Journal of Functional Foods 16 (2015) 403–413

Available online at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/jff

Use of metabolomics and fluorescence recovery after photobleaching to study the bioavailability and intestinal mucus diffusion of polyphenols from cauliflower waste

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ARTICLE INFO

Article history:
Received 17 February 2015
Received in revised form 22 April 2015
Accepted 24 April 2015
Available online 19 May 2015

Keywords:
Bioavailability
Polyphenols
Intestinal mucus
Caco-2 cells
In vitro digestion
Fluorescence recovery after photobleaching

ABSTRACT

The analysis of the bioaccessibility and bioavailability of polyphenols from cauliflower waste was achieved using targeted metabolomics and chemometric approaches. Changes in phenolic profile throughout the *in vitro* digestion and Caco-2 transport were investigated using LC-MS combined with principal components analysis and orthogonal partial least squares–discriminant analysis, and diffusion of polyphenols through intestinal mucus was monitored using fluorescence recovery after photobleaching (FRAPb). Results showed that recovery of polyphenols in the gastric phase was approximately 76–106% whilst losses of up to 70% were observed after the intestinal phase. Kaempferol-3-*O*-diglucoside and kaempferol-3-*O*-diglucoside-7-*O*-glucoside were found to permeate intact through the Caco-2 cells in very small amounts (0.3% recovery). Polyphenols also diffused rapidly through intestinal mucus without altering their biophysical properties. We conclude that targeted metabolomics and FRAPb are rapid and convenient tools to study the recovery of polyphenols during *in vitro* digestion, mucosal diffusion and Caco-2 transport.

1. Introduction

Polyphenols have long been studied due to their health-promoting properties, such as antioxidant, antiviral, hepatoprotective, and immune-regulatory activities (Middleton, Kandaswami, & Theoharides, 2000). In fact, daily consumption of polyphenol-rich foods has been associated with improved cardiovascular health and protection against cancer and other degenerative diseases (Baboota et al., 2013; Vita, 2005).

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http://dx.doi.org/10.1016/j.jff.2015.04.031
1756-4646/

However, from a physiological perspective, ingested polyphenols are subjected to gastrointestinal digestion and extensive metabolism by the epithelium. Several papers have been devoted in understanding the metabolic fate of polyphenols along the digestive tract and intestinal absorption, especially in the case of polyphenol-rich foods.

Aside from food products, it has earlier been reported that agricultural waste products are an excellent source of polyphenols, which could be recovered and used as functional ingredients. By-products from cauliflower harvest, for example, have previously been reported as an excellent source of polyphenols (Gonzales, Raes et al., 2014; Gonzales, Smagghe, Raes, & Van Camp, 2014; Llorach, Espín, Tomás-Barberán, & Ferreres, 2003; Llorach, Gil-Izquierdo, Ferreres, & Tomás-Barberán, 2003). However, little is known about the fate of these polyphenols during digestion.

Conventionally, bioaccessibility assessment was done by measuring polyphenol recovery at various stages of *in vitro* digestion using the Folin method, which provides the total phenolic content that is often expressed as gallic acid equivalents (or equivalents of other more closely related polyphenols). Although widely used, this method does not provide detailed information on the changes in the phenolic composition at every step of the digestion process. Because of this, HPLC techniques are often done to gather detailed information on the recovery of each of the polyphenols. In HPLC, polyphenols are characterized and quantified using equivalents due to the lack of standards for most phenolic compounds. Whilst this is an accepted method when using diode array detection (DAD), quantification using mass spectrometry requires exact standards since different compounds have different abundances at certain ionization conditions. Hence, quantification using DAD is preferred for complex matrices. However, polyphenols are often difficult to separate in liquid chromatography especially when the plant matrix contains many different compounds causing co-elution, and subjecting it to *in vitro* digestion presents more peaks and noise to the chromatogram. Therefore, relying on the HPLC chromatogram to analyze the recovery of individual compounds may not be easy and accurate. Also, integrating each chromatographic peak may be cumbersome when dealing with plant extracts due to the high number of polyphenol species present. Methods to analyze the recovery of polyphenols and the changes in its distribution in a reliable and convenient manner therefore need to be sought.

After intestinal digestion, polyphenols are faced with an immovable mucus layer that protects the epithelium from luminal contents whilst allowing the entry of nutrients (Mackie, Round, Rigby, & Macierzanka, 2012). Therefore, polyphenols need to diffuse through the mucus layer efficiently prior to reaching the epithelium. However, studies on the diffusive properties of polyphenols through intestinal mucus and their interactions have not been previously reported.

The use of Caco-2 cells to assess intestinal uptake of polyphenols is widely practiced (Farrell, Poquet, Dew, Barber, & Williamson, 2012; Gonzales, Van Camp et al., 2014; Németh et al., 2003). However, no report on the Caco-2 metabolism and transport of cauliflower polyphenols could be found in the literature. This information is crucial to ascertain the final bioavailability of the polyphenols and to determine which polyphenols survive the entire gastrointestinal digestion and absorption process.

Given these open questions, this study was performed to assess the bioavailability of cauliflower waste polyphenols through digestion using an *in vitro* digestion model and Caco-2 transport experiments. More importantly, the use of multivariate analysis techniques, principal component analysis (PCA) and orthogonal partial least squares–discriminant analysis (OPLS-DA), to determine the changes in the polyphenolic profile at every stage of digestion was explored. Further, the study aimed to measure the diffusion of the polyphenols in the intestinal digesta through an *ex vivo* intestinal mucus and to investigate the interaction of the polyphenols and the intestinal mucus.

2. Materials and methods

2.1. *Materials*

U(H)PLC–MS grade methanol and formic acid were acquired from Biosolve (Valkenswaard, The Netherlands). Analytical grade methanol used for extraction, HCl and NaOH were purchased from VWR International (Leuven, Belgium). Kaempferol, calcium chloride, pepsin, pancreatin, 2-aminoethyl diphenylborinate and 500-nm fluorescent polystyrene beads were purchased from Sigma-Aldrich (Poole, UK; Diegem, Belgium).

For the cell culture experiments, Dulbecco's modified Eagle's medium (DMEM) supplemented with Glutamax™ was purchased from Gibco (Langley, VA, USA) whilst foetal bovine serum was obtained from Greiner Bio-One (Wemmel, Belgium).

2.2. *Extraction of* Brassica oleracea *polyphenols*

Wastes from cauliflower (*B. oleracea L. var. botrytis*) left after harvest (July 2012) in the field were obtained in Ieper (Belgium) and stored at –20 °C, lyophilized and ground into fine powder. Extraction of the polyphenols was based on the method of Gonzales, Raes et al. (2014). Briefly, approximately 2 g of leaves were placed in 50 mL tubes and homogenized with 15 mL methanol at 10,000 rpm with the use of an ultraturrax for 45 s and then placed on ice for 15 s. The mixture was centrifuged at 13,000 × *g* for 10 min at 4 °C to obtain the supernatant (a) and the residue was re-extracted with 80% MeOH using the same procedure. After another round of centrifugation, the supernatant was collected (b) and added to (a). The volume of the extract was adjusted to 50 mL and was stored at –20 °C until further analysis for a maximum of five days.

2.3. *Simulated* in vitro *digestion*

An *in vitro* digestion model that mimicked the upper gastrointestinal stage of human digestion was adapted from Minekus et al. (2014). The methanolic extract was dried under reduced pressure and redissolved in distilled water to a concentration of approximately 100 μg/mL. A 1.5 mL aliquot was added with 1.2 mL of salivary fluid, 7.5 μL of 0.3M $CaCl_2$, 0.1 mL of amylase solution and 0.1925 mL of distilled water to attain a final volume of 3 mL. The mixture was mixed for 2 min in a