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DIGESTION AND ABSORPTION OF PHENOLIC COMPOUNDS ASSESSED BY *IN VITRO* SIMULATION METHODS. A REVIEW

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ABSTRACT

Phenolic compounds are a group of key plant metabolites found abundantly in fruit and vegetables. Because of their antioxidant properties, they play a significant role in preventing various degenerative illnesses, tumours or cardiovascular disease. In nature, they are present in foods mainly as esters, glycosides and polymers which need to undergo enzymatic hydrolysis in the digestive tract or by the gut microflora before becoming absorbed. The biological properties of these phenolic compounds undergoing this degradation, are thus governed by their absorption as well as metabolism. Many methods are used to assess the rates and the degrees to which these substances are digested and absorbed, both *in vivo* and *in vitro* ones, where the former are the most reliable, although they suffer from various limitations. For this reason, many *in vitro* models have now arisen to simulate the function of human digestion in the attempt to faithfully re-create real-life conditions. Mechanisms of polyphenols absorption have been principally studied by intestinal epithelial cell models, in particular, those using the Caco-2 cell line.

Key words: *polyphenolic compounds, gastrointestinal tract, Caco-2 cells, in vitro digestion*

STRESZCZENIE

Związki fenolowe są grupą roślinnych metabolitów występujących obficie w owocach i warzywach. Dzięki swoim właściwościom antyoksydacyjnym odgrywają istotną rolę w prewencji chorób degeneracyjnych, np. nowotworów lub chorób układu krwionośnego. W naturze większość związków polifenolowych jest obecna w pożywieniu w postaci estrów, glikozydów i polimerów, które aby ulec absorpcji muszą zostać zhydrolizowane przez enzymy trawienne bądź mikroflorę jelitową. Absorpcja oraz metabolizm związków polifenolowych zachodzące w przewodzie pokarmowym człowieka wpływa na ich właściwości biologiczne. Istnieje wiele metod *in vivo* oraz *in vitro* oceniających stopień i szybkość trawienia oraz przyswajanie związków polifenolowych. Metodami zapewniającymi uzyskanie najbardziej pewnych wyników są metody *in vivo*, jednak posiadają one wiele ograniczeń. Dlatego też powstało wiele modeli in vitro symulujących pracę układu pokarmowego człowieka i odtwarzających wiernie warunki w nim panujące. Do badań nad mechanizmami absorpcji związków polifenolowych przez nabłonek jelit używane są modele kultur nabłonkowych *in vitro*, w szczególności z wykorzystaniem linii komórkowych Caco-2.

Słowa kluczowe*: związki polifenolowe, układ pokarmowy, komórki Caco-2, trawienie in vitro*

INTRODUCTION

The naturally abundant phenolic compound metabolites found in plants, principally arise from the shikimate synthesis pathway as well as during the metabolism of phenylpropanoids. There are many reasons why plants make polyphenols; the main one being for various mechanisms of self defence. They are active in a plant's reaction to stress and they also exhibit a wide spectrum of antibacterial, antiviral and antifungal action [23]. Furthermore, polyphenols safeguard plant cells from the harmful effects of UV light as well as participating in cellular repair mechanisms [34]. Some polyphenols also confer organoleptic properties to plants such as colouration and taste. These compounds are usually divided into 4 structural groups, based on a common carbon skeleton, that consist of phenolic acids, flavonoids, stilbens and lignans [10].

Due to their antioxidant properties, the physiological roles of polyphenols include preventing various con-

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ditions, like cancer, coronary artery and cardiovascular diseases [12, 26, 36]. This stems from their ability to act as reducing agents, (ie. electron or hydrogen donors). They also possess metal-chelating properties, especially for iron and copper ions. The particular structure of polyphenols permits them to scavenge free radicals even more effectively than the antioxidant vitamins A and C [29]. The ability of flavonoids to arrest tumour formation is significant, as demonstrated by a twice greater probability of contracting cancer in persons whose diet is poor in fruit and vegetables compared to those with a high consumption. One way this is achieved is through the scavenging of free radicals by flavonoids which prevents DNA damage and thus the development of any changes that could result in pathologies so arising. Another way is by activating phase I and II enzymes which increases carcinogen excretion. If cellular changes leading to tumour formation have already occurred, then flavonoids can also assist apoptosis, thus resulting in the cell death of those that have mutated [3, 14, 20].

The physiological effect of polyphenols is wide ranging in humans. They inhibit certain enzymes of mitochondrial respiration and possess anti-inflammatory action due to inhibition of 5-lipoxygenase and cyclooxygenase. Through various mechanisms, they regulate the function of certain blood factors and that of smooth muscle. Liver protection is also afforded and isoflavones, isolated from legumes, are active ingredients of many products that women use to ameliorate the symptoms of the menopause [13].

Negative health effects of polyphenols are however seen when an upper dose limit is exceeded. Consuming 1-1.5 g of flavonoid based medicines is toxic, resulting in haemolytic anaemia, liver inflammation, fever etc. Teas that contain high amounts of polyphenols, especially tannin, can through their chelating action decrease iron uptake during digestion thereby leading to iron deficiency. Nevertheless, if a varied diet is normally consumed such toxic effects of polyphenols are extremely unlikely [5].

DIGESTION AND ABSORPTION OF POLYPHENOLS

As aforementioned, the absorption and metabolism of polyphenols in the digestive tract governs their biological properties. Only those released from the food matrix in the small and large intestine are digested. Polyphenols occur in foods mainly as esters, glycosides and polymers which cannot be absorbed in these native forms and thus require hydrolysis by digestive system enzymes or intestinal microflora. It is estimated that 48% of polyphenols are digested in the small intestine and 42% in the large intestine. Just 10% are undigested and remain intact within the food matrix. Of all the polyphenols, only aglycones are able to pass through biological membranes on account of being highly lipophilic. Another factor influencing the bioavailability of polyphenols is the nature of the food matrix itself. Polyphenols can react with some food constituents, eg. certain proteins and carbohydrates which can significantly affect how they are absorbed from digestion. Other important factors are the intestinal environment such as pH or the presence of bile salts [19].

Hydrolysis of glycoside flavonoids already starts in the mouth by means of β-glycosidase action, however its effectiveness is dependent on the types of sugars present in the molecule. Glucose conjugates are rapidly hydrolysed as opposed to others such as those of rhamnose [10]. In the stomach, due to the low pH, flavonoids oligomers degrade to smaller units. Of all the flavonoids, the flavon-3-ols exist as aglycones and pass intact into the duodenum. Deglycosylation, glucuronidation, methylation, sulphonation and hydroxylation of flavonoids then occurs in the small intestine. In the high pH conditions now therein, the flavonoid epigallocatechin gallate (EGCG), may become oxidised to very potent forms for scavenging free radicals and chelating ionic iron [32]. In addition, absorption of free phenolic acids occurs, which then conjugate with glucuronic acid. Esters of phenolic acids are however degraded by microbial esterases present in the large intestine [31].

Undigested polyphenols then pass into the large intestine, where they are subjected to further degradation into phenolic acids by colonic microflora as has been demonstrated in many studies [7, 17, 28, 31]. Glycosides are hydrolysed by bacteria to aglycones which are then transformed into various acids through the action of β-glucosidase, β-rhamnosidase and esterases. In contrast to native enzymes, those of the microflora are able to catalyse the degradation of flavonoid chains into simple units. Furthermore, they can also perform hydrolysis, dehydroxylation, demethylation and decarboxylation. So depending on the structure of polyphenols, a large variety of substances can thus be produced. Flavonols give rise to hydroxyphenylacetic acids whereas flavones and flavanones degrade to hydroxyphenylpropionic acids. Flavanols are however degraded to both phenylvalerolactone and hydroxyphenylpropionic acids. The metabolites of all these compounds are stages that ultimately lead to benzoic acid. Their presence is due to the action of gut microflora and they can be absorbed into the circulation where they bind to albumin and are transported to the liver. Here they undergo hydroxylation, demethylation, o-methylation as well as conjugation to glucuronide and sulphated derivatives through phase I and II enzymes. A significant part of these can, in later stages, be secreted together with bile back into the gut

where they again undergo hydrolysis and are either absorbed back or excreted via the faeces.

In the large intestine, the metabolism of anthocyanins depends on spliting glycosidic bonds and breaking the heterocyclic anthocyanin chain [2, 15], whereas metabolism of linseed lignans proceeds through microflora action, forming enterolactone and enterodiol products. The hydrolysis of one of isoflavones - daidzein leads to equol production that demonstrates high oestrogen receptor affinity. Conjugation of isoflavones to glucuronide and sulphated derivatives also occurs in the liver by UDP-glucuronosyltransferase and sulfotransferase enzymes, respectively. It is also assumed that flavonoids can be metabolised, without the aid of gut bacteria, through the action of phlorizin hydrolase, (glycosylceramidase), an enzyme of the small intestinal brush-border membrane [6].

METHODS FOR SIMULATING THE DIGESTION AND ABSORPTION OF POLYPHENOLS

There are many methods available to determine the rate and to what degree polyphenols are digested and absorbed; both *in vivo* and *in vitro*. The former are considered to be the most reliable, where quantitative analyses of suitable biomarkers found in urine, blood or faeces can be performed [27, 30]. Nevertheless, they are time consuming and difficult owing to very low analyte concentrations, coupled with high background interference. For these reasons many functional models were developed so that the conditions of the digestive tract could be faithfully simulated; a principal example being the TIM (TNO-Intestinal Models), as developed by the Netherlands Organization for Applied Scientific Research. Such a computer backed system allows studies to be performed on nutrient absorption, interactions between nutritional and functional food compounds, how food processing affects the nutritional/functional qualities of food and the effectiveness of prebiotics' action throughout the digestive tract. The system is available in two complementary parts. TIM-1 simulates the action of the stomach and small intestine, amongst which it is possible to control parameters like, temperature, digestive juices flow (eg. saliva, gastric/pancreatic juice) etc. The release of digestive enzymes and bile is simulated and the stomach and small intestine pH can be regulated. Adjustments can also be made for factors such as subject age, (ie. infant, adult or elderly), or the type of foodstuff(s) consumed. The TIM-2 simulates colonic conditions through regulation of pH, anaerobiosis and the metabolically active and diverse human gut microflora, where metabolites can be suitably removed from the system [21].

Several other models for simulating human digestion have been developed. This includes the Dynamic Gastric Model (DGM), developed by the UK Institute of Food Research that allows control in the stomach, amongst other parameters, over acidity, the quantity and rate of digestive enzyme release and physical mixing of stomach contents [21]. *In vitro* simulation, performed by *Gil-Izguierdo* et al. [8], studied polyphenols assimilation from orange and strawberry juice and strawberry jam. In the stomach, polyphenol digestion lasted 2 hours at 37ºC under pepsin-HCl conditions, (pH=2). Small intestinal digestion however took 2.5 hours with pancreatin and bile salts at 37ºC, pH=7. Simulated conditions were arranged so as to ensure correct pH control and maximum contact of the digesting mixture with the dialysis membrane. Results showed no effects of gastric digestion on polyphenols, whereas strawberry anthocyanins were transformed to chalcones and ellagitannins into free ellagic acid in the small intestine [8].

The bioconversion of polyphenols through gut microflora digestion was studied by *Gross* et al. [11] using combined faecal samples, as a microflora source, collected from 10 healthy volunteers. The fermentation of polyphenol compounds was thus studied, under anaerobic conditions of the large intestine, that were present in black tea, wine and grape juice. Subsequent analyses by MR (Magnetic Resonance) and GC/MS (Gas Chromatography/Mass Spectrometry), spectrometry revealed a large range of metabolites so arising. Metabolites such as pyrogallol and gallic acid were only present before fermentation in black tea whereas vanillic acid was almost undetectable. The latter was however found to be a metabolite of polyphenols in the wine/grape juice mixture. It was suggested that colonic microflora diversity amongst the population may result in various polyphenols metabolites occurring, with consequently varying effects on the human body [11]. Another study, *Steer* et al. [33] looked at the effect of gut microflora cultures (taken from the colons of volunteers) on genistin metabolism, also under anaerobic fermentation conditions. The assessment used a three-stage continuous culture system representative of conditions found in the proximal, transverse and descending parts of the human colon. The pH in each part was held at 5.5, 6.2, 7.0, respectively. Fermentation samples were then subjected to HPLC analysis. Within the first 24 hours, genistin was found to be rapidly metabolised where gut bacteria such as *Lactobacillus spp*., *Bacteroides spp*., *Bifidobacterium spp* exhibit β-glucosidase activity, resulting in increased concentrations of the metabolite aglycone form of this compound, genistein, to occur [33].

The studies mentioned so far, deal only with the separated parts of polyphenolic digestion, however a study by *Aura* [2] uses a complete *in vitro* model, ie.

taking gastric and small intestine digestion together with colonic fermentation by microflora. This was focused on the metabolism of dietary plant polyphenols such as quercetin, anthocyanins and lignans found in rye or linseed bran. Due to the presence of other digestible nutrients present in the studied raw material, it was also necessary to ensure conditions, biochemical and physical, prerequisite for their removal through being digested; this especially concerning the maximal removal of starch. These for instance included appropriate pH, temperature (37 ºC), presence of pepsin/pancreatin so as to represent, as far as possible, true life conditions. As a result, around 89-95% of the starch was removed through digestion and proteins became partially hydrolysed. Microbial fermentation of the polyphenols was achieved using inocula of human faeces, in various doses, obtained from healthy volunteers and anaerobic conditions were strictly maintained throughout the experiments. It was also necessary to use low concentrations of polyphenols to limit their inherent antibacterial action, thus preventing such effects occurring in the test inocula. It was found that the bacteria enzymatically degraded the flavonoids into their corresponding phenolic acids. Quercetin aglycones became microbially converted to hydroxyphenylacetic acids through splitting of the aromatic ring. In similar fashion, anthocyanins underwent extensive metabolism however the turnover was low (5%) compared to quercetin (60%). Thus, anthocyanins can be excreted intact and will therefore have a low bioavailability. The main metabolite of cyanidin was identified as being protocatechuic acid whilst enterolactone was identified as being the metabolic product of lignans using a dense dosing of colonic inocula. The latter process was however inhibited in the presence of the rye-matrix through, it was suggested, the microbiotica being sensitive to non-physiological low pH due to presence of acidic rye bran components. The relatively long time (36-48 hours) required for conversion to enterolactone, attests to a low amount of those microorganism responsible being present [2].

In vivo studies on polyphenol absorption mechanisms are seriously hampered by the lack of access to the human intestinal epithelium. The alternative is to use experimental animal or cell culture models or the popular *in vitro* cell culture techniques [10]. When studying antioxidant absorption, a frequently used cell line is Caco-2 in which the following enzymes are produced; disaccaridase, carboxylase, peptidase, CYP450 isoenzymes, glutathione-S-transferase, sulphotransferase, carboxylesterase, UDP-glucuronosyltransferase and glucuronidase [10].

Using HPLC analysis, a study by *Liu* and *Hu* [18] investigated the absorption and metabolism of flavonoids using the Caco-2 cell line, under conditions closely resembling those of the human digestive tract. The absorption of flavonoid aglycones was found to be very rapid, whereas glycoside uptake by the monolayer of Caco-2 cells, via the SGLT1 transporter, was weak and slow. Another study, *Brand* et al. [4], used a 2-compartment transwell monolayered Caco-2 cell system to mimic the intestinal barrier for investigating the metabolism of the citrus flavonoid hesperetin. Analyses were performed by HPLC with photodiode array detection. Results demonstrated very intensive metabolism of hesperetin into hesperetin 7-O-glucuronide and hesperetin 7-O-sulphate which were chiefly transported to the apical side of the monolayer; unconjugated hesperetin moved to the basolateral side [4]. A further uptake study by *Murota* et al. [24] was performed using the Caco-2 line epithelium model on intestinal transport of genistein, daidzein and their glycoside equivalents, genistin and daidzin. This showed that the isoflavone aglycones forms were much more efficiently taken up by enterocytes than the glycoside ones. The inefficient transport of the latter was due to a lack of binding affinity to the Caco-2 cellular membranes. It is also equally likely that because of the location of the ring within the diphenylpropane structure, intact aglycones forms of isoflavonoids can be transported towards the basolateral side; as opposed to the aglycone flavonoids [24].

SUMMARY

All the aforementioned methods that mimic degradation and uptake of polyphenols, provide an assessment on how these compounds are transformed by human digestion. Essentially this consists of releasing polyphenols from consumed foodstuffs, their degradation/digestion, uptake or metabolism [9]. Throughout this, direct study of the human digestive tract is impossible and any *in vivo* investigations are fraught with difficulty, and are thereby confined to the analysis of blood, urine or faeces. The main factors confounding reliable analyses are the presence of contaminants and low analyte concentrations, as well as rapid polyphenol metabolism. When using animal models, a main drawback is to find an equivalent, appropriate to human physiology and metabolism. These types of studies chiefly use rats, nevertheless they do not fully reflect the conditions of the human digestive tract. However, by combining complementary studies with the animal experiments, it then becomes possible to achieve acceptable outcomes. *Liu* and *Hu* [18] studied flavonoid transport in Caco-2 layers simultaneously with their absorption and metabolism in rat intestines. Here, the Caco-2 model reflected membrane transport whilst the animal experiments accounted for the otherwise lack of the phase I and II enzymes. It should be noted that all

animal experiments require ethical committee approval which is not always easy to obtain [10]. The artificial TIM system model however mimics human digestion perfectly well, where all the necessary digestive enzymes are present and released nutrient components can be absorbed through membranes, during which intestinal peristalsis is also simulated. Despite this the equipment remains prohibitively expensive.

Another system imitating human digestion is that used by *Gil-Izquierdo* [8] which accurately reflects the conditions found in the stomach and small intestine; the microbial fermentation stage of the large gut is however missing. This is a major disadvantage for studying polyphenols, as a large part of their metabolism occurs there. In addition the recycling of absorbed compounds is unaccounted for by this model. Cell culture model systems of the intestinal epithelium do however possess many advantages. These include a short experimental time, convenient multiple replication and the ability to analyse a large number of metabolic compounds. It is assumed that effective absorption of compounds *in vitro* is matched to that *in vivo*.

The Caco-2 system has also many significant disadvantages. Epithelium *in vivo* is many times more dense than the monolayer cellular layers used which accounts for subsequent differences in permeability. A study by *Artursson* et al. [1] demonstrated a 20- and 100-fold slower transport of compounds through the Caco-2 layer compared respectively to the large and small intestine. Another cause for these differences is the lack a blood capillary network in the monolayer together with the absence of any central nervous system (CNS) control. Furthermore, the Caco-2 cells in most studies are subjected to either native or extract-rich aglycone polyphenols which are not the forms that tissue or blood are exposed to *in vivo*. The concentrations of these analytes also need to be closely related to that found in plasma following a meal rich in polyphenols [16]. Work on cultured epithelial cell lines which increase their similarity to those of the intestine is thus being undertaken; the introduction of genes encoding for various enzymes and membrane transport proteins now holds great future promise [9].

It is however not always necessary to use such complicated and automated equipment for modelling and assessing the digestion and uptake of nutrients. Systems like the TIM can be replaced by cheaper models such those using standard laboratory apparatus. This was exploited in studies by *Miller* et al. [22] and *Wolfgor* et al. [35], where various conditions present, such as pepsin, bile salts, appropriate temperature (37ºC), pH (2.0 or 7.4) etc., were arranged to mimic those of the stomach or small intestine. Instead of hollow fibre membranes, as used by the sophisticated systems, dialysis tubing was used (pore sizes ranging 6000-8000 Da) [25].

It can be seen that new methods for studying and analysing the metabolism of consumed nutrients are all leading towards full automation. Such improvements can thereby, in the future, allow precisely defined studies to be undertaken which more closely mirror *in vivo* conditions.

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