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Statistical modelling coupled with LC-MS analysis to predict human upper intestinal absorption of phytochemical mixtures

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ABSTRACT

A diet rich in phytochemicals confers benefits for health by reducing the risk of chronic diseases via regulation of oxidative stress and inflammation (OSI). For optimal protective bio-efficacy, the time required for phytochemicals and their metabolites to reach maximal plasma concentrations (T_{max}) should be synchronised with the time of increased OSI. A statistical model has been reported to predict T_{max} of individual phytochemicals based on molecular mass and lipophilicity. We report the application of the model for predicting the absorption profile of an uncharacterised phytochemical mixture, herein referred to as the 'functional fingerprint'. First, chemical profiles of phytochemical extracts were acquired using liquid chromatography mass spectrometry (LC-MS), then the molecular features for respective components were used to predict their plasma absorption maximum, based on molecular mass and lipophilicity. This method of 'functional fingerprinting' of plant extracts represents a novel tool for understanding and optimising the health efficacy of plant extracts.

1. Introduction

Phytochemicals, also referred to as secondary metabolites, are the non-nutrient compounds in fruits, vegetables and other dietary plants which have been associated with reductions in the risk of major chronic diseases including cancer (Key, 2011), cardiovascular (Dauchet, Amouyel, & Dallongeville, 2009) and neurodegenerative diseases (D'Onofrio et al., 2016). More than 200,000 phytochemical structures have been identified but only a small percentage have been investigated with regard to their application in medicine, making them interesting candidates as pharmaceutically active agents (Hartmann, 2007). The health benefits of phytochemicals have been linked with their capacity to regulate oxidative stress and inflammation (OSI), which occurs as part of normal metabolism, but is also involved in the aetiology of most chronic diseases (Calder et al., 2009). The regulation of OSI by phytochemicals may occur by direct antioxidant activity or by an indirect mechanism via induction of antioxidant stress defence (Selby-Pham et al., 2017). Cells in the human body are continuously exposed to oxidising agents from the environment, foods or those produced by metabolic activities within cells. Maintaining the balance between oxidants and anti-oxidants is crucial for optimal physiological conditions in the body (Calder et al., 2009). Over-production of oxidants can cause OSI and unregulated OSI can damage large biomolecules such as proteins, DNA and lipids, which in turn results in an increased risk of chronic diseases (Kryston, Georgiev, Pissis, & Georgakilas, 2011). Therefore, regulating transient and cumulative OSI associated with daily activities and chronic diseases is important to lower OSI-related mortality.

The bio-efficacy of phytochemicals to protect human health is dependent on their absorption into circulation and delivery to the target cells (D'Archivio, Filesi, Varì, Scazzocchio, & Masella, 2010). However, phytochemicals are only transiently present in circulation after consumption because they are recognised as xenobiotics by the human body (Holst & Williamson, 2008). After consumption of dietary plants, some phytochemicals are absorbed in the small intestine and enter the circulatory system (D'Archivio et al., 2010). These phytochemicals may be modified by the liver and their hepatic metabolites re-enter the circulatory system (D'Archivio et al., 2010). The unabsorbed phytochemicals reach the large intestine and are subjected to structural transformation by the colonic microbiota. These microbial metabolites can also be absorbed via the colon (Holst & Williamson, 2008). The time required for phytochemicals or their metabolites to reach their maximal

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concentrations in the circulatory system (T_{max}) can be an important factor to understand and optimise the health benefits of plant foods.

The importance of T_{max} was demonstrated in a recent study where healthy volunteers consumed a strawberry drink two hours before, during, or two hours after a high fat meal (Huang, Park, Edirisinghe, & Burton-Freeman, 2016). The strawberry drink was observed to reduce the OSI associated with the high fat meal only when being consumed at two hours before the meal (Huang et al., 2016). Considering that T_{max} of the measured phytochemicals in strawberry was approximately 1–2 h (Sandhu et al., 2016), consumption of the drink two hours before the high fat meal ensured that maximal concentration in the circulatory system coincided with the post-prandial OSI to minimise the OSI damage (measured by plasma concentration of the biomarker interleukin-6) triggered by the meal (Burton-Freeman, 2010).

The bioavailability of phytochemicals is dependent on their chemical structures and dietary intake forms (D'Archivio et al., 2010). We have developed a statistical model, the Phytochemical Absorption Prediction (PCAP) model, to predict T_{max} of dietary phytochemicals absorbed in human upper intestine based on their molecular mass, lipophilicity (expressed as log P, the logarithm of the partition coefficient between water and 1-octanol) and dietary intake forms (Selby-Pham, Miller, Howell, Dunshea, & Bennett, 2017). Application of this model allows direct calculation of values of T_{max} of phytochemicals using molecular mass and log P. However, the PCAP model can only be applied to individual (known) phytochemicals. In order to expand the practical usefulness of the model, it is necessary that molecular mass and log P of complex mixtures of phytochemicals, reflecting their typical mode of consumption, can be identified. Accordingly, further development of methods to predict the T_{max} range arising from uncharacterised phytochemicals mixtures is required. Chemical identification, which presents additional challenges such as the need for a previously purified, synthesised or characterised chemical standard of specific phytochemical, is not required for this purpose.

The retention of compounds on C18 reverse phase columns during liquid chromatography (LC) is controlled by lipophilicity and therefore correlated with log P (Valko, 2004). This feature of reverse phase LC allows for the development of multiple methods to estimate the log P of drug compounds (Valko, 2004) and natural products (Camp, Campitelli, Carroll, Davis, & Quinn, 2013). Further, LC may be coupled with mass spectrometry (MS) so that, in addition to allowing the determination of log P from retention time, MS identifies the accurate molecular mass of the compound, referred to as a 'molecular feature', until the chemical identity of the compound is confirmed (Flamini et al., 2013; Tsao & Li, 2013).

The aim of this research was to apply LC-MS methodology to simultaneously determine values of log P and molecular mass of individual phytochemicals present in extracts of selected dietary plants. A further aim was to develop a data processing workflow to convert the LC-MS data output to individual T_{max} values of phytochemicals absorbed in the human upper intestine, using the PCAP model. The T_{max} values were then used to generate a characteristic 'functional fingerprint' which represents the human upper intestinal absorption kinetic profile of the tested plant extract. Finally, validation of predicted functional fingerprints was investigated by comparison with published clinical evidence of plasma T_{max} and regulation of OSI, for similar extracts.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and reagents including chloroform, methanol, Na_2CO_3, gallic acid, formic acid, acetonitrile, L-histidine, (S)-dihydroorotate, shikimate, 4-pyridoxate, 3-hydroxybenzyl alcohol, 2,5-dihydroxybenzoate, 3-hydroxybenzaldehyde, trans-cinnamate, estradiol-17α, deoxycholate, retinoate, oleic acid and heptadecanoate were from Sigma-Aldrich (St Louis, MO, USA). Folin-Ciocalteu reagent was from Merck (Darmstadt, Germany).

2.2. Preparation of plant extracts

Fresh forms of dietary plant materials were purchased from local retailers (Woolworth, Werribee, VIC, Australia) and included broccoli (*Brassica oleracea* var. *italica*), carrot (*Daucus carota* ssp. *sativus*), red sweet potato (*Impomoea batatas*), rhubarb (*Rheum rhabarbarum*), squash (*Cucurbita pepo* var. *ovifera*), eggplant (*Solanum melongena*), kale (*Brassica oleracea* var. *acephala*) and Vietnamese coriander (*Persicaria odorata*). All samples were subjected to Stage 1 of a three-stage generic processing as described previously (Bennett & Muench, 2011). Briefly, plant material was homogenised in a food processor (Breville, Sydney, NSW. Australia) with water (1:2 ratio w/v) before cooking by microwave at 800 W (Sharp Carousel, Huntingwood, NSW, Australia) for 10 min to a final temperature of ~70 °C. After cooling to room temperature, the mixture was ultrasonicated at 300 W for 11 min (Hielscher 400UPS, Hielscher, Germany) before bag filtration (1 μm pore size, Sefar Filtration Inc., Depew, NY, USA). The filtrate was freeze-dried, ground to a fine powder and stored with a desiccant at −18 °C. Processed products were referred as "project extracts".

Commercial plant products were used as "reference extracts" and were obtained from the following suppliers: blueberry (Super Sprout, Campbellfield, VIC, Australia), green tea powder (Absolute Green, DeDu Pty Ltd., Ermington, NSW, Australia), olive leaf powder (Austral herbs, Uralla, NSW, Australia) and tomato powder (Herbies's Spices, Rozelle, NSW, Australia).

2.3. Proximate composition analysis of plant extracts

Proximate composition of the plant extracts was determined using standard analytical methods. Moisture content was determined using a halogen moisture analyser (Model HR73, Mettler Toledo, Columbus, OH, USA). Nitrogen analysis was determined by LECO Trumac® N analyser (LECO Corporation, Michigan, USA). Protein content was calculated by multiplying nitrogen content by the recommended conversion factor of 6.25 for plant foods (Sosulski & Imafidon, 1990). Total lipid content was quantified by Waite Analytical Services (The University of Adelaide, SA, Australia) using gas chromatography (GC) after extraction with chloroform: methanol (9:1 v/v) and subsequent methylation, as described previously (Makrides, Neumann, & Gibson, 1996). Mineral analysis quantified Al, B, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, P, Pb, S, Se, Si, Ti and Zn using inductively coupled plasma atomic emission spectroscopy (ICP-AES). The ICP-AES analyses were conducted using a Varian Vista Pro (Varian Australia, Melbourne, Australia) as described previously (Bennett, Singh, & Clingeleffer, 2011). Total ash content was estimated as the sum of all mineral contents of plant extracts (James, 1995). The total carbohydrate content was determined by subtracting the sum of protein content, fat content, ash content and moisture from 100 (Njinkoue et al., 2016). All analyses were performed in duplicate.

2.4. Total phenolic content of plant extracts

Total phenolic content of plant extracts was quantified using a modified Folin-Ciocalteu spectrophotometric methodology (Singleton & Rossi, 1965). In brief, 20 μL plant extract (2 mg/mL in 20% methanol) was mixed with 1 mL of 0.2 N Folin-Ciocalteu reagent and 180 μL of Milli-Q water for 15 s. The mixture stood for 3 min before the addition of 800 μL of 7.5% Na_2CO_3. The mixture was further shaken for 15 s and then incubated in the dark at 37 °C for 1 h. The absorbance at 765 nm was measured using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The total phenolic content of plant extracts was expressed as gallic acid equivalent (GAE) by plotting the gallic acid calibration curve (from 0 to 500 μg/mL in 20% methanol).