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"Plants are known for their phenols", painted in oil on canvas in 2005 by Virpi Jokinen, the author's sister.

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Abstract

The aim of this work was to develop *in vitro* digestion models for mimicking the physiological conditions of upper intestine and microbial conversions in the colon. The main emphasis was on the microbial metabolism of plant phenolic compounds: pure quercetin derivatives, pure anthocyanins and lignans from rye bran and flaxseed.

When cereal samples are introduced to an *in vitro* colon model a removal of digestible components is needed. An enzymatic *in vitro* digestion model was developed for maximal starch removal from cereal samples. Pepsin, pancreatin and bile concentrations were optimized using an experimental design. Surprisingly, pepsin and bile also affected the extent of starch hydrolysis in synergy with pancreatin. 5–11 % of the original amount of starch remained in the residues of cereal products. Proteins were also partly hydrolysed. The *in vitro* enzymatic digestion model was used for the pretreatment of rye bran and flaxseed samples.

An anaerobic *in vitro* colon model, conventionally used for the fermentation of non-digestible carbohydrates, was developed further for studying pure phenolic compounds. Human faecal microbiota from several healthy donors was used in the preparation of an inoculum. A low inoculum concentration was used for decreasing the metabolite concentration from the faecal background in the studies concerning pure flavonoids. A dense faecal suspension was suitable for the conversion of rye bran and flaxseed lignans to enterolactone when the plant matrix was present.

Flavonoids were deconjugated and degraded to phenolic acids by faecal microbiota. Specific activities of the deconjugative enzymes from the

faecal inocula reflected the deconjugation rates of flavonoids. Quercetin aglycone was converted to hydroxyphenylacetic acids, but not to methylated phenolic acids. The extent of metabolism was 60 %, showing that ring-fission was a dominating route in the microbial metabolism of quercetin. Anthocyanins also underwent similar conversion, but the estimated extent of metabolite formation was low (less than 5 %). Protocatechuic acid was identified, and a phenoxyacid or a phenoxyaldehyde was proposed, as ring-fission products of cyanidin. In addition, it was suggested that anthocyanins undergo conjugation with an unknown moiety of 85 mass units. This conjugate was observed for several anthocyanins.

Enterolactone production from plant lignans proceeded steadily and slowly for 48 hours in the *in vitro* colon model using the dense (16.7 %) faecal suspension. Flaxseed lignan conversion to enterolactone was suppressed by the presence of rye matrix. The enterolactone-producing microbiota may be sensitive to non-physiological, low pH values caused by acidic components from rye bran in the presence of microbiota. The presence of rye bran matrix did not interfere with enterolactone formation in an *in vivo* rat model. The difference in the response to the rye bran matrix may be due to the absorption of the released and metabolised compounds in rats. Rats may also adapt to the diet during their feeding period. This may have enhanced the enterolactone production, and may have further increased the difference between the bioactivity of the microbiota in the *in vitro* and *in vivo* models used in this study.

Clinical human and animal trials describe end-point metabolism after adaptation to the test diet. The *in vitro* colon model assists in elucidation of the role of microbiota in the metabolical network of human digestive system and it helps in identification of the crucial reactions. Applications of this method can be extended from the studies of food components to pharmaceutical research.

Preface

Studies presented in this thesis were carried out at VTT Biotechnology during the years 1995–2004. The research was supported by the National Technology Agency of Finland (Tekes programme Innovation in Foods), the European Commission, the VTT programmes Future Foods and Tailored Technologies for Future Foods, Fazer Bakeries Ltd and Vaasan & Vaasan Ltd, which are gratefully acknowledged. I also express my gratitude to collaborators within the Tekes-project "Non-digestible carbohydrates and polyphenols in gut health": the Universities of Helsinki and Kuopio and to collaborators in the project EU-POLYBIND.

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My parents, Maija and Jaakko Räsänen, I thank you for your love, your care, your wisdom and your belief in constant education, which you have shared with me during my entire life. I also thank you for taking care of my sons, when needed. I thank my sister, Virpi Jokinen for her support and especially her artistic talent, a token of which can be seen in the cover of this book. I thank my sons, Matias (11 years) and Thomas (7 years) for being the lights of my life, and for teaching me the concept of controlled chaos. I thank you, Anna, Marjo, Päivi, Taina and Terhi, for your friendship. Without you my life would be much harder. I am also grateful for having friends in music. Torpparit choir, I am happy to share with you the enjoyment of making music of our own in Hannu´s tender care. Especially, I thank you, Hannu, for your touching compositions. Family, friends and music make my life worth living.

Espoo June 2005

Annukka

List of publications

This work is based on following publications (I–V), which are referred to in the text by their Roman numerals. Additional unpublished data is also presented.

I Aura, A.-M., Härkönen, H., Fabritius M. and Poutanen, K. 1999. Development of an *in vitro* enzymic digestion method for removal of starch and protein and assessment of its performance using rye and wheat breads. *J. Cereal Sci.* 29: 139–152.

II Aura, A.-M., O´Leary, K.A., Williamson, G., Ojala, M., Bailey, M., Puupponen-Pimiä, R., Nuutila, A.M., Oksman-Caldentey, K.-M. and Poutanen, K. 2002. Quercetin derivatives are deconjugated and converted to hydroxyphenylacetic acids but not methylated by human fecal flora *in vitro*. *J. Agric. Food Chem.* 50: 1725–1730.

III Aura, A.-M., Martin-Lopez, P., O´Leary, K.A., Williamson, G., Oksman-Caldentey, K.-M., Poutanen, K. and Santos-Buelga, C. 2005. *In vitro* metabolism of anthocyanins by human gut microflora. *Eur. J. Nutr.* 44: 133–142.

IV Aura, A.-M., Karppinen, S., Virtanen, H., Forssell, P., Heinonen, S.-M., Nurmi, T., Adlercreutz, H. and Poutanen, K. 2005. Processing of rye bran influences both the fermentation of dietary fibre and the bioconversion of lignans by human faecal flora *in vitro*. *J. Sci. Food Agric.* In press.

V Aura, A.-M., Oikarinen, S., Mutanen, M., Heinonen, S.-M., Adlercreutz, H.C.T., Virtanen, H. and Poutanen, K.S. 2005. Suitability of an *in vitro* fermentation model using human faecal microbiota in prediction of conversion of flaxseed lignans to enterolactone in reference to an *in vivo* rat model. *Eur. J. Nutr.* In press.

The author of the present thesis is the first author in charge of the experimental work, design and development of the studies above.

Abbreviations

DF	dietary fibre
RS	resistant starch
SCFA	short-chain fatty acid
GI	gastrointestinal
LPH	lactase phlorizin hydrolase
PA	proanthocyanidins
Cy3g	cyanidin-3-glucoside
Cyrut	cyanidin-3-rutinoside
HPLC	high-pressure liquid chromatography
ESI	electrospray ionisation
DAS	photodiode array spectrometry
LC	liquid chromatography
CEAD	coulometric electrode array detection
GC	gas chromatography
TR-FIA	time-resolved fluoroimmunoassay
MS	mass spectrometry

Contents

Appendices of this publication are not included in the PDF version. Please order the printed version to get the complete publication (http://www.vtt.fi/inf/pdf/)

1. Introduction

Epidemiological studies have shown that consumption of cereals, fruit, berries and vegetables is associated with lowered risk of some chronic diseases. Dietary recommendations are partly based on this epidemiological evidence. A balanced diet can be illustrated with a "Food pyramid", indicating which foods should be eaten in abundance and which should be consumed only in small amounts.

Most vitamins were identified between the years 1910 and 1950. Biologically active components that may diminish chronic disease risk have been recognized later, and their mechanism of action is still under investigation. Intake, availability and bioavailability (defined as absorption, transport into the circulatory system and delivery to the appropriate site of action) are important in estimating the potentially beneficial effects of dietary components on human health (Messina and Messina 1996; McGhie et al. 2003).

The word "bioaccessibility" is defined as the amount of ingested compounds that are available for absorption in the gastrointestinal tract. Thus bioaccessibility refers to availability and luminal changes prior to absorption and is a part of bioavailability. The amount of bioaccessible compounds can be equal to or less than the amount that is released from the food matrix (Stahl et al. 2002). The release affects absorption of dietary components and the amount entering the colon either as free components or within the matrix. In addition, non-digestible components can be degraded by intestinal microbiota. Thus there are several phenomena which may change the structure of ingested compounds.

When the metabolism of dietary compounds is studied *in vivo*, the overall metabolism of the ileum, liver, colon microbiota and intestinal tissues is included. In order to distinguish microbial conversions from those occurring in the tissues, it is necessary to isolate colonic microbes from the host. Consequently the present thesis is focused on the development and use of *in vitro* digestion models. The emphasis is on an *in vitro* colon model for studying microbial conversion of dietary phenolic compounds. When the phenolic compounds are introduced into the model within a

cereal matrix, digestible components need to be removed. An enzymatic digestion model is used for this purpose.

1.1 Dietary fibre and phenolic compounds in plant foods

1.1.1 Plant foods

Plant foods consist of cereal grains, vegetables, legumes, fruits and berries. Their high intake is recommended because of their substantial content of dietary fibre (DF), vitamins, antioxidants and other non-nutrients (ECP consensus panel on cereals and cancer 1998). The outer layers of cereal grains rich in DF also contain putatively health-promoting compounds such as phytate, lignans (diphenolics), phenolic acids, alkylresorcinols and polyphenols (McIntosh et al. 2003). Dietary phenols are ubiquitous plant-derived components. They can be divided into flavonoids, phenolic acids, stilbenes and lignans (Scalbert and Williamson 2000).

DF was defined as "the skeletal remains of plant cells that are resistant to digestion by enzymes of man" (Trowell 1972) and this hypothesis was later extended also to include storage polysaccharides (Trowell et al. 1976). Because oligosaccharides resist digestion in the upper intestine (van Loo et al. 1999), they were included in the most recent definition (AACC 2001). After the first definitions of DF, a hypothesis concerning the association of bioactive non-nutrients with the fibre complex was proposed in 1984 (Adlercreutz 1984). Thereafter, interest in the role of phenolic compounds in health and nutrition has been continuously expanding.

The recommended daily intake of DF is 3 g/MJ, and according to the national FINDIET 2002 study the mean intake of DF in Finland is 2.9 g/MJ and 2.4 g/MJ for women and men, respectively (Männistö et al. 2003). The intake of plant phenols is dependent on the types of plant foods consumed. Thus the total daily intake of plant phenols is estimated to be between 150 and 1000 mg (Stahl et al. 2002).

1.1.2 Dietary fibre (DF)

DF is most often quantified enzymatically by hydrolyzing starch and defining the soluble fraction by ethanol precipitation and the residual insoluble fraction gravimetrically (McCleary 2001; Asp et al. 1983; AOAC 2000a and b). The analyzed DF does not include small soluble oligosaccharides, which are not captured from the ethanol solution in the assay. Oligosaccharides are quantified separately by high-performance liquid chromatography (AOAC 2000c; McCleary et al. 2000; McCleary 2001). Due to the evolution of the DF concept methods of analysis are also the subject of continuous debate and development.

Chemically DF is heterogeneous, comprising of carbohydrates (cellulose, hemicellulose, arabinoxylans and β-glucans), polymeric uronic acids (pectic polysaccharides) and lignin (Englyst and Kingham 1990; McDougall et al. 1996; Truswell 1993). Cellulose, an insoluble DF component, has a high degree of ordering and pronounced crystallinity, which is caused by hydrogen bonding between parallel cellulose molecules (McDougall et al. 1996). Highly branched polymers, such as arabinoxylans and β-glucans, tend to be more soluble than less branched, linear polymers (Southgate 1995). Lignins are cell-wall polymers formed from phenyl propane units and they are highly insoluble (Begum et al. 2004). Furthermore, resistant starch (RS) is one subclass of DF, and it can be physically inaccessible starch (for example in partly milled grains and seeds), raw starch granules (for example in raw potato and banana), or retrograded starch, which is formed in cooked and cooled potato, in bread and in corn flakes (Englyst et al. 1992).

The major part of DF is carbohydrates, the primary structure of which consists of the intermolecular bonds between neutral monosaccharides. These glycosidic linkages are formed with two orientations (α or β) at any of the hydroxyl groups available. The orientation of bonds determines the secondary structure. In the α configuration the chain adopts a helical form and in the β configuration the chain has a flat ribbon-like appearance (McDougall et al. 1996; Southgate 1995). In their tertiary structure polysaccharides are cross-linked to each other with hydroxycinnamic acid ester dimers and structural proteins, which cause the development of

fibrous appearence (Iiyama et al. 1994; McDougall et al. 1996). These structures form the physical barrier enclosing a diverse pool of free and bound phenolic components.

When DF is degraded, a wide range of substances are released along the gastrointestinal tract, but a part still remains within the matrix. Thus intact cells and cell fragments can effectively act as transport vehicles for these substances (Ratcliffe et al. 1997). DF has many functions in the gastrointestinal tract (McDougall et al. 1996). However, in the present work DF is only considered as a carrier of phenolic compounds and as a co-substrate for the colon microbiota.

1.1.3 Phenolic compounds

In nature, phenolic compounds occur mostly as glycosides. Flavonoids are the most abundant polyphenols in our diets (Scalbert and Williamson 2000). Their flavonoid structure is based on the C6-C3-C6 skeleton. Flavonoids can be divided into several classes according to the degree of oxidation in the oxygen heterocycle and according to the degree of saturation in the C-ring (Clifford 2000a; Tomás-Barberán and Clifford 2000a; Hollman and Arts 2000).

The structures of phenolic acids are also diverse. Hydroxybenzoic acids are based on a C6-C1-skeleton (Tomás-Barberán and Clifford 2000b). Cinnamic acids are a series of trans-phenyl-3-propenoic acids with C6–C3 structures differing in their ring substitution. They are commonly found as conjugates. Caffeic acid and its esters and ferulic acid are the most frequently encountered phenolic acids in plant foods. For example chlorogenic acid is an ester of caffeoyl and quinic acids, whereas ferulic acid is esterified with hemicelluloses (Clifford 2000b; Andreasen et al. 2000).

Tannins have been defined as water-soluble, complex flavonoid polymers having a molecular weight between 500 and 3000 D (Chung et al. 1998). Condensed tannins can be formed for example from flavanols (proanthocyanidins) or phenolic acids esterified with a sugar (ellagitannins, gallotannins), possessing 12–16 phenolic groups and 5–7 aromatic rings

per 1000 units of relative molecular weight (Santos-Buelga and Scalbert 2000; Scalbert and Williamson 2000). Structures for different flavonoids, phenolic acids and condensed tannins are presented in Figure 1 and their typical sources are summarized in Table 1.

Table 1. Phenolic compounds and examples of their typical sources.

Phenolic compounds	**Typical sources**
Flavones Apigenin, luteolin	Red pepper, cellery
Flavonols Quercetin, myricetin	Onion, kale, broccoli, beans, apple and tea
Isoflavones Genistein, daidzein	Soy
Anthocyanidins Pelargonidin Cyanidin Delphinidin Peonidin Malvidin	Red fruits (plum, grape) and berries (strawberry, raspberry, bilberry, blackberry, red and black currant)
Flavanols Catechin Epicatechin Galloylated derivatives of above	Tea, red wine, chocolate
Flavanones Naringin, hesperetin	Citrus fruits
Phenolic acids Chlorogenic acid Ferulic acid	Fruits, vegetables, coffee Cereals
Tannins Ellagitannins Proanthocyanidins	Cloudberry, raspberry, strawberry, nuts, pomegranate, plum, apricot, peach, grape Apple, pear, grape, tea, red wine, chocolate
Stilbenes Resveratrols, viniferins	Grapes and peanuts
Plant lignans Syringaresinol, pinoresinol, isolariciresinol, lariciresinol, secoisolariciresinol, matairesinol	Flaxseed, rye, berries, fruits

Chlorogenic acid is present in most fruits, vegetables and coffee and ferulic acid is abundant in cereals (Clifford 2000b; Andreasen et al. 2000). Ellagitannins are present in some nuts, pomegranate, plum, apricot, peach and grape and are abundant in cloudberry, raspberry and strawberry (Clifford and Scalbert 2000; Kähkönen et al. 2001).

Stilbenes are 1,2-diarylethens substituted with several hydroxy groups and sometimes a methoxy group. They are abundant in grapes, wine, peanuts and related products (Cassidy et al. 2000). Plant lignans are diphenolic compounds with a 1,4-diarylbutane structure and are found in cereals, berries and fruits (Cassidy et al. 2000; Mazur and Adlercreutz 1998; Mazur et al. 2000). The structures of lignans are shown in Figure 2. The lignan content is highest in the outer layers of the kernel, where the DF content is also highest (Nilsson et al. 1997). Secoisolariciresinol and matairesinol were long considered to be the most important plant lignans in rye, but recently new lignans have been found. Thus, syringaresinol, pinoresinol, lariciresinol and isolariciresinol have also been quantified in rye. In rye bran the syringaresinol content is almost ten-fold the secoisolariciresinol and matairesinol contents (Heinonen et al. 2001). The quantified plant lignans of flaxseed include only secosisolariciresinol and matairesinol (Thompson et al. 1991), but pinoresinol, isolariciresinol and demethoxy-secoisolariciresinol have also been identified in flaxseed (Meagher et al. 1999; Sicilia et al. 2003). Flaxseed is still the richest source of plant lignans due to its high content of secoisolariciresinoldiglucoside.

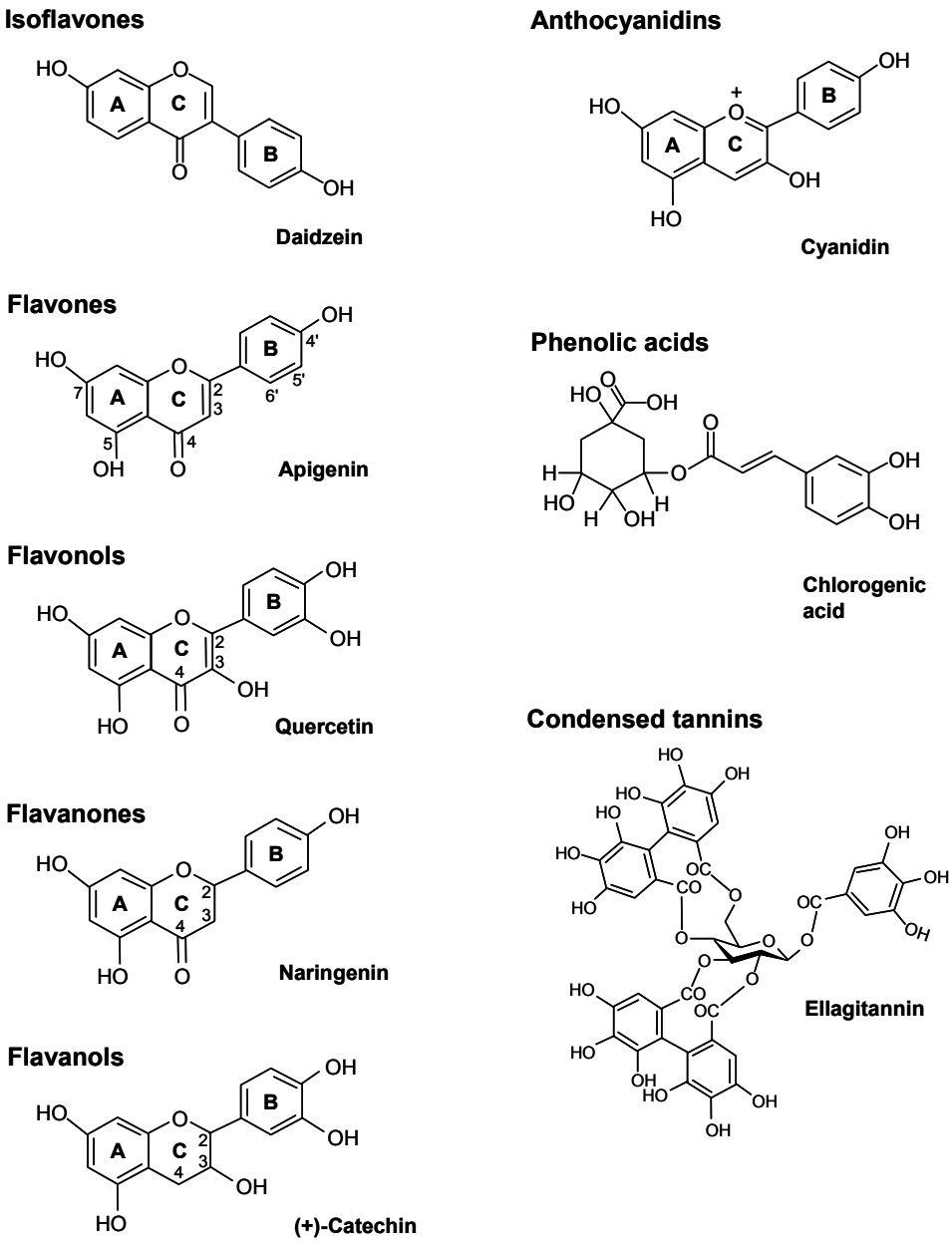
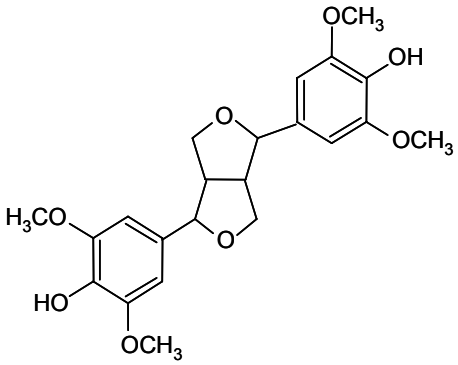
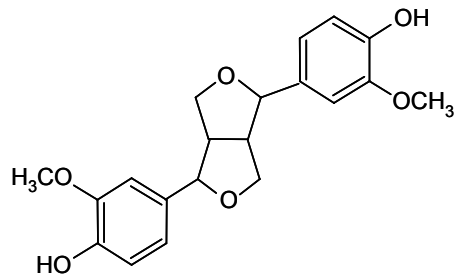


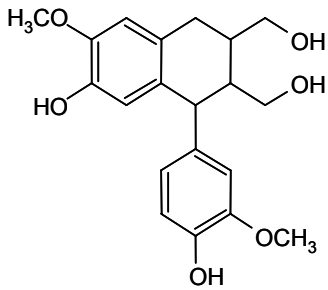
Figure 1. Structures of some flavonoids, phenolic acids and condensed tannins.



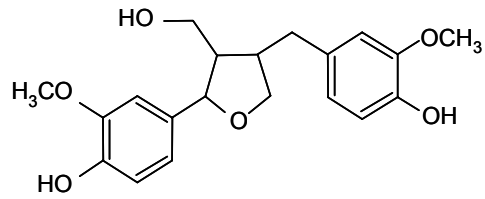
Syringaresinol



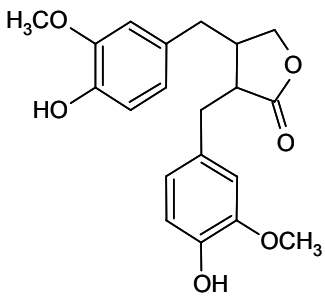
Pinoresinol



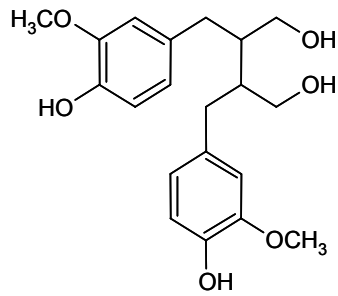
Isolariciresinol



Lariciresinol



Matairesinol



Secoisolariciresinol

Figure 2. Structures of plant lignans in rye.

1.1.4 Significance of plant foods, DF and phenolic compounds to human health

According to a recent report published by The World Health Organization (WHO) there is varying strength of evidence for a correlation between consumption of DF, fruit, vegetables, whole grains and berries and reduced risk of various chronic diseases (WHO 2003). According to the report, reduced risk of cardiovascular diseases is associated with high consumption of fruit, vegetables and berries. The evidence for this correlation was considered as convincing, whereas the evidence for a correlation with DF consumption was merely rated as probable (WHO 2003). Several epidemiological studies support the association of DF, vegetable and fruit intakes with lowered risk of coronary heart disease (Pereira et al. 2004; Mozaffarian et al. 2003; Liu et al. 2002a; Kris-Etherton et al. 2002; Hu 2003; Rimm et al. 1996; Steinmetz and Potter 1996; Pietinen et al. 1996).

The association of DF intake and colon cancer has been under investigation in several epidemiological studies. The results of these studies have been contradictory, because some have failed to show an association of DF intake with reduced risk of colon cancer (Fuchs et al. 1999; Pietinen et al. 1999; Terry et al. 2001), whereas others have provided supportive evidence (Bingham et al. 2003; Peters et al 2003; Slattery et al. 2004). In one study DF intake was inversely related to large bowel cancer incidence. Individuals with the lowest incidence of cancer consumed 35 g of total fibre per day and 12 g cereal fibre (Bingham et al. 2003). A high intake of DF was also associated with a reduced risk of prevalent adenomas, particularly aggressive ones and those occurring in the colon (Peters et al. 2003). An inverse association of rectal cancer with an intake of plant food and fibre was seen at a threshold of approximately 5 servings per day (Slattery et al. 2004). Bound phytochemicals, for example antioxidants in grains, can survive the stomach and the intestinal digestion and reach the colon. This may partly explain the correlation between grain consumption and the prevention of colon cancer as supported by epidemiological studies (Johnson 2002; Adom and Liu 2002).

The term phytoestrogen has been used for lignans and isoflavones due to their structural similarity to estradiol-17β (Cassidy et al. 2000). Some phytoestrogens have particular affinity to the β-estrogen receptor (ERβ), causing an estrogenic or antiestrogenic effect and their intake has been associated with a decreased risk of hormone-related cancers (Tham et al. 1998; Adlercreutz 2002). In the highest tertile of total fibre intake breast cancer risk was decreased by 29 % among premenopausal women. The effect was probably mediated by phytoestrogens (Bonilla-Fernandéz et al. 2003). Plant lignans are converted to mammalian lignans, enterodiol and enterolactone, in the gastrointestinal (GI) tract (Setchell et al. 1981; Borriello et al. 1985; Thompson et al. 1991; Heinonen et al. 2001). One recent study has shown that intake of whole grains and vegetables determines the plasma enterolactone concentration (Johnsen et al. 2004). An association between high enterolactone concentration in urine and serum and reduced risk of breast cancer has been proposed (Ingram et al. 1997; Pietinen et al. 2001; Hultén et al. 2002).

A high intake of fruit and vegetables was associated with reduced risk of developing cancers. The evidence for this correlation was rated as probable in the report by the WHO (WHO 2003). It has been shown that the antioxidative properties of flavonoids and phenolic compounds are related to their structure (Rice-Evans et al. 1996). Antioxidants scavenge reactive oxygen species, which mediate signals in pathways initiating programmed cell death, apoptosis (Gee and Johnson 2001; Lopaczynski and Zeisel 2001). It has been shown that antioxidation did not correlate with *in vitro* antiproliferation activity (Meyers et al. 2003). At high doses flavonoids may act as mutagens, pro-oxidants that generate free radicals, and they can act as inhibitors of key enzymes involved in hormone metabolism (Skibola and Smith 2000). When the biological activities of phenolic compounds are studied *in vitro*, it is important to take into account the fact that the dietary phenols are present in the human body as conjugates, and at concentrations of 0.1–10 μmol/l (Kroon et al. 2004). Despite the epidemiological evidence that various phenolic compounds lower the risk of some chronic diseases, the mechanism of their action is still under debate.

1.1.5 Biological activities of colonic metabolites

The importance of DF intake is associated with the rate and extent of fermentation. If the extent of fermentation is low, DF increases faecal bulk, reduces the transit time and binds and removes harmful components (mutagens, steroids, bile acids and xenobiotics) (McDougall et al. 1996). If the fermentation rate and extent are high, the health effects are mediated by the SCFA formation, for example by that of butyrate. Butyrate production *in vivo* was enhanced by the consumption of rye (Gråsten et al. 2000 and 2002; McIntosh et al. 2003), of resistant starch (Martin et al. 1998; Brouns et al. 2002) and of wheat pentosans (Gråsten et al. 2003). The butyrate concentration in the gut contents of a healthy individual can reach 12–20 mM (Smith and German 1995). It has been shown that the increased production of butyrate in the colon further increases the circulating level of butyrate in the portal vein (Bach Knudsen et al. 2003b).

As the colonic metabolites pass through the epithelium during absorption, local effects in the colon can be expected. The effect of butyrate may depend on the state of the colon epithelium. Butyrate has been shown to induce cell proliferation of the crypt base, and to induce apoptosis, programmed cell death, enhancing the healthy tissue turnover in normal colonocytes. In neoplastic cells butyrate inhibits proliferation at the crypt surface, the site of potential tumour development (Brouns et al. 2002; Johnson 2002). Furthermore, butyrate induces apoptosis in human colon cancer cell lines (Avivi-Green et al. 2002). Readily fermentable, soluble DF promotes the growth of adenomas in APC ^{Min} mice (Oikarinen et al. 2003; Mutanen et al. 2000), which might suggest that excessive production of SCFA may have adverse effects in the animal models, in which the apoptosis is already disturbed.

A high enterolactone concentration has been associated with a high intake of vegetables and cereals with antioxidant activity and is considered to be a biomarker of their intake (Kilkkinen et al. 2003; Johnsen et al. 2004). A higher concentration of plasma enterolactone has been associated with a lower risk of acute coronary events (Vanharanta et al. 1999) or with a lower risk of breast cancer (Ingram et al. 1997; Pietinen et al. 2001;

Adlercreutz 2002). However, enterodiol and enterolactone have less *in vitro* antioxidant activity than their precursor, secoisolariciresinol diglucoside (Kitts et al. 1999). When the microbial metabolites of rutin and quercetin (3,4-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid) were compared with their precursors, the microbial metabolites had more anti-platelet aggregation activity and less mutagenicity than their parent compounds (Kim et al. 1998; Kim et al. 1996).

A considerable amount of information is available on the putative biological effects of the dietary phenolic compounds present in food. This interest in the food components has been promoted by the *in vitro* antioxidative acitivity of the dietary phenolic compounds. However, the biological activities are structure-related and when the structure of the molecule changes, it is likely that these properties also change.

1.2 Development of *in vitro* digestion models

The *in vitro* digestion models mimic conditions and luminal reactions which occur in the mouth, stomach, duodenum, ileum and colon. The *in vitro* upper intestinal models are used for removal of digestible components and for detection of changes in the non-digestible ones. The colon model elucidates the role of microbiota in the metabolism of all non-digestible parts of the diet, DF and phenolic compounds. Membrane-bound activities are not taken into consideration in these models. The aim of the *in vitro* digestion models is to characterize and clarify the structural changes of food components under physiological conditions, caused by alimentary enzymes and by the colonic microbiota, and to obtain information going beyond that gained by the chemical analysis of food.

1.2.1 *In vitro* enzymatic digestion of starch

Starch is classified into several classes according to the rate of hydrolysis. Resistant starch (RS) is defined as the fraction of starch which is fermented in the colon (Englyst et al. 1992). Several methods have been adopted for the measurement of RS (Englyst et al. 1992; Berry 1986;

Muir and O´Dea 1992; Champ 1992). Enzymatic starch removal is performed in DF analyses, in which starch can be removed under non-physiological conditions for example using Termamyl (a heat-resistant α-amylase) in a boiling waterbath (Prosky et al. 1992). The sample can be gelatinized by boiling prior to the enzymatic digestion at 60°C (Asp et al. 1983). With each of these methods, gelatinized and solubilized samples show a high degree of starch hydrolysis. Another method of DF analysis was developed by Englyst and Cummings (1988) using α-amylase digestion at 42°C.

In addition, starch has been removed using several enzymatic *in vitro* batch methods in order to obtain a DF residue for *in vitro* fermentation experiments (Hoebler et al. 1991; Muir and O´Dea 1993; Lebet et al. 1998). Hoebler and co-workers used Termamyl and a boiling waterbath and the starch removal was complete, but during the incubation cell-wall polysaccharides were also solubilised possibly due to the high temperature used in the assay (Hoebler et al. 1991). Later, a dialysis step was adopted to remove the digested components and to retain the soluble fibres in the retentate, which was subsequently freeze-dried prior to the *in vitro* fermentation (Lebet et al. 1998).

Ileal effluents obtained from ileostomy patients have also been used as substrates in the *in vitro* colon models. The ileal effluents and dietary fibre isolates showed a similar extent of total SCFA production in 24 h, although the extents differred from those of the whole foods (McBurney et al. 1988). The metabolizable energy after fermentation of the ileal effluents correlated with the corresponding calculated energy values in the diets. These studies supported the combined use of the ileal *in vivo* model and the *in vitro* fermentation model in studies of the energy balance of DF (McBurney and Thompson 1989a). However, when the DF contents of the samples from ileally cannulated pigs were compared with those from *in vitro* enzymatic digestion, degradation of soluble DF components was observed in the *in vivo* samples but not in those from the *in vitro* treatment (Hoebler et al. 1998).

A multi-compartmental, continuous, computer-controlled *in vitro* digestion model (TIM-1, TNO gastro-Intestinal Model) simulates the

secretion, motility and absorption of the upper intestine (Minekus et al. 1995). The model includes compartments mimicking the stomach, the duodenum and the ileum, and reproduces accurately the pre-set data on the transit time, the pH and the bile concentrations, which have been obtained from *in vivo* studies with healthy human volunteers. The model has been used for studying the digestion and the absorption of glucose, for estimation of the bioavailability of iron and phosphorus, for studies of the coagulation and digestion of proteins, and for studies of the survival of lactic acid bacteria (Minekus et al. 1995).

Enteromix® research tools (Danisco) include clinical assays, simulations and screening assays. Physiological, microbiological and immunological responses to food and feed ingredients can be simulated. These simulation models are used for the evaluation of efficient doses of active ingredients in the food and feed and for studies of the effect of the food matrix on the tested response (www.enteromix.com).

1.2.2 *In vitro* colon models

The *in vitro* colon models include continuous, semi-continuous and batch culture systems, which maintain colonic microbiota, usually obtained from human faeces, under strictly anaerobic conditions. The methods differ from each other in the structure of the fermentor, substrate-to-inoculum ratios, media, operating conditions and sampling (Rumney and Rowland 1992). Different fermentation methods have been compared in different laboratories (Edwards et al. 1996). Media have varied from a simple mineral salt solution to a complex medium containing vitamins, hemin, SCFA, yeast extract and trypticase, a reducing agent and different buffers. The cultures have been static or several mixing methods have been used: regular swirling, a shaking water bath or periodic mixing. All these variables affect the fermentation of starch and the comparision of the results is very difficult (Edwards et al. 1996).

Different types of colon models can be used for specific purposes. Batch cultures are suitable for the studies of microbial metabolism of DF, RS and isolated phenolic compounds (Barry et al. 1995; Edwards et al. 1996;

Déprez et al. 2000; Heinonen et al. 2001). However, during the incubation nutrients are consumed and products are accumulated, causing altered culture conditions which may result in a distorted interpretation of microbial metabolism in batch cultures at later time points. This can be avoided in semi-continuous systems (Campbell et al. 1992). Continuous and semi-contiuous systems are also suitable for study of the colonic ecosystem and bacteriology (Miller and Wolin 1981; Allison et al. 1989). A single-stage continuous system cannot reproduce the heterogeneity of physiological conditions and nutrient availabilities that occur at different parts of the colon *in vivo*. However, this reproduction of conditions can be achieved in a multi-stage chemostat (Allison et al. 1989).

Molly and co-workers presented a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. This so-called SHIME model consists of two vessels simulating the duodenum/jejunum, and ileum, respectively, and the 3rd, 4th and 5th vessels corresponding to the caecum and ascending colon, transverse colon and descending colon, respectively (Molly et al. 1993). A three-stage compound continuous culture system was investigated in terms of carbohydrate metabolism and amino acid metabolism at the University of Reading. Carbohydrate breakdown and SCFA production occurred mainly in the first reactor, whereas amino acid fermentation producing branch-chain fatty acids occurred mainly in the reactors 2 and 3. Thus, the reactions in the three reactors corresponded to reactions occurring in the ascending, transverse and descending colon *in vivo*, respectively (Macfarlane et al. 1989; Macfarlane et al. 1992; Macfarlane et al. 1998; Probert et al. 2004).

The latest development of a continuous, anaerobic colon model is a computer-controlled *in vitro* system (TIM-2) with peristaltic mixing, water absorption and absorption of fermentation products (Minekus et al. 1999). When the substrate is passed from one compartment to another through the model, the temperature, pH, peristaltic movements, absorption of water and the fermentation products are continuously monitored and controlled. The model used a high-density complex microbiota and it has been used for the fermentation of DF and phenolic compounds, for studies of the formation of ammonia, for the investigation of probiotic bacteria and for studies of the bioconversion of glucosinolates

(Venema et al. 2004). The continuous flow models require high technical and scientific expertise and they have high costs in terms of both hardware and running expenses.

The Enteromix® tools also include a small-scale, semi-continuous colon simulator. This model consists of four sequentially attached anaerobic vessels representing the ascending, transverse, descending and distal colon, respectively. All fluid transitions and pH adjustments are computer-controlled. Because of the small working volumes of the vessels, only a small amount of substrate is needed for a 48-hour simulation (Mäkivuokko et al. 2005). When valuable isolated compounds are applied to the colon model *in vitro*, it is also relevant to use small batch cultures with adequate stirring. Furthermore, this is a simple way of studying accurately the time course of formation of the microbial metabolites.

A European interlaboratory study was performed for testing a simple, reproducible batch system for DF fermentation (Barry et al. 1995). The inoculum was composed of fresh human faeces mixed with a carbonate-phosphate buffer supplemented with trace elements and urea. Five different carbohydrate sources were compared in five laboratories on three occasions in order to determine the pH, residual non-starch polysaccharide and SCFA production during the *in vitro* fermentation. The observed interlaboratory differences could be reduced either by reducing the amount of substrate or by using a dense inoculum. The optimal amount of carbohydrates was 10 g/l in a faecal suspension of 167 g/l. There was also a close correspondence between the *in vitro* data and an *in vivo* rat experiment using diets supplemented with the same sources of DF (Barry et al. 1995).

Another interlaboratory study was conducted for quantification of RS. A similar amount of fresh human faeces (160 g/l) was suspended to a buffer in a batch *in vitro* fermentation model in order to compare substrates (100 mg) containing different amounts of total and resistant starch (Edwards et al. 1996). The buffer delayed the decrease of pH caused by SCFA production. The maximum incubation time was 24 h, which was considered to correspond to the transit time through the proximal colon (Edwards et al. 1996).

1.2.3 Faeces as an inoculum

The number and the composition of the microbiota vary considerably along the human gastrointestinal tract. Due to the low luminal pH the total bacterial count in gastric contents is usually below 10^3 cells/g. In the small intestine, numbers of bacteria range from 10^4 cells/ml in the proximal ileum to 10^6–10^7 cells/ml in the ileocaecal region. The human colon typically contains 10^{11}–10^{12} cells/ g contents, the majority of which are strict anaerobes (Salminen et al. 1998; Kleessen et al. 2000). The colon resembles a continuous, anaerobic fermentor with a volume of 500 ml and with a content of 220 g of digesta. The microbiota are diverse, consisting of 400–500 different bacterial species, as well as yeast, fungi and protozoa, which are poorly quantitated (Conway 1995). In order to become established in the intestine, the microbiota must be able to multiply at a faster rate than their elimination rate. An acidic environment and excessive peristalsis disturb colonisation by the microbiota (Kleessen et al. 2000). Colonic microbiota is closely associated with the colonic epithelium, preventing the invasion of pathogenic bacteria and interacting with the immune system via lymphocytes in the epithelia and mucosa (Salminen et al. 1998).

The most common way of obtaining colonic microbiota is by collecting faecal samples, because the use of faecal donors is non-invasive and there are no ethical objections (Conway 1995). Obligately anaerobic bacteria, *Bacteroides* spp., *Eubacterium* spp., *Bifidobacterium* spp., clostridia, lactobacilli, anaerobic cocci and *Fusobacterium* spp., are detected in high numbers in faeces by bacterial culture. In addition, facultative anaerobic organisms such as *Escherichia coli*, enterococci and streptococci are commonly encountered. The investigation of colonic microbiota by culture-based methods has several limitations. The lack of culture media supporting the growth of some components of the dominant microbiota, difficulties in the identification by phenotypic methods and continuous changes in the taxonomy complicate the studies (Kleessen et al. 2000). However, the systematic determination of gut microbiota is now possible by high-resolution molecular genetic techniques such as ribosomal RNA sequencing (Salminen et al. 1998; Tannock 2001).

The enumeration of different microbial species resident in the colon is difficult. Selective plating underestimates the abundance of many species and new tools have been developed for characterization of the microbiota. The isolation of the genome of faecal microbiota and guanin-cytocin fractionation (%G-C profiling) together with denaturing gradient gel electrophopresis enhance the diversity assessment and detection of minority populations of bacteria (Apajalahti et al. 2003; Apajalahti et al. 2004; Holben et al. 2004). Furthermore, the importance of analysing the biological activity of the faecal microbiota in terms of microbial metabolites has been emphasized (Conway 1995; Salminen et al. 1998). It has been postulated that the microbiota in the feaces has a higher similarity to the luminal than to the mucosal microbiota in the colon, due to the higher proportion of facultative anaerobes near the mucosa. However, since dietary constituents are first encountered by the luminal microbiota *in vivo*, it is relevant and convenient to use the faecal microbiota in the *in vitro* colon model (Rumney and Rowland 1992). Although the composition of the bacterial community is an important issue, the aims of the present thesis did not include the identification and characterization of the faecal microbiota. The target of the present thesis was focused on the metabolic activity of the faecal microbiota.

Individual variation of faecal samples is caused by the age or the diet of the donor, by the time of collection of the faeces, by intestinal diseases and by medication, especially antibiotics (Conway 1995; Salminen et al. 1998; Rowland et al. 1999; Kilkkinen et al. 2002). Both intra-individual and inter-individual variations of faecal microbiota occur and the variations are of similar magnitude independently of their origin (Barry et al. 1995). Most bacterial species are capable of several metabolic activities. In order to improve the reproducibility of metabolic studies using faeces, a pooled faecal sample is required from at least three different donors. Thus the metabolic activity can be stabilised by diversifying the microbial composition (Weaver et al. 1989; Mortensen et al. 1991; McBurney and Thompson, 1989b). In the colon 1500 ml of the chyme entering the caecum is reduced to 80–200 ml, which is discarded as faeces (Guyton and Hall 1996a). From this data the “physiological” concentration of the faecal suspension could be calculated as 5–13 % (v/v) in the caecum.

The microbes were not isolated from faecal matter, because a selection of the microbiota would occur. Particularly, cultivation increases the relative abundance of the facultative anaerobes (Bearne et al. 1990). Furthermore, different carbohydrate sources in the *in vitro* fermentation cause changes in the microbiota (Slade et al. 1987). A long incubation time decreases the viability of the microbiota due to the severely nutrient-limited conditions (Allison et al. 1989).

Faecal dry matter contains 30 % bacteria, 10–20 % fat, 10–20 % inorganic matter, 2–3 % protein, 30 % undigested roughage from food and dried consituents of digestion fluids and epithelial cells (Guyton and Hall 1996a). Faecal matter provides nutrients for the microbial population obtained from the donors. The chemical and physical nature of the dense inoculum is also closer to that present in the caecum and reduces the need for adding components to the medium, such as bile acids and micronutrients, which are naturally present in faeces. Therefore, only a simple buffer is sufficient as a medium.

A high inoculum concentration allows the best survival of bacterial species, and also produces a lower redox potential and a higher final osmolality. However, each inoculum is different and only relative comparisons can be made between series (Edwards et al. 1996; Barry et al. 1995). Nevertheless, a simple rapid procedure in the preparation of the pooled, dense inoculum from several donors can overcome the problems of individual variation and the selection of microbiota during the incubation in the *in vitro* colon model. An advantage of a batch model is that the same faecal inoculum devoid of substrates can be incubated simultaneously and its metabolite profile can be presented as a reference to those of the substrates.

1.3 *In vitro* digestion models in relation to gastrointestinal physiology, digestion and metabolism of phenolic compounds

The human gastrointestinal physiology, the digestion of carbohydrates and the metabolism of phenolic compounds are described in the following sections in order to elucidate the choices regarding the reagents, enzymes, operating pH values and incubation times of each stage of the *in vitro* digestion models. The sites of the gastrointestinal tract which are simulated by the *in vitro* enzymatic model and the colon model are illustrated in Figure 3.

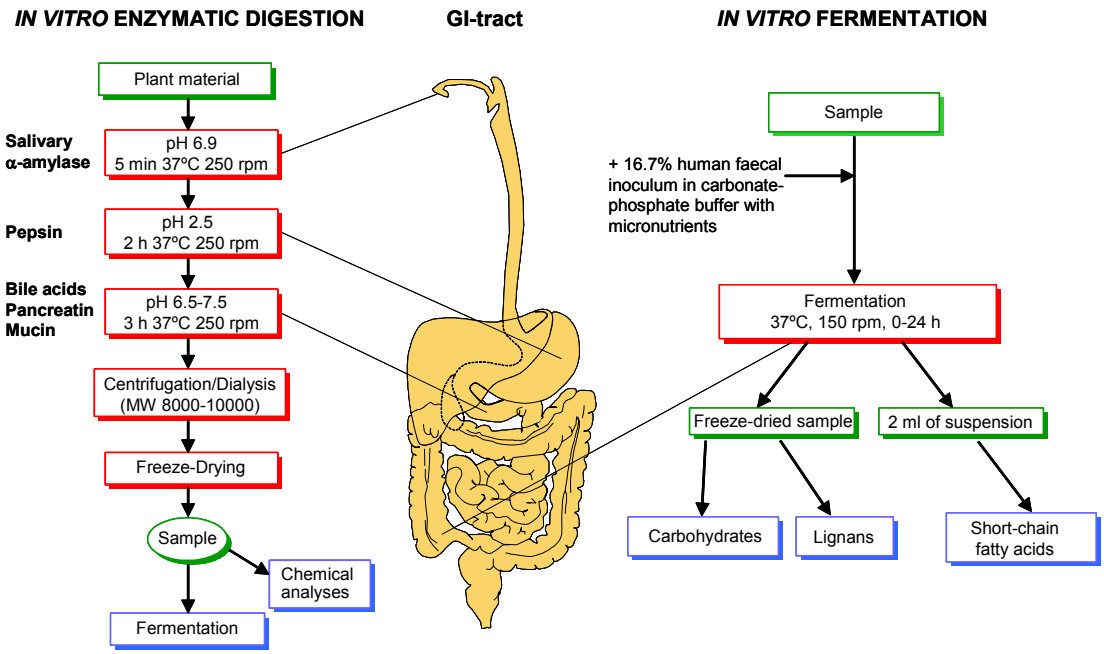


Figure 3. The sites of the gastrointestinal tract simulated by the developed in vitro *enzymatic digestion and colon models.*

1.3.1 Mouth

The digestion of food starts in the mouth, where teeth grind the plant food with a force of 300–1000 N (Guyton and Hall 1996a). The disintegrated plant matrix contains cell-wall tissue pieces with a large proportion of intact cells (McDougall et al. 1996). The hydrolysis of starch starts in the mouth, where maltose, maltotriose and α-limit dextrins are formed. However, only 5 % of all the starch eaten is hydrolysed by the time the food is swallowed (Guyton and Hall 1996c). During mastication the principal salivary glands start their secretion of saliva, which ranges daily between 800 and 1500 ml. Saliva contains two major types of proteins: ptyalin, an α-amylase for starch digestion, and mucin for lubrication and surface protection purposes. Saliva also contains a high concentration of potassium and bicarbonate and some sodium and chloride (Guyton and Hall 1996b).

The disintegration and starch hydrolysis in the mouth have been simulated in digestion models in several ways. In one *in vitro* digestion procedure commercial salivary α-amylase was applied in 20 mM phosphate buffer at pH 6.9 in the presence of sodium chloride (Lebet et al. 1998). When the availability of starch was measured, authentic saliva was diluted and used similarly in the presence of sodium chloride in potassium-sodium-phosphate buffer (50 mM, pH 6.9) (Björck et al. 1984). Two methods applied the mouth stage *in vivo* prior to *in vitro* digestion by pepsin and pancreatin. Samples were chewed for 15 seconds by human volunteers in order to measure the hydrolysis rate of starch and the extent of starch hydrolysis for estimation of RS. Several subjects were required in order to take into account inter-individual variation of the resulting particle size from different subjects (Granfeldt et al. 1992; Muir and O´Dea 1992).

1.3.2 Stomach

In the stomach, gastric glands secrete pepsin as pepsinogen, gastrin hormone and hydrochloric acid for protein digestion. Hydrochloric acid is secreted as a 160 mM solution at pH 0.8. Total daily gastric secretion is about 1500 ml (Guyton and Hall 1996b). The *in vivo* profile measured

radiotelemetrically showed the gastric pH to be 1.6 (Gee et al. 1999). Pepsinogen is not active until it is in contact with hydrochloric acid and is transformed to pepsin, the active protease. The optimum pH for pepsin is between 1.8 and 3.5, and there is only little proteolytic activity at pH 5. Gastrin stimulates the secretion of enzymes, and active pepsin further enhances the activation of pepsinogen. When the acidity of gastric juice decreases below pH value 3.0, the stimulation of secretion becomes blocked and the stomach is protected from excessive acidity (Guyton and Hall 1996b).

The hydrolysis of starch continues in the stomach for 1 hour and 30–40 % of starch is hydrolysed to maltose. Salivary amylase activity is inhibited by the acid when the pH of the chyme falls below 4.0 (Guyton and Hall 1996c). Many plant cells burst in the acidic conditions of the stomach due to breakage of acid-labile bonds, and polysaccharide residues may be released. The solubility of carbohydrates is enhanced and the viscosity of the stomach contents increases causing the feeling of satiety (McDougall et al. 1996). The movements of the gastric muscles mix and disintegrate food further to produce a semifluid chyme with gastric secretions. The stomach stores food and regulates the chyme at a suitable rate for digestion and absorption (Guyton and Hall 1996a).

To simulate the conditions in the stomach, the pH was adjusted in both the batch and continuous models by addition of hydrochloric acid until the pH decreased to 1.5–2.0. A commercial pepsin preparation was used for the hydrolysis of proteins in carbohydrate foods (Muir and O´Dea 1992; Minekus et al. 1995; Goni et al. 1997; Lebet et al. 1998). The effects of the low pH and the presence of enzyme proteins on the detection of phenolic compounds were studied by adding hydrochloric acid and pepsin (pH 2.0) or a simulated gastric juice containing sodium chloride, pepsin and hydrochloric acid (pH 1.2) to the samples (Martinez-Ortega et al. 2001; Spencer et al. 2000).

Methods for simulation of the upper intestine have also been adopted for the study of phenolic compounds. Malvidin-3-O-glucoside was incubated in the presence of procyanidin dimer in darkness under anaerobic conditions and an anthocyanin-flavanol adduct was formed at pH 2.0 and

3.8 (Salas et al. 2003). Furthermore, proanthocyanidin oligomers were hydrolyzed to mixtures of epicatechin monomer and dimer in the gastric milieu (pH 2.0) in the absence of enzymes (Spencer et al. 2000).

Residence time in the human stomach *in vivo* varies according to the particle size (Guyton and Hall 1996a), but incubation times used in the different *in vitro* batch models have been fixed, ranging from 30 min to 2 hours depending on the method (Muir and O´Dea 1992; Lebet et al. 1998; Gil-Izquierdo et al. 2002). In one multi-compartmental dynamic continuous model either slow or fast transit time was simulated. The half-time of the delivery was 35 min and 70 min for the fast and the slow transit time, respectively. The pH of the stomach varied from 4.8 to 1.7 in 120 min. The simulation of both the half-time and the pH were based on the *in vivo* data (Minekus et al. 1995).

1.3.3 Duodenum and ileum

In the human body the amount and the characteristics of pancreatic juice are determined by the presence of chyme and its food components in the upper portions of the small intestine. Pancreatic juice contains proteolytic enzymes (trypsin, chymotrypsin, carboxypolypeptidase, elastase and nuclease), amylase for starch and glycogen hydrolysis, lipase for neutral fat digestion, phospholipases which split fatty acids from phospholipids, and cholesterol esterase (Guyton and Hall 1996b). The daily pancreatic secretion is approximately 1000 ml. Bicarbonate ions are also secreted in order to increase the duodenal pH. Proteolytic enzymes are in an inactive form and are activated enzymatically by enterokinase or by active trypsin. Pancreatic juice also contains a trypsin inhibitor.

Bile salts and sodium bicarbonate are secreted into the duodenum from the biliary gland of the liver and stored in the gall bladder, which has a volume of 30–60 ml. The bicarbonate solution increases the volume of the secretion and further increases the duodenal pH. The normal daily biliary secretion is between 600 and 1200 ml. Most of the bile salts (94 %) are recirculated by the enterohepatic circulation. Bile salts have an important role in emulsifying fat, in enhancing the digestion by lipase and in the transport of

the hydrolysed products through the intestinal mucosa (Guyton and Hall 1996b). A radiotelemetric measurement has shown that the pH between the duodenum and distal ileum is 6.2–7.9 (Gee et al. 1999).

Virtually all the available starch is digested within 15–30 seconds after chyme enters the duodenum and mixes with the pancreatic juice and bile (Guyton and Hall 1996c). The particle size of plant foods is reduced and the cell-wall polymers are solubilised concomitantly in the ileum (McDougall et al. 1996). The membrane-bound enzymes in the ileal epithelium, namely sucrase, maltase, isomaltase and lactase, hydrolyse the released maltose and small glucose polymers to monosaccharides, which are then actively transported through the brush-border membrane with a transport mechanism aided by sodium ions. Insulin accelerates the transport of glucose to the cells and permits rapid uptake, storage and use of glucose especially by muscles, adipose tissue and liver (Guyton and Hall 1996b, c, d). Peptidases split small peptides into amino acids and intestinal lipase degrades neutral fats into glycerol and fatty acids (Guyton and Hall 1996b). Soluble DF increases the viscosity of the digesta and ensures the steady and slow absorption of glucose (McDougall et al. 1996).

When the starch digestion in the duodenum was simulated in batches, the pH was adjusted by addition of sodium hydroxide to reach a pH between 5.0 and 6.9 depending on the enzymes used in the hydrolysis (Muir and O´Dea 1992; Granfeldt et al. 1992; Goni et al. 1997; Lebet et al. 1998). The variation of the pH was measured in the continuous model. In the duodenal compartment the pH was initially 8.0 and it was stabilized during the residence time to vary between 6.5 and 7.0 (Minekus et al. 1995). Starch was hydrolysed mainly by pancreatin only (Muir and O´Dea 1992; Granfeldt et al. 1992; Goni et al. 1997; Lebet et al. 1998), but some models also used amyloglucosidase (Muir and O´Dea 1992; Goni et al. 1997).

When phenolic compounds are consumed in the diet, they are released from the matrix after mastication, the released part is absorbed through the ileal epithelium and the rest is released, metabolised and absorbed in the colon (Figure 4). Absorption is affected by the structure of phenolic compounds (glycosylation, molecular weight and esterification) (Scalbert et al. 2002). Phenolic compounds can be transported through the

epithelium as glycosides by sugar transporters. In the epithelial cells, cytosolic β-glucosidase hydrolyzes these glycosides, and aglycones are formed after absorption (Figure 5). Aglycones can also be formed in the lumen by the action of membrane-bound lactase phlorizin hydrolase (LPH) and they are absorbed passively through the epithelium (Scalbert and Williamson 2000).

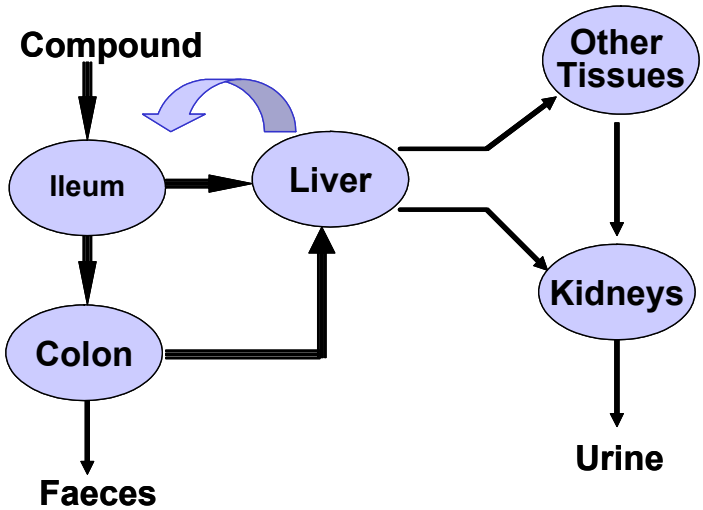


Figure 4. General metabolism of phenolic compounds, modified from Scalbert and Williamson (2000).

Once absorbed, aglycones undergo a conjugation in the ileal epithelium or in the liver. Hepatic metabolites (methylated, sulphated or glucuronidated conjugates) are returned to the luminal side via bile (enterohepatic circulation) (Figure 4) (Nemeth et al. 2003; Scalbert and Williamson 2000).

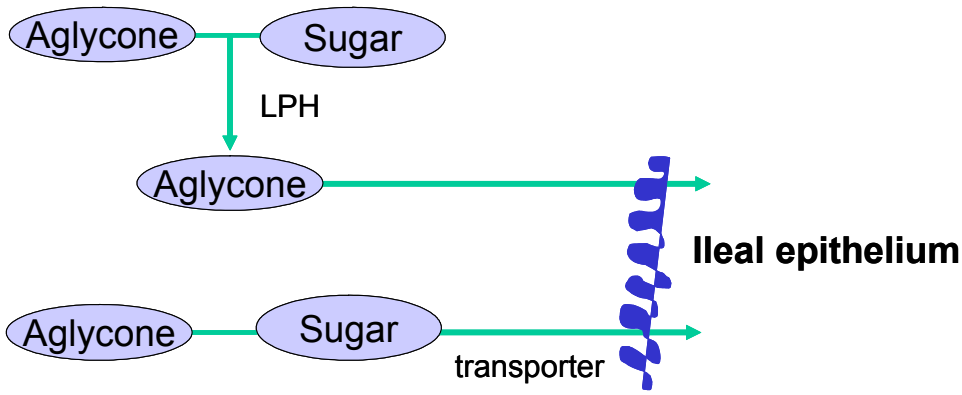


Figure 5. The deglycosylation of phenolic compounds, modified from Day et al. (2000).

The effects of pH and the alimentary enzymes were studied on the structure and the detection of phenolic compounds in various *in vitro* batch models. Phenolic compounds have been subjected to the dissolution test according to the United States Pharmacopeae using a sodium chloride solution with HCl and pepsin in the stomach stage, and sodium hydroxide with pancreatin in the duodenum stage (Martinez-Ortega et al. 2001; Goh and Barlow 2004). Martinez-Ortega and co-workers (2001) measured changes in phenolic compounds in wine in gastric and intestinal media using a paddle method for mixing. The digestion of wines increased the concentration of gallic acid, caffeic acid, quercetin and catechin, while the concentrations of resveratrol, caffeoyltartaric acid and ethyl gallate were stable in the gastric and intestinal media. Goh and Barlow (2004) studied flavonoid recovery and stability in the digestion of *Gingko biloba* leaves. The digestion increased the recovery of flavonols from the leaves. The content of (-)-epigallocatechin gallate was decreased in authentic intestinal juice as a consequence of an oxidative dimerization (Yoshino et al. 1999).

A sophisticated method for liquid samples containing phenolic compounds was developed by Gil-Izquierdo and co-workers (2002). The method consisted of an initial pepsin-HCl incubation for 2 h at 37°C. A dialysis tube containing water and sodium bicarbonate was added to the pepsin incubation, and pancreatin with bile salts was added after the pH reached a value of 5. After

2.5 h at 37°C, the dialysis tube was removed and the dialysate was analysed for phenolic compounds (Gil-Izquierdo et al. 2002). Sodium bicarbonite from the dialysis tube increased the pH and the phenolic compounds had diffused into the tube. Thus the absorption of phenolic compounds was also performed *in vitro*. The method was used in several studies regarding the availability, the structural transformations and the effects of industrial processing methods on phenolic compounds (Gil-Izquierdo et al. 2001, 2002, 2003; Pérez-Vicente et al. 2002; Vallejo et al. 2004).

The residence time of food in the duodenum and ileum is between 3 and 5 hours, during which it is digested and absorbed (Guyton and Hall 1996c). The simulated residence times in the duodenum stage of the batch digestion models varied from 1.5 hours to 2–3 hours (Lebet et al. 1998; Martinez-Ortega et al. 2001; Gil-Izquierdo et al. 2002). When transit through the ileum was also included in the model, the incubation time was prolonged to 6 hours (Muir and O´Dea 1992). In the continuous digestion model, the half-time of the ileal delivery was 85 and 160 min for the fast and the slow application, respectively (Minekus et al. 1995).

1.3.4 Colon

The colon is divided into the caecum, ascending colon, transverse colon, descending colon, sigmoid and rectum. Due to the active absorption only about 1500 ml of chyme is emptied from the ileum to the caecum daily (Guyton and Hall 1996a). The only secretory function in the colon is the mucus secretion with bicarbonate. The epithelial cells contain enzymes only in small amounts. Mucus holds the faecal matter together, protects the colonic wall from the microbial activity and bicarbonate provides a barrier against the acidic metabolites formed by the microbial action in the colon (Guyton and Hall 1996b). Radiotelemetric measurements have shown that the pH at the ileo-caecal valve is 7.9, but decreases rapidly to 5.4 in the transverse colon, indicating a rapid formation of acidic metabolites (Gee et al. 1999). Study of these conversion reactions is the main interest in the *in vitro* colon model and these aspects are discussed in detail in the following sections.

The colon also has the two following functions: absorption of water, electrolytes and SCFA, and the storage of faecal matter before defecation. Sodium absorption occurs by an active transport mechanism and it is responsible for the large electrical gradient across the mucosa (Guyton and Hall 1996a).

The saccharolytic microbiota have several hydrolysing enzyme activities. Xylanases, arabinanases β-glucanases, galactomannanases, polygalacturonases, mucinases and cellulases degrade polysaccharides to oligomers and monomers, which are further converted to short-chain fatty acids (SCFA) (Salyers and Leedle 1983; Hudson and Marsh 1995; Cummings 1995). Gastrointestinal mucus, mucin, is a protective gel-forming glycoprotein (Neutra and Forstner 1987), which is also fermented to SCFA by the colonic microbiota (Macfarlane et al. 1989).

During the fermentation of carbohydrates some 40 % of the energy escaping digestion can be transformed to an available form by SCFA production (Livesey 1995). According to Cummings (1995) SCFA are the major products of fermentation in the human colon, arising from dietary and endogenous carbohydrates and proteins which reach the large bowel. The predominant SCFA found in human colon are acetic acid, propionic acid and butyric acid. The minor fatty acid related anions of the human colon are formic acid, lactic acid, valeric acid, caproic acid, isobutyric and isovaleric acids. Fermentation of different polysaccharides results in typical relative proportions of acetate, propionate and butyrate (Cummings 1995). The measurements of microbial fermentation products from sudden death victims have shown that the products from carbohydrate fermentation (SCFA, lactate, ethanol) are formed in the caecum and in the ascending colon, and that the concentrations of metabolites from protein fermentation (ammonia, branched chain fatty acids and phenolic compounds) increase progressively from the right to the left colon (Macfarlane et al. 1992). Furthermore, gaseous products, hydrogen and carbon dioxide, are formed as primary gases, and methane or hydrogen disulphide as secondary gas products, the formation of which scavenges hydrogen. Only a certain proportion of human individuals (30-60 %) are methane producers (Levitt et al. 1995).

Those phenolic compounds which enter the colon are unabsorbed glycosides, such as quercetin-3-rhamnoglucoside, and conjugates after ileal and hepatic metabolism via enterohepatic circulation (Scalbert and Williamson 2000). Compounds can also be enclosed in the food matrix and thus absorption can be prevented (Nielsen et al. 2003). When compounds reach the caecum, they are subjected to the microbial metabolism. Many flavonoids undergo ring-fission, in which the B-ring is degraded and phenolic acids are formed (Figure 6) (Scalbert and Williamson 2000; Scalbert et al. 2002; Rechner et al. 2002; Manach et al. 2004). *Butyrivibrio* sp. C_3, *Clostridium orbiscindens* sp. nov. and *Eubacterium ramulus* were capable of the ring-fission of quercetin, whereas only *Enterococcus casseliflavus* was able to utilize glucose from quercetin-3-glucoside and it did not attack the C-ring (Krishnamurty et al. 1970; Winter et al. 1991; Schneider et al. 1999). Microbial metabolites are absorbed from the colon and are again subjected to the metabolism of the liver resulting in glucuronidated and sulphated conjugates (Axelson and Setchell 1981; Adlercreutz et al. 1995; Lampe 2003). Microbial metabolites also appear in plasma and urine (Sawai et al. 1987; Gross et al. 1996; Kilkkinen et al. 2003; Juntunen et al. 2000; Nesbitt et al. 1999). Microbial metabolites of several flavonoids have been identified either from *in vivo* animal trials or from clinical trials involving human subjects. Furthermore, phenolic compounds have been incubated with animal caecal or human faecal microbiota. This *in vivo* and *in vitro* evidence is collated in Appendix A.

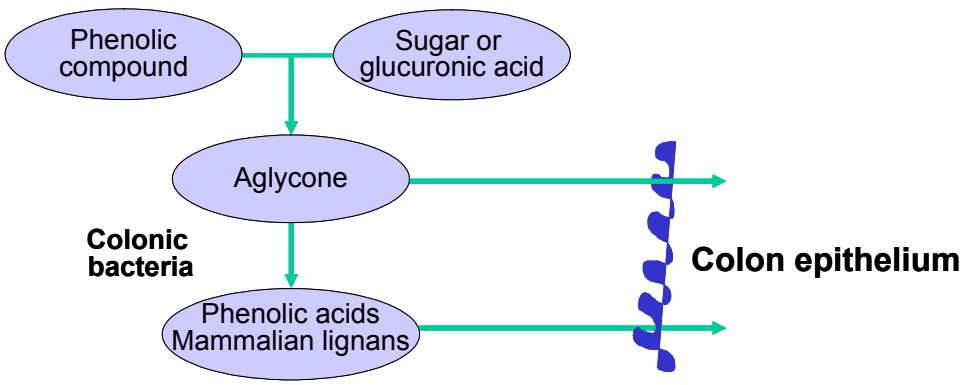


Figure 6. The microbial metabolism of phenolic compounds in the colon.

Another example of colonic metabolism of phenolic compounds is the bioconversion of plant lignans. After their release from the plant matrix, plant lignans undergo conversion to enterodiol and enterolactone (Setchell et al. 1981; Borriello et al. 1985; Thompson et al. 1991; Heinonen et al. 2001; Wang et al. 2000; Xie et al. 2003). The involvement of colonic microbiota in the conversion was confirmed by a study using germ-free and human microbiota-associated rats (Bowey et al. 2003). Secoisolariciresinol is converted to enterodiol and enterolactone via several intermediates. Pinoresinol and lariciresinol appeared to have a similar metabolism to that of secoisolariciresinol and matairesinol. However, syringaresinol and isolariciresinol were also suggested to have alternative routes of metabolism by human faecal microbiota *in vitro* (Heinonen et al. 2001). Furthermore, synthetic, labelled lignin has been shown to be converted to enterolactone in rats (Begum et al. 2004). Lignin is structurally related to lignans, but differs from them in its polymeric nature. Only two species have hitherto been identified to take part in the metabolism of secoisolariciresinol, *Peptostreptococcus* sp. SDG-1 and *Eubacterium* sp. SDG-2 (Wang et al. 2000). *Enterococcus faecalis* PDG-1 was responsible for biotransformation of pinoresinol diglucoside to (+)-lariciresinol (Xie et al. 2003). Thus enterolactone production is a complex phenomenon including several putative precursors and intermediary metabolites.

The amount of substrate is a crucial factor in fermentation studies. There is a requirement to maintain the microbiota unchanged in the *in vitro* fermentation. This can be achieved by adjusting the amount of carbohydrates to an optimal level and by using a dense faecal inoculum, which is less susceptible to changes in the course of fermentation. When pure phenolic compounds are introduced to the *in vitro* fermentation model, the molecular weight and the solubility of the substrate compound vary according to their degree of conjugation. The more sugar moieties are attached, the more soluble are the compounds. Therefore, it is important to measure the amount of the substrate on a molar rather than on a mass basis.

The substrate-to-inoculum ratio describes the concentration of the substrate in relation to the faecal suspension concentration. Substrate-to-inoculum ratios in some earlier investigations have been shown to vary

from 1.4 to 590 μmol/g fresh faeces (Table 2). The highest ratio was used for the identification of minor metabolites and the medium was saturated with one phenolic compound (Justesen and Arrigoni 2001). A lower ratio than that above was used for the identification of metabolites from catechin derivatives by a nuclear magnetic resonance technique (Meselhy et al. 1997). Conversion efficiencies cannot be studied when the faecal suspension is saturated with the compound under investigation. When ^{14}C-labelled proanthocyanidins (PA) were used in the study of Déprez et al. (2000), only 2.7 % of the ^{14}C-label from the substrate was recovered from the metabolites, suggesting other possible routes of degradation. Since the inoculum concentration was low, the microbiota may have been affected by the substrates. The proposed microbial metabolites from these experiments are collated in Appendix A.

Comparison of conversion efficiencies has been possible with the lowest substrate-to-inoculum ratio in the study of enterolactone formation from pure plant lignans. Because the substrates were dosed on a mass basis and the molecular masses of these plant lignans vary, the substrate-to-inoculum ratios on a molar basis were slightly different for each compound. The inoculum concentration was also high, which meant that a high concentration of the rather expensive pure lignans was required (Heinonen et al. 2001).

Table 2. The substrate-to-inoculum ratios applied for isolated phenolic compounds in faecal fermentation experiments in vitro.

Substrate	**Substrate concentration** **μmol/l**	**Faecal suspension concentration** **g/l**	**Substrate-to-inoculum ratio** **μmol/g fresh faecal matter**	**Reference**
Flavonols Rutin Isoquercitrin Quercetin	 **16 000** **22 000** **30 000**	 **50** **50** **50**	 **320** **440** **600**	(Justesen and Arrigoni 2001)
Condensed catechins Proanthocyanidin (PA) as catechin unit eq. PA polymers Labelled PA polymers	 **5 000** **5 000**	 **9.5** **9.0**	 **530** **560**	(Déprez et al. 2000)
Catechins (-)-epicatechin and its galloylated derivatives	**1 000**	**50**	**20**	(Meselhy et al. 1997)
Lignans Mat, Seco, Pin, Syr, HMR, IsoLar, Lar[1]	**235–350**	**167**	**1.41–2.10**	(Heinonen et al. 2001)

[1] Mat: Matairesinol; Seco: Secoisolariciresinol; Pin: Pinoresinol; Syr: Syringaresinol; HMR: Hydroxymatairesinol; IsoLar: Isolariciresinol; Lar: Lariciresinol

2. Aims of the study

The overall target of the study was to develop *in vitro* digestion models for mimicking the physiological conditions of the upper intestine and microbial conversions in the colon. Furthermore, the aim was to study the degradation of plant phenolic compounds in these models.

The specific aims of the present study were

- to develop an *in vitro* enzymatic digestion model for the removal of starch prior to the *in vitro* fermentation of cereal samples (I)

- to develop and use an anaerobic *in vitro* faecal fermentation model for the bioconversion of pure phenolic compounds in order to identify individual microbial metabolites and to follow the time course of their formation (II, III)

- to assess the role of faecal microbiota in the overall metabolism of phenolic compounds (II, III)

- to assess the role of rye matrix in relation to the microbial metabolism of plant lignans (IV, V).

3. Materials and methods

Only a summary of the materials and methods used in this work is presented here. Detailed information is included in the original publications I–V.

3.1 Substrate preparation

3.1.1 Pure phenolic compounds

Rutin (quercetin-3-O-rhamnoglucoside) was used in the development of a faecal fermentation method for isolated phenolic compounds (II). Rutin trihydrate and isoquercitrin (quercetin-3-O-glucoside) were purchased from Extrasynthese (France) (II, III). A mixture of quercetin glucuronides was prepared as described by O´Leary and collaborators (2000). The composition of the quercetin glucuronide mixture was 85 % quercetin-4´-O-glucuronide, 6.5 % quercetin-3´-O-glucuronide and 6.5 % quercetin-3-O-glucuronide and/or quercetin-7-O-glucuronide (II). A list of standards used in the analyses of the quercetin study is provided (II).

In order to study the deglycosylation of anthocyanin glycosides and to identify microbial metabolites of anthocyanins, cyanidin-3-glucoside (Cy3g) and cyanidin-3-rutinoside (Cyrut) were obtained from the skins of red plums (*Prunus domestica* L.). Malvidin-3-glucoside, delphinidin-3-glucoside, petunidin-3-glucoside and peonidin-3-glucoside were isolated from the skins of red grapes (*Vitis vinifera* L.). The anthocyanins were isolated from the plant material by repeated extractions with methanol containing 0.1 % HCl, and purified by column chromatography (Silicagel 60- Polyclar AT; 80:20) and by semi-preparative high-pressure liquid chromatography (HPLC) (II). A list of standards used in the study of the colonic metabolism of anthocyanins is provided in III.

3.1.2 Cereal samples

The enzymatic *in vitro* digestion model was developed using alimentary enzymes (salivary α-amylase, pepsin, and pancreatin) for the digestion and hydrochloric acid, sodium bicarbonate and bile salts for adjustment of the pH. The released glucose (response) was illustrated as a three-dimensional area with contour lines (response surface). Development of the enzymatic digestion model was performed using cooked wholemeal rye flour (cv. 'Anna') in the experimental design 1. In the second design (experimental design 2) when enzyme concentrations were increased response surfaces could not be obtained with cooked rye flour and therefore native rye flour was used. The assessment of the model was performed using bread samples. The rye bread was commercial, soft, wholemeal sourbread containing 20% wheat flour. Wholemeal wheat bread and white wheat bread were baked at the VTT test bakery. The breads were freeze-dried, milled and sieved (particle size 800 μm) before passing through the model. The small particle size was selected to avoid the effect of particles on the digestion (I).

Rye bran and flaxseed meal were donated by Melia Ltd (Raisio, Finland) and Elixi Oil Ltd (Somero, Finland), respectively (IV, V). Thermomechanical (extrusion) and xylanase treatments were used for the partial solubilisation of cell-wall structures to enhance the release of plant lignans (IV: Figure 1). After processing all the cereal samples were additionally digested in the enzymatic *in vitro* digestion model.

The pre-digested rye bran and flaxseed meal were used when the supplements for the *in vitro* colon model were composed. For the rye bran-flaxseed (R&F) supplement the amounts of the pre-digested samples were fixed to 180 mg of rye bran and 20 mg of flaxseed meal. The DF content of the R&F supplement was 96.7 mg/ 200 mg. Rye bran (R) and flaxseed meal (F) supplements were standardized to the same DF content by addition of pharmaceutical grade cellulose, mimicking the composition of the diets consumed by rats in the *in vivo* model (V).

3.2 *In vitro* digestion models

3.2.1 Development of the enzymatic digestion model and its application to cereal samples

A pre-treatment method for cereal samples was developed in order to obtain a DF residue for the *in vitro* colon model. The effect of alimentary enzymes on the extent of glucose release was also studied (I). The batch method was based on a continuous model described by Molly et al. (1993). The experimental designs 1 and 2 (I; Tables II and III) were developed in order to study the synergistic effects of the alimentary enzymes pepsin and pancreatin, and of pancreatin and bile. Enzyme doses per 1500 mg cereal sample varied in the first design as follows: salivary α-amylase 10–40 U; pepsin 0.6–1.4 mg; pancreatin 10–40 mg and bile 0.4–0.6 g, and in the second design as follows: pepsin 1–2 mg, pancreatin 25–75 mg and bile 0.5–0.7 g. The final enzyme and bile doses were adjusted to the following levels: pepsin 2 mg, pancreatin 75 mg and bile 0.6 g, which allowed the maximal extent of starch hydrolysis (784 mg) measured as released glucose. The day-to-day variation of the final *in vitro* method (I; Figure 1) was studied using raw wholemeal rye flour in 12 replicates on six different days. The 12 replicates were combined and the dried residues were analysed for starch, protein and DF contents (I). In the assessment of the final procedure, whole meal rye flour, wholemeal rye bread, wholemeal wheat bread and white wheat bread were subjected to the enzymatic *in vitro* digestion in triplicate on four different days. The insoluble residues of each sample were washed, freeze-dried and combined for their characterization in order to avoid day-to-day variation (I).

In the study of lignan conversion in the *in vitro* colon model the starch and protein contents of rye bran, its fractions and flaxseed were reduced by the *in vitro* enzymatic digestion and the digestion products were removed by a dialysis procedure using a CelluSep H1 membrane (cut-off 10 000; Membrane Filtration Products Ltd, San Antonio, TX, USA) for 26 hours according to Karppinen et al. (2000). After the dialysis the retentates were freeze-dried (IV, V).

3.2.2 Development of the *in vitro* colon model and its application to phenolic compounds

To study the role of faecal microbiota in the metabolism of phenolic compounds the *in vitro* faecal fermentation method according to Barry and co-workers (1995) was modified for pure phenolic compounds. The *in vitro* colon model for fermentation of pure compounds is illustrated in Figure 7. In order to save the valuable, purified phenolic compounds, there was a need to adjust both the inoculum and substrate concentrations. Faecal suspensions were prepared under strictly anaerobic conditions using faeces from at least four healthy donors as described in the publications: In the quercetin study (II) faecal suspension was prepared in the buffer (0.11 M carbonate - 0.02 M phosphate) at pH 6.0 (A) or pH 6.9 (B). Fermentation was performed for rutin in a small batch (10 ml) and in a large scale (1000 ml) with pH control (II). To define the optimal substrate-to-inoculum ratio the faecal suspension (B; 5 %, w/v) was spiked with 0.5, 1.0, 1.5 and 2.0 μmol of rutin in methanol (II). In the anthocyanin study (III) two concentrations (5 or 1 %; w/v) of the faecal suspension were prepared in the same buffer at pH 5.5 in order to enhance the stability of anthocyanin substrates.

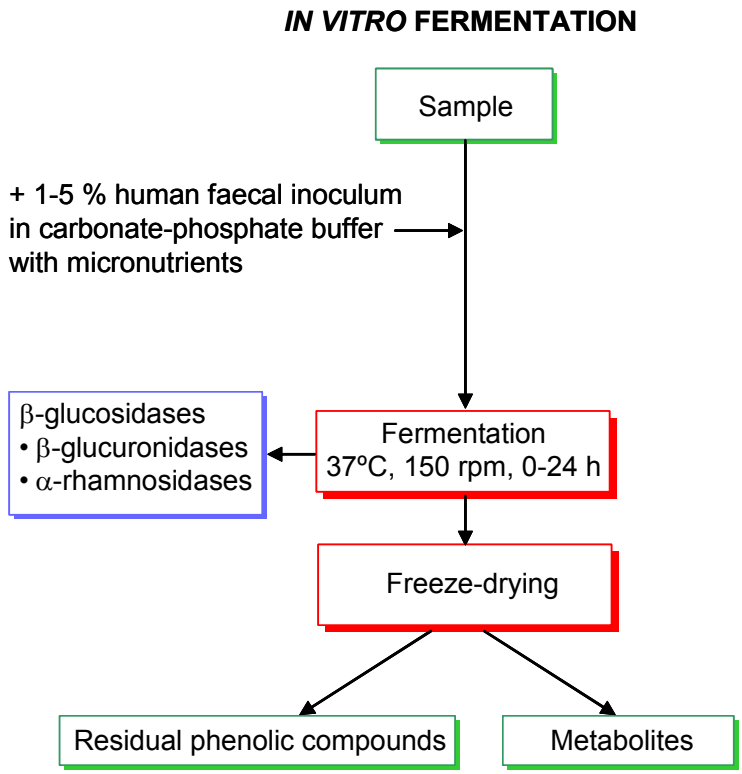


Figure 7. The in vitro *colon model for pure phenolic compounds.*

3.2.3 Application of the colon model to lignans within plant matrix

The amount of pre-digested rye bran and its fractions in the fermentation model was 100 mg (IV) as recommended by Barry et al. (1995) and 109–200 mg in the rye bran and flaxseed experiment (V: Table 1). A faecal suspension was prepared under strictly anaerobic conditions using faeces from four healthy donors (IV, V). When rye bran and its fractions were subjected to the *in vitro* colon fermentation model, the inoculum concentration was first diluted to 10 % (w/v) to reduce enterolactone formation in the faecal background and the time course was followed for up to 24 h. The *in vitro* fermentation was then repeated for pre-digested

rye bran using a dense (16.7 %, w/v) inoculum and the incubation time was prolonged to 48 h (IV). These conditions were applied when rye bran and flaxseed plant lignans were subjected to the microbial conversion (V). The dense microbial population (16.7 %, w/v) was used to avoid changes in the microbiota of the inoculum, as recommended by Edwards et al. (1996) and to ensure a diverse microbial population for the enterolactone formation.

The samples were incubated in triplicate in a waterbath at 37°C with magnetic stirring (250 rpm) and the time courses of the microbial metabolite formation and the consumption of neutral sugars were monitored (Figure 3). Results were calculated per 10 ml of the faecal suspension.

3.3 Characterization of the samples

3.3.1 Alimentary and faecal enzyme activities

The α-amylase activity in the pancreatin preparation was measured using the Ceralpha method of Megazyme (Australia) (I). The extraction was made at room temperature at pH 5.2 and the activity was measured at 40°C for 10 min using p-nitrophenylmaltoheptaoside as a substrate.

Enzymes needed for the deconjugation of flavonoid glycosides and glucuronides were (α, L)-rhamnosidase, (β, D)-glucosidase and (β, D)-glucuronidase. Enzyme solutions were prepared from the faecal inocula (1 % and 5 %, w/v). In the quercetin study faecal microbes from a 5 % suspension were washed before the lysis. Thus the specific activities represented only the intracellular enzyme activities (II). In the case of anthocyanins a 1 % suspension was used and the specific activities included both extracellular and intracellular enzymes (III). The cells were lysed with lysozyme and the cell debris was removed by centrifugation. The susbtrates of the enzyme activity measurements were p-nitrophenylderivatives and the absorbances were measured at 405 nm (II, III). The protein concentration was measured spectrophotometrically (Bradford 1976). The specific activities of the enzymes in the faecal suspensions were expressed as nmol/min · mg protein.

3.3.2 Cereal samples

Glucose released in the *in vitro* enzymatic digestion model was analysed by liquid chromatography (LC) with a refractive index detector after preliminary acid hydrolysis (I). The contribution of solubilized β-glucan to released glucose was considered constant.

Characterization of the cereal samples was performed as follows: The contents of total and digestible starch, β-glucans and fructans were detected spectrophotometrically using specific enzymatic kits (Megazyme, Ireland). The resistant starch (RS) content was calculated as a remainder after subtraction of the digestible starch content from the total starch content. Pentosan contents were measured spectrophotometrically after acid hydrolysis (Douglas 1981). Contents of soluble and insoluble DF were measured gravimetrically in duplicate according to Asp et al. (1983). Protein content was analysed with the Kjeldahl method (6.25 x nitrogen; Analytica-EBC, 1998) and the content of ash gravimetrically after incineration (550°C). Moisture content was determined with a Karl-Fischer titrator (DL18, Mettler, Switzerland). Analyses were performed in triplicate, if not otherwise stated, for rye flour and bread samples (I) and in duplicate for rye bran, its fractions and flaxseed meal (IV, V).

When the performance of the *in vitro* enzymatic digestion method was assessed, the microstructure of the bread samples before and after the *in vitro* enzymatic digestion was investigated by bright-field microscopy. The average molecular weights of β-glucan in wholemeal rye bread before and after *in vitro* enzymatic digestion were detected by size-exclusion-HPLC (SEC-HPLC) using a fluorescence detector (I).

3.3.3 Phenolic compounds and microbial metabolites

Rutin, quercetin conjugates and hydroxyphenyl acid metabolites were detected with HPLC. The verification of the microbial metabolites of quercetin was performed by HPLC with electrospray ionisation-mass spectrometry (ESI-MS) (II). Anthocyanins and their metabolites were extracted with methanol containing 0.2 % hydrochloric acid and analysed

by HPLC with photodiode array spectrophotometric (HPLC-DAS) and mass detection (LC-MS) (III).

Volatile SCFA were measured from faecal suspensions. SCFA were extracted with diethyl ether and analyzed by gas chromatography (IV, V). The time courses of mammalian lignan and neutral sugar concentrations were followed from the freeze-dried samples. Plant lignans from rye bran and flaxseed meal (IV, V) were determined by gas chromatography-mass spectrometry (GC-MS) (Mazur et al. 1996) with modifications (IV, V). Duplicate analyses were carried out for each sample. Mammalian lignan contents of the caecal and the ileal contents of rats and from the faecal fermentation samples at time points 0, 24 and 48 h were determined as presented for the food samples. Mammalian lignans in the fermentation samples were analyzed using HPLC with coulometric electrode array detection (CEAD) as described by Heinonen et al. (2001). Time-resolved fluoroimmunoassay (TR-FIA) was used for analysis of the enterolactone concentration from rat plasma after incubation with *Helix pomatia* enzyme mixture. The determination of neutral sugars was carried out by DIONEX-HPLC. The extent of metabolite formation was calculated by using areas under the curves for enterodiol and enterolactone, for the total amount of SCFA (the sum of acetate, propionate and butyrate) and for butyrate alone (V).

3.3.4 Statistical analyses

All the statistical analyses (V) were performed with the SPSS software (SPSS Inc., Chicago, IL). Values for the rat study were expressed as median (minimum, maximum) or mean ± SD and values for the *in vitro* fermentation study as mean ± SD.

4. Results and discussion

4.1 Development and application of the enzymatic *in vitro* digestion model

The main focus of this thesis is on the *in vitro* colon model. However, when the colon model is applied to food containing digestible components, enzymatic digestion is required in order to obtain a residue resembling the chyme, which enters the colon. Therefore an *in vitro* enzymatic digestion model was developed with special emphasis on starch hydrolysis by the means of physiological pH and temperature (37°C) and using an enzyme combination as close to the *in vivo* conditions as possible (I). Although *in vitro* digestion of starch has been studied thoroughly in several ways, the effects of pepsin, bile and pancreatin on the extent of starch hydrolysis have not been studied previously. Similarly the enzyme concentrations have not been varied using an experimental design.

The variables in the first design were the doses of salivary α-amylase, pepsin, pancreatin and bile. Since the contribution of salivary α-amylase to starch hydrolysis was small, its dose was fixed (50 U per 1500 mg cereal sample) in later experiments and only the doses of the three latter enzymes were investigated in the second design.

The two designs resulted in equations, the coefficients of which described the significance of each variable and their possible cross-effects on the released glucose. In the first design (I; Table II), the resulting equation I[2]:

Glucose (mg) = 1498.9 + 18.5 X – 7.1 Y + 2956.2 Z + 2977.1 Z^2 + 1.9 XY + 12.1 YZ

showed that the coefficient for the bile dose (2956.2) was high and the relative importance of the other terms (-7.1 to 18.5) in the equation was

[2] X = pepsin dose (mg/1500 mg sample); Y = pancreatin dose (mg/1500 mg sample); Z = bile dose (g/1500 mg sample).

small. Synergistic effects of pepsin and pancreatin and pancreatin and bile were both positive (1.9 and 12.1, respectively), but not very strong.

The corresponding equation II[3] obtained from the second design (I; Table III):

Glucose (mg) = 82.8 + 164.8 X + 15.3 Y + 32.0 Z – 0.1 Y2 – 2.1XY

showed that the positive effect of pepsin (coefficient 164.8) on the released glucose was higher than those of pancreatin and bile (coefficients 15.3 and 32.0, respectively) (I; Eq. 2; $P < 0.05$).

The influence of pepsin and bile on the extent of starch hydrolysis was evident. Pepsin degraded the protein matrix, which enhanced the starch availability for its hydrolysis by pancreatin. The result was in agreement with that of Holm et al. (1985), who showed that starch hydrolysis of wheat samples was substantially enhanced by pepsin. The effect of pancreatin concentration on the extent of starch hydrolysis was pronounced only when sufficient amounts of emulsifying bile salts were present, which may have increased the interaction between starch and pancreatin.

The fit of the model (R^2) desribes how well the calculated values obtained from the equation fit to the measured responses. In the determination of the predictivity of the model (Q^2) each point of the response surface is predicted using the response of the other points of the response surface. The fit and the predictivity of the model in the second design were high (0.96 and 0.93, respectively). The difference between measured and predicted amounts of released glucose was within the limits of ±2×RSD (35.1 mg) with 95 % probability. Pepsin, bile and pancreatin concentrations were finally set to 2 mg, 0.6 g and 75 mg per 1500 mg cereal sample, respectively. Day-to-day variation of the digestion procedure was very low (less than 1.5 %) when measured as residual

[3] X = pepsin dose (mg/1500 mg sample); Y = pancreatin dose (mg/1500 mg sample); Z = bile dose (g/1500 mg sample).

starch content in the native digested rye flour in the second design. Standard deviation between predicted and measured values (RSD: 17.6 mg) was smaller than sample-to-sample standard deviation (STD: 21 mg).

When the developed method was assessed with whole meal rye flour, whole meal rye and wheat breads and white wheat bread, 5–11 % of total starch remained in the residue (I; Table VI). Several values can be found in the literature for the amount of residual starch after digestion in the *in vivo* ileostomy model: 3 and 12 % for hot and cold potato starch, respectively (Englyst and Cummings 1987), 2.5 % for white wheat bread (Englyst and Cummings 1985), and 10 and 8 % for white and wholewheat breads, respectively (Wolever et al. 1986). Furthermore, when residual carbohydrates were measured as the amount of hydrogen in the breath of ileostomy patients after consumption of starchy foods, residual starch was very similar: 11% for white bread and 8 % for wholewheat bread (Wolever et al. 1986). Cummings and Englyst compared the estimation of RS surviving the digestion of several foods by two methods: studying starch content from ileal effluents and using the hydrogen breath test for healthy subjects. In conclusion, it was estimated that 10 % of all the digested starch in Western diets is resistant to hydrolysis in the upper intestine (Cummings and Englyst 1991). Thus the *in vitro* enzymatic digestion model used in the present study was able to mimic the extent of starch hydrolysis in the *in vivo* data obtained from the literature.

A high extent of starch removal (89–95 %) was observed in this study. This could partly be due to the small particle size (800 μm) of the samples and the actions of bile and pepsin (I). In the methods measuring the hydrolysis rate of starch, the pancreatin concentrations are rather low, which results in a varying extent of starch hydrolysis (20–80 %) depending on the source of starch (Snow and Dea 1981; Björck et al. 1984; Goni et al. 1997; Granfeldt et al. 1992). Hoebler and co-workers (1991) used proteolytic enzymes at 50°C, thermostable α-amylase at 100°C and finally amyloglucosidase at 60°C, and obtained 84–91 % starch hydrolysis. In the present study the optimization of the enzyme concentrations could have contributed to an even higher extent of starch hydrolysis using physiological temperature and concomitant actions of enzymes and bile, similarly to the conditions in human digestion.

In the present study the bread samples were studied by microscopy before and after *in vitro* enzymatic digestion. Residual starch was partly in ungelatinized starch granules and partly enclosed in the cell wall matrix (I, Figures 4a–h). The hydrolysis of cereal starch granules starts from the specific susceptible zones (Gallant et al. 1992), which can be observed as holes in the centre of the ungelatinized starch granules (I; Figures 4b and 4f).

When the extent of starch hydrolysis is maximized, the further hydrolysis should occur in the slowly digestible starch, because readily digestible starch is rapidly hydrolysed and in the remaining starch fraction RS should not be susceptible to further hydrolysis at all. Thus it could be assumed that the amount of RS would be constant before and after digestion. However, 1500 mg of wholemeal rye bread sample contained 33 mg RS before digestion and its residue after *in vitro* digestion contained only 5.6 mg RS per 1500 mg original sample. Similarly, 1500 mg of white wheat bread sample contained 10 mg RS and after digestion its residue contained only 2.7 mg RS per 1500 mg original sample. Cell-wall matrix swelled in the digestion procedure and a part of RS might have become more available for the hydrolysis. Thermostable α-amylase at 95°C was used in the starch assay. Crystalline starch resists the hydrolysis, but high temperature results in porous, swollen and gelatinized granules with an increased susceptibility to amylolysis (Gallant et al. 1992; Colonna et al. 1992). This may have reduced the amount of RS after digestion. The results presented here also indicate that the starch categories are specified by the method of analysis.

When the DF contents of the residues were analysed from wholemeal rye and wheat breads after centrifugation, 84% and 75 % of the soluble DF, respectively, was lost (I; Table VI). The loss was observed as decreased amounts of pentosan (71 %) and β-glucan (83 %), especially in the wholemeal rye bread (I; Table VI). Furthermore, the average molecular weight of β-glucan increased from 190 000 in wholemeal rye bread to 260 000 in the corresponding residue after digestion and the lost β-glucan (MW 50 000) was observed in the supernatant (I; Figure 5). Of the original β-glucan and pentosan 99 % and 98%, respectively, were retained in the residue after digestion and dialysis (Karppinen et al. 2000; Lebet et al. 1998). Therefore the dialysis step was used later in this work for

removal of digested components of rye fractions, rye bran and flaxseed meal (IV, V).

During the assessment of the enzymatic *in vitro* digestion model, 10–25 % of protein remained in the residues of wheat and rye breads (I; Table V). Although complete removal was not achieved, degradation of the protein matrix was observed (I; Figure 4a–h). The method was applied to the pre-treatment of rye bran and flaxseed meal prior to the *in vitro* fermentation model (V). The residual protein content in the combined rye bran and flaxseed (R&F) supplement was 14.4 % (V, Table 3), the fermentation of which resulted in 60 μmol of branched chain fatty acids of 200 mg sample per 10 ml batch by the faecal microbiota in 48 h. In the same sample the production of acetate, propionate and butyrate derived from carbohydrates was 1520 μmol per 10 ml batch. Thus the amount of branched chain fatty acids was negligible.

4.2 Development and application of the *in vitro* colon model to metabolism of phenolic compounds

4.2.1 Substrate-to-inoculum ratios

The *in vitro* fermentation models have been widely applied to measurements of the rate and extent of carbohydrate fermentation. However, the microbial metabolism of phenolic compounds has not received similar attention. The development of modern analytical methods has made the application of the *in vitro* colon model relevant again and has enabled quantitative measurement of the metabolites. As a consequence, the *in vitro* colon model was applied to the present study. However, the method designed for the study of the carbohydrate fermentation needed adjustment of the substrate and the inoculum concentrations to ensure detection of the microbial metabolites from the faecal suspension, which contains the same metabolites at low concentrations. An additional benefit of finding a suitable substrate-to-inoculum ratio was to save valuable pure compounds and to ensure the solubility of the compounds in the medium. Several substrate-to-inoculum ratios were used in the present thesis for the quercetin derivatives,

anthocyanins and plant lignans from rye bran, from its preparations and from flaxseed meal.

The faecal suspensions were prepared under strictly anaerobic conditions by pooling fresh faeces from at least four healthy donors. A special emphasis was put on the protection of the faecal microbiota from oxygen over the period from donation to the end of the incubation in the *in vitro* model. The viability of the obligate anaerobic microbiota had been tested previously in a carbohydrate fermentation study, in which the number of anaerobes (10^9–10^{10} cells/ g) exceeded the number of aerobes (10^6–10^7 cells / g) after 4 hours of incubation (Karppinen et al. 2000). The inocula in the present study were prepared using the same method.

Rutin was chosen as a model substrate, because it is a stable compound and commercially available. It is also considered to be one of the quercetin derivatives reaching the colon due to its rhamnoglucoside moiety (Scalbert and Williamson 2000). The suitable substrate-to-inoculum ratio for pure phenolic compounds was found by diluting the faecal suspension to 5 % (w/v), which is within the limits of physiological caecal concentrations (5–13 %) (Guyton and Hall 1996a). The suspension was later spiked with rutin (5–200 μM). A rutin concentration of 100 μM was chosen, because it was high enough to be detected from the background and clearly below the saturation point of rutin (205 μM; Budavari et al. 1996; II). Thus, some selection of microbiota must have occurred during the incubation with rutin, because the background metabolite levels were slightly lower in the samples containing rutin (II; Fig. 3b). Phenolic compounds have antimicrobial properties (Puupponen-Pimiä et al. 2001). It is important to use the compounds at low concentrations so as not to suppress the activities of the microbiota. The same substrate concentration (100 μM) and different inoculum concentrations were used for the studies of anthocyanins: 1 % was used for the deglycosylation of cyanidin glycosides and 5 % for the detection of microbial metabolites (III).

Rye bran was processed with cell-wall degrading enzymes and soluble and insoluble residues were obtained. A faecal suspension concentration of 10 % (w/v) was applied for the rye bran preparations in order to

decrease the amount of enterolactone background and the same concentration was compared with a 16.7 % (w/v) suspension concentration in the fermentation of rye bran (IV). Since enterolactone concentration was higher (20 nmol) using the 16.7 % faecal suspension than when using the 10 % suspension (13.5 nmol) (IV: Figure 5b), the higher concentration 16.7 % was applied in further studies with rye bran and flaxseed meal (V).

A substrate dose of 100 mg per 10 ml of faecal suspension is optimal for studying SCFA formation from carbohydrates (Barry et al. 1995) and this dose was also applied to the fermentation of rye bran fractions. The plant lignan contents of rye bran and its preparations were rather low (3.2–24.6 nmol per 100 mg of sample; IV). It was speculated that enterolactone formation could be enhanced by increasing the amount of plant lignans in the substrate dose. Thus the amount of rye bran was increased to 180 mg per 10 ml of faecal suspension in rye bran and flaxseed meal (R&F) and rye bran (R) supplements (V). The area under the curve of the enterolactone formation for R supplement (740±4 nmol×h) was lower than that for the feacal background (1520±70nmol×h), indicating either that the level of plant lignans in the pre-digested rye bran susbtrate was still too close to the faecal background to be detected, or that the formation was suppressed (V; Figure 1B). Thus flaxseed (20 mg per 10 ml of faecal suspension) served as the main source of plant lignans in the R&F and flaxseed (F) supplements and rye bran mainly as the source of the rye bran matrix for the R and R&F supplements.

Substrate-to-inoculum ratios used in all the experiments of this thesis are presented in Table 3. These results indicated that metabolites are detectable from the faecal background if the substrate-to-inoculum ratio is between 0.2–10 μmol phenolic compounds per gram fresh faeces (II–V). The enterolactone formation in 24 hours was 18 nmol per 10 ml of faecal suspension for the pre-digested and dialysed rye bran extract, and 14 nmol per 10 ml for the faecal suspension alone. Thus the substrate-to-inoculum ratio for the pre-digested rye bran extract (0.025 μmol phenolic compounds per gram fresh faeces) was the smallest ratio still showing the enterolactone formation from the faecal background (IV).

Table 3. The substrate-to-inoculum ratios used in the different experiments.

Reaction **Substrate**	**Substrate concentration** **µmol/l**	**Inoculum** **g/l**	**Substrate-to-inoculum ratio** **µmol/g fresh faeces**	**Reference**
Deglycosylation				
Rutin	**100**	**10**	**10**	(II)
Cyanidin-3-rutinoside and -glucoside	**100**	**10**	**10**	(III)
Ring-fission				
Rutin and isoquercitrin	**100**	**50**	**2**	(II)
Cyanidin-,	**100**	**50**	**2**	(III)
Delphinidin-,	**100**	**50**	**2**	(III)
Petunidin-,	**100**	**50**	**2**	(III)
Pelargonidin- and	**100**	**50**	**2**	(III)
Malvinidin-3-glucoside	**100**	**50**	**2**	(III)
Quercetin glucuronides	**20**	**50**	**0.40**	(II)
Plant lignan conversion				
Rye bran	**1.17**	**167**	**0.007**	(IV)
	1.17	**100**	**0.012**	(IV)
Rye bran extract	**2.46**	**100**	**0.025**	(IV)
Supplements:				
Rye bran (R)	**2.19**	**167**	**0.013**	(V)
Flaxseed (F)	**39.5**	**167**	**0.24**	(V)
R&F	**41.7**	**167**	**0.25**	(V)

4.2.2 Deconjugation of flavonoids

A deconjugation is the first step in the microbial bioconversion of phenolic compounds in the colon. In the deconjugation both glycosides and ileal or hepatic glucuronides are transformed to corresponding aglycones, which are further metabolised in the colon. In a previous unpublished study faecal suspension (5 %, w/v) was incubated with rutin to determine whether deconjugative enzymes (α,L-rhamnosidase, β,D-glucosidase, β,D-glucuronidase) were inducible. Bacteria were isolated from the faecal suspension, cells were disrupted with lysozyme as desribed in (II) and the cell debris was removed. The specific activities of α,L-rhamnosidase, β,D-glucosidase, β,D-glucuronidase were 1.0, 6.2–8.0 and 2.0–2.7 nmol/min · mg protein, respectively, and they were rather stable during incubation from 0 to 4 hours in the *in vitro* colon model. The small changes in the specific activities at different time points were due to the changes in the protein concentrations and not due to the changes in enzyme activities (unpublished data).

Rutin was deglycosylated completely in 60 min by α,L-rhamnosidase (1.2 nmol/min · mg protein) and β,D-glucosidase (6.2 nmol/min · mg protein). Rutin was deglycosylated at a slower rate (within 60 min) than isoquercitrin (quercetin-3-glucoside; within 20 min). The low specific activity of α,L-rhamnosidase explained the slower deglycosylation of rutin. In the deglycosylation of isoquercitrin only β,D-glucosidase activity was required. A transient appearance of quercetin aglycone was observed (II; Figure 2). The mixture of quercetin glucuronides was deconjugated within 10 min by β,D-glucuronidase (2.0 nmol/min · mg protein). Thus all quercetin derivatives were rapidly deconjugated, but the rate was dependent on the moiety attached to the aglycone (II). The specific enzyme activities of the cell-free extract from the isolated bacteria reflected the *in vitro* deconjugation rates of phenolic compounds by faecal suspension.

Since the deconjugation of quercetin derivatives was rapid using the 5 % (w/v) faecal suspension (II), the inoculum concentration had to be diluted to a 1 % (w/v) faecal suspension for the study of deglycosylation of anthocyanins, cyanidin-3-rutinoside and cyanidin-3-glucoside. The

deglycosylation of rutin was repeated and used as a reference. Initial concentrations of the added pure phenolic compounds were 100 μmol/l.

The deglycosylation of cyanidin-3-glucoside was faster than that of cyanidin-3-rutinoside and cyanidin aglycone was identified as one of the microbial metabolites (Cy-1). Rutin was also deglycosylated at a slow rate and quercetin aglycone appeared concomitantly. The heat-inactivated suspension did not deglycosylate any of the flavonoids under investigation (III; Figures 3 and 5). The specific activities of the bacterial enzymes in 1 % faecal suspension were low: 0.23±0.05 and 0.63±0.09 nmol/min · mg protein for α,L-rhamnosidase and β,D-glucosidase, respectively, and they were in accordance with the hydrolysis rates of the corresponding glycosides shown in the *in vitro* colon model. The lysed faecal enzyme solution included both intra- and extracellular enzyme activities, because microbial cells were not washed prior to the lysis. The 1 % faecal suspension was too dilute to allow isolation of the microbes from the suspension.

4.2.3 Ring-fission of quercetin

The aim of the application of the anaerobic *in vitro* colon model for pure phenolic compounds was to identify and quantitate their microbial metabolites and to assess the role of the faecal microbiota in the overall metabolism of phenolic compounds. The microbial metabolites of quercetin were confirmed and the time course and the extent of ring-fission were studied (II). The microbial metabolites of quercetin were identified as 3,4-dihydroxyphenylacetic acid, the primary C-ring-fission product, and its dehydroxylation product: 3-hydroxyphenylacetic acid (Figure 8). These metabolites are derived from the B-ring and from the remnants of the C-ring (Hollman and Katan 1998). Phloroglucinol, an A-ring metabolite, detected from the incubations with pure colon bacteria (Krishnamurty et al. 1970; Winter et al. 1991; Schneider et al. 1999) was not detected from the freeze-dried faecal samples with the complex microbiota. Another proposed colonic metabolite, 4-hydroxy-3-methoxyphenylacetic acid, was detected from the faecal background and from samples with rutin, which indicated that the methylated product

originated from the donors (II). Hollman and Katan (1998) suggested O-methylation to occur in the liver after microbial metabolism of quercetin. The same metabolites, including the methylated one, have been identified from *in vivo* samples (Appendix A: Table 2). Sawai and co-workers (1987) reported that human serum concentrations of quercetin metabolites increased 4 to 8 h after oral intake, reached a maximum level at 8 to 12 h and returned to the baseline between 20 and 35 h after the oral dose. The same metabolites were also identified from human urine (Gross et al. 1996).

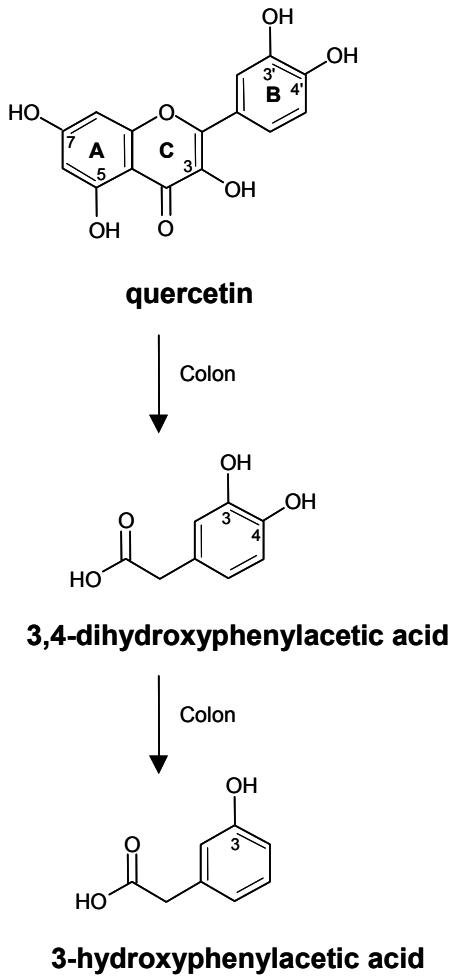


Figure 8. In vitro *formation of quercetin metabolites by human faecal microbiota.*

The maximum concentration of 3,4-dihydroxyphenylacetic acid was observed 2 hours from the start of the incubation, after which it decreased steadily and the concomitant appearance of 3-hydroxyphenylacetic acid was observed. The conversion was completed within 8 h (II; Figure 3a). Maximally 60 % of the initial amount of rutin (100 μM) was converted to the microbial metabolites during the anaerobic fermentation *in vitro*.

Other alternative routes of degradation could also explain the low recovery of the metabolites. A substantial part (23.0–81.1 %) of the radio-labelled quercetin was recovered as carbon dioxide (Walle et al. 2001), part of which may have come from the quercetin and part from the phenylacetic acids. Schneider and co-workers (1999) observed that quercetin or its metabolites may be degraded to acetate and butyrate. According to Sawai et al. (1987), side chains of 3,4-dihydroxyphenylacetic acid may also be shortened to 3,4-dihydroxytoluene. In conclusion, the aims of identification of the microbial metabolites of quercetin and assessment of the role of the faecal microbiota in its metabolism were partly achieved.

4.2.4 Microbial metabolites of anthocyanins

When the microbial metabolism of anthocyanins was studied (III), two faecal suspension concentrations (1 % and 5 %, w/v) were used: cyanidin-3-glucoside and cyanidin-3-rhamnoglucoside were subjected to 1 % (w/v) feacal suspension and malvidin-, delphinidin-, petunidin-, peonidin- and cyanidin-3-glucoside and cyanidin-3-rutinoside were subjected to 5 % (w/v) faecal suspension. Finally, the extent of metabolite formation was studied in reference to the amount of the substrate at the intial time point (III).

Four metabolites were found from the incubations of cyanidin-3-glucoside and cyanidin-3-rutinoside. Cy-1, cyanidin aglycone, and Cy-2, protocatechuic acid (3,4-dihydroxybenzoic acid), were identified by comparing the UV-visible spectrum and elution characteristics with phenolic standards of the putative metabolites (III). A third metabolite (Cy-3), was possibly a phenoxyacid or a phenoxyaldehyde. However, no mass spectra were obtained for Cy-2 and Cy-3 due to the mild ionization conditions used. Furthermore, the fourth metabolite (Cy-4) was detected

after 2 h of incubation using 1 % faecal suspension, and as the only microbial metabolite when the suspension concentration was 5 % (w/v) (III). The microbial metabolites of cyanidin glycoside are illustrated in Figure 9.

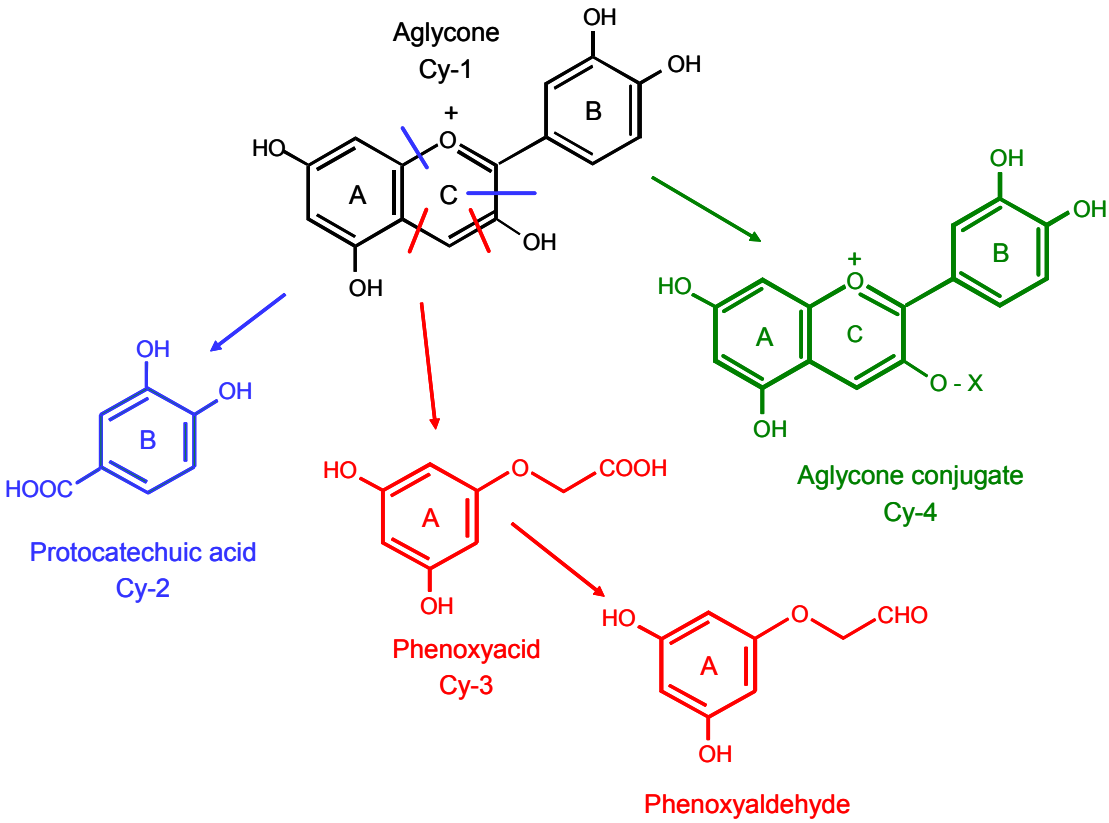


Figure 9. Formation of metabolites from cyanidin aglycone by human faecal microbiota in vitro. *X: an unknown moiety of 85 mass units.*

Cy-4 was 85 mass units larger than the cyanidin aglycone. A similar molecular mass difference was also detected as in the case of the metabolites of 3-glucosides of delphinidin, petunidin, pelargonidin and malvidin. It could be assumed that the metabolite was the product of a conjugation of the aglycone with a moiety containing either sulphur or nitrogen. A sulphate conjugation is possible due to arylsulphotransferases

present in the faecal microbiota, as has been shown for phenolic antibiotics by human intestinal bacteria (Kim et al. 1992). A structural transformation involving nitrogen was also considered, because harpagoside, an iridoid (a monoterpenoid lactone), was reported to undergo replacement of oxygen with nitrogen in the aromatic ring either by the human faecal microbiota or by the action of β-glucosidase in the presence of ammonia (Harborne et al. 1999; Baghdikian et al. 1999). Both β-glucosidase and ammonia can be present in the colon contents, as the activity of the enzyme was detectable from the suspensions used in this thesis and ammonia can be formed from undigested proteins by the colonic microbiota (Macfarlane and Macfarlane 1995).

Different metabolite profiles were observed using the 1 % and 5 % faecal suspensions in the bioconversion of cyanidin-3-rutinoside (III, Figures 8A and B). In the 1 % faecal suspension, cyanidin aglycone (Cy-1) appeared transiently and protocatechuic acid (Cy-2) was the major metabolite. The amount of the phenoxyacid or of the aldehyde (Cy-3) increased steadily during the 2 h incubation and the cyanidin conjugate (Cy-4) began to appear after 90 min. The latter was the only detected metabolite using the 5 % faecal suspension or incubation times longer than 2h for the samples with the 1 % faecal suspension.

The concentrations of the microbial metabolites were estimated from the amount of substrate, cyanidin-3-rutinoside, detected at the initial time point from the corresponding faecal suspensions. The extent of microbial metabolite formation was less than 5 μM. It should also be noted that the concentration of cyanidin-3-glucoside derived from the deglycosylation of cyanidin-3-rutinoside did not exceed 7 μM and that it was quantitated by reference to a standard and not estimated from the amount of substrate (III; Fig. 4).

The low recovery of the microbial metabolites may have been caused by an underestimation of the initial amount of anthocyanins, as they may have been bound to the faecal matrix. The faecal suspension concentration of 5 % (w/v) resulted in a low recovery of substrates (42–66%). The average recovery of the substrate from the 1 % active faecal suspension was 75 % and 83 % for cyanidin-3-glucoside and cyanidin-3-rutinoside, respectively.

Anthocyanin aglycone can open spontaneously to a chalcone structure in neutral or mildly basic solutions. Colourless pseudobases and chalcones are already the dominating forms of anthocyanins at pH values 5.5–5.8 (Clifford 2000a; III). In the anthocyanin study, the initial pH of the buffer was 5.5. The addition of faeces to the buffer resulted in a faecal suspension with a pH of 5.75, which was lower than that used in the quercetin study. The pH equilibrium may have caused underestimation of the concentration of the substrate anthocyanins at the initial time point (0 hour).

Furthermore, alternative routes of metabolism are possible. Anthocyanins may be converted to carbon dioxide or SCFA by a complete degradation of either the C6-C3-C6 skeleton or the microbial metabolites, as was earlier shown for quercetin (Walle et al. 2001; Schneider et al. 1999).

In conclusion, the aim of the identification of anthocyanin metabolites was only partly achieved. The extent of the microbial conversion of anthocyanins was substantially lower than that shown for quercetin derivatives. However, the overall metabolism of anthocyanins is largely unknown and protoctechuic acid, as the microbial metabolite of cyanidin, has not been reported before. The identification of even a single microbial metabolite elucidates the possible route of the overall metabolism and may also facilitate studies of bioconversions *in vivo*.

Protocatechuic acid has been reported to have anti-oxidative and anti-inflammatory characteristics and it blocks stress signal transduction. The intake of protocatechuic acid decreased *tert*-butylhydroperoxide-induced lesions in the rat liver *in vivo*. A histopathological evaluation revealed that hepatocyte swelling, leukocyte infiltration and necrosis were all reduced. Protocatechuic acid also inhibited *tert*-butylhydroperoxide-induced tyrosine phosphorylation, implying activation of a stress signal pathway, in the rat liver (Liu et al. 2002b). Furthermore, protocatechuic acid in concentrations of 500 and 1000 ppm has been able to inhibit cell proliferation *in vivo* induced by carcinogens in the colon and liver in rats (Tanaka et al. 1993 and 1995). Protocatechuic acid can be formed in the colon, it can be absorbed through the intestinal epithelia and it can be transported to the liver for further metabolism as reported for many phenolic compounds

(Scalbert and Williamson, 2000). Thus protocatechuic acid could be found at sites where it could exhibit the chemopreventive properties mentioned above.

4.2.5 Enterolactone formation from plant lignans

Rye bran was extruded and fractions were prepared by hydrolysing the cell walls by xylanase and isolating the soluble rye bran extract and its residue. Extruded rye bran, its hydrolysate, rye bran extract and its residue were compared in terms of the enterolactone formation. The enterolactone formation was studied in the *in vitro* colon model after subjecting the rye bran products to the *in vitro* enzymatic digestion procedure (IV). Furthermore, the suitability of the *in vitro* fermentation model for prediction of the enterolactone formation was assessed by comparing the approaches of the *in vitro* colon model and an *in vivo* rat model. Rye bran (R), flaxseed (F) and their combination (R&F) were introduced into the two models (V). The influence of the rye bran matrix on the conversion of plant lignans to enterolactone was discussed (IV, V).

It was easier to distinguish the intermediary enterodiol formation of rye bran from that of the faecal background using the 10 % faecal suspension than using the 16.7 % faecal suspension, (IV; Fig. 5a). In the experiments with quercetin derivatives and anthocyanins, it was possible to show the intermediary steps in the microbial metabolism of phenolic compounds by diluting the inocula (II, III). However, rye bran lignans were converted to enterolactone to a higher extent (up to 20 nmol) using a 16.7 % faecal suspension concentration and a longer incubation time than using a 10 % faecal suspension (14 nmol) for 24 h (IV; Fig. 5b). Thus dense suspension concentration and a maximum duration of 48 h were also applied in the incubations of rye bran and flaxseed samples (V).

The enterolactone production was found to be a steadily proceeding slow process in all the experiments (Figure 10; IV: Fig. 4b and 5b; V: Fig. 1B) as compared with the production of enterodiol (Figure 10; IV: Fig. 4a and 5a; V: Fig. 1A) or the bioconversions of flavonoids (II: Fig. 3a; III: Fig. 8). This result is supported by the findings of Kilkkinen and co-workers

(2001), who found a positive association in men between the serum enterolactone concentration and constipation, and by those of Johnsen and co-workers (2004), who showed that frequent bowel movements in women had a negative effect on the plasma enterolactone concentration. Human subjects with long colonic transit time may have more time to convert plant lignans to enterolactone than those subjects whose colon motility is very active and the subsequent transit time of the colonic contents is shorter.

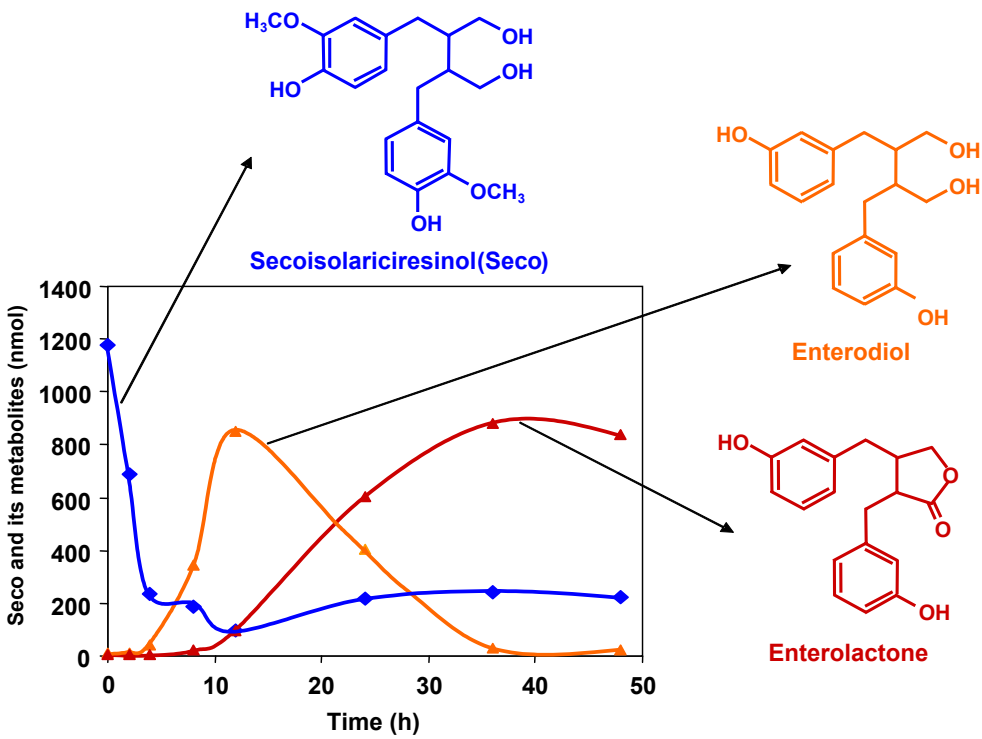


Figure 10. In vitro *formation of enterodiol and enterolactone from flaxseed secoisolariciresinol (Seco) by the 16.7 % (w/v) human faecal suspension (10 ml).*

When rye plant lignans were released enzymatically by cell wall-degrading xylanase, the xylanase hydrolysed the arabinoxylan backbone and released phenolic compounds from rye bran. Especially the plant lignan conversion from pre-digested soluble rye bran extract was enhanced by these treatments. Maxima of enterodiol formation in 10 ml samples were 16 nmol, less than 10 nmol and 4 nmol for rye bran extract, other rye fractions and for the faecal background, respectively. Maxima of enterolactone formation in 10 ml samples were 18 nmol for rye bran extract, 13.5–14 nmol for native rye bran before and after the extrusion and for the faecal background, and 7–8 nmol for xylanase-treated rye bran and insoluble rye bran residue. When plant lignans were in the presence of either intact or xylanase-treated rye bran matrix, plant lignans were converted *in vitro* to enterodiol, but enterolactone formation in 24 h was suppressed (IV: Figures 4a and 4b). There may have been a suppressing component in the rye bran matrix, which was retained in other fractions than the rye bran extract. Especially, the dialysis after the enzymatic digestion *in vitro* could have removed some soluble rye bran components from the extract, because suppression of enterolactone formation was not observed in the dialysed extract. The suppression was also observed in the *in vitro* fermentation experiment with rye bran (R) supplement, the enterodiol and enterolactone formations of which were below those of the faecal background (V).

In the *in vitro* colon model the highest enterodiol concentration in a 10 ml sample of the R&F supplement reached 220 nmol in 12 hours, whereas enterodiol formation from the F supplement was only 120 nmol in 6–8 hours. The enterolactone formation occurred steadily for 36 hours in both the supplements containing flaxseed, but there was a huge difference in the enterolactone concentrations reached at the end of the incubation depending on the presence of the rye matrix. For the F supplement the maximal enterolactone formation per 10 ml faecal suspension was 200 nmol and for the R&F supplement it was only 90 nmol (V; Figures 1A and B). Thus the suppression caused by the rye matrix also influenced enterolactone formation from the flaxseed lignans.

In the *in vivo* rat model the presence of rye bran in the diet enhanced significantly ($P < 0.05$) the caecal enterolactone formation from flaxseed

in the R&F group (407.1 nmol/g) compared with the groups consuming flaxseed (F group: 260.2 nmol/g) or rye bran alone (R group: 72.4 nmol/g) (V: Table 2). The R group differed significantly from the control (C) group (23.8 nmol/g). The caecal enterodiol concentrations were significantly higher in the F group (50.0 nmol/g) than in the other test groups (0.5–3.8 nmol/g), suggesting an influence of the rigid flaxseed matrix, which is able to retain plant lignans and slow down the conversion rate. The rye bran components in the diet enhanced the plant lignan conversion from the diets containing flaxseed. The conversion of rye lignans to enterolactone has been observed in cannulated pigs (Glitso et al. 2000; Bach Knudsen et al. 2003a) and in human subjects (Juntunen et al. 2000), which suggests that components of the rye bran matrix do not interfere with the enterolactone formation in the *in vivo* experiments. Thus the *in vivo* enterolactone formation presented here is in agreement with the previous *in vivo* data, but the *in vitro* data regarding enterolactone formation in the presence of rye bran matrix in the present thesis is in contradiction to the data regarding the *in vivo* enterolactone formation from rye. This discrepancy required further investigation.

In a yet unpublished study a phenolic fraction from rye bran was prepared and it was fermented in the *in vitro* colon model using a high dose of the substrate (1 g/ fermentation bottle). The phenolic fraction from rye bran contained 2 % of phenolic acids and 50 % of carbohydrates, which were readily fermented in all experiments. The pH of the faecal samples containing the rye phenolic fraction decreased below 5.0 in 2 hours and enterolactone formation was not observed at all. Plant lignans remained intact during 48 hours of incubation using the dense 16.7 % faecal suspension. When the *in vitro* fermentation experiment was repeated using a decreased amount of the rye phenolic fraction (0.25 g), the pH values were within physiological limits (above 5.4) and enterolactone was formed during the incubations.

It is possible that the components interfering with enterolactone formation could be phenolic acids or SCFA formed from the fermentation of carbohydrates. They are both released or fermented during the *in vitro* fermentation, when the rye matrix is present (IV, V). As small molecules, phenolic acids may be removed in the dialysis of rye bran extract (IV) or

absorbed *in vivo*. If they are released during the fermentation, their removal does not occur in the *in vitro* colon model and consequently the pH in the model decreases and can suppress the enterolactone formation. It is also possible that phenolic acids are converted to other microbial metabolites, which were not analysed in the present thesis.

The different influence of rye matrix on enterolactone formation in the *in vivo* rat model and in the *in vitro* colon model could also be explained by differences in the microbiota. There are in general such great differences in the metabolical activities of the rat and the human microbiota that human microbiota-associated rats are used for metabolical studies (Rumney and Rowland, 1992; Bowey et al. 2003). A dense faecal suspension (16.7 %; V) is assumed to maintain stability during the fermentation (Edwards et al. 1996), whereas the four-week feeding period used in the rat model in the present study is such a long time that adaptation of the microbiota cannot be avoided (V). This interpretation is supported by earlier findings from a feeding experiment, in which a pre-adaptation period with ferulic acid enhanced the excretion of enterolactone in rats after consumption of a diet containing wheat bran and ferulic acid (Nicolle et al. 2002). Since rye bran is a good source of ferulic acid and its dimers (Andreasen et al. 2000), it is possible that ferulic acid in the R and R&F diets further enhanced enterolactone formation in the *in vivo* rat model, increasing the natural difference between the microbiota in the human faecal inoculum and in the rat caeca.

Soluble carbohydrates were also abundantly present in the phenolic fraction of rye, causing SCFA formation and decrease of pH (unpublished data). It may be postulated that the consumption of nutrients, the release of phenolic acids and the accumulation of SCFA results in the low pH, which may have affected the activity of the microbiota. These observations suggest that enterolactone-converting microbiota is sensitive to low pH. Consequently, the conditions may not be physiologically relevant in static batch cultures at late time points after 24 hours (Campbell et al. 1992).

The consumption of glucose during the fermentation occurred at the slowest rate in the samples containing the flaxseed (F) supplement (Figure 11), the pH of which decreased more slowly than that of the samples

containing rye bran (R and R&F supplements) (Figure 12). In addition to flaxseed meal, the F supplement contained powdered cellulose, which is known to have a crystalline structure and a high resistance to fermentation by colonic bacteria (McDougall et al. 1996).

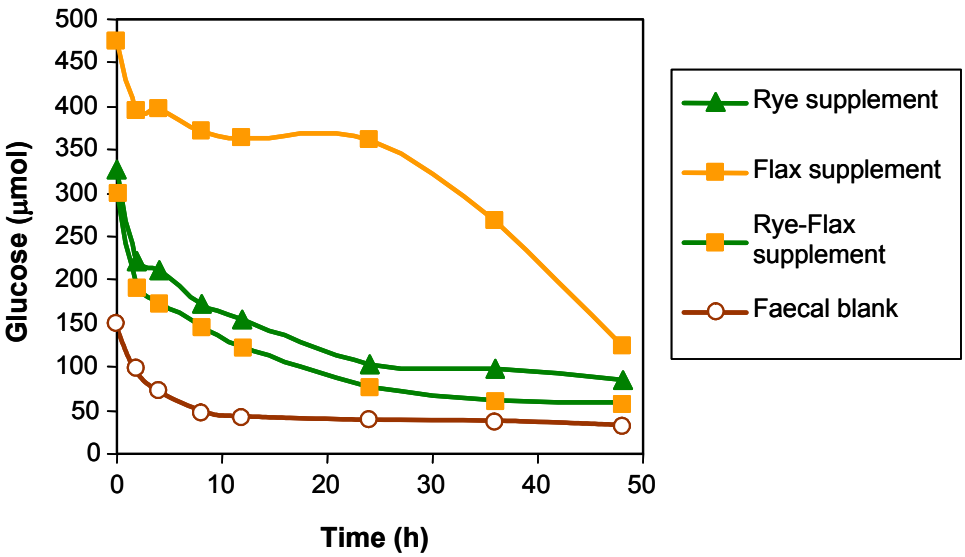


Figure 11. Consumption of glucose in the in vitro *colon model with the rye bran (R), flaxseed (F) and rye bran and flaxseed (R&F) supplements and in the faecal background.*

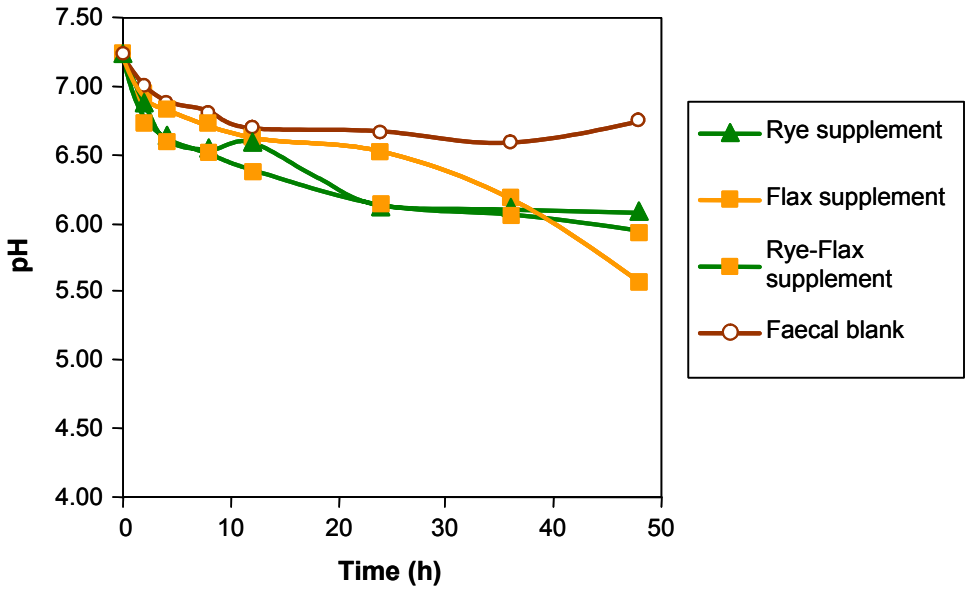


Figure 12. Changes in pH in the in vitro *colon model with the rye bran (R), flaxseed (F) and rye bran and flaxseed (R&F) supplements and in the faecal background.*

Rye bran contained readily fermentable carbohydrates, which was evident from the rapid SCFA formation (IV: Figures 3a; V: Figure 1C). The presence of rye bran enhanced the butyrate formation both in the *in vitro* fermentation and in the *in vivo* rat model (IV: Figure 3d; V: Figure 2). A high proportion of butyrate has been associated with consumption of rye in human subjects (Gråsten et al. 2000) and in animals (Gråsten et al. 2002; Bach Knudsen et al. 2003b). The SCFA and butyrate productions showed a good correlation in our studies in both the *in vitro* and *in vivo* models. In the case of the SCFA formation, the *in vitro* fermentation model predicted well the responses observed in the *in vivo* rat model.

4.3 Reproducibility of the results from the *in vitro* colon model

The biological activity of the *in vitro* colon model originates from pooled faecal samples from at least four invidual donors. The biological activity of the different inocula is presented below by collating the primary data from several experiments. Pre-digested rye bran and pre-digested flaxseed meal were used as reference substrates. Means and standard deviations of the enterolactone, the enterodiol and the SCFA (as a sum of acetate, propionate and butyrate) formations were calculated from triplicate measurements (Table 4). The enterolactone formation was at the level of that of the faecal background in four individual experiments with pre-digested rye bran as a substrate (using two 10 % (w/v) and two 16.7 % faecal suspensions). High enterolactone formation was observed from three experiments with pre-digested flaxseed meal (using one 10 % (w/v) and two 16.7 % faecal suspensions). High standard deviations of enterodiol and enterolactone formation were observed for flaxseed meal. Flaxseed has a rigid cell-wall structure (Freeman 1995), which may have affected the release of plant lignans and their metabolism to enterodiol and enterolactone.

Table 4. Formation of enterodiol, enterolactone and short-chain fatty acids (SCFA) (average±standard deviation) from pre-digested rye bran and flaxseed meal, and the faecal background in the specific experiments. All analyses were performed in triplicate using the 10 % or 16.7 % (w/v) faecal suspensions for 24h or 48 h, respectively.

Sample Experiment (Faecal suspension %, w/v)	**Enterodiol (nmol)**	**Enterolactone (nmol)**	**SCFA (μmol)**
Pre-digested rye bran			
Exp 1 (10 %)	**6±0**	**14±0**	**820±20**
Exp 2 (10 %)	**2±0**	**16±1**	**720±20**
Exp 3 (16.7 %)	**15±1**	**19±1**	**920±80**
Exp 4 (16.7 %)	**14±3**	**21±1**	**870±120**
Pre-digested flaxseed meal			
Exp 2 (10 %)	**46±70**	**20±20**	**760±10**
Exp 3 (16.7 %)	**22±1**	**840±30**	**1100±40**
Exp 5 (16.7 %)	**540±250**	**450±280**	**1000±80**
Faecal background			
Exp 1 (10 %)	**4±0**	**14±1**	**470±40**
Exp 2 (10 %)	**0±0**	**14±3**	**340±50**
Exp 3 (16.7 %)	**17±1**	**13±0**	**680±50**
Exp 4 (16.7 %)	**18±0**	**44±1**	**490±12**
Exp 5 (16.7 %)	**0±0**	**27±0**	**1150±180**

When the microbial bioconversion of quercetin was assessed (II), the volume of the fermentation vessel or the initial pH of the buffer caused some quantitative differences between the series, although the profiles of 3,4-dihydroxyphenylacetic acid and 3- hydroxyphenylacetic acid were

similar (II: Fig. 3a). The profiles of the background metabolites were different and they were strongly dependent on the inoculum (II; Fig. 3b). All this data shows that the metabolite profiles are qualitatively reproducible for each substrate. However, individual variation of the microbiota is reflected in the quantitative data. Thus, the results should always be shown in reference to the formation of the metabolites in the faecal background of the particular experiment.

The biological activities reflect the composition of the colonic microbiota. The quantitation of bacterial species is difficult, because for example selective plate-count methods underestimate the abundance of species (Apajalahti et al. 2003). Isolation and characterization of the total community DNA and additional guanin-cytocin fractionation (%G+C profiling) could provide a novel approach to studying differences between the inocula (Apajalahti et al. 2004; Holben et al. 2004). These methods could be added to the quality management of the *in vitro* colon model.

Calculation of means and standard deviations is the only way of evaluating the data in triplicate measurements. In order to perform a statistical evaluation of the metabolite formation, the number of replicates should be increased. Non-parametric tests can be used if the number of replicates is higher than six, and the parametric tests require as many as twenty replicates. However, the dimensions of the anaerobic chamber limit the total number of fermentation bottles in one experiment and one inoculum. Thus the number of replicates can be increased only if the number of tested substrates is decreased. A fermentation study could be designed so that the metabolite profiles first are screened for a large number of substrates. The experiment could be repeated with the relevant substrates, at crucial time points and with several replicates in another experiment, the results of which could be evaluated statistically using for example the non-parametric methods.

Each specific inoculum has a biological activity of its own, which is shown as quantitative differences between the experiments. Large individual variation has been reported in the biological activities in the human colon (Rowland et al. 1999; Lampe 2003). One of the benefits of the *in vitro* colon model is that it avoids the individual variation within

each experiment. Despite the quantitative differences between the series, it is possible to repeat the response of a substrate with reference to the faecal background. An *in vivo* response can be studied from biological fluids in a clinical experiment. After receiving the *in vivo* metabolite profile, microbial metabolites *in vitro* can be observed against the *in vivo* data, and the role of the microbial bioconversions in the overall metabolism of the substrate compound can be observed.

5. Conclusions

Dietary components undergo several tranformations during digestion. These reactions affect their bioavailability including the absorption, metabolism, transport to the target organs and potential biological effects of the active molecules. Clinical trials provide evidence of the health effects, but the specific bioconversions occurring at the specific sites cannot easily be characterized from the *in vivo* data. The *in vitro* digestion models facilitate investigation of the roles of specific sites in the gastrointestinal tract in digestion and microbial metabolism. The microbial metabolism can be studied only by isolating the colonic microbiota from the other active sites of metabolism, which justifies the development and the use of the *in vitro* faecal fermentation model. It is also very important to introduce the food samples to the *in* vitro fermentation model without such digestible components which would be absorbed in the human digestive system. This justifies the use of the enzymatic *in vitro* digestion model. The main target of this thesis was the development and use of these two *in vitro* digestion models.

The first specific aim was to develop the *in vitro* enzymatic digestion model for the removal of starch from cereal substrates prior to their *in vitro* fermentation. This aim was fulfilled, because the *in vitro* enzymatic digestion model was able to mimic the extent of starch hydrolysis in the *in vivo* data reported in the literature. Furthermore, when dialysis was used instead of centrifugation in the separation of the DF residue, soluble DF components were retained and the residue resembled better the chyme entering the human colon. The enzymatic digestion model is essential as a pre-treatment if the sample is introduced to the fermentation model as food and not as an isolated DF fraction. In this manner the non-physiological fermentation of components which would already have been absorbed in the small intestine *in vivo*, could be avoided.

The second specific aim was the application of the *in vitro* fermentation model using human microbiota to the bioconversion of pure phenolic compounds, to the identification of the microbial metabolites and to monitoring the time course of the metabolite formation. This aim was partly achieved. Two main microbial metabolites were identified and

quantitated for quercetin using a substrate-to-inoculum ratio which enabled the detection of the metabolites from the faecal background. The microbial degradation of anthocyanins occurred only to a much smaller extent than that of quercetin. Four microbial metabolites were distinguished from the faecal background, but only protocatechuic acid was identified with certainty. In addition, there was an unidentified compound, a phenoxyacid or a phenoxyaldehyde, present as a ring-fission metabolite, and an unidentified aglycone conjugate. This conjugate was formed from all the tested anthocyanins, and it was the only microbial metabolite when the faecal suspension was dense. The microbial population may be more dilute in the distal parts of the ileum in man and protocatechuic acid could be formed before the caecum. The anti-proliferative effect of protocatechuic acid on cancer cell-lines may suggest that degradation of anthocyanins by microbiota may be beneficial as an anti-cancer agent in the liver and in the colon, the organs which participate in phenolic metabolism.

When the time courses of the microbial metabolites were compared, phenolic compounds were converted to their metabolites in the *in vitro* colon model at different rates. The formation of the intermediary metabolites could be shown by using diluted inocula. Deconjugation occurred within 2 hours using a 1 % faecal suspension, and ring-fission of quercetin within 2 hours and subsequent dehydroxylation within 8 hours using a 5 % faecal suspension. Enterodiol formation required a dense faecal suspension (16.7 %) and a long incubation time (up to 12 h) to reach its maximal concentration. Enterolactone conversion was the slowest process and the maximum was reached towards the end of the incubation time (36–48 hours) with the dense (16.7 %) faecal suspension *in vitro*. This may suggest that the microbial population capable of enterolactone conversion is not abundant.

The third aim was to assess the role of the faecal microbiota in the overall metabolism of phenolic compounds. This aim could be achieved for quercetin, and to a minor extent for anthocyanins. To fully achieve this aim all the microbial metabolites should have been identified from the *in vitro* faecal fermentation model and from human biological fluids *in vivo*. Quercetin was degraded to hydroxyphenylacetic acids by microbes, but

not methylated. However, there could be other routes of degradation such as formation of SCFA or gases, which were not quantitated. Quantitation would only have been possible using radiolabelled substrates. In the case of anthocyanins identification of one metabolite and proposals for two others were achieved. The low extent of the microbial degradation suggests that the concentrations of these metabolites in the body fluids may be low. It is also possible that most of the anthocyanins are removed from the body intact or as conjugates, which may further explain the low bioavailability suggested for anthocyanins. In the case of plant lignans, the role of microbiota in enterolactone formation has already been established in earlier studies and other intermediary metabolites than enterodiol were not reported in this study.

The fourth aim was to assess the role of the rye matrix in the metabolism of plant lignans. This aim was partly achieved, because the release and the formation of acidic components (phenolic acids and SCFA) from the rye matrix decreased the pH of the samples, and consequently suppressed the enterolactone formation. When the pH values of the samples were either neutral by removal of soluble components after enzymatic hydrolysis (rye bran extract), or mildly acidic by decreasing the amount of sample (phenolic fraction from rye bran), enterolactone formation was observed. The released components or their metabolites are not removed during the *in vitro* fermentation and they may interfere with the microbiota. However, we can only speculate which component causes the interference. Most probably there are several affecting factors. Furthermore, very little is known about the bacterial species which are capable of enterolactone formation. If the enterolactone-converting species are sensitive to the acidic pH, this observation may help in the isolation of this particular microbiota.

The evidence of the reduced risk of chronic diseases is based on epidemiological studies. Case-control studies have been performed in order to identify correlations between a disease incidence and a habitual conduct and its consequencies (smoking, diet, weight reponse etc.). Human interventions or animal trials describe the end-point of the metabolism often after a period of adaptation to the components under investigation. The *in vivo* animal models can be used for studies of the

mechanisms of action of the biologically active compounds. However, a living body is such a complex network of biochemical reactions that simplification may facilitate recognition of the crucial reactions occuring in the body. The biological activities of the different sites can be studied *in vitro* by isolating for example the epithelial cells, some parts of the intestine, the hepatocytes, the colonocytes or microbial populations from the host. The *in vitro* digestion models are a bridge between the analysis of food composition and structure on the one hand and human metabolism on the other.

Although the microbial metabolites of flavonoids, phenolic acids and phytoestrogens were identified in the 1960s and 1970s, it is surprising that this metabolism did not receive much attention between the 1980s and 2000. Consequently, surprisingly little interest has been focused on the biological effects of the microbial metabolites. The recognition of the active metabolism in the ileum and the liver has increased the level of interest in microbial metabolism. The development of modern analytical equipment has enabled quantitation of the metabolites, which is important in order to elucidate their relevance to human health. Studies of biological activity and bioavailability have been performed for components detected from food. It has been suggested that compounds should be introduced to the *in vitro* assays of biological activity in forms and at concentrations which are relevant *in vivo*. In addition, the lack of knowledge concerning the biological activities of the microbial metabolites demands attention from the scientific community.

In the future the *in vitro* digestion models could also be applied to pharmaceutical research. Traditional dissolution tests, comparable to the enzymatic *in vitro* digestion model, are designed for medicinal formulations. Furthermore, the enzymatic *in vitro* digestion model could be used for studying the interactions between food components and medicines. The *in vitro* colon model has not been used extensively for pharmaceutical research. However, the microbial metabolism of controlled release medicinal compounds could be studied in the *in vitro* colon model. The whole metabolome could be analysed by observing a broad range of putative metabolites from the *in vitro* fermentation model with the aid of bioinformatics. Furthermore, the adverse effects of the

candidate molecules could be predicted by identifying the toxic metabolites. The *in vitro* colon model could be validated by comparing the *in vitro* results of known medicines with the data from human clinical trials.

The real benefit of the colon model is demonstrated if the model facilitates identification of the metabolites circulating in the human body. Time and resources can be saved in the screening of new candidate molecules if precursors for the toxic metabolites can be ruled out or if the precursors of the therapeutic metabolites can be included before a clinical trial. Thus the *in vitro* colon model could be used as a pilot trial in the pre-clinical stage of drug development.

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In vitro digestion models for dietary phenolic compounds

Abstract

The aim of this work was to develop *in vitro* digestion models for mimicking the physiological conditions of upper intestine and microbial conversions in the colon. The main emphasis was on the microbial metabolism of plant phenolic compounds: pure quercetin derivatives, pure anthocyanins and lignans from rye bran and flaxseed.

When cereal samples are introduced to an *in vitro* colon a removal of digestible components, is needed. An enzymatic *in vitro* digestion model was developed for maximal starch removal from cereal samples. Pepsin, pancreatin and bile concentrations were optimized using an experimental design. Surprisingly, pepsin and bile also affected the extent of starch hydrolysis in synergy with pancreatin. 5–11 % of the original amount of starch remained in the residues of cereal products. Proteins were also partly hydrolysed. The *in vitro* enzymatic digestion model was used for the pretreatment of rye bran and flaxseed samples.

An anaerobic *in vitro* colon model, conventionally used for the fermentation of non-digestible carbohydrates, was developed further for studying pure of phenolic compounds. Human faecal microbiota from several healthy donors was used in the preparation of an inoculum. A low inoculum concentration was used for decreasing the metabolite concentration from the faecal background in the studies concerning pure flavonoids. A dense faecal suspension was suitable for the conversion of rye bran and flaxseed lignans to enterolactone when the plant matrix was present.

Flavonoids were deconjugated and degraded to phenolic acids by faecal microbiota. Specific activities of the deconjugative enzymes from the faecal inocula reflected the deconjugation rates of flavonoids. Quercetin aglycone was converted to hydroxyphenylacetic acids, but not to methylated phenolic acids. The extent of metabolism was 60 %, showing that ring-fission was a dominating route in the microbial metabolism of quercetin. Anthocyanins also underwent similar conversion, but the estimated extent of metabolite formation was low (less than 5 %). Protocatechuic acid was identified, and a phenoxyacid or a phenoxyaldehyde was proposed, as ring-fission products of cyanidin. In addition, it was suggested that anthocyanins undergo conjugation with an unknown moiety of 85 mass units. This conjugate was observed for several anthocyanins.

Enterolactone production from plant lignans proceeded steadily and slowly for 48 hours in the *in vitro* colon model using the dense (16.7 %) faecal suspension. Flaxseed lignan conversion to enterolactone was suppressed by the presence of rye matrix. The enterolactone-producing microbiota may be sensitive to non-physiological, low pH values caused by acidic components from rye bran in the presence of microbiota. The presence of rye bran matrix did not interfere with enterolactone formation in an *in vivo* rat model. The difference in the response to the rye bran matrix may be due to the absorption of the released and metabolised compounds in rats. Rats may also adapt to the diet during their feeding period. This may have enhanced the enterolactone production, and may have further increased the difference between the bioactivity of the microbiota in the *in vitro* and *in vivo* models used in this study.

Clinical human and animal trials describe end-point metabolism after adaptation to the test diet. The *in vitro* colon model assists in elucidation of the role of microbiota in the metabolical network of human digestive system and it helps in identification of the crucial reactions. Applications of this method can be extended from the studies of food components to pharmaceutical research.

Keywords

phenolic compounds, flavonoids, plant lignans, rye, flaxseed, *in vitro* digestion models, alimentary enzymes, faecal fermentation

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Plants offer a rich source of bioactive compounds, including a diverse group of plant phenols. Epidemiological studies have shown the importance of plant-based foods in the risk reduction of many chronic diseases. However, our knowledge on the role of individual plant compounds, their metabolites and mechanisms of action is far from complete. This thesis presents the development of an *in vitro* model mimicking enzymatic digestion in the upper intestine and application of an *in vitro* colon model to pure phenolic compounds and to phenolics within plant matrices. The *in vitro* enzymatic digestion model was used to obtain a dietary fibre residue for an *in vitro* colon fermentation model using a faecal inoculum. The microbial metabolites of pure phenolic compounds were identified and quantified and the role of microbiota in the overall metabolism of phenolics was discussed. It was also shown that the presence of rye matrix influenced the formation of mammalian lignans, possibly through the acidic components either released from the matrix or converted by microbiota. These methods offer a bridge between chemical food analyses and human physiology by predicting the *in vivo* behaviour of complex foods and their components.

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VTT PUBLICATIONS 575 *In vitro* digestion models for dietary phenolic compounds

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