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Simulated gastrointestinal digestion and *in-vitro* colonic fermentation of date (*Pheonix dactylifera* L.) seed polyphenols

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Abstract

Date seeds are a by-product of date fruit industry and a rich source of polyphenols. In this study, *in-vitro* bioaccessibility and colonic fermentation of major polyphenols from date seed powder (DSP) and DSP fortified yoghurt (DSPY) were investigated using HPLC. Catechin, epicatechin, and procyanidin A2, B1 and B2 were stable during simulated gastric and sequential intestinal digestion. Bioaccessibility was significantly ($P<0.05$) higher for all compounds from DSPY compared with DSP. After *in-vitro* colonic fermentation of insoluble digestion materials, most of the target compounds were metabolised by faecal bacteria to ferulic acid, 3-hydroxyphenylacetic acid, 3-phenyl-propionic acid and 3-(4-hydroxyphenyl) propionic acid. DSPY contained significantly ($P<0.05$) higher level of free polyphenols as indicated by higher bioaccessibility, however, the stability of the polyphenols and their fermentation products from DSPY were similar to that of DSP alone. These data would be useful in product developments incorporating DSP as a source of polyphenols in food products.

Introduction

Polyphenols are secondary plant metabolites and form an integral component of the human diet. The importance of polyphenols as bioactive compounds is widely appreciated. One mechanism of their bio-activity is related to their contribution to the body antioxidant defence and to modulating biochemical activities against oxidative stresses (Forman *et al.,* 2014; Mateen *et al.,* 2016). Nonetheless, bioaccessibility of polyphenols from food matrixes and their bioavailability, which is affected by chemical stability and absorption kinetics in the gastrointestinal tract, metabolic modification in the liver and pharmacokinetics of plasma clearance, are the major determinants of the health efficacy of polyphenols from foods or as supplements. For example, the interactions of polyphenols with food matrix components such as proteins, lipids and dietary fibre that influenced their accessibility and chemical stability for intestinal absorption and colonic fermentation have been widely documented (Mandalari *et al.,* 2016; Fu *et al.,* 2016; Oliveira and Pintado, 2015), and that polyphenol glycosylation and esterification also impact on their bioavailability (Scalbert *et al.,* 2002).

Polyphenols, if released from food matrix, can undergo substantial structural changes before and after absorption in the small intestine. These may include de-glycosylation and further structural changes at the intestinal cell membranes by various enzymes, including lactase-phlorizin hydrolases. Some polyphenols can be absorbed through the intestinal epithelial cells *via* active, passive, or facilitated transport (Bohn et al., 2015). Inside the epithelial cells, they are transformed into various metabolised forms by phase-II enzymes such as cytosolic β-glucosidase, glucuronosyltransferase, sulfotransferase, catechol-O-methyltransferase (Gleichenhagen & Schieber, 2016). These sulphated, methylated, or glucuronidated metabolites (referred to as phase-II metabolites) then enter the enterohepatic circulation, and may undergo further similar transformations in the hepatic cells. Finally they reach the systemic circulation, having access to blood, lymph, and various organs such as the brain, or enter kidneys leading to urinary excretion (Gleichenhagen & Schieber, 2016). Unabsorbed polyphenols such as large proanthocyanidins or those bound to food matrix components are inaccessible to digestive enzymes, and therefore reach the colon, where they are metabolised by the colonic microbiota. Subsequently, the health efficacy of polyphenols also depends on their intestinal solubility that affects bioavailability (Fu *et al.,* 2015), and from the generation of readily absorbable phenolic acids from undigested materials due to colonic bacterial fermentation (Aura, 2008).

Date palm is a unique crop as it grows in arid and semi-arid climates, thus seeds of the date fruits are able to survive these harsh conditions until germination (Chao, 2007). Secondary metabolites such as polyphenols are produced in response to such environmental stresses, and in addition serve as a major defence against microbial and herbivorous attacks. Date seeds as a by-product of date fruit industry are produced in large quantities and have been identified as a rich source of proanthocyanidins, glycosylated flavonoids (Habib *et al*., 2014; Sirisena *et al*., 2017) and dietary fibre (Al-Farsi and Lee, 2008). Therefore it is a potential low-cost ingredient which can be added to various food products to increase polyphenol and dietary fibre contents. The polyphenol contents in date seeds contained organic solvent extractable polyphenols (EPP) and non-extractable polyphenols (NEPP) (Sirisena *et al*., 2017). The EPP included flavan-3-ol monomers, dimers and oligomers, as well as glycosylated flavones and flavonols. The NEPP contained insoluble tannin-like materials, which was partly made up of procyanidin monomeric constituents.

Commercially, date seed powder (DSP) is available as caffeine free, health promoting hot drink catering to niche markets (Brouk & Fishman, 2016). A roasting process for making a 'coffee-like' powder from date kernels has been patented by Cohen *et al*. (2011). Ghnimi *et al*. (2015) studied the sensory properties and chemical attributes of roasted date seed powder. The same authors as well

as Sekeroglu *et al*. (2012) reported the absence of caffeine in DSP. In addition, food products with enhanced level of polyphenols and dietary fibre have been developed in laboratory scale by incorporating DSP. Platat *et al.* (2015) reported that addition of DSP (Khalas variety) improved the dietary fibre and phenolic compounds contents, and lowered the acrylamide levels of regular pitta bread. Similarly, a previous study by Bouaziz *et al.* (2010) incorporated a defatted date seed fibre concentrate from Deglet nour variety seeds into pitta bread, and noticed improvement in cellulose and hemicellulose (50 and 20% dry weight basis, respectively). They reported that course-defatted DSP can improve dietary fibre contents without significant negative effects to the bread quality.

In-vitro and animal studies on the bioactivity of date seed polyphenols have provided valuable information on their potential health benefits (Dehghanian *et al*., 2017; Saryono et al., 2017). However, whether these bioactive polyphenols are readily accessible for absorption in the intestine (bioavailable), remains to be established. It is known that food matrix properties have a considerable effect on the release of polyphenols during digestion (Bohn *et al.,* 2015; Padayachee *et al.,* 2012). It is also known that the amount of polyphenols escaping absorption in the small intestine and reaching the colon is high, such that microbial fermentation products may significantly contribute to the dietary phytochemicals from polyphenol intake (Monagas *et al.,* 2010).

In this paper we reported on the stability and *in-vitro* bioaccessibility of the EPP in date seed during simulated gastric and sequential gastric-intestinal digestions, followed by identification of the polyphenol metabolites from *in-vitro* colonic fermentation using HPLC. We also reported on the influence of a food matrix on bioaccessibility using DSP incorporated into yoghurt. The data obtained gave valuable insight on polyphenol-food matrix interactions, as well as useful information for potential functional food development using date seed powder.

Materials and Methods

Materials

Calcium chloride dihydrate, hydrochloric acid (HCl), magnesium sulphate heptahydrate, sodium chloride, sodium hydroxide, potassium phosphate (mono basic), potassium phosphate (di-basic), sodium hydrogen carbonate were ACS reagent grade and purchased from Bio21 Stores, University of Melbourne, Victoria, Australia.

Nutrient Agar (NA) and MRS Agar (Oxoid®), AnaeroGen® sachets (AN 0010W, Oxoid ®), Anaerobic indicator strips (BR55, Oxoid®), peptone (LP0034B) and tryptone (LP0042R) were purchased from Thermo Fisher scientific, Vic, Australia. Nitrogen gas (purity 4.0, O_2 free N_2) was purchased from Coregas pty Ltd, NSW, Australia. BD Gas pak™ 3.5 L anaerobic jars were purchased from BD Australia, NSW, Australia.

Bile extract from porcine (B8631), casein (C3400), guar (G4129), L-cysteine HCl (C7880), mucin from porcine stomach (M1778), pancreatin from porcine pancreas (P6976), pectin from citrus peel (P9135), pepsin from porcine gastric mucosa (P6887), phosphate buffered saline (P3813), soluble potato starch (S2004), and yeast extract (09182) were purchased from Sigma-Aldrich, NSW, Australia.

Preparation of date seed powder

Date seed powder (DSP) for the preparation of DSP yoghurt (DSPY), and *in-vitro* digestion were prepared as previously described by Sirisena *et al*. (2016). Briefly, seeds were separated from date fruits (Deglet nour variety) harvested from date palms cultivated in Arid Zone Research Institute in Alice Springs, Australia. Date seeds were washed with warm water and air dried at room temperature in the laboratory for 5 days. Seeds were grounded into a powder fine enough to pass a 1 mm sieve (cutting mill Retsch SM100, Germany).

Preparation of yoghurt containing date seed powder

Yoghurt fortified with 5% (w/w) date seed powder (DSPY) was prepared, adapting the methods of Mudannayake (2015). In this method, 13 g of skim milk powder (Devondale, Victoria, Australia) was dissolved in 100 mL of milliQ water to obtain 13% (w/v) reconstituted skim milk with a protein content of 35 mg/mL. The reconstituted skim milk was distributed into 50 mL sterile centrifuge tubes (20 ml each), and DSP was added at 5% (w:v). These milk samples were then heated to 90 °C for 10 min in a water bath, cooled to 40 °C, and inoculated with pre-cultures La-5 and Bb-12 at a concentration of 1% (w/v) each. The mixture further incubated at 40 °C for 6 hr to set the yoghurt. The pH was monitored before and after incubation to track fermentation progress and yoghurt formation. The set yoghurt was stirred using a tube homogeniser to obtain uniform distribution of DSP throughout the system before subjecting to digestions. Controls of yoghurt without adding DSP and DSP in milk without heat treatments were also prepared and treated similarly for digestion.

Effect of heat-treatment on polyphenol release

In order to examine the effect of heat-treatment during yoghurt preparation on polyphenol release, DSP was added to the reconstituted skim milk (5%, w:w) and subjected to similar heat treatment but

without the fermentation step. The heat treated unfermented milk containing DSP (DPSM +heat) was then stirred vigorously to uniformly suspend the DSP, and 5 g sample was subjected to gastric digestion. DSP in milk without going through the heat treatment (DSPM -heat) was also prepared and samples subjected to gastric digestion as a control (no heat treatment).

Gastric and intestinal digestion

Preparation of Simulated Gastric Fluid (SGF) and Intestinal Fluid (SIF)

SGF and SIF were prepared following US pharmacopeia Convention (2009). For SGF, 2 g NaCl and 7 mL HCl (37%, w/v) were mixed with 800 mL MilliQ water, followed by pH adjustment to 1.2 using 1M NaOH. For each experiment, SGF with pepsin was prepared fresh by adding pepsin at a concentration of 6.4 mg/mL. The solution was stirred 15 min before use. To prepare SIF, 5 mM phosphate buffered saline was prepared with NaCl and $CaCl_2$ to concentration of 0.4 M and 15 mM. pH was adjusted to 6.8 using 1 M HCl, and stirred using magnetic stirrer immediately prior to use in order to keep $CaCl_2$ in solution. Fasted and fed state bile salt concentrations for this study were selected based on published literature (Holm, Müllertz, & Mu, 2013). Two bile concentrations were selected to simulate the fasted and fed conditions. Fasted state SIF was prepared with 2.5 mg/mL and fed state SIF with 40 mg/mL bile concentrations (Fu *et al.,* 2015). To each fasted and fed state SIF, pancreatin was added immediately before experiment to a concentration of 10 mg/mL. Prepared solutions were incubated with constant shaking at 37 °C prior to experiment. Pancreatin was prepared freshly in 5 mM phosphate buffer on the same day of the experiment and was also kept at 37 °C with shaking.

Digestions

Gastric digestion and sequential gastric-intestinal (hereafter referred to as intestinal) digestion methods were adapted from (Fu *et al,.* 2015). All digestions were carried out in triplicates.

Gastric digestion was carried out by adding the SGF to DSP and DSPY in 50 mL sterile centrifuge tubes. The tubes were place in a tube shaker inside an incubator set at 37 °C, and incubated with constant gentle shaking programme for 2 h. The tubes were then centrifuged at 8422 g for 10 min to separate soluble and insoluble fractions for HPLC analysis of the polyphenols. SGF were also added to DSPM +heat and DSPM -heat samples and similarly treated and separated into soluble and insoluble fractions for HPLC analysis.

Intestinal digestion was carried out by adding fasted or fed state SIF to separate sets of gastric digest after 2 h of incubation as described before, with pH adjustment to 6.8 using 1 M NaOH to carry the digestion forward. This was followed by addition of pancreatin (in buffer phosphate buffer 5 mM, pH

7.0) to a final concentration of 1.2 mg/mL in the digestion mixture. The tubes were then placed in a portable tube shaker and incubated for 3 hr at 37 °C with gentle shaking, followed by centrifugation at 8422 g (Jouan C3i Q5, California, U.S.A.) to separated soluble and insoluble fractions for HPLC analysis.

Colonic fermentation

Basal media preparation

For basal medium, 5 g soluble starch, 5 g peptone, 5 g tryptone, 4.5 g yeast extract, 4.5 g NaCl, 4.5 g KCl, 2 g pectin, 4 g mucin, 3 g casein, 1.5 g $NaHCO_3$, 0.8 g L-Cysteine HCl, 1.23 g $MgSO_4.7H_2O$, 1.0 g guar, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.4 g bile salts, 0.11 g $CaCl_2$ and 1 mL Tween 80 were dissolved and made up to 1 L in MilliQ water. pH was and adjusted with HCl (1 M) or NaOH (1 M) at 25 °C, and sterilized at 121 °C for 20 min in the autoclave (Fu 2015).

Faecal slurry preparation

Freshly void faeces from a healthy female aged 30 yr, who did not have previous intestinal disease, maintained a diet free of any polyphenol supplements, and has not consumed probiotics or antibiotics for 3 months, was used to prepare the faecal slurry. Ethical approval was obtained from the Veterinary and Agricultural Sciences Human Ethics Advisory Group, University of Melbourne (Ethics Approval ID: 1545922). Although the limitation in using one donor is acknowledged, this preliminary report is focussed on biotransformation and the primary emphasis is on detection of biotransformation of the polyphenols. Briefly, 20 g faeces were weighed into a stomacher bag and 80 g sterilized pre-N_2 flushed 0.1M phosphate buffer (pH = 7.0) was added to make 20 % w/w faecal slurry. It was then homogenized for 5 min in a stomacher mixer and filtered through sterile muslin cloth to remove particulate matter. Faecal slurry was then transferred to 50 mL sterile, pre-N_2 flushed tubes with 5 mL aliquots using sterile pipette. The tubes were used for experiment on the same day, or stored in -80 °C for analysis within a week. All work involving faecal samples were carried aseptically under bio-safety chamber (Fu 2015). As the aim of this study was to investigate if some of the date seed bound polyphenols reach the colon and undergo fermentation, identification was the bacterial species were not carried out. Also, the fermentation results are related to the microbiota activities from a single individual, since the experiment only aim to show fermentability DSP.

Bacterial enumeration

Aerobic and anaerobic bacteria were enumerated before the start of the fermentation and after 24 hr of fermentation. For the initial enumeration, 5 mL faecal slurry was mixed with 5 mL of sterile

basal medium, and serially diluted to 10^{-10} in sterile buffered peptone water (0.1%) containing 0.5g/L L-cysteine HCl. The same was done after 24 hr fermentation samples in triplicate.

From each dilution 100 µL was transferred onto either MRS or NA plates in triplicate, using spread plate method. The NA plates were incubated aerobically at 37 °C for 48 h. MRS plates were incubated anaerobically using anaerobic chamber containing anaerobic gas generator and indicator, at 37 °C for 48 hrs. Selected dilutions were used for enumeration.

In-vitro colonic fermentation

Colonic fermentation was carried on the insoluble fraction (8422 g precipitate) from intestinal digesta. Previously prepared tubes containing 5 mL faecal slurry was mixed with 5 mL of basal media, both pre-warmed to 37 °C. To this, 0.5 g sediments from intestinal digestion (DSP or DSPY) were added. All treatments were prepared in triplicate. All tubes were then flushed with O_2 free N_2 and then lids were closed with hand-tight. They were then placed in an anaerobic chamber with anaerobic gas generator and anaerobic condition indicator. The chambers were placed in a shaking incubator (ZWYR-240, Labwit, Shanghai, China) maintained at 100 rpm and 37 °C, and the fermentation was carried out for 24 hrs. Blanks were prepared without the sample, and also sample and basal media without any faecal slurry. After 24 hr fermentation the tubes were stored at -20 °C and processed for HPLC analysis.

HPLC analysis

Preparation of samples for HPLC analysis

The gastric and intestinal digestions as well as colonic fermentation samples were centrifuged at 8422 g for 10 min at 20 °C and separated into soluble and precipitate fractions. Polyphenols were then extracted from the soluble (8422 g supernatant) and insoluble fractions (8422 g precipitate) of gastric and intestinal digestions using 20 mL acetone/methanol (1:1, v:v). Organic extracts from each fraction were evaporated to a small volume under vacuum at 40 °C (Buchi Vac V500 system, Flawil, Switzerland), reconstituted to 5 mL with the extraction solvents, and filtered through 0.45 µM nylon filter disc for HPLC analysis. Additionally, gastric and intestinal digestion mixtures without separation into supernatant and precipitate by centrifugation were also extracted following the same procedures.

HPLC methods

HPLC separation and quantitation of polyphenols and polyphenol fermentation products were performed using a Waters 2690 Alliance HPLC separation module (Waters, NSW, Australia), equipped with a Gemini C18 Silica 250 × 4.6 mm, 5 µM column (Phenominex Inc., NSW, Australia),

and a Waters 2998 Photodiode Array (PDA) detector. The mobile phases used were; MilliQ water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B).

HPLC analysis of gastric and intestinal digested samples was based on a method described by Sirisena *et al.* (2017) for analysis of date seed free polyphenols. A linear gradient of 5-30% of B in A over 60 min was used for the elution. PDA detection was set at λ 280 nm. For colonic fermentation samples, a linear gradient of 2-100% B in A over 60 min was used, with the PDA detector set at λ 270 nm (Appeldoorn *et al.,* 2009a). HPLC analyses were carried out for 3 extracted samples per each treatment.

Quantification of polyphenols

We focused on 5 major DSP polyphenols (catechin, epicatechin, procyanidin B1, procyanidin B2, and procyanidin A2) that were previously identified by HPLC-MS (Sirisena *et al*., 2017). Their stability and *in-vitro* bioaccessibility were monitored in gastric and intestinal digestion stages. HPLC peaks corresponding to the polyphenols were confirmed by an internal standard spiking method: the putative peak was spiked with added standard and the increased in peak area of the target peak indicates spiking of peak. Quantification of individual polyphenol in the sample was based on its peak area and the peak area of standard curve generated with the standard compounds, and was expressed as µg/g DSP.

Statistical analysis

Statistical analyses were performed using SAS software (Version 9.2). The collected data were subjected to one-way analysis of variance (ANOVA), and the means were separated using Least Significant Difference (LSD) test ($p < 0.05$). Final results were reported as mean ± SD (n=3) with 2 significant figures according to EURACHEM guidelines (Ellison and Williams, 2012).

Results and Discussion

Bioaccessibility is define as the accessibility of a nutrient from food matrixes that are available for absorption during digestion, thus is a prerequisite for its bioavailability in the body. It is known that in addition to free polyphenols, significant amount are associated with or weakly bound to food matrix components such as protein and dietary fibre (Padayachee *et al*., 2012). The release of bound polyphenols from the food matrix into intestinal fluid during digestion requires their solubilisation, which is also a prerequisite for their absorption and thus bioavailability (Fu *et al*., 2015). Additionally, plant materials in general, including DSP, contain significant amount of tannin like high molecular weight (HMW) polymeric polyphenolic materials that are not soluble, thus are not accessible during

digestion (Arranz *et al.,* 2010). Free and bound low molecular weight (LMW) polyphenols that escaped absorption in the small intestine are passed next into the colon where bacterial fermentation may occur, generating secondary absorbable products (Bohn, 2014). Fermentation of HMW polyphenols may also occur but to a much lesser extent (Aura, 2008).

In this study, two monomeric and 3 dimeric polyphenols from the seeds of *Pheonix dactylifera* L. were monitored in gastric and intestinal digestion stages. These polyphenols were identified in a previous study using HPLC-MS as date seeds extractable polyphenols (EPP) and included, catechin, epicatechin, procyanidin B1, procyanidin B2, procyanidin A2 (Sirisena *et al*., 2017). These were the most abundant date seed polyphenols, and those confirmed with HPLC-MS and internal standard spiking, therefore were selected to be monitored in this digestion study.

The reason for not comparing the initial quantities of polyphenols in the DSP to the polyphenol quantities in SGF is due to the effect of extraction process on the quantities. The quantities extracted by organic solvents from the raw DSP cannot be directly compared with the quantities released in the gastric juice, which acts somewhat as an extraction itself. Therefore it makes more sense to compare the quantities of DSP polyphenols released in the gastric stage and how much is available in the soluble intestinal stage (i.e. bioaccessible).

We determined the level of free (therefore accessible) and matrix bound (therefore not accessible) LMW polyphenols from DSP and DSPY after *in vitro* gastric or sequential gastric-intestinal digestion. Free polyphenols were defined by their availability in the supernatant fraction of the digesta as detected by HPLC after centrifugation of the digestion fluid at 8422 g for 10 min (Figure 1). Similarly, bound LMW polyphenols associated with the DSP matrixes were recovered from the 8422 g pellet by organic solvent extraction, and detected by HPLC. The recoveries of the target polyphenols from the combined free and bound fractions were high, indicating most of polyphenols in the digestion protocol can be accounted for. This good recovery conclusion was based on comparison with the polyphenol recoveries from DSP (84-99%) and DSPY (81-99%) in gastric or intestinal digesta without centrifugation (Supplementary Tables 1 and 2).

Bioaccessibility and stability of polyphenols from DSP during gastric and intestinal digestion

Accessible polyphenols can be defined as polyphenols that are released from the food matrix into digestion fluids during digestion so that absorption can occur (Fu *et al.,* 2015). The accessibility of the target polyphenols from DSP ranged widely (Table 1). Accessibility of catechin, epicatechin, procyanidin B1 and procyanidin B2 ranged from 31.6-49.6% in gastric digest, 19.4-40.5% in fasted

303 and 27.1-47.2% in fed intestinal digests. By contrast, procyanidin A2 was not released during gastric
304 or intestinal digestions of DSP, indicating it may not be accessible for absorption.

305 In terms of absolute quantities, procyanidin B1 was the most abundantly accessible polyphenol from
306 DSP in gastric digest (191.6 ± 2.4 μg/g DSP), which was followed by procyanidin B2 (95.8 μg/g DSP),
307 catechin (45.43 ± 0.81 μg/g DSP) and epicatechin (18.25 ± 0.56 μg/g DSP). The levels in intestinal
308 digest were smaller, suggesting that most of these polyphenols were released during gastric
309 digestion. This indicated that the majority of these polyphenols were released in gastric fluid, which
310 was then carried forward to the intestinal digestion state. The intestine is the main site of absorption
311 for both polyphenolic monomers and dimers (Appeldoorn *et al.,* 2009a). The 4 polyphenols were
312 most likely released from their associations with proteins and fibres matrix within the DSP due to the
313 low pH conditions of the gastric fluid which loosen fibre matrixes (Oliveira and Pintado, 2015), and to
314 the action of gastric pepsin in unravelling polypeptide structures in digestions. For example, Işık *et*
315 *al.,* (2014) reported a 70% release of polyphenols embedded in pea protein isolate after 1 h
316 simulated gastric digestion with pepsin treatment. Still, a significant amount of the polyphenols in
317 DSP was associated with the DSP matrixes as indicated by their percentage accessibility. These
318 bound polyphenols were released from the matrix by solvent extraction with acetone/methanol and
319 quantified by HPLC, but covalently bonded polyphenols to matrix materials would not be extracted
320 by the procedure.

321 Most of the simulated digestion studies on polyphenols have used extracts or pure compounds, and
322 studies involving food matrices are limited. Properties on individual food matrices can have a
323 substantial effect on bioaccessibility of polyphenols as shown by our results. A solid food matrix such
324 as DSP has to be disrupted and the polyphenols solubilized into digestive fluids to be bio-accessible.
325 Most polyphenols are located in the plant cell vacuole and apoplast where they are usually
326 conjugated to monosaccharides, polysaccharides and proteins (Bohn *et al.,* 2015). Once they are
327 released from the cell upon cell wall break-down due to processing or metabolism, free polyphenols
328 may form interactive associations with proteins and dietary fibre in the food matrix (Padayachee *et*
329 *al.,* 2012).

330 The slightly lower polyphenol levels in intestinal fluids indicated loss of polyphenols had occurred
331 transiting from gastric to intestinal digestion. The levels of free polyphenols in the intestinal digest
332 compared to the gastric digest ranged from 64.3-92.3% for the fasted state to 91.9-96.5% for the fed
333 state (Table 1). Polyphenols are known to undergo irreversible structural changes by auto-oxidation,
334 isomerisation and conjugation in alkaline pH, but some species are more stable than others
335 dependent on the structure (Friedman and Jürgens 2000). Losses of polyphenols between gastric

and intestinal digestion stages have been observed before; for example, Correa-Betanzo *et al*., (2014) reported a loss of 44% in wild blue berry. The higher level of polyphenols in the fed state compared to the fasted state was also observed, and pointed to the enhanced solubility effect due to bile salts (Bohn, 2014). This effect has been attributed to the ability of bile salts to form micelles with the polyphenols enhancing their solubility and chemical stability (Silva *et al.,* 2014).

Bioaccessibility and stability of polyphenols from DSP yoghurt (DSPY) during gastric and intestinal digestion

Yoghurt is a good model system for fibre and polyphenol enrichment. DSP itself is a complex food matrix, and incorporation of yogurt protein and protein gel increases the complexity of the system to digestion. In addition to the dietary fibres present in DSP, milk proteins in yoghurt are also known to interact with polyphenols. The non-covalent association between polyphenols with α and β caseins is known to involve hydrophobic interactions and H-bonding, and a positive correlation has been observed between binding affinities and number of OH groups in polyphenol (Hasni *et al.,* 2011). Whey protein β-lactoglobulin, which exists as a dimer with each polypeptide containing a hydrophobic grove, is known to bind polyphenols strongly at that site (Jianbo *et al.,* 2011, von Staszewski *et al.,* 2012).

Yoghurt has a possible impact on the accessibility of polyphenols from DSP as a consequence of yoghurt culture fermentation and heat treatment during yoghurt manufacturing process. Accessibility for all 5 polyphenols in the gastric digestion of DSPY was higher than for DSP, and ranged from 44.2-95.8% (Table 2). The increase in accessibility of procyanidin B1, procyanidin B2, and epicatechin from DSPY, compared to DSP were 1.5, 1.7 and 2.8 folds, respectively. In particular, procyanidin A2, which was not accessible from DSP, was highly accessible from DSPY. Appeldoorn *et al.* (2009b) reported that procyanidin A2 is better absorbed compared to procyanidin B1 and B2 in the rat intestine, indicating its' potential contribution to procyanidin related health benefits.

Another notable effect of yoghurt on accessibility was the large increase in the absolute quantities of free catechin, epicatechin, procyanidin B2 and procyanidin A2 in gastric digest, which were 76.0 ± 2.1, 150.1 ± 1.1, 431.7 ± 7.9, and 327.8 7.8 µg/g DSP, respectively (Table 2). Level of procyanidin B1 in DSPY gastric digest (71.9 ± 1.8 µg /g DSP) was however lower than from DSP gastric digestion (95.8 ± 1.2 µg/g DSP). This could be a result of the loss of procyanidin B1 in gastric digest due to its characteristic chemical instability at very low pH (Dallas *et al*., 2003).

The higher absolute quantities of free polyphenols would suggest that yoghurt culture fermentation has increased the level of monomeric and dimeric polyphenols from DSP and also have a positive effect on polyphenol release from DSP matrix. These effects have been observed before in other studies on polyphenol enrichment with yoghurts. Sun-Waterhouse *et al.* (2013) reported that pre-fermentation affected the profile and quantity of accessible polyphenols by over 3 folds compared to post fermentation with the addition of polyphenols to yoghurt.

Since DSP was added prior to heat treatment in yoghurt production, it might be possible that heat treatment has contributed to the higher level of free polyphenols. Indeed, adding DSP to the same milk mixture used to make the yoghurt and subjected it to yoghurt making heat treatment similar to, but without the bacterial fermentation step, increased the released of the polyphenols by 10-25% compared to unheated control (data not shown). Thus, heat treatment in yoghurt making may increase the free polyphenol levels in gastric digest.

Similar to DSP, the levels of the free polyphenols in DSPY intestinal digestion did not exceed the levels in DSPY gastric digest, indicating that they were completely released during gastric digestion. However, the stability in DSPY intestinal digestion was significantly ($p<0.05$) lower than their stability in DSP intestinal digestion for some polyphenols, but was similar for others in this complex system. It ranged from 31.5-96.5% in fasted state, and 35.4-99.5% in fed state when compared to the levels in DSPY gastric digest (Table 2). Epicatechin was least stable with less than 35% remaining while procyanidin B2 and A2 were the most stable with >87% remaining in intestinal digest. These differences could be due to different affinities of polyphenols towards milk proteins, which affected their stability in the intestinal pH. For example, Arts *et al.* (2002) observed that β-casein strongly masked the antioxidant activity of epicatechin compared with catechin at pH 7.4, indirectly indicating differences in binding affinities.

Colonic fermentation

The insoluble materials from DSP and DSPY intestinal digestion were carried forward to the colonic fermentation phase. The soluble materials were not carried forward as they are commonly assumed to be absorbable from the intestine (Bohn, 2014). In addition to monitoring fermentation products from polyphenols, pH changes and bacterial counts were also recorded (Supplementary Tables 3 and 4). The initial pH of faecal slurry was at pH 6.9, but after 24 h of fermentation the pH was decreased in all samples. As expected, the decrease in pH was more pronounced for DSP yoghurt, due to continuing activity of lactic acid bacteria from the yoghurt on the yoghurt contents (Fu *et al.,* 2016).

Aerobic and anaerobic bacterial counts in the original faecal slurry were 4.92 ± 0.05 and 8.61 ± 0.10 $\log_{10}$ CFU/mL, respectively. After 24 h fermentation, anaerobic counts increased by approximately 1 log CFU/mL, while aerobic counts showed a smaller increase, indicating that the fermentation conditions were more favourable towards anaerobic bacteria.

A number of bacterial fermentation peaks, not present in control faeces, were tentatively identified in DSPY based on spiking of the chromatographic peaks with commercial standards, which showed that polyphenol fermentation metabolites were produced (Supplementary Fig. 1). These included simple phenolic compounds such as 4-hydroxyphenyl acetic acid, 3-hydroxyphenyl acetic acid, 3-(4-hydroxyphenyl) propionic acid, ferulic acid, 3-(2-hydroxyphenyl)propionic acid and 3-propionic acid (Table 3). All these compounds have been previously identified in *in-vitro* human faecal fermentation of polyphenols (Aura, 2008).

3-Phenylpropionic acid was the most abundant phenolic acid, detected at 306.8 ± 9.6 - 488 ± 12 µg/g and 313.53 ± 7.6 - 371.3 ± 8.2 µg/g from DSP and DSPY, respectively after colonic fermentation. It is a known major metabolite from human gut microbial fermentation of flavanols, flavanones, flavones and polymeric flavan-3-ols (proanthocyanidin) and a final product of ring fission metabolic pathway of catechins (Aura, 2008). In addition, 3-(4-hydroxyphenyl)propionic acid was detected at 117.0 ± 5.8 - 123.9 ± 1.8 µg/g and 96.9 ± 1.0- 107.8 ± 4.1 µg/g DSP from DSP and DSPY, respectively. Rechner *et al.* (2004) reported this propionic acid derivative to be a fermentation metabolite of catechin and naringin.

B-type procyanidin dimers which are present in date seeds are known to produce 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone as the main fermentation metabolites (Appeldoorn *et al*., 2009a). The same authors reported also 3-hydroxyphenyl acetic acid, 4-hydroxyphenylacetic acid, 3-hydroxyphenylpropionic acid, phenylvaleric acids, monohydroxylated phenylvalerolactone, and 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl) propan-2-ol in the B-type procyanidin dimer fermentation products. In this study, 3-hydroxyphenyl acetic acid was a major metabolite detected from both DSP (189.5 ± 3.5 - 213 ± 13) and from DSPY 114.9 ± 5.3 -188.6 ± 3.1), while 4-hydroxyphenyl acetic was detected at much lower levels (1.60 ± 0.10 - 45.2 ± 4.2 and 48.95 ± 0.54 - 59.0 ± 2.8 for DSP and DSPY, respectively).

Ferulic acid was detected at fairly high levels in DSP (140 ± 9.1 - 216 ± 6.3 µg/g DSP) and DSPY (372 ± 8.2 - 456 ± 10 µg/g DSP). Free ferulic acid has previously been detected in some date seed varieties (Al-Farsi and Lee, 2008), however not in the Deglet nour (Habib *et al*., 2014; Sirisena *et al*., 2017). Ferulic acid is usually found esterified with hemicelluloses (Aura, 2008), and it has been reported

that fibre-bound ferulic acid is released in human colon by the action of human colonic microbiota (Kroon *et al*., 1997; Rondini *et al*., 2004). Furthermore, ferulic acid was found in human urine as a colonic metabolite of condensed tannin (proanthocyanidin) (Aura, 2008). We can therefore speculate that ferulic acid found in the colonic fermentation samples were microbial metabolites of the high molecular weight proanthocyanidins, or those bound to hemicelluloses.

According to our results, none of the five major polyphenols we were monitoring in this study were detected in the colonic fermentation samples. Therefore remaining amounts were not reported. It can be assumed that apart from some amount of loss in extraction, majority was available in the supernatant intestinal fluid, and the remaining were transformed/fermented to other compounds during colonic fermentation.

Conclusions

This study provides good preliminary evidence that validates the claim of DSP as a functional ingredient. The major date seeds polyphenols, catechin, epicatechin, and procyanidin A2, B1 and B2, were stable during *in-vitro* gastric digestion. While some losses occur with follow up intestinal digestion, substantial amounts were bio-accessible in the intestinal stage as soluble materials. Yoghurt culture, heating, and fermentation significantly increased the bioaccessibility of the polyphenols during gastric and intestinal digestion. Procyanidin A2, which was not accessible from DSP, was highly accessible from DSPY during digestion. Major phenolic metabolites from faecal bacteria fermentation of the digestion insoluble polyphenols were detected, and included 3-phenylpropionic, 4-hydroxyphenyl acetic, 3-(2-hydroxyphenyl) propionic acid, 3-(4-hydroxyphenyl)-propionic, 3-hydroxyphenyl acetic acid and ferulic acid. Thus, DSP can release substantial amounts of polyphenols upon gastro-intestinal digestion, and therefore can potentially be added to food products without further extraction. DSP may provide further health benefits by the production of phenyl derivatives of short chain fatty acid as a result of colonic fermentation of the insoluble polyphenols.

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Conflicts of Interest: None

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Legend to Figures

Figure 1: HPLC chromatograms of soluble fraction of digesta; (A) Date seed powder (DSP) gastric digest, (B) DSP intestinal fasted states digest, (C) DSP intestinal fed states digest, (D) Date seed powder yoghurt (DSPY) gastric digest, (E) DSPY intestinal fasted states digest, (F) DSPY intestinal fed states digest. Compounds were identified by a HPLC internal standard spiking method which was correlated by UHPLC-MS analysis in a separate study (Sameera et al. 2017): (1) procyanidin B1, (2) catechin, (3) procyanidin B2, (4) epicatechin (5) procyanidin A2.

Legend for supplementary files

Supplementary Figure 1: HPLC chromatograms colonic fermentation products of date seed powder (DSP) (A) fasted state (B) fed state and date seed powder yoghurt (DSPY) fasted (C) and fed (D) states. Compounds were tentatively identified by a HPLC internal standard spiking method: (1) 4-hydroxyphenyl acetic, (2) 3-hydroxyphenyl acetic, (3) 3-(4 hydroxyphenyl)propionic acid, (4) ferulic acid, (5) 3-(2-hydroxyphenyl) propionic acid, (6) 3-phenylpropionic acid. Insert shows a blow up region of the chromatogram.

Supplementary Table 1: Recovery of polyphenols from date seed powder (DSP) after gastric and intestinal digestion

Supplementary Table 2: Recovery of polyphenols from date seed powder yoghurt (DSPY) after gastric and intestinal digestion

Supplementary Table 3: pH changes in date seed powder (DSP) and date seed powder yoghurt (DSPY) colonic fermentations and control faecal slurry fermentation

Supplementary Table 4: Aerobic and anaerobic bacterial counts before and after colonic fermentation of date seed powder (DSP), date seed powder yoghurt (DSPY) and control samples

Table 1: Quantification of free and bound polyphenols from DSP after SGF and SIF-Fasted and SIF-Fed digestion

	Free Polyphenols* FPP (μg/g DSP)	Matrix Bound Polyphenols** BPP (μg/g DSP)	Total FPP + BPP (μg/g DSP)	Accessibility FPP/(FPP+BPP) (%)
SGF				
Catechin	45.43 ± 0.81^{b}	50.75 ± 0.87^{b}	96.18	47.2
Epicatechin	18.25 ± 0.56^{a}	39.3 ± 1.5^{a}	57.55	31.6
Procyanidin B1	191.6 ± 2.4^{d}	473.8 ± 5.3^{d}	665.4	28.7
Procyanidin B2	95.8 ± 1.2^{e}	97.0 ± 1.2^{e}	192.8	49.6
Procyanidn A2	ND	25.51 ± 0.28^{f}	25.51	0
SIF-Fasted				
Catechin	40.3 ± 1.1^{g} (88.8%)***	59.2 ± 1.0^{h}	99.5	40.5
Epicatechin	11.75 ± 0.33^{f} (64.3%)	48.6 ± 1.0^{g}	60.35	19.4
Procyanidin B1	177.0 ± 1.5^{i} (92.3%)	506.9 ± 3.7^{j}	683.9	25.8
Procyanidin B2	69.6 ± 3.0^{j} (72.6%)	112.5 ± 2.0^{k}	182.1	38.2
Procyanidn A2	ND	23.5 ± 1.3^{l}	23.5	0
SIF-Fed				
Catechin	43.88 ± 0.95^{l} (96.5%)	49.00 ± 0.59^{b}	92.88	47.2
Epicatechin	16.93 ± 0.85^{k} (92.7%)	41.4 ± 1.1^{a}	58.33	28.9
Procyanidin B1	181.6 ± 1.7^{o} (94.7%)	488.2 ± 5.6^{n}	669.8	27.1
Procyanidin B2	88.15 ± 0.62^{p} (91.9%)	98.83 ± 0.45^{e}	186.98	47.1
Procyanidn A2	ND	23.62 ± 0.56^{l}	23.63	0.00

*Solubilised polyphenols associate with the 10 000g supernatant of the digesta, which was extracted from the supernatant materials into methanol/acetone and analysed by HPLC

** Insoluble polyphenols associate with the 10 000g precipitate of the digesta, which was extracted from the precipitated materials into methanol/acetone and analysed by HPLC

***Stability of polyphenol expressed as %: (free polyphenol in SIF)/(free polyphenol in SGF)

$^{a,b,c...}$ Means followed by different superscript letters within each column indicates statistically significant values ($P<0.05$)

Table 2: Quantification of free and bound polyphenols from DSPY after SGF and SIF Fasted and SIF-Fed digestion

	Free Polyphenols* FPP (μg/g DSP)	Matrix Bound Polyphenols** BPP (μg/g DSP)	Total FPP + BPP (μg/g DSP)	Accessibility (%) FPP/(FPP+BPP) (%)
SGF				
Catechin	76.0 ± 2.1^{b}	61.4 ± 1.2^{b}	137.4	55.3
Epicatechin	150.1 ± 1.1^{a}	14.67 ± 0.56^{a}	164.77	91.1
Procyanidin B1	71.9 ± 1.8^{d}	90.7 ± 2.8^{d}	162.7	44.2
Procyanidin B2	431.7 ± 7.9^{e}	56.5 ± 1.0^{e}	488.2	88.4
Procyanidn A2	327.8 ± 7.8^{f}	42.48 ± 0.44^{a}	1026.08	95.8
SIF-Fasted				
Catechin	48.2 ± 1.8^{g} (63.2%)***	80.8 ± 2.8^{f}	129.0	37.3
Epicatechin	47.4 ± 0.25^{g} (31.5%)	80.2 ± 1.4^{f}	127.6	37.1
Procyanidin B1	69.5 ± 2.1^{d} (96.5%)	71.9 ± 1.4^{h}	141.4	49.1
Procyanidin B2	309.5 ± 3.4^{i} (71.6%)	162.0 ± 2.2^{i}	471.5	65.6
Procyanidn A2	285.53 ± 1.3^{j} (87.0%)	44.07 ± 1.6^{j}	329.6	86.6
SIF-Fed				
Catechin	55.7 ± 2.5^{k} (73.2%)	88.1 ± 1.3^{k}	143.8	38.7
Epicatechin	53.2 ± 0.95^{k} (35.4%)	93.8 ± 5.5^{d}	147.0	36.1
Procyanidin B1	71.6 ± 3.0^{bd} (99.5%)	97.13 ± 1.4^{m}	168.73	42.4
Procyanidin B2	229.03 ± 4.2^{f} (53.0%)	149.06 ± 3.1^{n}	378.09	60.5
Procyanidn A2	298.8 ± 3.9^{l} (91.1%)	31.13 ± 0.85^{g}	329.93	90.5

* Solubilised polyphenols associate with the 10 000g supernatant of the digesta, which was extracted from the supernatant materials into methanol/acetone and analysed by HPLC

** Insoluble polyphenols associate with the 10 000g precipitate of the digesta, which was extracted from the precipitated materials into methanol/acetone and analysed by HPLC

***Stability of polyphenol expressed as %: (free polyphenol in SIF)/(free polyphenol in SGF)

$^{a,b,c...}$ Means followed by different superscript letters within each column indicates statistically significant values ($P<0.05$).

Table 3 Quantification of polyphenol metabolites from colonic fermentation of bound polyphenols in DSP and DSPY after intestinal digestion

Peak no.	fermentation product	DSP colonic fasted (µg/g DSP)	DSP colonic fed (µg/g DSP)	DSPY colonic fasted (µg/g DSP)	DSPY colonic fed (µg/g DSP)
3	4-hydroxyphenyl acetic	1.60 ± 0.10[a]	45.2 ± 4.2[b]	48.95 ± 0.54[b]	59.0 ± 2.8[c]
4	3-hydroxyphenyl acetic	213 ± 13[a]	189.5 ± 3.5[b]	114.9 ± 5.3[c]	188.6 ± 3.1[d]
5	3-(4-hydroxyphenyl)-propionic acid	117.0 ± 5.8[a]	123.9 ± 1.8[a]	107.8 ± 4.1[b]	96.9 ± 1.0[b]
6	Ferulic acid	216.2 ± 6.3[a]	140.8 ± 9.1[b]	372.4 ± 8.2[c]	456 ± 10[d]
7	3-(2-hydroxyphenyl) propionic acid	5.25 ± 0.58[a]	4.89 ± 0.31[a]	0.850 ± 0.020[b]	1.8600 ± 0.0010[c]
8	3-phenylpropionic	306.8 ± 9.6[a]	488 ± 12[b]	313.53± 7.6[a]	371.3 ± 8.2[c]

[a,b,c...] Means followed by different superscript letters within each row indicates statistically significant values ($P<0.05$).

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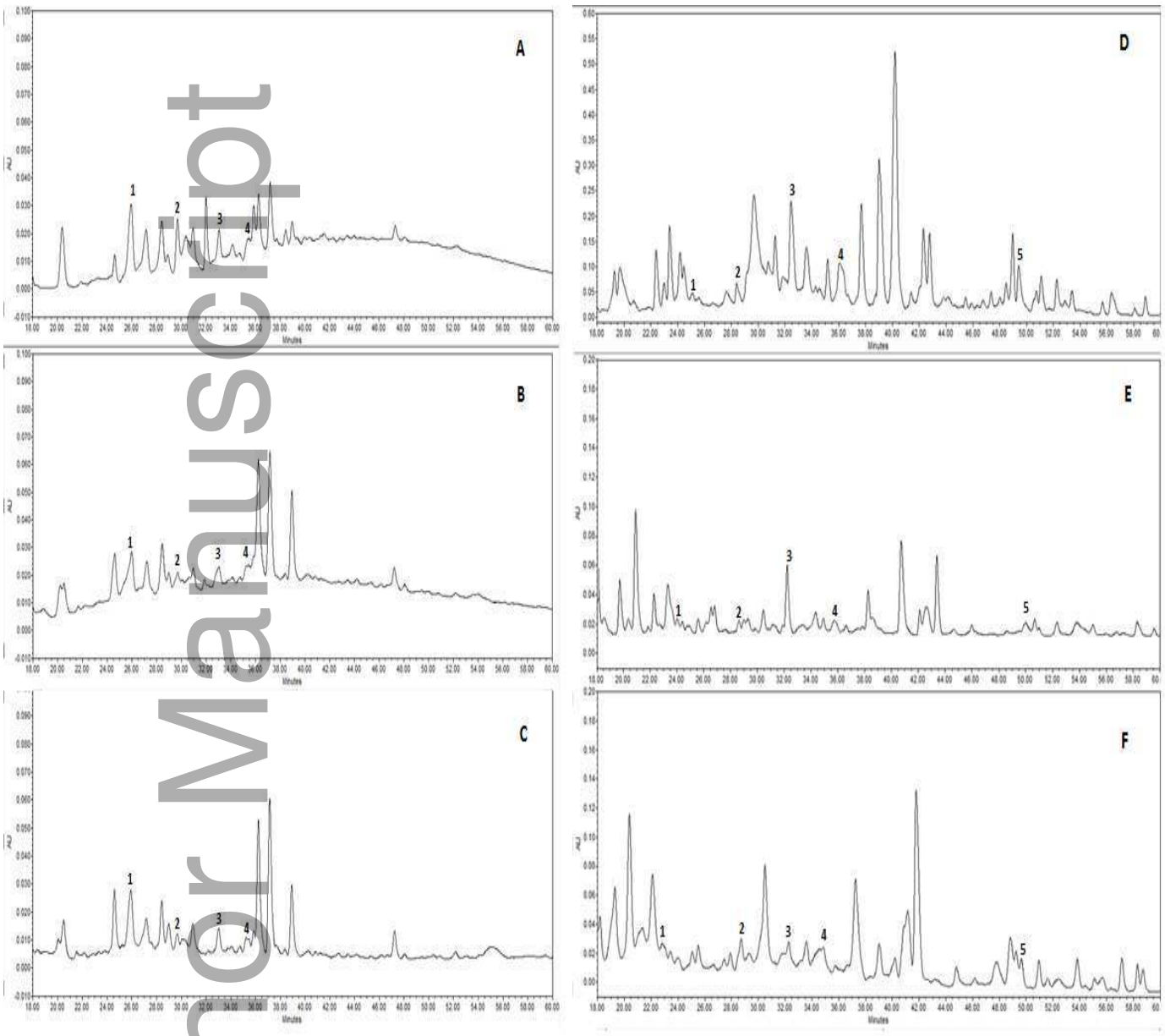

Figure 1: HPLC chromatograms of soluble fraction of gastric and intestinal digesta. (A) DSP gastric digest, (B) DSP intestinal fasted states digest, (C) DSP intestinal fed states digest, (D) DSPY gastric digest, (E) DSPY intestinal fasted states digest, (F) DSPY intestinal fed states digest. Compounds were identified by a HPLC internal standard spiking method which was correlated by UHPLC-MS analysis in a separate study (Sameera et al. 2017): (1) procyanidin B1, (2) catechin, (3) procyanidin B2, (4) epicatechin (5) Procyanidin A2.