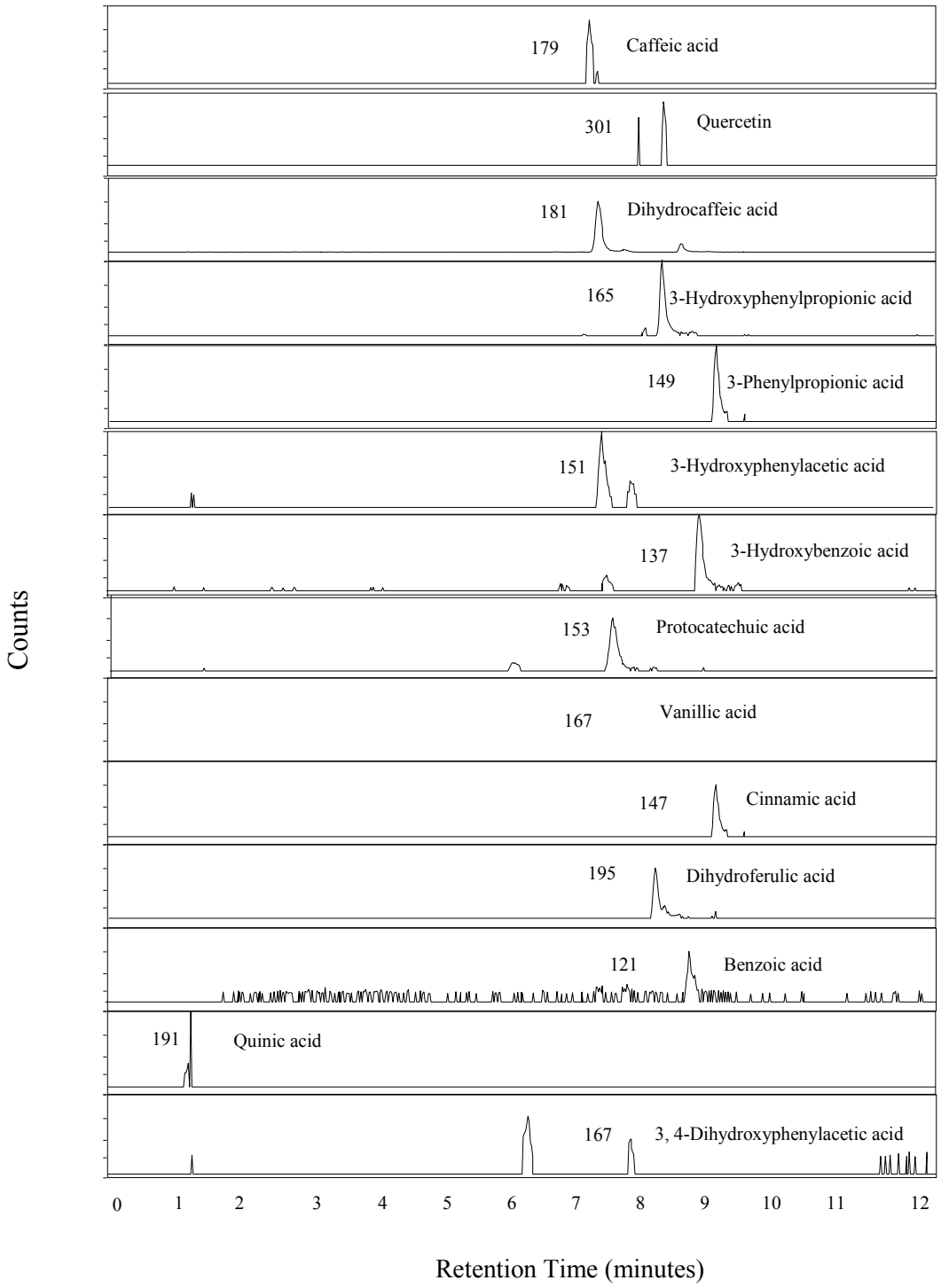
A



B


193
Ferulic acid
301
Quercetin
181
Dihydrocaffeic acid
165
3-Hydroxyphenylpropionic acid
149
3-Phenylpropionic acid
151
3-Hydroxyphenylacetic acid
153
Protocatechuic acid
167
Vanillic acid
147
Cinnamic acid
121
Benzoic acid
191
Quinic acid
167
3,4-Dihydroxyphenylacetic acid
163
Coumaric acid
Counts
0
1
2
3
4
5
6
7
8
9
10
11
12
Retention Time (minutes)

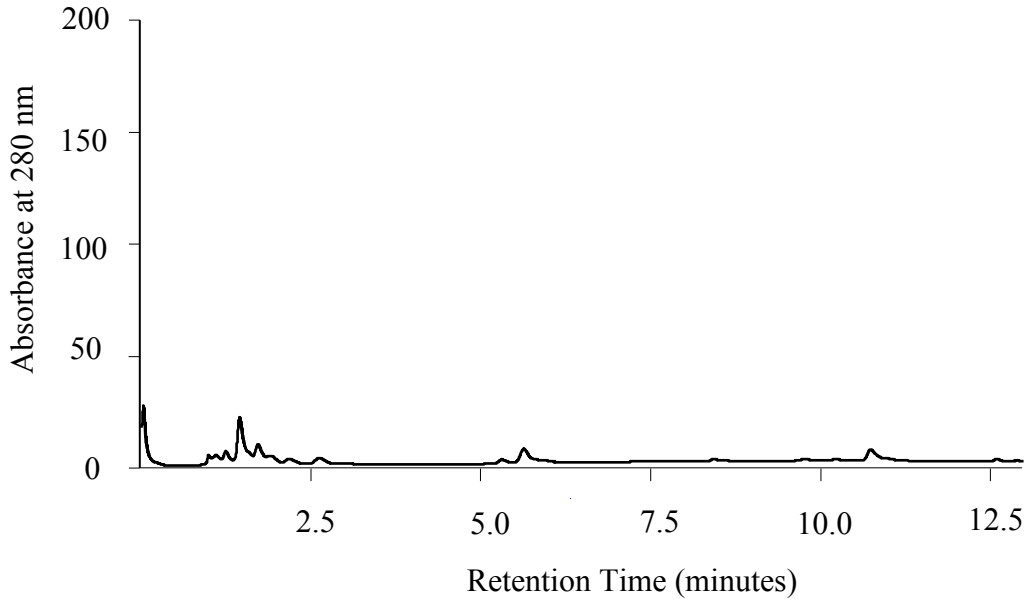
C


179
Caffeic acid
301
Quercetin
181
Dihydrocaffeic acid
165
3-Hydroxyphenylpropionic acid
149
3-Phenylpropionic acid
151
3-Hydroxyphenylacetic acid
137
3-Hydroxybenzoic acid
153
Protocatechuic acid
167
Vanillic acid
147
Cinnamic acid
195
Dihydroferulic acid
121
Benzoic acid
191
Quinic acid
167
3, 4-Dihydroxyphenylacetic acid
Counts
0
1
2
3
4
5
6
7
8
9
10
11
12
Retention Time (minutes)

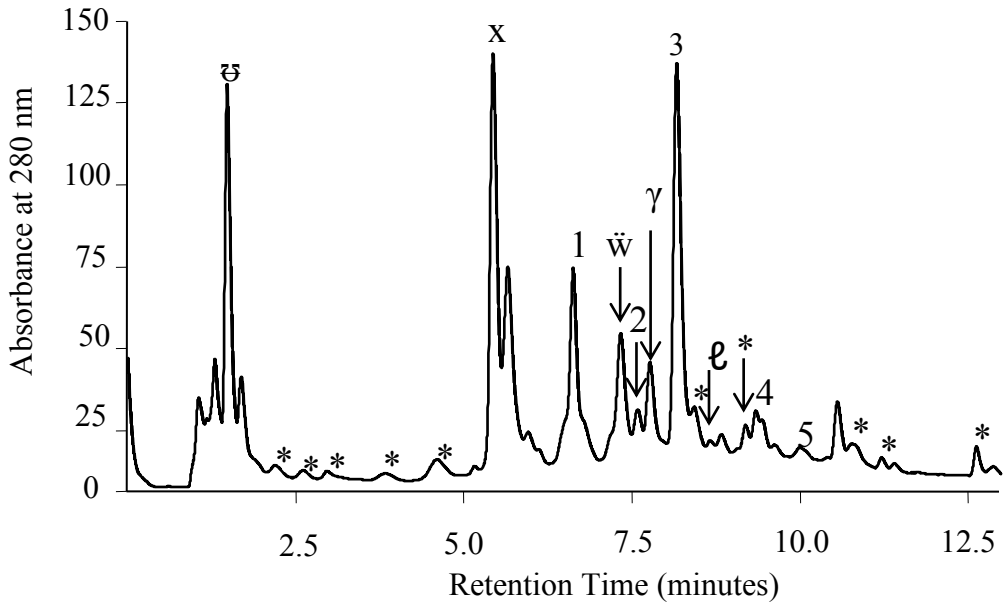
LC-MS polyphenolic profile of the colonic vessels (A) ascending colon (V3); (B) transverse colon (V4); and (C) descending colon (V5) after 24 hours of fermentation.

Figure 3.3 Chromatogram of polyphenols in colonic vessels at baseline and after 24 h of fermentation from reverse phase HPLC monitored at 280 nm.

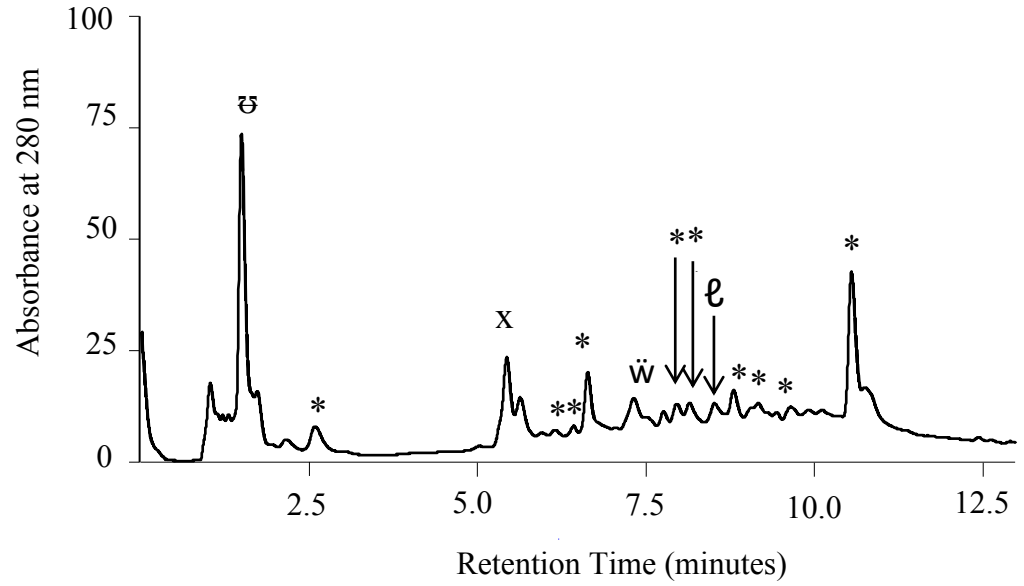
A



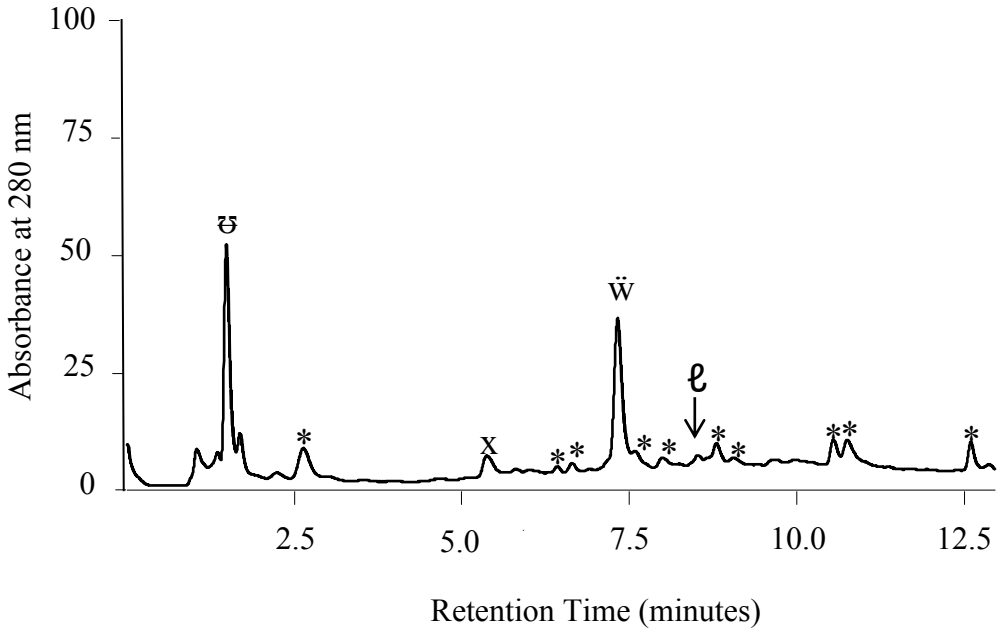
B


Absorbance at 280 nm
150
125
100
75
50
25
0
2.5
5.0
7.5
10.0
12.5
Retention Time (minutes)
x
3
γ
1
ẅ
2
4
5

C


Absorbance at 280 nm
100
75
50
25
0
2.5
5.0
7.5
10.0
12.5
Retention Time (minutes)
x
ẅ

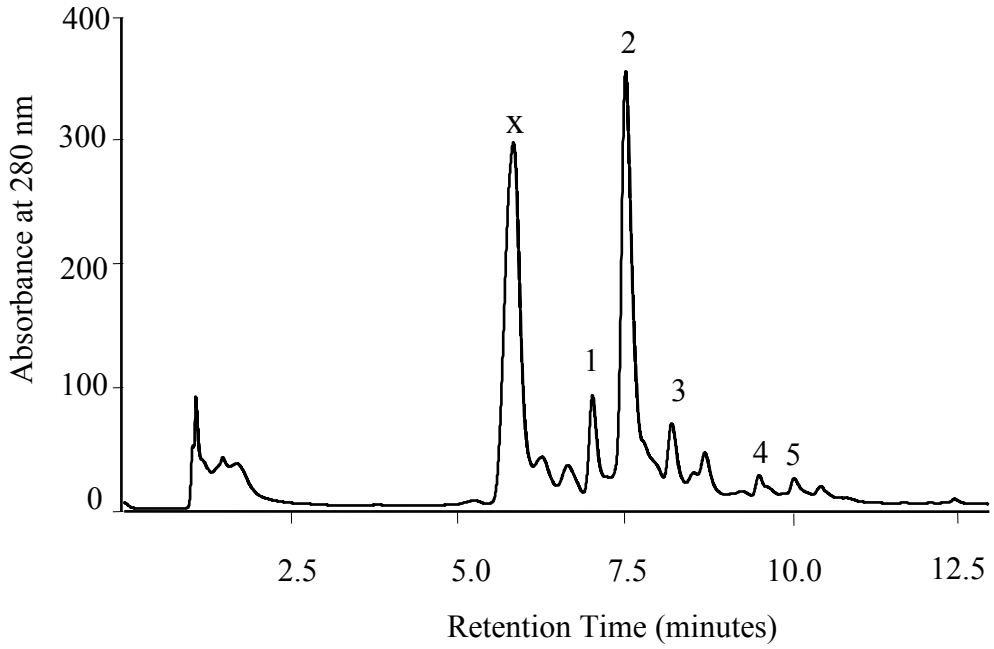
D



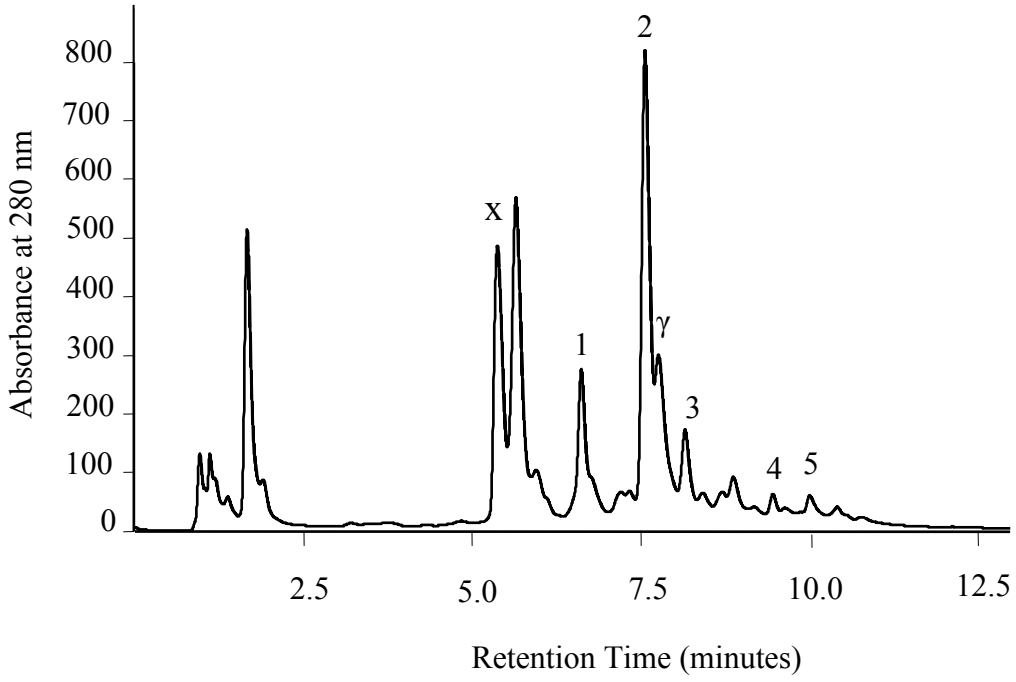
Polyphenolic profile of (A) vessels at baseline, (B) 'Onaway' potato extract after digestion in vessel 3 (ascending colon), (C) vessel 4 (transverse colon), and (D) vessel 5 (descending colon): (1) neoCGA, (2) CGA, (3) CA, (4) FA, (5) RU, (x) tryptophan, (ʊ) quinic acid, (γ) cryptoCGA (ẅ) dihydrocaffeic acid, (ℓ) 3-Hydroxyphenylpropionic acid, (*) new peak formed upon microbial biotransformation of polyphenols.

Figure 3.4 Chromatogram of polyphenols in digested potato extract, stomach vessel, and small intestine vessel after 24 h of digestion from reverse phase HPLC monitored at a wavelength of 280 nm.

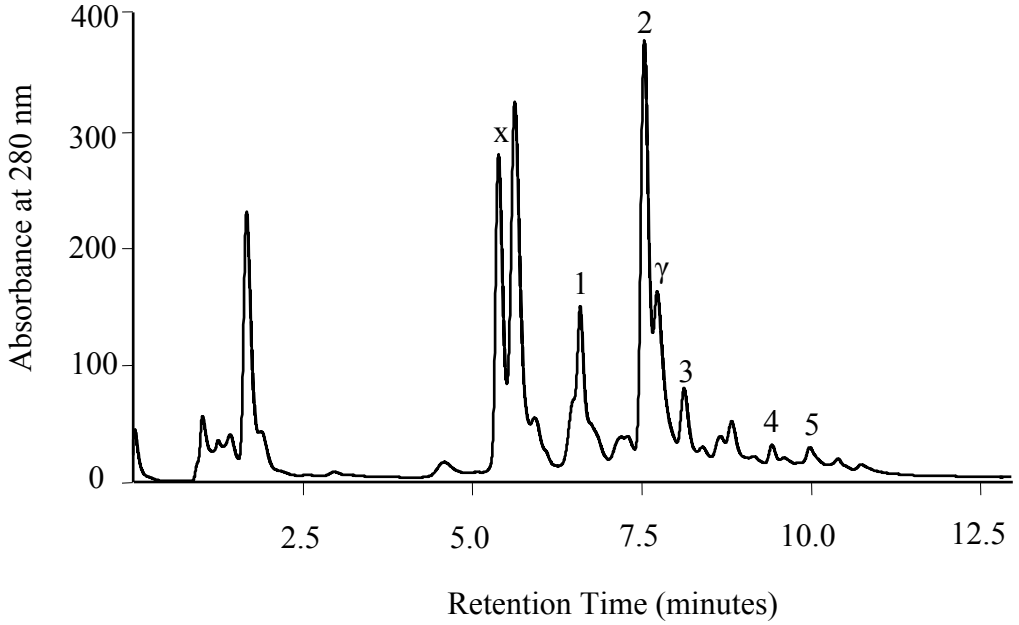
A



B

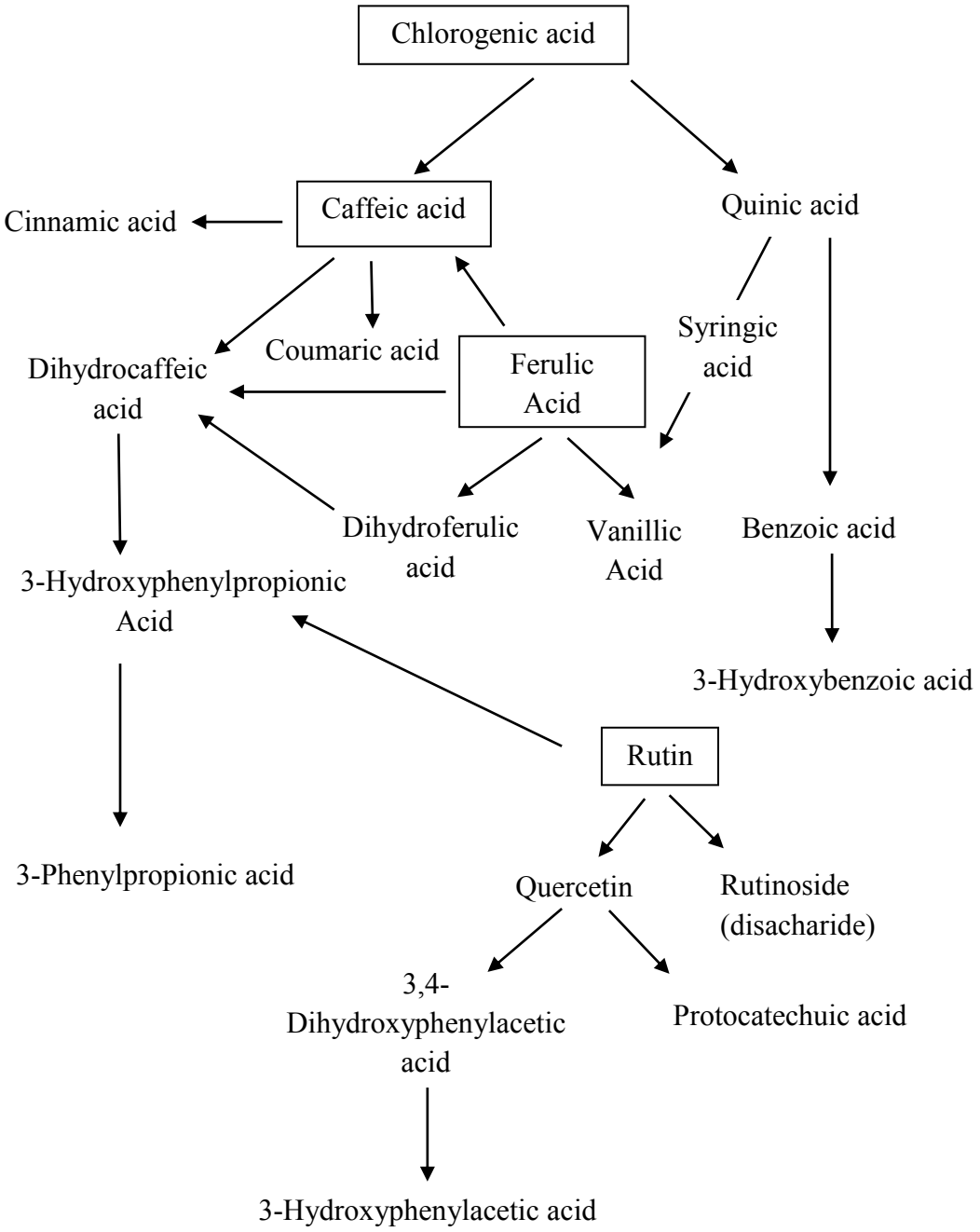


C



Polyphenolic profile of (A) 'Onaway' potato extract before digestion, (B) potato extract after digestion in vessel 1 (stomach), and in (C) vessel 2 (small intestine): (1) neoCGA, (2) CGA, (3) CA, (4) FA, (5) RU, (x) tryptophan, (γ) cryptoCGA .

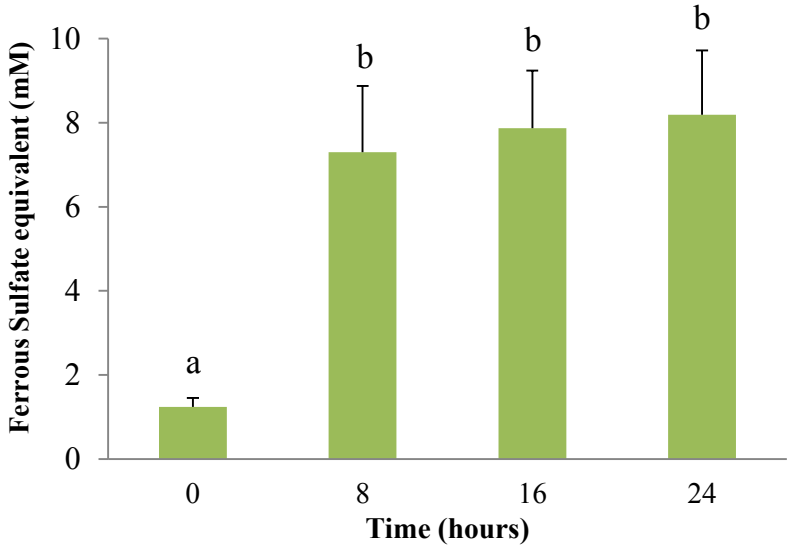
Figure 3.5 Suggested pathway of colonic biotransformation of parent compounds in the potato extract into microbial metabolites.



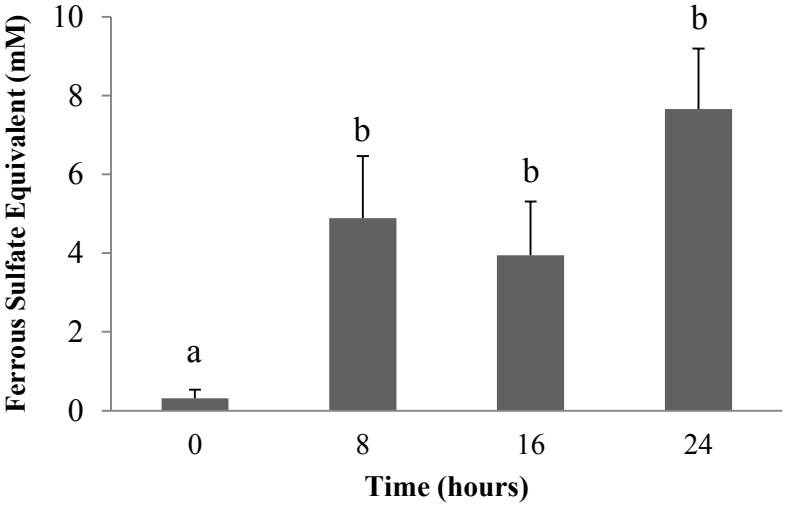
Parent compounds are shown in boxes. References: Farah et al, 2008; Rechner et al, 2004; Olthof et al, 2003; Gonthier et al, 2006; Gonthier et al, 2003; Braune et al 2009; Russel et al, 2008; Rechner et al, 2001; Stalmach et al, 2010; Aura et al, 2002.

Figure 3.6 Change in FRAP antioxidant capacity with time in the 5 vessels that simulate the different gastrointestinal compartments of the Computer Controlled Dynamic Human Gastrointestinal Model.

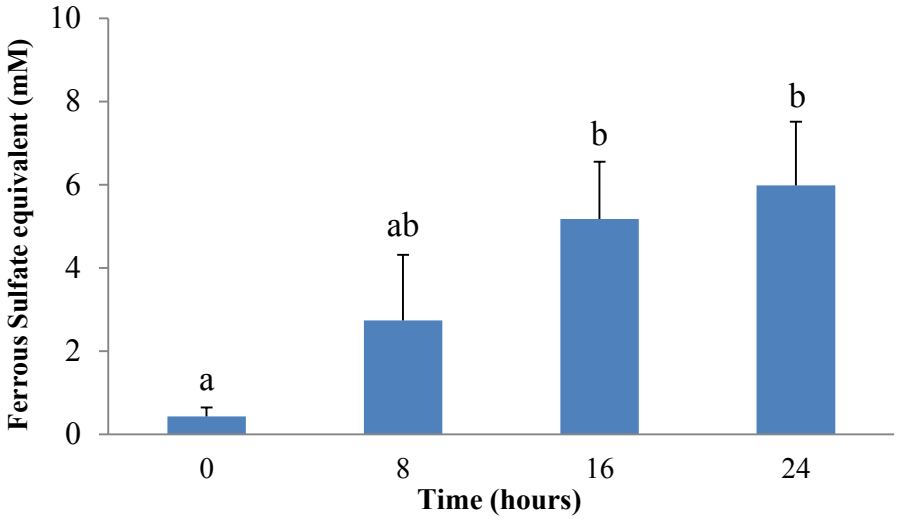
A



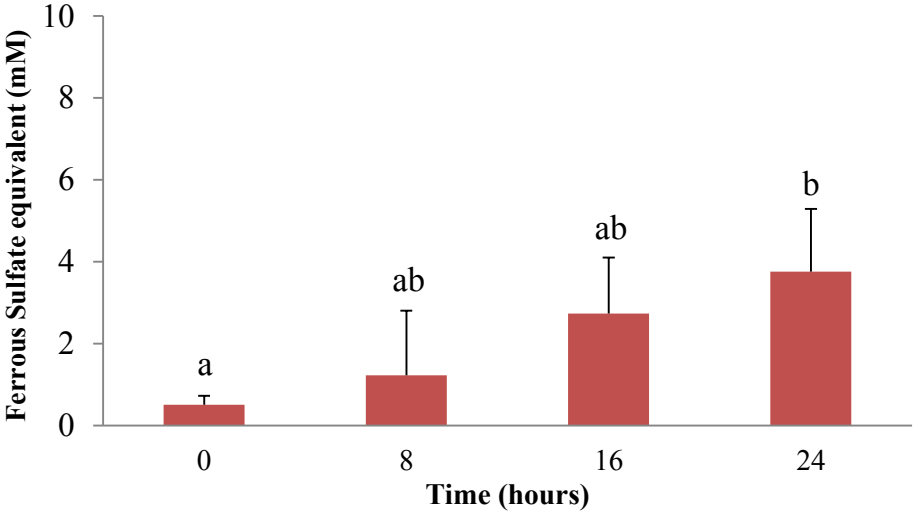
B



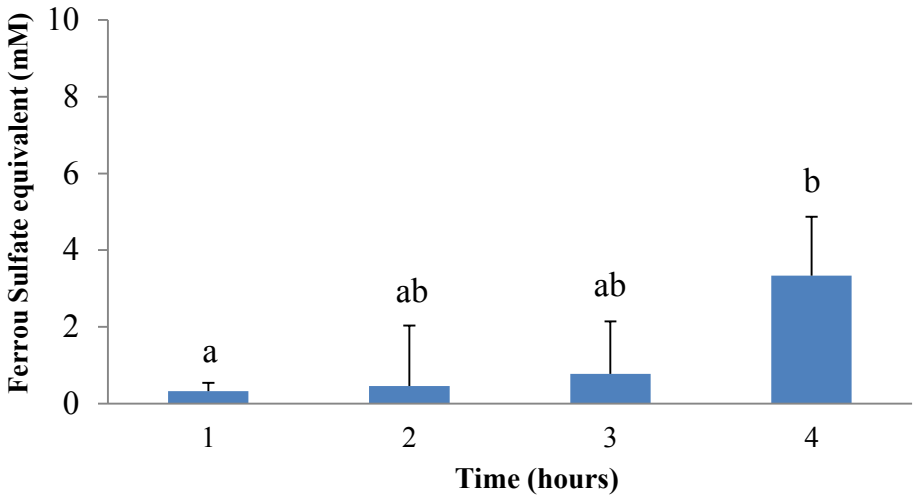
C


Ferrous Sulfate equivalent (mM)
10
8
6
4
2
0
a
ab
b
b
0
8
16
24
Time (hours)

D


Ferrous Sulfate equivalent (mM)
10
8
6
4
2
0
a
ab
ab
b
0
8
16
24
Time (hours)

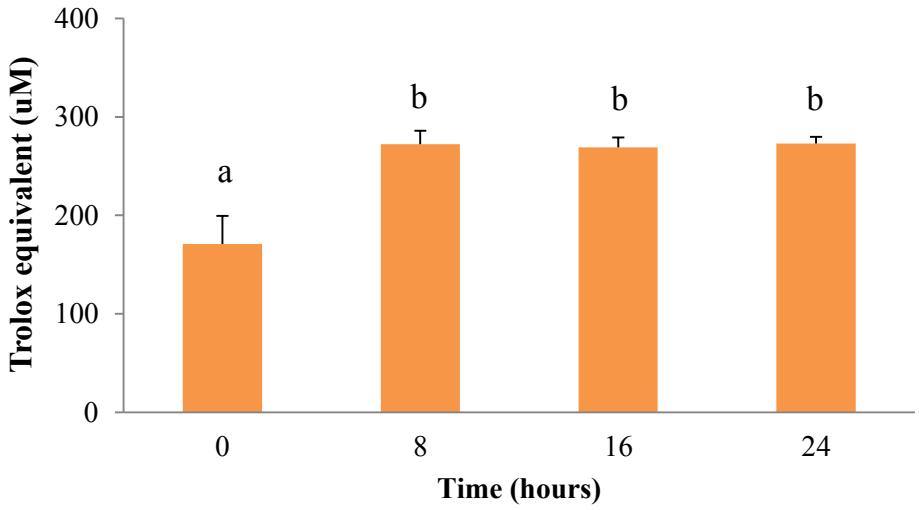
E



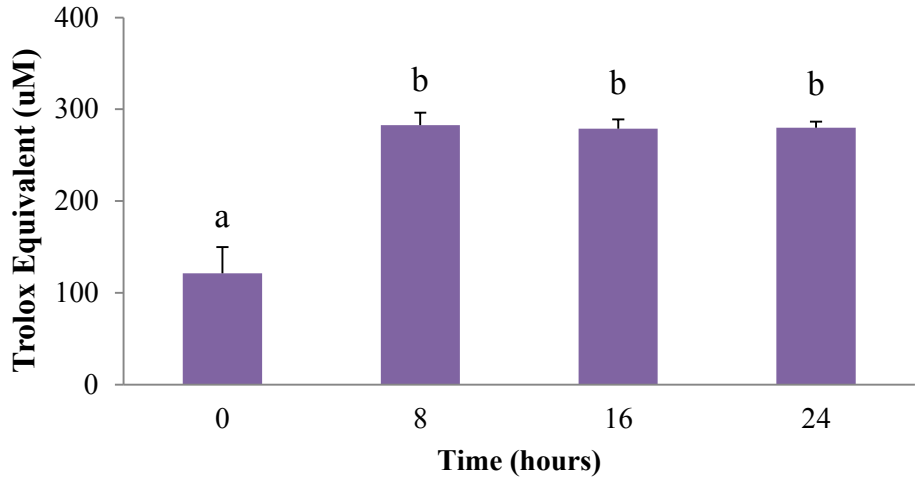
Increase in FRAP antioxidant capacity with time in vessels representing (A) vessel 1 (stomach), (B) vessel 2 (small intestine), (C) vessel 3 (ascending colon), (D) vessel 4 (transverse colon), and (E) vessel 5 (descending colon). Data are represented as LSmeans ± SE. LSmeans having the same letter are not statistically different from each other.

Figure 3.7 Change in ABTS antioxidant capacity with time in the 5 vessels that simulate the different gastrointestinal compartments of the Computer Controlled Dynamic Human Gastrointestinal Model.

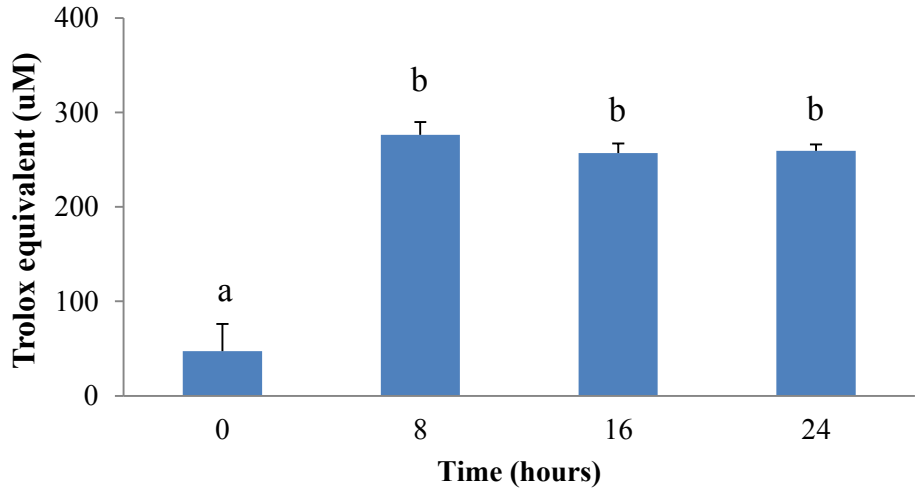
A



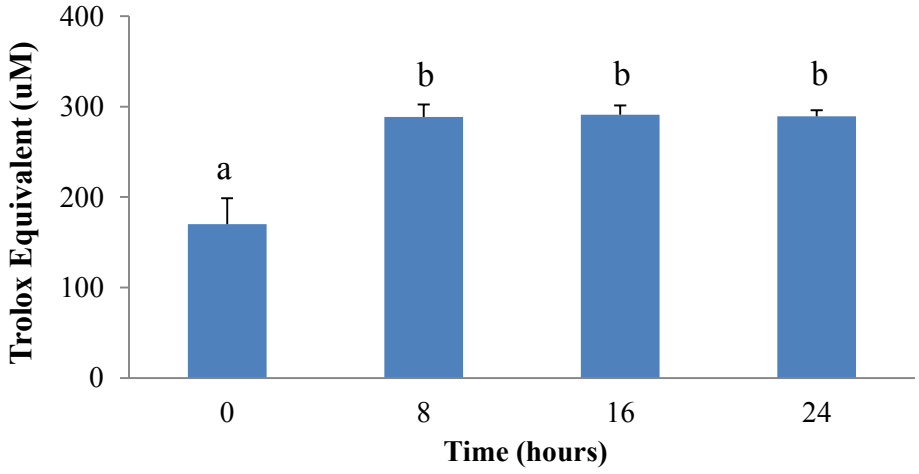
B



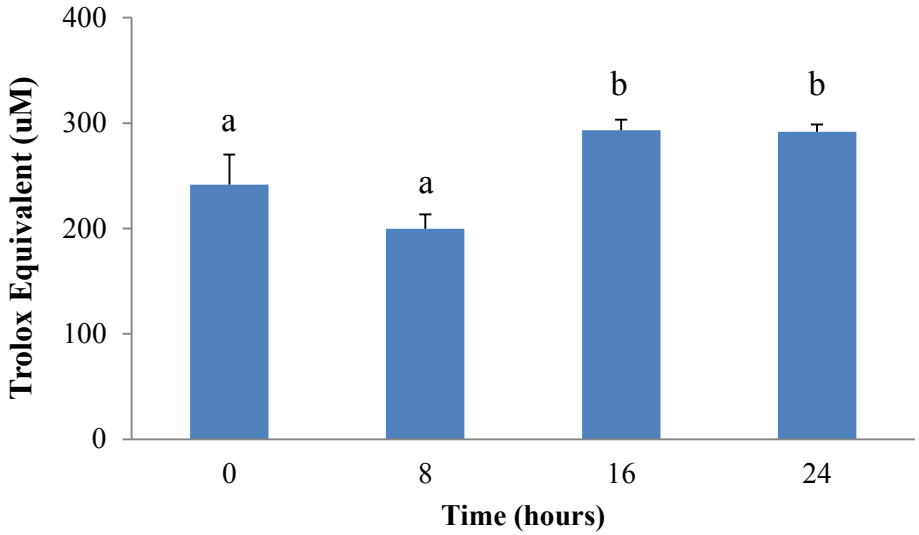
C


400
300
200
100
0
Trolox equivalent (uM)
a
b
b
b
0
8
16
24
Time (hours)

D


400
300
200
100
0
Trolox Equivalent (uM)
a
b
b
b
0
8
16
24
Time (hours)

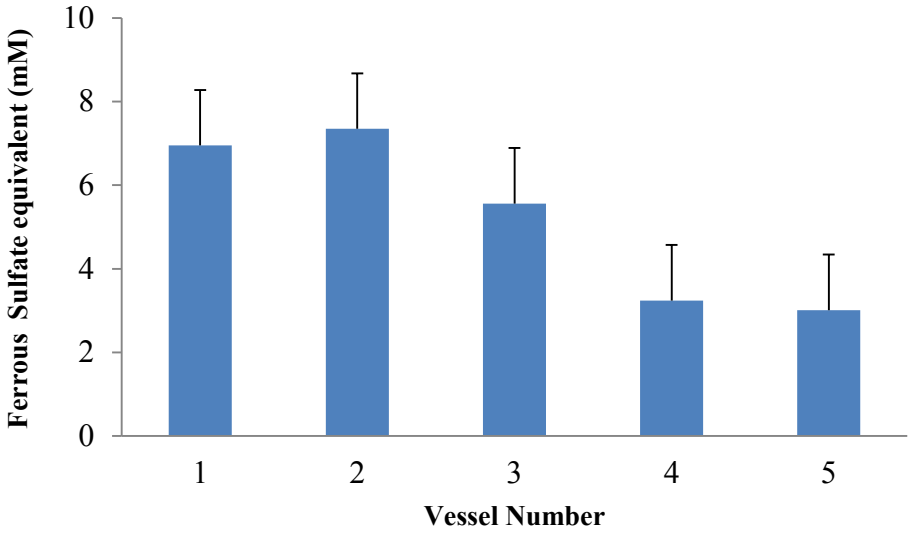
E



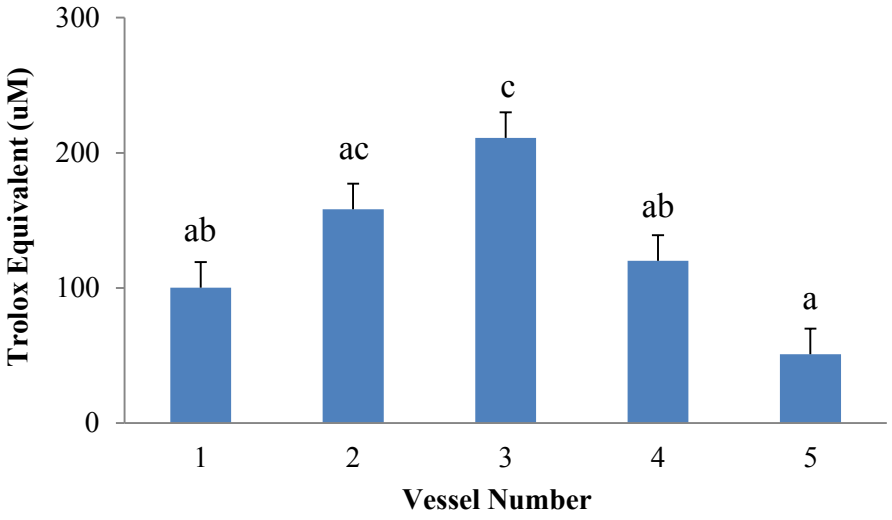
Increase in ABTS antioxidant capacity with time in vessels representing (A) vessel 1 (stomach), (B) vessel 2 (small intestine), (C) vessel 3 (ascending colon), (D) vessels 4 (transverse colon), and (E) vessel 5 (descending colon). Data are represented as LSmeans ± SE. LSmeans having the same letter are not statistically different from each other.

Figure 3.8 The increase in FRAP and ABTS activity from baseline in the 5 vessels that simulate the different gastrointestinal compartments of the Computer Controlled Dynamic Human Gastrointestinal Model, after 24 hours of digestion and fermentation.

A



B



Increase in (A) FRAP antioxidant activity in the vessels of the GI measured at 24 hours. (B) Increase in ABTS antioxidant activity in the vessels of the GI measured at 24 hours. Baseline values are subtracted. Data are represented as LSmeans ± SE. LSmeans having the same letter are not statistically different from each other.

Chapter 4

Final Conclusion

4.1 General Summary and Conclusion

Polyphenols are the major dietary source of antioxidants in the human diet supplied primarily by staple food commodities (D' Archivio et al., 2007; Crozier et al., 2009). Dietary polyphenols are found as esters, polymers, or glycosides that mostly require further digestion into simpler forms to get absorbed (Manach et al., 2004). Once ingested, polyphenols undergo enzymatic and microbial digestion (Manach et al., 2004; Saura-Calixto et al., 2007). A large amount of polyphenols escape absorption in the upper gastrointestinal (GI) tract and reach the large intestine (LI) to be metabolized by the microfloral species producing secondary microbial metabolites (Graefe et al., 2001; Olthof et al., 2001; Farah et al., 2008; Renouf et al., 2010; Stalmach et al., 2010).The potential health benefits of polyphenols can partly be attributed to the microfloral metabolites produced in the GI tract as they are absorbed at the colon and appear in the blood and urine after phase I and II metabolism, rather than the parent compounds originally ingested (Gonthier et al., 2003; Cerdá et al., 2004; Stalmach et al., 2010).

To assess the effect of gut digestive conditions involving digestive enzymes and human microflora on polyphenol structure and function, more novel complex continuous models have recently been implemented as an improved alternative to earlier static models using only one basic fermentor. These complex models can represent different segments of the human colon (van Duynhoven et al., 2011). Information regarding changes in antioxidant capacity and structures of polyphenols upon enzymatic and microfloral digestion, particularly as related to their biotransformation in different GI segments, is limited.

The Computer Controlled Dynamic Human Gastrointestinal Model (GI model) (Fig. 3.1) was used in this thesis study as a continuous five compartment GI system to assess the impact of colonic microflora on polyphenol structure and function following polyphenol exposure to stomach and intestinal digestive processes. Specifically, a polyphenol-rich potato extracts (PE) containing the phenolic acids chlorogenic acid (CGA), caffeic acid (CA), and ferulic acid (FA) and the flavonoid rutin (RU) was obtained from ‘Onaway’ potato and was subject to digestion in the GI model. The change in the profile of the polyphenols and

their metabolites and the antioxidant capacity was then assessed in the digestates from different compartments of the GI model.

Isomerization of CGA was the only change in HPLC phenolic profiles detected after gastric and pancreatic digestion of the polyphenols (Fig. 3.4), whereas isomerization of CGA has only been reported after simulated in vitro intestinal conditions only (Bermúdez-Soto et al., 2007; Kahle et al., 2011). Major changes in LC-MS and HPLC phenolic profiles occurred in the colonic vessels by microbial action as exhibited by the presence of newly formed microbial metabolites in the colonic vessels (Figs. 3.2 and 3.3); compared to the minimal changes in HPLC polyphenolic profiles among the upper GI vessels (Fig. 3.4). Therefore, microbial action is required for biotransformation to occur.

Different LC-MS and HPLC profiles were detected among the 3 colonic segments (Figs. 3.2 and 3.3; Table 3.2). While previous studies did not characterize the different metabolites produced in the different colonic segments, derivatives of phenylpropionic acid, phenylacetic acid and benzoic acid, detected in the vessels of the GI model, were previously reported to be produced following in vitro fermentation of polyphenols in a basic fermentor (Rechner et al., 2004; Gonthier et al., 2006; Gumienna et al., 2011). Unlike the basic fermentor, the GI model can account for microbial effects of different colonic segments that contain different microfloral species as mediated by different pH ranges that mimic those in the colonic segments in vivo. Therefore, the model may be more physiologically relevant in comparison to other studies that used a basic fermentor to assess the microbial effect on polyphenol bioaccessibility and transformation (Saura-Calixto et al., 2007; Gumienna et al., 2011).

Different microbial species possess different esterases, hydrogenases, dehydroxylases, and isomerases that can cause different dehydroxylation, reduction, and isomerisation reactions leading to the production of different polyphenolic metabolites (Selma et al., 2009). Although bacterial speciation among the 3 colonic vessels that represent the ascending, transverse, and descending colon was not assessed, it is possible that, due to pH differences among the colonic vessels, different microbial species present in each vessel,

resulted in the major differences in polyphenolic profiles and microbial metabolites (Figs. 3.2 and 3.3; Table 3.2). The assessment of the different bacterial species present could have added a better highlight of the reactions taking place within each vessel.

In the ascending colon, all the parent compounds in the PE were detectable but microbial biotransformation was initiated leading to polyphenol degradation together with the formation of microbial metabolites (Figs. 3.2A and 3.3B). CGA was the primary polyphenol biotransformed in the ascending colon as it isomerized into neoCGA and cryptoCGA and also degraded into CA and quinic acid (Figs. 3.2A and 3.3B). Interestingly, the CGA isomers were not detectable in the transverse or descending colon as they likely get further metabolized by the microbial species into degradation products (Figs. 3.2B, 3.2C, 3.3C, and 3.3D; Table 3.2). Likewise, it appeared that RU, FA, and the relatively large amounts of CA produced in the ascending colon were absent in the segments of the transverse and descending colon, with trace amounts of FA and CA in the transverse and descending colon respectively (Table 3.2). Hence, it is conceivable that the possible health benefits that might be attributable to the presence of parent compounds such as CGA, CA, RU and FA as well as the CGA isomers and CA that are formed in the ascending colon might not be available for the other segments of the colon due to their further microbial breakdown. Similarly, the metabolites formed in the subsequent segments of the colon (V4 and V5) like quercetin, 3-Phenylpropionic acid, or cinnamic acid (Table 3.1) can exert their health benefits in the transverse and descending segments of the colon.

Being unique in measuring the antioxidant capacity in different segments of the colon, results showed that the antioxidant capacity in the colonic vessels as measured by the FRAP assay was significantly increased after 16 h of fermentation (Fig. 3.6). This is likely the time required for the polyphenols to reach the colonic vessels and for subsequent microfloral metabolism of the polyphenols to generate the metabolites. The above factors may explain partly the relatively lower polyphenolic content and antioxidant capacity in the last two colonic vessels. However, the results portrayed here show that the antioxidant

capacity measured in the first segment of the colon is not the same as that in the subsequent ones. To study the effect of time on the generation of polyphenols in our samples, a longer testing period of 36 or 48 h, might have shown a greater increase in phenolic content and antioxidant capacity in the last two colonic vessels.

The type of microbial metabolites present in the transverse and descending colonic vessels might also affect their antioxidant capacity since the change in the phenolic structure such as the loss of the hydroxyl group can decrease its antioxidant capacity (Rice-Evans et al., 1996; Cao et al., 1997). In particular, biotransformation of the most abundant polyphenol in the PE, CGA, led to the production of 3- Phenylpropionic acid (Table 3.2 and Figure 3.5), which has no hydroxyl group attached on the aromatic ring. This metabolite is the end product of 3-Hydroxyphenylpropionic acid, which is a common intermediate metabolite for all the parent compound of the PE as portrayed by the pathway of biotransformation in Fig. 3.5.

Parent compounds in the upper GI vessels and microbial metabolites in the colonic vessels showed similar trends with the increase in ABTS (Figure 3.7). Moreover, no significant difference in the increase in FRAP activity from baseline was detected between the colonic segments and the vessels with parent compounds (Fig. 3.8A). Hence, despite the different factors that affect the antioxidant capacity, it appears that the various accumulated microbial metabolites produced were similarly effective towards antioxidant capacity measures. The presence of both; the parent compounds and the microbial metabolites, in the ascending colon segment contributed to the greatest increase in ABTS activity (Fig 3.8B); possibly because both showed a similar trend with ABTS (Fig 3.7). Therefore, the presence of parent phenolics and metabolites simultaneously can add up to the health benefits and antioxidant capacity.

In addition to the antioxidant activity of microbial metabolites, these are also shown to have anti-inflammatory and anti-carcinogenic effects. The colonic metabolites, 3-Hydroxyphenylpropionic acid and 3-(4-Hydroxyphenyl)propionic acid were detected among the major metabolites in the fecal water of 20

individuals and were able to decrease the COX-2 protein levels by causing 15-62% inhibition in colon cancer cells, hence decreasing inflammation (Karlsson et al., 2005). When tested with colon cancer cell lines (HCT116) and prostate cells (LNCaP), the microbial metabolite 3, 4-Dihydroxyphenylacetic acid mostly occurring upon microbial biotransformation of RU, showed an antiproliferative activity (Gao et al., 2006).

4.2. Suggestions for Future studies

This thesis provides useful information regarding the biotransformation that occurs upon the enzymatic and microbial digestion of the important polyphenols, CGA, CA, FA and RU, in different segments of the GI involving biotransformation by microbial species obtained from the fecal water of healthy individuals. It would be interesting to examine the metabolites obtained following digestion of polyphenols using fecal samples from individuals with common GI diseases like Crohn's disease or ulcerative colitis. Assessment of the biotransformation patterns of polyphenol metabolism by the microflora from individuals with intestinal diseases could illustrate how the microbial composition of individuals with GI disease might influence the generation of bioactive polyphenolic metabolites. Similar studies could also be performed using fecal samples from individuals who are taking prebiotics in order to asses if the intake of prebiotics contributes in improving the polyphenolic profile or producing new metabolites following polyphenol intake. The digestates can be used to study the in vitro antioxidant effect of polyphenols post-digestion in cells induced with oxidative stress. Another consideration would be to simulate the digestion of cooked polyphenol-rich foods to assess if biotransformation is altered after heating processing.

Future studies can also study in more detail the effect of polyphenols on colonic microflora. Bacterial species interact differently with the polyphenols and their metabolites that can cause changes in the microbial ecosystem (Lee et al., 2006). It is possible that polyphenols could improve the disturbed colonic microfloral balance associated with inflammation in chronic inflammatory

diseases such as colitis and colon cancer (Parkar et al., 2008). For example, tea phenolics and their aromatic acids including CA, catechin, epicatechin, gallic acid, 3-Phenylpropionic acid, significantly affected the growth of the pathogenic bacteria species *Clostridium perfringens*, *Clostridium difficile* and *Bacteroides spp.* with relatively little effect on probiotic bacteria such as *Bifidobacterium spp. and Lactobacillus spp.* (Lee et al., 2006). Also, RU increased the adhesion of the probiotic *Lactobacillus rhamnosus* to Caco-2 cells in a dose dependent manner, which could contribute to GI health (Parkar et al., 2008). Thus, the use of polyphenols as prebiotics seems promising, where their interactions with the microflora and the produced metabolites can cause growth to the probiotic beneficial bacteria and inhibit the growth of pathogenic bacteria.

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