Development and Validation of an *in vitro* Experimental GastroIntestinal Dialysis Model with Colon Phase to Study the Availability and Colonic Metabolisation of Polyphenolic Compounds*

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Abstract

The biological effects of polyphenols depend on their mechanism of action in the body. This is affected by bioconversion by colon microbiota and absorption of colonic metabolites. We developed and validated an *in vitro* continuous flow dialysis model with colon phase (GastroIntestinal dialysis model with colon phase) to study the gastrointestinal metabolism and absorption of phenolic food constituents. Chlorogenic acid was used as model compound. The physiological conditions during gastrointestinal digestion were mimicked. A continuous flow dialysis system simulated the one-way absorption by passive diffusion from lumen to mucosa. The colon phase was developed using pooled faecal suspensions. Several methodological aspects including implementation of an anaerobic environment, adapted Wilkins Chalgren broth medium, 1.10^8 CFU/mL bacteria suspension as inoculum, pH adaptation to 5.8 and implementation of the dialysis system were conducted. Validation of the GastroIntestinal dialysis model with colon phase system showed a good recovery and precision (CV < 16%). Availability of chlorogenic acid in the small intestinal phase (37 ± 3%) of the GastroIntestinal dialysis model with colon phase is comparable with *in vivo* studies on ileostomy patients. In the colon phase, the human faecal microbiota deconjugated chlorogenic acid to caffeic acid, 3,4-dihydroxyphenyl propionic acid, 4-hydroxybenzoic acid, 3- or 4-hydroxyphenyl acetic acid, 2-methoxy-4-methylphenol and 3-phenylpropionic acid. The GastroIntestinal dialysis model with colon phase is a new, reliable gastrointestinal simulation system. It permits a fast and easy way to predict the availability of complex secondary metabolites, and to detect metabolites in an early stage after digestion. Isolation and identification of these metabolites may be used as references for *in vivo* bioavailability experiments and for investigating their bioactivity in *in vitro* experiments.

Abbreviations

CFU/mL:	colony forming units/mL
FIP-Unit:	Fédération Internationale Pharmaceutique
GIDM-colon:	GastroIntestinal dialysis model with colon phase
USP-Unit:	United States Pharmacopeia
WCA:	Wilkins Chalgren agar
WCB:	Wilkins Chalgren broth

Introduction

Over the past 30 years the health effects of polyphenols have been studied intensively. Their interesting antioxidant properties have been related to the protecting effect of diets rich in fruit and vegetables against several chronic diseases such as cardiovascular disease, breast-, prostate and colon cancer [1,2]. However, there are insufficient data to provide conclusive evidence on these health effects. The biological effects of polyphenols depend on the origin, the amount consumed in diets and – as most important factor – their bioavailability. Intestinal absorption, distribution in body tissues, bioconversion by the colonic microbiota with formation of colonic metabolites and elimination are main efficacy influencing factors [3,4]. An extensive review comparing bioavailability and bioefficacy of polyphenolic compounds showed that the most well-absorbed polyphenols (after intake of a 50 mg dose of agly-

* Dedicated to Professor Dr. Dr. h.c. mult. Adolf Nahrstedt on the occasion of his 75th birthday.

cone equivalent) are the benzoic acid derivative gallic acid (C_{max} = 4 µM), the isoflavones (genistein and daidzein) (C_{max} = 2 µM), the flavanones, and quercetin glucosides (C_{max} = 0.8 µM) [5].

90 to 95% of polyphenols are not absorbed in the small intestine and reach the colon where the microflora hydrolyses the glycosides, glucuronides, sulfates, amides, esters, as well as fission of aromatic rings, reduction, decarboxylation, demethoxylation, demethylation and dehydroxylation reactions occur [6]. The resulting phenolic acids are well absorbed through the colonic epithelium and may be subjected to phase II metabolism in the enterocyte and/or the liver before being excreted in urine [7]. Compounds that appear in the plasma after 3 hours and with a T_{max} of more than 5 hours are absorbed in the colon [8]. The bioavailability and biological activity of colonic metabolites remains uncertain and is a challenging research area.

The aim of this study was the development and validation of an *in vitro* GIDM-colon to study the availability of plant secondary metabolites like polyphenolic compounds. Earlier work by Shen et al. (1994) and Bosscher et al. (2000, 2001) led to the development of this continuous flow dialysis system specifically simulating digestion and absorption in gastric and small intestinal stages, but without colon phase [9–11]. Physiological conditions (digestion pH, digestion time, enzymes and temperature) were mimicked and the continuous removal of the dialysed compounds through a dialysis membrane simulated the one-way pathway in the gastrointestinal tract from lumen to mucosa.

In the current study the initial gastrointestinal simulation system was extended with a colon phase. Several methodological aspects like the implementation of the colon phase in the system were investigated. The colon phase was developed using pooled faecal suspensions. The optimal conditions with respect to growth medium, bacterial growth phase, initial concentration of bacteria, the concentration of the pancreatin-bile mixture and optimisation of the dialysis system were investigated. This GIDM-colon was optimized and validated using the hydroxycinnamic polyphenol chlorogenic acid (5-O-caffeoylquinic acid) as model compound.

Results

In this study the initial *in vitro* continuous flow dialysis system by Bosscher et al. (2001), simulating gastric-small intestinal digestion, was adapted to adult digestion conditions [11]. The pH and enzyme concentrations in gastric and small intestinal phase were adapted to adult conditions (gastric conditions: pH 2, pepsin activity 1729 FIP/3 mL; small intestinal conditions: pH 7.5, pancreatin activity 480 FIP-U lipase, 2154 FIP-U amylase, 96 FIP-U protease and 0.876 mmol bile/L). Moreover an important addition to the GIDM, was the extension with a colon phase resulting in the GIDM-colon system. Different parameters, including adaptation of the concentration of the pancreatin-bile mixture, the influence of bacterial growth on the metabolisation of chlorogenic acid, most optimal cultivation of faecal slurries, and digestion time were studied. In order to create an anaerobic environment, the GIDM-colon system was installed in a glove box.

As 95% of bile acids are reabsorbed in the ileum and recycled back to the liver via the enterohepatic circulation, only 5% of bile acids will enter the colon. Thus, in order to investigate the impact of different concentrations of bile acids (0 to 0.3%) on the viability of bacteria, the formation of caffeic acid was followed using a monoculture of *Lactobacillus acidophilus* (**Fig. 1**). The pancreatin-bile mixture consisting of 0.052% (w : v) pancreatin and 0.1% (w : v) bile was selected as the optimal mixture, since 13 ± 1.2% caffeic acid was found after one day of chlorogenic acid digestion. With the initial bile concentration of 0.3% (w : v) as recommended by Shen et al. and Bosscher et al., no caffeic acid was formed and the growth curve of *L. acidophilus* showed the fastest fall [9, 11].

The lower the concentration of bacteria, the slower the metabolisation of chlorogenic acid. With a faecal bacteria suspension of 10^5 CFU/mL, 6 h after digestion of 70 mg (199.4 µmol) chlorogenic acid still 81.7 ± 4.9 µmol chlorogenic acid persisted and 22.5 ± 1.8 µmol caffeic acid was measured (**Fig. 2**). Increasing the faecal bacteria suspension to 10^8 CFU/mL resulted in the complete metabolisation of chlorogenic acid and its first metabolite caffeic acid to other metabolites ($p < 0.05$).

With regard to optimization of faecal suspension cultivation, the metabolisation of chlorogenic acid (197.7 µmol; 0.070 g) was investigated. This resulted in an availability of 92.9 ± 8.5 µmol chlorogenic acid and 27.7 ± 3.9 µmol caffeic acid for uncultivated faeces (10^8 CFU/mL), and 10.5 ± 2.9 µmol chlorogenic acid and 15.0 ± 2.9 µmol caffeic acid for an overnight faecal suspension (17 h, 10^8 CFU/mL). Moreover, metabolites other than caffeic acid were analysed in the colon dialysate sample after digestion with an overnight faecal suspension. In order to stabilize the suspension, the addition of 80% (v : v) fresh WCB during cultivation of the faecal slurry, was essential and prevented a drop in bacteria concentration by a factor 100 (to 10^6 CFU/mL). The digestion time in the colonic phase was 6 h for chlorogenic acid. During those 6 h metabolites were formed and were dialysed (**Table 1**). At 4 h chlorogenic acid was completely metabolised.

The GIDM-colon was validated using chlorogenic acid. Using the HPLC conditions described, 3 peaks were produced after digestion and dialysis of chlorogenic acid in the small intestine, corresponding to 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid and 5-O-caffeoylquinic acid (= chlorogenic acid) respectively (**Fig. 3** Peaks A*, D* and E*). After further digestion of chlorogenic acid in the colon phase (during 6 h), different metabolites were formed and dialysed: 3,4-dihydroxyphenyl propionic acid, 4-hydroxybenzoic acid (B), 3- or 4-hydroxyphenyl acetic acid (C), caffeic acid (F), 2-methoxy-4-methylphenol (G), 3-phenylpropionic acid (H).

The mean availability of chlorogenic acid in the dialysate fraction after the small intestinal phase was 37 ± 3% (mean ± SD), mimicking the absorption from lumen in the small intestine to mucosa. 126.6 ± 0.6 µmol chlorogenic acid remained in the retentate fraction and was subjected to colonic degradation. Different digestion times were investigated and are shown in **Table 1**. Chlorogenic acid and its metabolite caffeic acid weren't detectable anymore after 4 h of colonic digestion, indicating a complete degradation of this compound. After 24 h, only 3-phenyl-propionic acid was measured. **Table 1** summarizes the availability of the metabolites and in **Fig. 4** the degradation pathway of chlorogenic acid is depicted.

Chlorogenic acid was recovered almost completely after incubation and dialysis of the standards in the *in vitro* gastric and small intestinal stage. The overall recovery of chlorogenic acid at concentrations of 2.01 mmol/L averaged 100 ± 1.5% (n = 9). A recovery *t*-test (one sample *t*-test) for each sample concentration did not show any significant difference ($p < 0.05$) from 100% recovery. The within-day precision values of chlorogenic acid in the dialysate and retentate fraction of the small intestine were 6.1%

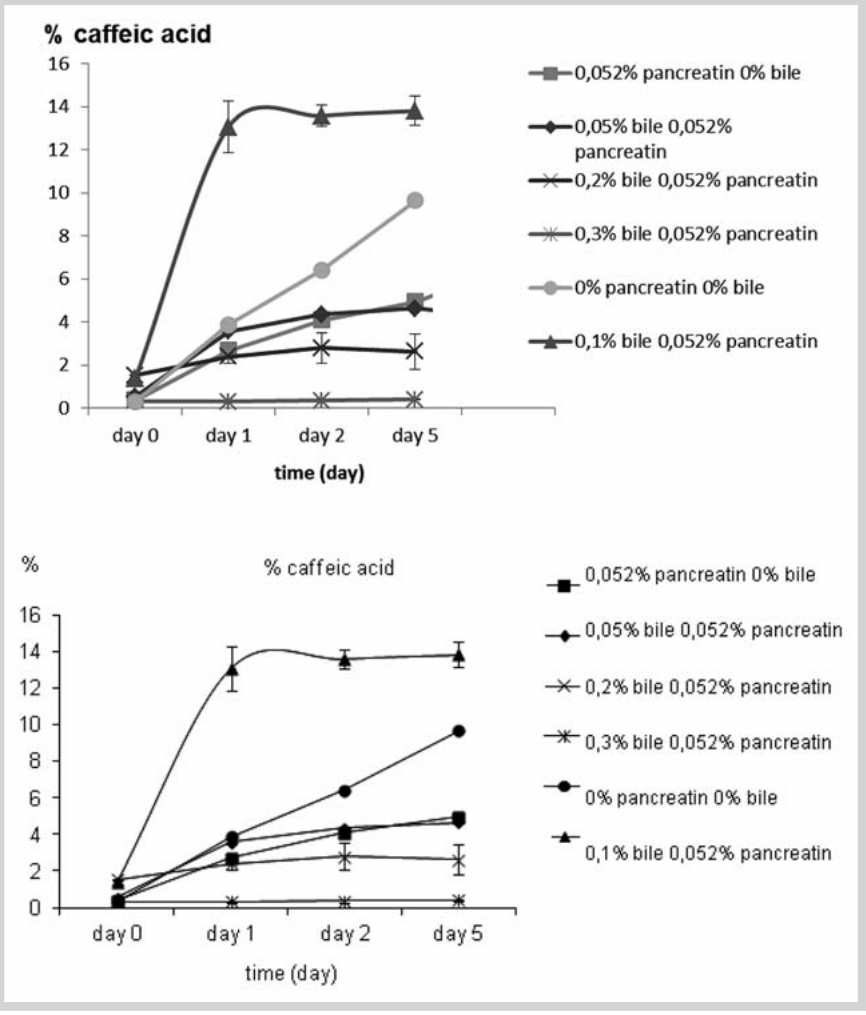

Fig. 1 Formation of caffeic acid (%) after digestion of chlorogenic acid by *L. acidophilus* using different concentrations of bile and pancreatin. The combination of 0.052% (w:v) pancreatin and 0.3% (w:v) bile was the initial concentration, used in the GIDM model with colon phase [9, 11, 36].

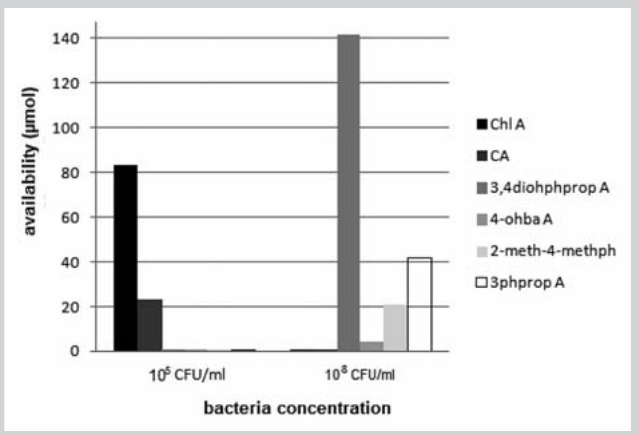

Fig. 2 Digestion of chlorogenic acid (Chl A) in the colon phase, using 10^5 CFU/mL and 10^8 CFU/mL faecal bacteria. After a digestion time of 6 h, the availability of chlorogenic acid and its metabolites (CA = caffeic acid; 3,4diohphprop A = 3,4-dihydroxyphenyl propionic acid; 4-ohba = 4-hydroxybenzoic acid; 2-meth-4-methph = 2-methoxy-4-methyl phenol; 3phprop A = 3-phenylpropionic acid) were measured and expressed in µmol.

and 10.9% (n = 9) respectively, whereas the between-day precision values of the dialysate and retentate fraction were 5.3% and 15.7% respectively (n = 9). Within- and between-day precision values of the metabolites formed after digestion of chlorogenic acid in the colon phase are predicted in ▶ **Table 2**. The results of the mean availability of chlorogenic acid (in the small intestine) and the metabolites (formed after colon phase), obtained on three different days, were not significantly different (ANOVA, p = 0.05).

Discussion

Due to the complexity of measuring the bioavailability of polyphenols *in vivo,* and their extensive metabolisation in the gut, there is a high need for good and simple *in vitro* gastrointestinal simulation models. *In vivo* experiments are expensive, time consuming and often lead to quite variable results. *In vitro* models are faster, well controlled and can be easily used to study large numbers of samples at different doses. Moreover they can be used as first screening to investigate the bioavailability of polyphenolic compounds and to identify potential metabolites. Therefore the aim of our study was to optimize and validate an *in vitro* experimental continuous flow gastrointestinal dialysis model with dialysis during the colon phase, to study the availability of polyphenols and their metabolisation. The GIDM-colon is a unique *in vitro* system mimicking the gastrointestinal tract, and has never been described in literature before. The continuous

Table 1 Mean availability of chlorogenic acid metabolites after GIDM-colon expressed as µmol ± SD.

	Mean available amount (µmol ± SD)				
	Dialysate			Retentate	
Metabolite	**2 h**	**4 h**	**6 h**	**6 h**	**24 h**
Chlorogenic acid	8.8 ± 0.3				
Caffeic acid	1.4 ± 0.2				
3,4-Dihydroxyphenyl propionic acid	2.6 ± 0.4	8.9 ± 0.5	14.2 ± 6.1	115.7 ± 8.1	
4-Hydroxybenzoic acid	3.8 ± 2.9	0.3 ± 0.7			
2-Methoxy-4-methylphenol	0.4 ± 0.4	0.5 ± 0.2	2.7 ± 0.2	17.4 ± 1.9	
3-Phenylpropionic acid	0.9 ± 0.5	2.2 ± 0.5	2.4 ± 0.2	36.5 ± 2.0	20.2 ± 2.8

Number of replicates = 9

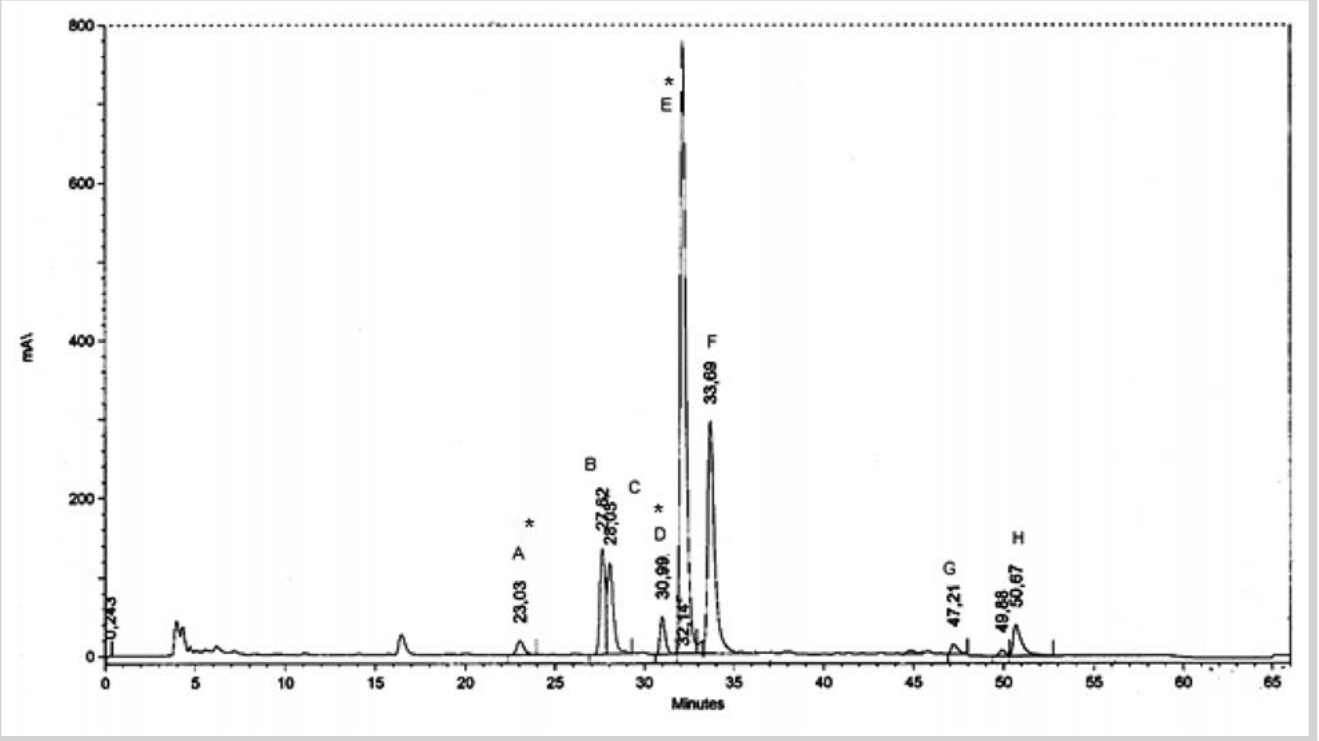

Fig. 3 HPLC chromatogram of chlorogenic acid and metabolites after 2 h of digestion in the GIDM-colon. Peak A: 3-O-caffeoylquinic acid (retention time RT = 23 min, wavelength = 310 nm), peak B: 4-hydroxybenzoic acid (RT = 27 min, wavelength = 286 nm), peak C: 3- or 4-hydroxyphenyl acetic acid (RT = 28 min, wavelength = 286 nm), peak D: 4-O-caffeoylquinic acid (RT = 30 min, wavelength = 310 nm), peak E: chlorogenic acid (RT = 32 min, wavelength = 310 nm), peak F: caffeic acid (RT = 34 min, wavelength = 310 nm), peak G: 2-methoxy-4-methylphenol (RT = 47 min, wavelength = 286 nm), peak H: 3-phenylpropionic acid (RT = 50 min, wavelength = 210 nm). RT, retention time, * detected in gastric-small intestinal phase dialysate.

flow eliminates the compounds diffusing through a semi-permeable membrane, thus simulating absorption by passive diffusion. The *in vitro* system mimics the physiological conditions of the stomach, small intestine and colon. Different aspects including the time dependent change in pH in the different compartments, the temperature (37 °C) and inoculation with human faecal suspensions to establish a microbial population resembling the human colonic microflora, were optimised. The concentration of the digestion enzyme pepsin was adapted to adult conditions. The digestion enzymes pancreatin and bile acids were also optimized [11]. Regarding the colon phase of the system, the anaerobic ascending colon environment (pH 5.8) was specifically simulated since only in the proximal half of the colon metabolisation and absorption are observed [12, 13]. Chlorogenic acid was used as a model polyphenol since it is the most predominant phenolic acid in the human diet [14, 15]. In addition, its absorption profile has been studied in ileostomy patients and is already well known, as well as its colonic metabolisation which has been studied in *in vitro* and in *in vivo* experiments [16–18]. Therefore this compound was the ideal reference for optimisation and validation of the GIDM-colon.

In this study the *in vitro* continuous flow dialysis method developed by Bosscher et al. and Shen et al., which was initially used for bioavailability studies of minerals in babies and toddlers, was adapted to adult digestion conditions and adaptations were made to extend the model with a colon phase [9, 11]. First, the concentration of bile acids [initial concentration: 0.3% (w : v)] in the small intestinal phase influenced the bacterial growth and metabolisation capacity negatively and was adapted to 0.1% (w : v). In the human gut, bile acids are absorbed by passive diffusion along the entire gut and by active transport in the ileum [19]. Our results [0.1% (w : v)] were also reported in literature us-

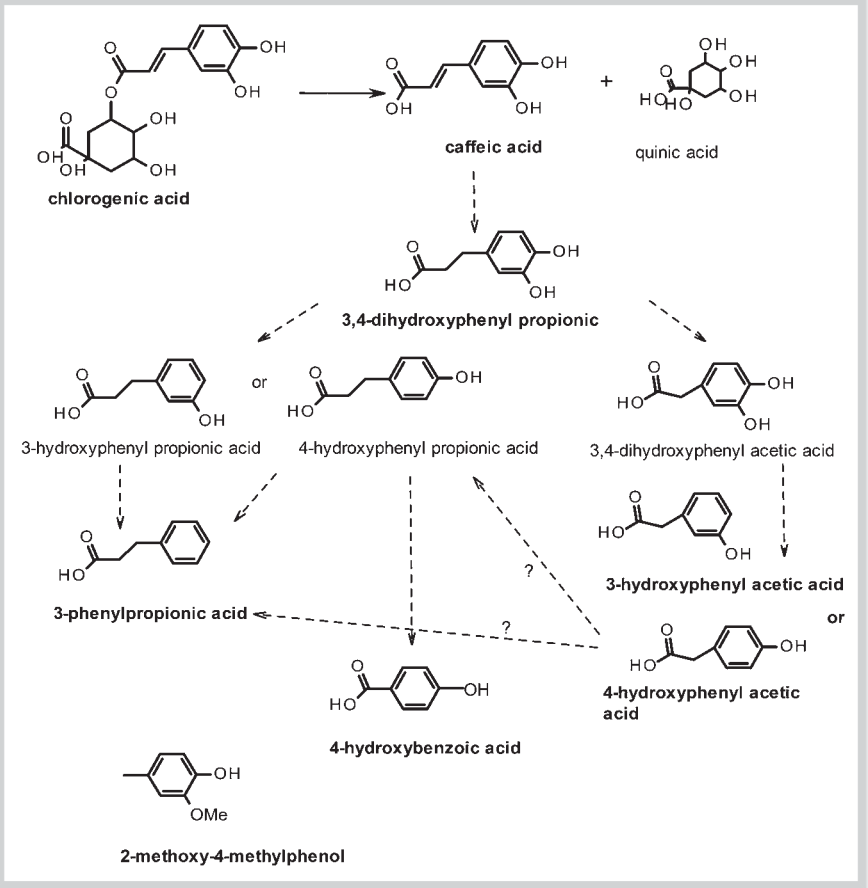

Fig. 4 The metabolites of chlorogenic acid after digestion in the GIDM-colon and identified by HPLC-dual UV detection. quin = quinic acid.

Table 2 Validation of the colon phase of the *in vitro* GIDM-colon after digestion of chlorogenic acid (2.01 mmol/L): within-and between-day precision of the dialysate fractions (4 h and 6 h of colon digestion) and the retentate fraction (6 h after colon digestion).

Metabolite	Intermediate precision	4 h Dialysate CV (%)	6 h Dialysate CV (%)	6 h Retentate CV (%)
3,4-Dihydroxyphenyl propionic acid	within-day	5.3	4.8	2.7
	between-day	7.2	3.0	13.3
4-Hydroxybenzoic acid	within-day	8.3	ND	ND
	between-day	5.8		
2-Methoxy-4-methyl-phenol	within-day	4.6	9.4	8.7
	between-day	11.8	4.1	15.9
3-Phenylpropionic acid	within-day	23.5	8.4	6.1
	between-day	20.5	10.7	3.9

Within-day precision: n = 3 in one day; between-day: 3 days, n = 9; CV: coefficient of variation; ND: not detectable

ing different bacteria strains [20–22]. As bile acids do not diffuse through the dialysis membranes of the GIDM-colon, an adaptation of the concentration was necessary in order to establish the colon phase.

A method to cultivate the faecal slurry was investigated. WCB growth medium was adapted for optimal bacteria growth by adding Tween 80, L-cysteine and resazurin. In order to ensure the bacteria to be at a concentration of 10^8 CFU/mL, frozen pooled faeces were cultivated in WCB for 17 h. 80% (v:v) of fresh medium was added to the cultivated faeces suspension which resulted in stabilisation and prevented a decrease in bacteria concentration.

The availability of chlorogenic acid in the GIDM-colon system and the metabolisation rate is influenced by the amount of bacteria. The lower the concentration of bacteria, the slower the metabolisation of chlorogenic acid. The digestion time for chlorogenic acid in the colon phase revealed 6 h, since its metabolites are detected within 6 h (at 2 h, 4 h and 6 h). A digestion time of 24 h resulted in the detection of only 3-phenylpropionic acid, the last metabolite of chlorogenic acid. Thus it is not necessary to digest chlorogenic for up to 24 h. However the digestion time of more complex polyphenolics, e.g. rutin, can be longer. This was observed for the metabolisation of the flavonoid rutin. Only after 20 h of digestion, the metabolites of rutin were detected and confirmed with literature (data not shown). Depending on the compound, digestion time in the GIDM-colon can vary from 2 h to 24 h.

In order to determine the reliability of the *in vitro* dialysis method, our *in vitro* availability data of chlorogenic acid and its metab-

olites were compared with results of *in vivo* studies. However, some *in vivo* studies are performed in animal models and one might question extrapolation to humans [23–25]. In addition, measurements of urine or plasma levels in animals and humans might underestimate the availability of the ingested compound due to metabolite formation (like glucuronides, sulphates). Data on the absorption of chlorogenic acid in humans are hampered by their metabolic conversion by the colon microbiota and/or body tissues, e.g. liver. Therefore, we compared our data obtained from the small intestinal phase with studies based on the absorption of chlorogenic acid in healthy ileostomy subjects, and our results were comparable with those availability data [17,26,27]. Only one third was absorbed in the small intestine after oral administration of chlorogenic acid supplements [17,18,27,28]. For calculation of the availability, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid and 5-O-caffeoylquinic acid were considered [24,25,27]. The pH change that occurs when shifting from gastric (pH 2) to small intestinal phase (pH 7.5) may result in the transesterification of chlorogenic acid to 3-O-caffeoylquinic acid and 4-O-caffeoylquinic acid metabolites. Other metabolites were not detected in the small intestine. This was expected since colonic microbiota are responsible for further metabolisation [24].

As the aim of the study was the development of a GIDM with colon phase we focussed on the main colonic metabolites of chlorogenic acid. As represented in **Fig. 4** the first metabolic event after digestion of chlorogenic acid in the GIDM-colon was the appearance of caffeic acid, indicating a cleavage of the ester bond between caffeic and quinic acid. Reduction of the double bond in caffeic acid results in 3,4-dihydroxyphenyl propionic acid. Dehydroxylation either at C4 or C3 of this metabolite leads to 3-(3-hydroxyphenyl) propionic acid or 3-(4-hydroxyphenyl) propionic acid which can be further dehydroxylated to 3-phenylpropionic acid [17,29–31]. β-oxidation of 3-(4-hydroxyphenyl) propionic acid could form 4-hydroxybenzoic acid. After 5 h 4-hydroxybenzoic acid could not be detected anymore. All the metabolites formed were dialysed during colonic digestion. However, comparison of our availability data with literature is difficult since we are the first group specifically investigating metabolite availability at the colon phase. The GIDM-colon was validated using chlorogenic acid and provided good recovery. With a CV less than 16%, the model has a good repeatability (within-day precision) and between-day precision for chlorogenic acid (2.01 mmol/L; dialysate and retentate fraction) in the gastric and small intestinal phase. Within- and between-day precision were also studied on the metabolites formed after microbial fermentation by faecal slurries in the colon phase. For 3,4-dihydroxyphenyl propionic acid, 2-methoxy-4-methyl-phenol and 4-hydroxybenzoic acid CV_{within} and $CV_{between}$ averaged < 10% and < 16%, respectively. Given the different stages (gastric, small intestine, colon) of the system, the dialysis system and the complexity of the human faecal slurries and possible interferences with bacterial growth, it can be concluded that the precision of this *in vitro* GIDM-colon system is very good. The GIDM-colon is a reliable *in vitro* system to investigate the metabolites of polyphenols as well as their absorption after digestion in the gut. The metabolites can be used as references for *in vivo* experiments and their biological activity can be investigated using *in vitro* experiments. The GIDM-colon is a non-expensive and easy to apply system. After an experimental time of 4 days, dialysate and/or retentate samples were ready for identification. Regarding the use of 4 dialysis-cells, different replicas and a blank sample can be performed in one experiment [32–35].

In conclusion, an *in vitro* continuous flow gastrointestinal dialysis model with a culture of pooled human faeces to study the colonic metabolisation and absorption of polyphenols was developed and validated. The system is very useful to study the colonic metabolisation of polyphenolic compounds or other complex plant secondary metabolites for predicting the availability by passive diffusion of polyphenols and their metabolites. Isolation and identification of colonic metabolites is easy which makes isolation from complex matrices, e.g. blood, after administration to animals or humans, unnecessary. In contrast to other *in vitro* models (reported in literature), our GIDM-colon is a fast, easy to operate system. LC-MS/MS analyses should be implemented in further metabolisation studies to identify metabolites of more complex polyphenolic compounds.

Materials and Methods

Chemicals and reagents

Deionised water (milliQ, Millipore) was used in all experiments. Chlorogenic acid (5-O-caffeoylquinic acid, 95%), caffeic acid (3,4-dihydroxycinnamic acid, > 99%), 4-hydroxybenzoic acid (99%), 3,4-dihydroxyphenyl propionic acid (98%), 3-hydroxyphenyl acetic acid (99%), 4-hydroxyphenyl acetic acid (99%), 2-methoxy-4-methylphenol (99%), and 3-phenylpropionic acid (98%), used as standards of fermentation metabolites, were obtained from Sigma-Aldrich. Methanol Chromasolve® HPLC grade, pepsin (P-7000, from porcine stomach mucosa, 800–2500 U/mg protein), bile salt (B-8631, porcine), pancreatin (76 190, from hog pancreas, 149 USP U/mg amylase), sodium dihydrogen phosphate anhydrous (NaH_2PO_4), disodium phosphate dihydrate ($Na_2HPO_4 \times 2H_2O$), sodium thioglycolate broth, L-cysteine, resazurine sodium salt, glycerol suitable for culture were purchased from Sigma-Aldrich. Hydrochloric acid (HCl, 32%), dimethylsulfoxide, *ortho*-phosphoric acid (H_3PO_4, 85%), sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH) and ethanol were obtained from Merck. All chemicals and reagents were of analytical grade. Tween 80 was ordered by Becton Dickinson. WCB[6] and agar WCA[6] were obtained from ThermoFischer Scientific.

Equipment

Dialysis tubing with a molecular weight cutoff of 12 to 14 kDa (Visking size 6 Inf Dia 27/32–21.5 mm: 30 M) was obtained from Medicell Ltd. Membranes were stored at 4 °C in a 20% (v : v) ethanol solution. Before use, the dialysis tubings were rinsed three times with deionised water (10 min each). Stirred ultrafiltration cells (model 8200, 200 mL, 63.5 mm diameter), the related controller (controller MF2 and a reservoir RC800) and the ultrafiltration discs (Ultracel molecular weight cutoff 1000, 63.5 mm diameter) were purchased from Millipore [36]. The dialysis membranes were soaked in 0.1 mmol/L NaOH (30 min) and washed three times with deionised water before use (10 min each). Disposable paper collection devices, Potocult® were from Ability Building Center, the recipient VR faeces D41 × 57mm, Bagpage® 400 mL sterile full page filter bags and the Stomacher Lab Blender were purchased from VWR. The globe-box (Jacomex Globe Box T_3) for creating the anaerobic environment was derived from TCPS.

Preparation of digestive juices

16% (w : v) of pepsin was dissolved in 0.1 mmol/L HCl (57 633 FIP-U/100 g). The pancreatin-bile mixture was prepared by dissolving 0.4% (w : v) pancreatin and 0.76% (w : v) bile in 0.1 mmol/L $NaHCO_3$ [32 000 FIP-U lipase, 143 600 FIP-U amylase, 6400 FIP-U protease and 58.4 mmol bile/L (for lipase = 1 FIP-unit = 1 USP-unit [United States Pharamcopeia]; for amylase = 1 FIP-unit = 4,15 USP-unit; for protease = 1 FIP-unit = 62,5 USP-unit).

Sample preparation

75 mg (0.201 mmol) of chlorogenic acid was mixed with 47 mL of deionised water.

Preparation of faecal slurry

Human faecal donors (n = 3) were selected, meeting the following inclusion criteria: 25 to 50 years, non-smoking, non-vegetarian, normal defecation, no history of gastrointestinal disease, and no intake of antibiotics 3 months prior to donation [18,37,38]. A faecal slurry of 10% (w : v) faeces was prepared by homogenizing each stool sample with a sterile phosphate-buffer solution (0.1 mmol/L, pH 7.0) in a stomacher (3 min). The sterile filter bags consisting of a full-page filter were able to filter the slurry and remove particulate food material. A mixed faecal pool was stored at − 80 °C prior to use. The phosphate buffer solution consisted of NaH_2PO_4 [0.58% (w : v)] $Na_2HPO_4 \times 2H_2O$ [1.03% (w : v)] and sodium thioglycolate solution [3.45% (v : v)]. After autoclaving (121 °C, 15 min), sterile glycerol 17% (v : v) was added.

Cultivation of the faecal slurry

Prior to its implementation in the GIDM-colon, the faecal slurry was cultivated. The composition of the basal growth medium was: WCB (33 g), L-cysteine (0.5 g/L), Tween 80 (2.8 mL/L), resazurine sodium salt (1.4 mg/L). The medium was autoclaved at 121 °C for 15 min. In the globe-box (5% CO_2, 5% H_2, 90% N_2), 10% (v : v) of the pooled frozen faeces in WCB were incubated for 17 h by continuously mixing using a magnetic stirrer. 80% (v : v) of fresh WCB was added and the bacteria suspension was incubated for another hour before addition to the retentates of the small intestinal digest. The viable cell concentrations of the anaerobic bacteria were determined before and after the experiment as $\log_{10}$ CFU/mL, by means of decimal dilution series of the bacteria samples plated onto the WCA. This viable cell concentration was an indication for the reliability of the GIDM-colon.

Experimental methods – the GastroIntestinal dialysis model with colon phase

The experimental set up was based on an *in vitro* continuous flow dialysis model, described by Shen et al. and Bosscher et al. [9,11]. The initial system only mimicked the gastric-small intestinal phase and was specifically focused on the gastrointestinal digestion in babies and toddlers. We now adapted the system to adult digestion conditions. We extended the system with a colon phase (performed in an anaerobic globe-box) in view of the study of the availability and metabolisation of polyphenolic compounds. Samples were made in fourfold. Briefly, during simulation of the gastric stage which is conducted outside the anaerobic globe-box in plastic jars, the sample's (47 g) pH was set to 2 using 6 mol/L HCl. 3 mL of the pepsin solution was added and samples were incubated for 1 h in a shaking water bath at 37 °C (120 strokes/min). The intestinal phase was performed in the anaerobic globe-box (5% CO_2, 5% H_2 and 90% N_2) creating an anaerobic environment at a temperature of 37 °C. The intestinal phase was simulated using Amicon stirred cells (dialysis cells; ▶ **Fig. 5**), equipped with a dialysis membrane. The gastric digest was transferred from the jars to the Amicon stirred cells in the anaerobic globe-box and fortified with deionised water to have a total amount of 100 mL. A small dialysis bag containing an amount of 1 mol/L $NaHCO_3$ according to the titratable acidity was added to the dialysis cell. The titratable acidity is the number of equivalents of NaOH (0.5 mol/L) required to titrate the amount of gastrointestinal digest to a pH of 7.5 [36]. Indirectly the pressure inside the dialysis cells was set at 2 bar (29 psi). The dialysis cells were continuously stirred and the dialysate was collected. After 30 min, 15 mL of pancreatin-bile mixture was added to the neutralised digest (pH 7.5) with an end concentration of 0.052% (w : v) of pancreatin and 0.1% (w : v) of bile in the dialysis cells. The pressure of 2 bar was directly set at the dialysis cells and dialysis was continued for another hour. Dialysate (the compounds absorbed in the small intestinal phase) and a fraction of the retentate samples (compounds unabsorbed) of the small intestine were analysed by HPLC-dual UV detection. Retentate samples of the small intestine were the substrates for the colonic microbiota. 50 mL of the faecal bacteria suspension was added (1.10^8 CFU/mL) and digestion pH was maintained at ± 5.8 (1 mmol/L HCl). Dialysis during the colon-phase was accomplished by a pressure of 0.6 bar directly set at the dialysis cells (▶ **Fig. 6**). Each sample dialysate of the colon,

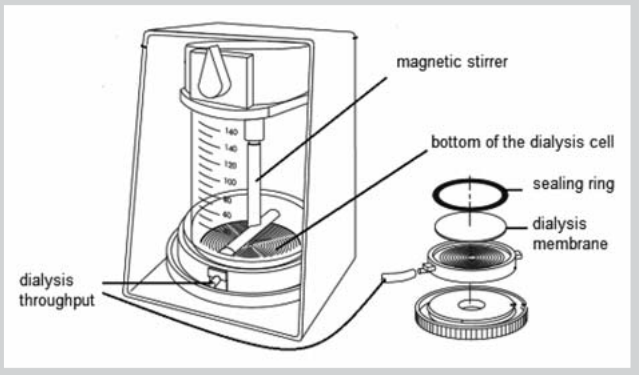

Fig. 5 Magnification of a dialysis cell, just before connecting to the system.

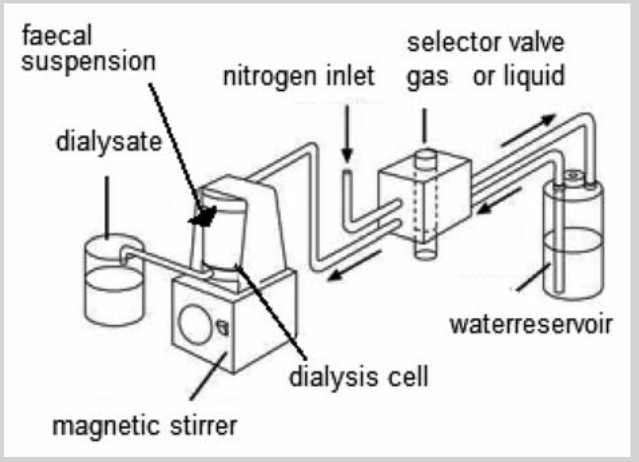

Fig. 6 Schematic representation of the colon-phase of the GIDM-colon.

taken after 2, 4 and 6 h of dialysis, and the final retentate of the colon were frozen (− 80 °C) prior to analysis by HPLC with dual UV-detection.

Bile-pancreatin concentration

To investigate the impact of different concentrations of bile acids (0%, 0.05%, 0.1%, 0.2%, 0.3%) in the small intestinal phase on the viability of bacteria, the formation of caffeic acid (the first metabolite of chlorogenic acid) was followed using a monoculture of *Lactobacillus acidophilus* (10^8 CFU/ml). Retentate samples of the small intestine were incubated for 5 days at 35 °C – 37 °C. At regular time points, samples were collected for analyses of metabolite formation by HPLC-UV detection and/or for the determination of the viability of *Lactobacillus acidophilus*. Each sample was made in duplicate.

Determination of optimal bacteria concentration

To investigate the influence of the bacteria concentration (total anaerobes) in the faecal suspension on the metabolisation of chlorogenic acid, addition of faeces with 10^5 CFU/ml or 10^8 CFU/ml bacteria to chlorogenic acid was investigated. The gastric-small intestinal phase was performed as described above. 50 mL of the faecal bacteria suspension was added (either 1.10^5 CFU/mL or 1.10^8 CFU/mL) to perform the colon phase. Digestion pH was maintained at ± 5.8 (1 mmol/L HCl). After 6 h of colon digestion, dialysate samples were analysed by HPLC with dual UV-detection.

HPLC-UV detection

To obtain absorbance signals within the linear range of the calibration curve, dialysate samples of the small intestine were diluted with 20% methanol. Retentate samples of the small intestine without faecal flora were diluted with WCB. Retentate samples of the colon and faecal suspension were centrifugated for 10 min at 14 000 rpm. The resulting supernatant was analysed by HPLC analysis using the Thermo Fischer Spectra system consisting of SCM 1000 Vacuum membrane degasser, P1000XR gradient pump, autosampler AS3000, UV 2000 detector with dual wavelength. Samples were analysed on a Xselect CSH C_{18} analytical column (3 mm × 250 mm, 5 µm) with a Xselect CSH C_{18} guard column (3 mm × 20 mm) from Waters. The Mobile phase A consisted of a mixture of 5% methanol and 0.05% H_3PO_4 (v : v) and the mobile phase B of 80% methanol and H_3PO_4 0.05% (v : v). The gradient applied was as follows, from 0 to 2 min 93% A and 7% B, from 2 to 50 min 20% A and 80% B, from 50 to 52 min 20% A and 80% B, from 52 to 55 min 93% A and 7% B. Run time was 66 min, injection volume 20 µL and flow rate 0.3 mL/min. Quantification of the identified compounds was undertaken at various wavelengths according to the absorption maximum of the different compounds. At 0 min wavelengths 286 nm and 310 nm were selected, at 48 min 286 nm and 210 nm, and at 65 min again 286 nm and 310 nm were set. Compounds were identified according to retention time, UV-spectra and spiking with commercially available relevant standards. The standard stock solutions (dissolved in 20% methanol), ranging from 2.6 µg/mL to 530 µg/mL, were diluted to final concentrations with 30% WCB in deionised water. The linearity of the standard curve was investigated by injecting different concentrations in duplicate and according to the ANOVA/LOF test and quality coefficient calculation the linear models were accepted ($r^2 \geq 0.999$). The calibration curve for chlorogenic acid is $y = 13\,256\,505.80\,x + 15\,947.49$, $y = 26\,991\,206.39\,x + 17\,479.93$ for caffeic acid, $y = 7\,421\,521.16\,x + 2815.10$ for 3,4-dihydroxyphenyl propionic acid, $y = 4\,441\,460.64\,x + 1911.04$ for 4-hydroxybenzoic acid, $y = 23\,059\,275.58\,x + 133\,230.11$ for 3-phenylpropionic acid, $y = 58\,114\,377.66\,x - 179\,953.84$ for 2-methoxy-4-methylphenol.

Validation of the GastroIntestinal Dialysis Model with colon phase system

Within-day precision of chlorogenic acid (2.01 mmol/L) was calculated according to the amount of each compound in the dialysate and retentate fraction of three replicates of a single day. The between-day precision for chlorogenic acid (2.01 mmol/L) was obtained from nine digestions during three subsequent days. The within- and between-day precision were calculated for the dialysate and retentate fractions of the small intestinal phase and the colon phase. The total amount of each compound in the dialysate and retentate of the small intestinal phase was determined in % and the recovery was calculated. The total amount of chlorogenic acid was defined by measuring 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid and 5-O-caffeoylquinic acid. Calculation of availability of compounds:

$$\text{Availability (mmol)} = ([(A/M) \times V]/D) \times 1000$$

A = total content of the element in the sample calculated on the basis of standard curve (g/L); M = the molecular weight of the compound (g/mol); V = the volume (L) and D = the dilution factor.

Statistical methods

Statistical evaluation of the data was done by SPSS, version 16.0. The mean availability (mean available fraction) in the retentate and dialysate fraction was compared by one-way ANOVA. The difference was considered significant if $p < 0.05$.

Conflict of Interest

The authors declare no conflict of interest.

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