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COMPREHENSIVE REVIEW

Nutrikinetic studies of food bioactive compounds: from *in vitro* to *in vivo* approaches

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ABSTRACT

Poor absorption is an important cause of costly late-stage failures in functional food development, and therefore, it has become widely appreciated that pharmacokinetic parameters should be considered as early as possible in the functional food development process. In many cases, the molecular structure of bioactive ingredients is known, but information is lacking on how they interact with other food components, what their fate is upon consumption, what they do in the body and what their target site is. This information is of major importance, as the biological effects of food bioactive compounds (CBAs) are ultimately determined by their bioavailability and their temporal and spatial distribution in the body. In this chapter, we propose the phases to perform nutrikinetic studies of food CBAs from the simplest *in vitro* assays, applicable in early stages of the development of a functional food, to human intervention studies, which are required by the European Food Safety Authority and are aimed to establish the dose–exposure relationship (pharmacokinetic studies) and at last the exposure–response relationship (pharmacodynamic studies).

Keywords

Bioaccessibility, compliance biomarkers, human intervention, metabolism, nutrikinetics, tissue distribution

History

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Introduction

Before the legal authorization of the health claims, the Panel on Dietetic Products, Nutrition and Allergies (NDA) of the European Food Safety Authority (EFSA) is the responsible for evaluating the evidence from all the studies presented, including human, animal and *in vitro* mechanistic studies and is asked to provide a scientific opinion. To assist applicants in preparing and presenting their applications for authorization of health claims related to the consumption of a food category, a food, or its constituents (including a nutrient or other substance, or a combination of nutrients/other substances), in 2011 the NDA panel prepared a technical guidance for applications for authorization of health claims under Regulation (EC) No. 1924/2006 on nutrition and health claims made on foods (EFSA, 2011a). According with the Regulation, the organization and content of the application should be organized into five parts. Part 2 contains information on the characteristics of the food/constituent (Part 2.1) or the food or category of food (Part 2.2) for which a health claim is made. Where applicable, this information should contain aspects considered pertinent to the claim, such as the composition, physical and chemical characteristics, manufacturing process, stability and bioavailability. The information required in this part of the application includes: (1) name and characteristics for food constituent or Name and composition for food, (2) manufacturing process, (3) stability information and (4) bioavailability data. Where applicable, the relevant data and rationale that the constituent for which the health claim is made is in a form that is available to be used by the human body (e.g. absorption studies) should be provided. If absorption is not necessary to produce the claimed effect (e.g. plant sterols, fibers and lactic acid bacteria), the relevant data and rationale that the constituent reaches the target site should be provided. If available, data on any factors (e.g. formulation and processing) that could affect the absorption or utilization in the body of the constituent for which the health claim is made should be provided (EFSA, 2011a).

In this sense, given that poor absorption is an important cause of costly late-stage failures in functional food development, it has become widely appreciated that pharmacokinetic parameters should be considered as early as possible in the functional food development process. In many cases, the molecular structure of bioactive ingredients is known, but information is lacking on how they interact with other food components, what their fate is upon consumption, what they do in the body and what their target site is (de Vos, 2006). This information is of major importance, as the biological effects of food bioactive compounds are ultimately determined by their bioavailability and their temporal and spatial distribution in the body.

The pharmacokinetics of food bioactive compounds includes the same steps as those for orally ingested drugs (liberation, absorption, distribution, metabolism and excretion) and faces some of the same challenges, including transporters and enzymes. However, the application of the classical concepts of pharmacokinetics and pharmacodynamics (action of a drug in the body for a period of time) to food constituents is a challenge. Whereas drugs generally contain a single effective component that is specific and only has one target site in the body, foods consist of a variety of

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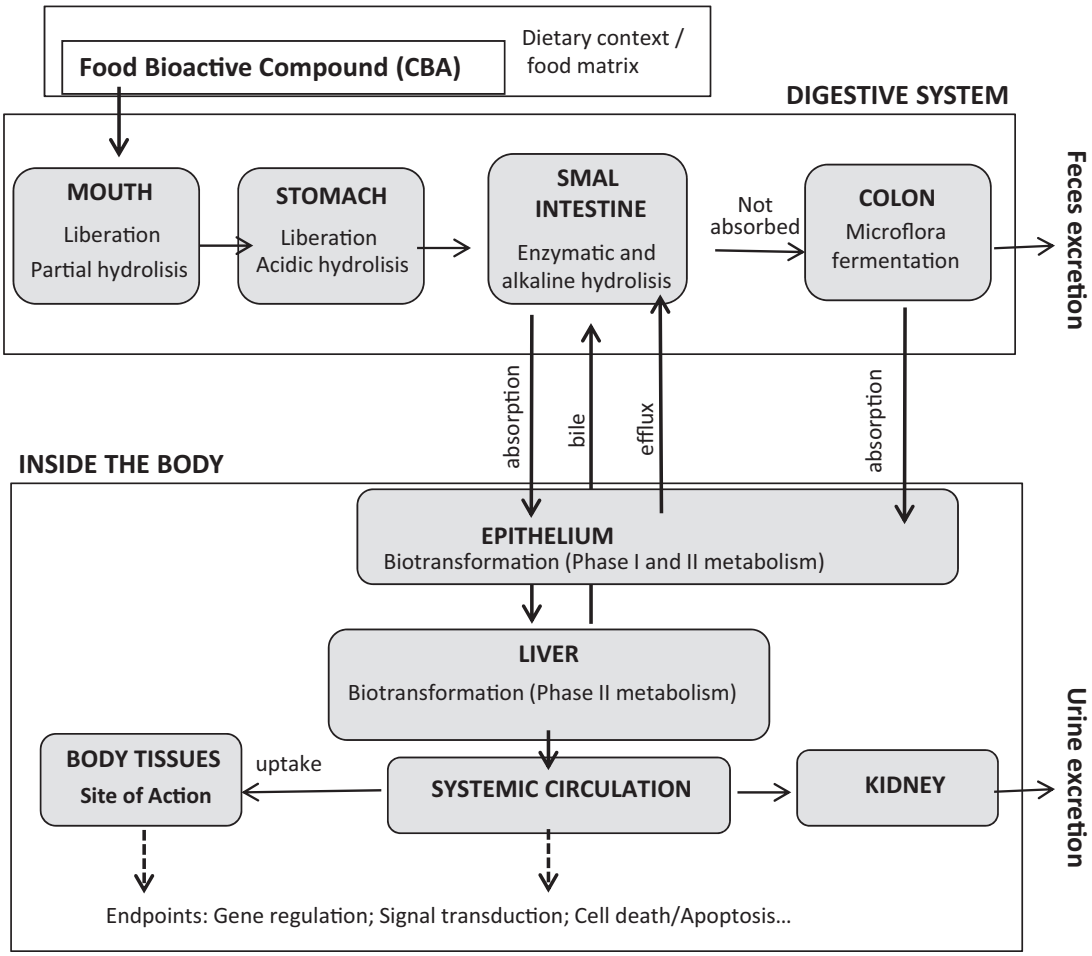

Figure 1. The journey of food CBAs from food through the human body.

components and can have multiple interactions with many human targets upon consumption. In addition, foods are consumed every day, whereas drugs are usually taken in small amounts for a short period of time. Moreover, food components show a wide range of interactions among each other, whereas drugs are delivered in a form that is as pure as possible in a formulation that specifically addresses bioefficacy. Finally, foods should also be tasteful, safe and convenient to be successful in the consumer market, what determines its formulation, in many cases. Considering this complexity, nutridynamics is proposed to *study how a food component is affected within the food matrix and by the food itself, as well as studying what it does in the human body* (de Vos, 2010). Hence, it addresses the fate, kinetics and interactions of food components embedded in a food matrix and their physiological effects upon consumption, including the mechanism of action, the effect of concentration and the interaction with other food components in the diet. In this way, nutridynamics couples food characteristics with processes in the body in a systematic way, including both quantitative and kinetic aspects, and hence links aspects of food and biotechnology on the product side with target delivery and nutrigenomics on the consumer side.

Therefore, to explore and determine the mechanisms of action of food bioactive compounds (CBAs) and their role in disease prevention, it is crucial an understanding of the factors that constrain their release from the foods and their extent of absorption and fate in the organism (Cadenas & Packer, 2002). The concept of ''bioavailability'' therefore incorporates: (i) availability for absorption or ''bioaccessibility''; (ii) absorption; (iii) tissue distribution and (iv) bioactivity (Stahl et al., 2002). It is important to emphasize that some CBAs do not need to enter to the systemic circulation to exert bioactivity. Indeed, many of these compounds may play an important role in the gastrointestinal tract without the necessity of being absorbed.

The journey of CBA or CBAs from food through the human body can be described in the following stages: consumption, gut luminal events, absorption and distribution (Figure 1). However, only a proportion (sometimes highly variable depending upon the food matrix composition, processing and storage) of these food components is absorbed and utilized. Understanding this concept of bioavailability is essential to all those involved in food production, nutritional assessment and determining diet–health relationships.

Based on the complexity of bioavailability studies, it is necessary to develop strategies that, in a first phase, allow carrying out the selection of a functional component, a functional ingredient or a functional food. Current food science and nutrition aim to understand the role of nutritional compounds at the molecular level (Herrero et al., 2012). This should be the basis for creating functional foods, which must be the result of a long period of research. The logical stages in the process of developing a food generally start from an intensive search for functional ingredients, usually guided by a bibliographic search or clear evidence of health benefits related to their ingestion. This preliminary stage is extremely useful for identifying possible functional ingredients, which will be tested *in vitro* to verify their potential functionality, with a preliminary screening to avoid carrying out human clinical trials needlessly. Subsequently, functional ingredients should be tested in animals as a preclinical

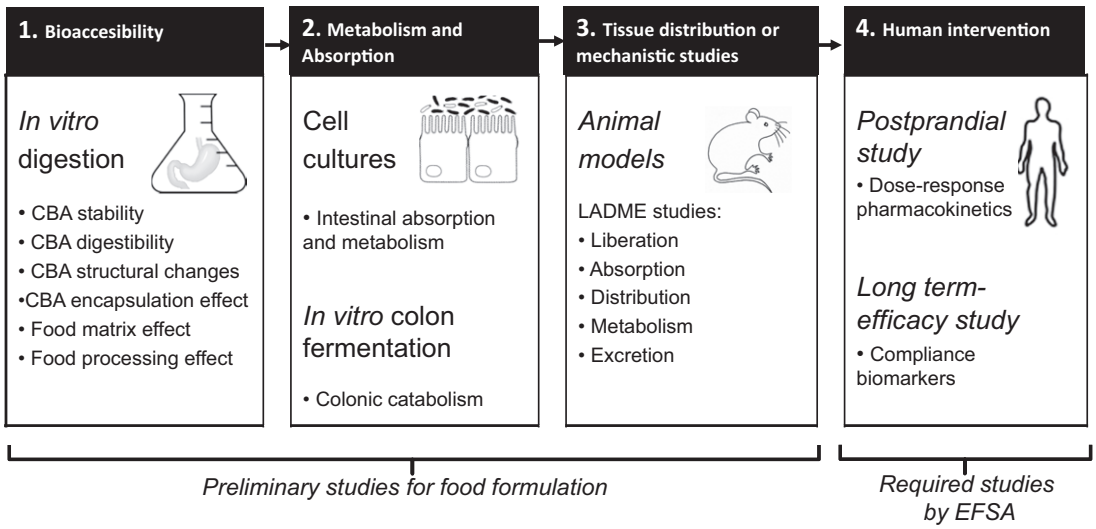

Figure 2. Practical recommendations to perform nutrikinetic studies with food CBAs: from the simplest *in vitro* assays, recommended in early stages of the development of a functional food, to human intervention studies, which are aimed to establish the dose–exposure relationship (pharmacokinetic studies) and at last the exposure–response relationship (pharmacodynamic studies).

model. Although human clinical trials are mandatory to test a functional ingredient, this involves an extremely complex organization of volunteers, generally chosen with a specific profile, and is a huge technical and economical investment. One important aspect of a functional food is the fact that the behavior of the active compounds when included in a food matrix is not the same as when the active compounds are tested as single molecules. The effect exerted by the food matrix during the digestion process may modify important parameters, such as the stability of the compounds during digestion and their release from the food, modulating their bioavailability and in last term their bioactivity.

Thus, prior to carrying out human clinical trial, *in vitro* models (e.g. enzymatic assays, cell cultures, genomic tests or *in vitro* simulation) are very useful techniques for mimicking biological situations and allowing the study of the food matrix effect. *In vitro* models are typically characterized by the fastness and excellent relation cost/effectiveness. The next step is *in vivo* preclinical trials using experimental animals, generally rats or mice. Nonetheless, the response obtained by *in vitro* models is not always directly extrapolated to *in vivo* systems. So, and according to the technical guidance for the preparation and presentation of an application for authorization of a health claim draw up by the EFSA, studies realized in animal or other model systems cannot substitute human data (EFSA, 2011a).

Next, we propose the phases to perform nutrikinetic studies CBAs from the simplest *in vitro* assays, applicable in early stages of the development of a functional food, to human intervention studies. These studies are only feasible once the product is already developed and it is necessary to establish the dose–exposure relationship (pharmacokinetic studies) and at last the exposure–response relationship (pharmacodynamic studies) (Figure 2).

In vitro digestion models

The aim of the *in vitro* simulations is to characterize changes that take place in dietary components during the digestion process under physiological conditions, adapting the temperature, the pH, the enzymatic concentrations and, specifically in the colonic fermentation models, the microbiota. In broad terms, there are two types of *in vitro* digestion simulations: the *in vitro* upper intestinal models and the colonic fermentation models. Briefly, the *in vitro* simulation models of the digestion process mimic the physiological conditions and luminal reactions that occur in the mouth, stomach and small intestine, regardless of the metabolism. The *in vitro* upper intestinal models (usually named digestion models) are commonly used to evaluate the digestibility of dietary components and detect changes in the non-digestive fraction. Besides, the colonic fermentation model includes an inoculum of colonic microbiota and is used to evaluate the colonic metabolism, usually using the non-digestible part obtained after the *in vitro* upper intestinal simulation (Aura et al., 2005) (described in the Section ''Colonic catabolism: *in vitro* models'').

In vitro digestion models are commonly used to evaluate the digestibility, structural changes and release of food components under gastrointestinal conditions representing a cheap and rapid alternative to animal and human studies. *In vitro* digestion models differ from one another mainly in the digestion steps represented in the model (mouth, stomach, small and large intestine), the composition of the biological fluids (e.g. salts, enzymes and buffers) and the fluid flows utilized in each step in the digestion sequence (Hur et al., 2011). In general, all the *in vitro* digestion simulations are performed at 37 °C and simulating the duration of the digestion times in humans, with slightly modifications, as occurs in the body, according to the nature of the sample being tested. Physiologically large food particles move through the stomach more slowly than smaller ones, and have to be less than 1 mm to cross the pylorus valve.

More than 80 studies related with *in vitro* digestion models have been carried out in the past 10 years and these were reviewed by Hur et al. (2011). The most appropriate enzymatic composition used in the *in vitro* simulations must be designed taking the composition of the tested samples into account. Related to this, only a few of the reported *in vitro* digestion methods have simulated the mouth stage, which implies an α-amylase treatment of the tested sample. Amylase, present in the mouth and stomach, is the enzyme responsible for the conversion of starches into oligosaccharides and monosaccharides. Amylase is usually related to the digestion of starch, lipase to the digestion of fatty product and pepsin or trypsin to protein digestion. Lipase, present in the stomach and pancreas, transforms the triacylglycerols and diacylglycerols into monoacylglycerols and free fatty acids (Hur et al., 2011) and, as occur with the application of amylase in the *in vitro* simulations, the application of lipase is not extensible to all of the reported *in vitro* digestion systems (Bublin et al., 2008;

Green et al., 2007; Versantvoort et al., 2005). Proteases, including stomach protease (pepsin) and small intestine protease (trypsin and chymotrypsin), are responsible for breaking proteins and peptides down into peptides and aminoacids, respectively, and their use is more widespread as they are included in practically all the *in vitro* digestion methods (Green et al., 2007; Laurent et al., 2007; Kedia et al., 2008; Savage & Catherwood, 2007). Nevertheless, *in vitro* digestion methods that use complex enzyme mixtures are more reproducible than those that use a single enzyme (Fatouros & Mullertz, 2008). To reinforce the interest of the enzymatic simulations, in 2011 EFSA NDA Panel (EFSA, 2011b) considered as appropriated an *in vitro* method developed by Englyst et al. (1996, 1999), based on incubation with pancreatic enzymes (invertase, amylase, amyloglucosidase) and applied to starch-containing foods.

Another biological parameter measurable through the use of *in vitro* upper intestinal models is the bioaccessibility, defined as *the amount of an ingested compound that becomes available for absorption in the gastrointestinal tract*. It is therefore an integration of all the luminal events. The amount that becomes bioaccessible may be equal to or less than the amount of the CBA that is liberated from the food matrix since: (i) it may not all transfer/convert to absorbable species, (ii) some may transfer to unabsorbable species and (iii) some may interact with other components of the luminal contents to become unabsorbable (Stahl et al., 2002). Overall, these are complex processes that depend on an array of variables including: (i) the state of the food (e.g. raw or processed), (ii) particle size, (iii) mode of size reduction (e.g. cell rupture or separation), (iv) digestive enzymes and products of digestion, (v) composition of the meal (i.e. fat/carbohydrate/protein content), (vi) the presence of bile acids/salts and (vii) time. All these factors, and others, will have an impact on: (i) the rate of delivery (i.e. gastric emptying) to the duodenum and jejunum, (ii) the sequence of digestion of nutrients, (iii) length of the ileum over which absorption will occur and (iv) the limit of absorption. This will, in turn, affect the profile of systemic delivery of the food component and, possibly, its metabolic fate. The bioaccessibility could be approximated *in vitro* by the implementation of a dialysis membrane by which small molecules are separated from the duodenal digestion mixture and considered as the bioaccessible fraction liable to be absorbed.

Practical recommendations for the *in vitro* digestion studies

Based on the interest of the application of the *in vitro* digestion test in the first phases of the development a functional food we propose a gastrointestinal *in vitro* digestion model based on the methodology described by Rubió et al. (2014a). The model describes a three-step procedure to mimic the digestive process in the mouth, stomach (gastric digestion) and small intestine (duodenal digestion). The digestion starts by adding amylase in phosphate buffer solution to food test (1–2 g), which is incubated for 5 min (mouth phase). The gastric digestion starts by adding 10 mg of α-amylase (100–300 ud/g) in a phosphate buffer solution (pH 6.9 with 0.04% of NaCl and 0.004% of $CaCl_2$) to mouth mixture (food test), which is incubated for 5 min. Then, the pH is adjusted to 2.0 by adding HCl concentrate and 15 mg of porcine-pepsin solution (24.750 ud/g of pepsin) in 1 ml 0.01 N HCl. The mixture is shaken in an incubator for 2 h at 37 °C. Then, to mimic the duodenal digestion, the pH is adjusted to 6.5 by adding $NaHCO_3$. Then, 5 ml of duodenal juice, which consisted of 2.5 ml of bile salts and 2.5 ml of pancreatin (8 g/l), is added and the mixture shaken for another 2 h at 37 °C. The bile salt concentration should be previously adjusted to the fat content of the food test: 50 g/l for samples with low fat content; and 80 g/l for samples with high fat content (Ortega et al., 2009). To evaluate the potential bioaccesibility of the CBAs, a continuous-flow dialyzed duodenal phase is recommended. The proposed system is designed with an adapted Liebig-West condenser and some end-fitting fluid connectors (Ortega et al., 2009). The first chamber contained the dialysis tub (molecular mass cut-off at 12 400 Da), through which the duodenal mixture flowed by using a peristaltic pump and a phosphate buffer solution, which covered the dialysis tub. A temperate water solution is pumped from a bath through the water jacket to keep the system's temperature constant, under 37 °C. At the end of the dialyzed duodenal digestion step, two fractions are collected and analyzed separately. These are the outside dialysis solution (OUT), which was considered the dialyzable fraction, and the inside dialysis tub content (IN), referring to the non-dialyzable fraction (Ortega et al., 2009). The dialyzable fraction (OUT) is considered to be the fraction that could be available for absorption into the systematic circulation by passive diffusion. Meanwhile, the non-dialyzable fraction (IN) is attributed to the digested fraction that would reach colon fermentation intact. To evaluate the CBAs stability during different digestion phases and the potential bioaccesibility all fractions are freezing or freeze-drying and stored until their analysis.

Metabolism and absorption test models

Colonic catabolism: *in vitro* models

Although gut was long described as an independent organ with immunostimulation properties (Bocci, 1992) and the majority of the studies carried out focused on identifying gut microbiota species, scientific studies have demonstrated the relation between various microbial species and some pathologies, such as Crohn's disease, irritable bowel syndrome, obesity or diabetes (Bischoff, 2011; Ley et al., 2005; Malinen et al., 2005; Manichanh et al., 2006; Nielsen et al., 2014; Turnbaugh et al., 2006). Moreover, the role played by colonic microflora in the metabolism of some dietary compounds, e.g. phenolic compounds and fiber, is no less important. Non-metabolized polyphenols, polyphenols bonded to the food matrix, and phase II metabolites that reach the colon may suffer the enzymatic action of colonic microflora and prior to their excretion, the new colonic metabolites may exert a specific function in the colon itself (Unno et al., 2003), or may be absorbed and returned to the blood stream, reaching centers of action (Lampe, 2003).

Several colonic fermentation models have been developed, although the basic principle of the colonic fermentation model is common to all. This is a single or multiple chemostats inoculated with fecal microbiota operating under physiological temperature and pH, in anaerobic conditions. The complexity of *in vitro* colonic fermentation models increases with the number of chemostats operating simultaneously, even mimicking the entire colon, imitating any of the individual sections of the large intestine (e.g. proximal, transverse and distal colon). Briefly, batch culture fermentation models are closed systems containing pure or mixed bacterial suspensions of fecal material in a selected medium under anaerobic conditions. They represent the easiest fermentation model and are generally used to perform fermentation studies and substrate digestion assessment (Dall'Asta et al., 2012; Gumienna et al., 2011; Lesmes et al., 2008; Pompei et al., 2008) with the drawback that only short-term fermentation studies can be performed. More complex fermentation models named continuous fermentation models due to being endowed with a continuous flow mimicking *in vivo* conditions have been carried out and are extensively used to assess long-term fermentation studies, with the aim of elucidating colon functions (Macfarlane & Macfarlane, 2007), metabolic activity (Possemiers et al., 2011)

or gut bacterial colonizations (Cinquin et al., 2004; Le Blay et al., 2009), among other applications. In addition, two types of continuous fermentation models may be differentiated according to their number of stages: single or multistage continuous fermentation models (Payne et al., 2012). Apart from the complexity derived from the number of stages, the technique used for fecal inoculation is a determining factor in their effectiveness in mimicking biological environments. The simplest inoculum is represented by the use of pure cultures or defined mixed cultures (Macfarlane & Macfarlane, 2007). Nevertheless, in most *in vitro* fermentation models, a liquid fecal suspension (Serra et al., 2010, 2011) has been used as the inoculum, this being a good technique for short-term fermentation simulations (less than 4 weeks) due to liquid fecal inocula generally experiencing a rapid washout of less competitive bacteria the longer the incubation time. To solve problems of inoculum washout, immobilized fecal microbiota have been developed (Cinquin et al., 2004, 2006; Zihler et al., 2010), in which fecal beads formed with a polysaccharide matrix are mixed with the growth medium generally in continuous fermentation models.

The common limitation of all of the described fermentation models is the absence of host functionality. This limitation was overcome by developing artificial digestive systems, which represent the most advanced attempt at fermentation models. Artificial digestive systems are able to simulate human digestive functions, including motility, bile secretion, pH and absorption for small intestinal models and peristaltic mixing and water and metabolite absorption for colonic models. The combination of artificial models results in a complex mimicking system of the digestive tract applied generally to advanced pharmaceutical or nutritional studies (Blanquet-Diot et al., 2009; Souliman et al., 2006, 2007).

The evaluation of the working mechanisms of probiotics, the metabolism of dietary components and the impact of dietary components on the gut microbiota are some of the applications of colonic fermentation models usually performed with batch or single-continuous fermentation models (Mennigen & Bruewer, 2009).

Practical recommendations to colonic catabolism studies

To evaluate the catabolism of CBAs by the gut microbiota, we propose a simple *in vitro* colonic fermentation model by human microflora (Mosele et al., 2014). Fecal samples are collected from healthy volunteers who followed normal dietary habits and declared they had not ingested antibiotics for at least 3 months prior to the sample collection. For each volunteer, 15 g of fresh feces is homogenized for 60 s in a stomacher with 300 ml of culture medium to make 5% fecal slurry. The fermentation medium is carbonate-phosphate buffer and this is prepared by mixing (all in grams per liter): 9.240 $NaHCO_3$, 3.542 $Na_2HPO_4{\cdot}2H_2O$, 0.470 NaCl, 0.450 KCl, 0.227 $Na_2SO_4{\cdot}10H_2O$, 0.055 $CaCl_2$ (anhydrous), 0.100 $MgCl_2{\cdot}6\ H_2O$, 0.400 urea with 10 ml of added trace element solution (trace element solution containing milligrams per liter) 3680 $FeSO_4{\cdot}7H_2O$, 1159 $MnSO_4{\cdot}H_2O$, 440 $ZnSO_4{\cdot}7H_2O$, 120 $CoCl_2{\cdot}6H_2O$, 98 $CuSO_4{\cdot}5H_2O$, 17.4 $Mo7(NH_4)6O_2{\cdot}4H_2O$. The medium is adjusted to pH 7.0 using hydrochloric acid and stored in an anaerobic chamber for 48 h to remove the oxygen before the fermentation experiment. The anaerobic environment is continuously monitored by anaerobic indicator strips. For each fermentation batch, 0.5:10 (w/v) dilution of the fecal slurry with the anaerobic buffer is prepared and homogenized in a stomacher (1 min). The resulting fecal suspension is distributed in disposable tubes (10 ml/tub/incubation time) and 0.1 g of the IN fraction collected after the duodenal digestion of test food (*in vitro* digestion model) are added. The mixtures are fermented at 37 °C in continuous shaking (60 rpm) under anaerobic conditions. To study the kinetic of colonic catabolism and the metabolic pathways, the incubation is stored at different fermentation times (0–72 h). The tubes are freeze or freeze-dried and stored until their analysis.

Absorption and metabolism models: Cell cultures

Cell cultures represent a predictive tool for the intestinal absorption of CBAs (Hur et al., 2011). Compared with *in vitro* models, the use of culture cells allows evaluation of the metabolism using human or animal tissue, preserving cell integrity and maintaining the interaction between the cells with good standardization and reproducibility, although it is not always easy to preserve the tissue structure and cellular differentiation in cell cultures (Sambruy et al., 2001). The typical polarized organization, the asymmetrical distribution of membrane proteins and lipids and the presence of highly organized structures of epithelial cells hinder the maintenance of cultured intestinal epithelium. A range of cell models has been developed to preserve the complex functional and morphological organization of the intestinal epithelium *in vitro* (Sambruy et al., 2001). Primary cell cultures are cell isolated from the small intestine (Evans et al., 1992) or the colon (Fonti et al., 1994) maintaining a variable degree of differentiation for 3-days in culture. Nevertheless, these kinds of culture cells rapid lose their differentiated characteristics. The second type of culture cells is the cell lines from normal tissues, obtained from primary cultures by isolating homogenous cell lines capable of proliferating and surviving for several *in vitro* passages generally using the small intestine of rats. Normal cell lines transfected with regulatory genes have been reported as the third type of culture cells used to maintain cultured intestinal epithelial cells, and obtained from crypt cell lines. The last type of culture cells, and probably the most widely used of these, is the cell lines of tumoral origin. The use of cell cultures is reinforced by the possibility of using tissue cultures to study the behavior of specific alive cells in a controlled environment (Mather & Roberts, 1998).

The Caco-2 cell line is an important example of cell lines of tumoral origin used in nutritional modeling for predicting intestinal absorption and metabolism (e.g. transport system – diffusion, endocytosis, transcytosis and conjugations) at the molecular and cellular level of nutraceuticals, food additives and dietary constituents, among other compounds and even for testing possible health benefits. Caco-2 cell lines were isolated from human colon adenocarcinoma (Fogh et al., 1977) during the 1970s, and the leading property of these cells is their capacity to differentiate themselves spontaneously in the culture starting at confluency and reaching it in 2–3 weeks. It allows the formation of a monolayer of highly polarized cells, joined by functional tight junctions, with well-developed and organized microvilli on the apical membrane. Caco-2 cells normally express hydrolase activities associated with the apical membrane. Some of the intestinal functions are also expressed and, despite their tumoral origin, they exhibit some biochemical characteristics of the normal adult intestine (Harris et al., 1992).

In the nutritional field, the human hepatoma cell line (HepG2) and the primary hepatocytes are other widely used families of culture cells. Cultured hepatocytes are the most suitable *in vitro* model for evaluating hepatic bio-transformations and are of great relevance in toxicological and pharmaceutical studies (Wilkening et al., 2003). Primary hepatocytes do not proliferate and lose their metabolic activity, although this is the best culture cell model for mimicking the hepatic metabolism *in vitro*. Nevertheless, HepG2 is an excellent approximation that provides a reproducible human system diminishing the problems of proliferation and differentiation.

Practical recommendations to absorption and metabolism studies

Based on the interest of the metabolism and potential absorption of the CBAs in the first phases of the functional food development, we propose the use of a co-culture of Caco-2 and HepG2 cells (Rubió et al., 2014a). The cells are used for the metabolism and transport studies after 21 days once the confluent monolayer had formed, and the cells expressed a constant transepithelium electrical resistance. Once the cells reach confluence, a double-layered co-culture of Caco-2 cells and HepG2 cells is prepared. For the experiments, double-layered co-culture is treated with the pure molecule or with the duodenal digestion mixture of food test submitted to *in vitro* digestion. After the cells treatment, the cell mediums (apical and basolateral) are recovered and metabolites are analyzed to determine the metabolism and transport.

Preclinical animal models of CBA metabolism and tissue distribution

Despite the technologic advances in the *in vitro* models, they cannot fully replace *in vivo* studies. The use of animal models is more widely accepted than *in vitro* models to bridge the gap between *in vitro* studies and the full human organism (Mortensen et al., 2008), although data from animal studies are controversially criticized due to the physiological differences between animal and human metabolism (Gomes et al., 2007). *In vitro* studies and animal experimentation are useful tools for obtaining a valid approximation to human *in vivo* processes (e.g. digestion, absorption, metabolism, tissue distribution and even health benefits of CBAs). Thus, the data from *in vitro* and *in vivo* studies with animals or other non-human models may be useful as supporting evidences, considering that human clinical trials cannot be replaced by *in vitro* trials as expressed in the EFSA scientific and technical guidelines for preparing and presenting an application for authorization of a health claim (EFSA, 2011a).

Animal studies can be performed in a short period of time, enabling the study from the *in utero* exposure to the animal's death, also including intergenerational experiments. The variability introduced by environmental factors, generally uncontrollable in human studies, is minimized by standardization of laboratory conditions and by the use of ''defined animals'' (animals with known genetic and health status) (Öbrink & Rehbinde, 2003). The choice of an animal model will depend on its validity with respect to the phenomenon studies (data obtained in the animal model predict the situation in the non-experimental conditions) and the expression of that phenomenon in the chosen model (Mortensen et al., 2008). Ideally, to perform nutrition research anatomy, the biochemistry and physiology of the digestive system should be close to those of humans, and the administered dose should be adjusted by an animal to human compensation (Freireich, 1966).

Although the innovative animal models have improved over recent years, the evaluation of the metabolism of food CBAs is generally performed by the use of rodents (rats and mice), which represent the overwhelming majority of all the laboratory animals (Hau & van Hoosier, 2003). The digestion and metabolism are similar to the human processes, including absorption, metabolism (phase I and phase II metabolism in liver and intestinal epithelial cells and colonic fermentation) and excretion. Among other reasons, including their low cost, easy reproduction from a single couple and easy handling, make rodent one of the best models for *in vivo* nutritional research. So, thousand of different nutrition experiments involving dietary polyphenols, as purified molecules (Konishi et al., 2006) and as a component of a food (Donovan et al., 2006; Neilson et al., 2010; Tsang et al., 2005) have been carried out using rat and mice models. Over the last decade, the use of chimeric mice with humanized tissues, specifically the liver (Taleno et al., 2004), has been a breakthrough in applied nutrition research. Their capacity to reproduce human-type metabolic responses reduces the species differences between experimental animals and humans, and minimizes the problems derived from the use of primary culture of human tissues (e.g. the inability of cells to proliferate, their quick degradation and the requirement for specific culturing or technical conditions) (Li et al., 1997).

In terms of metabolism and tissue distribution of the CBAs, the use of rodents is considered very interesting to determine the amount of CBA and its metabolites found *in vivo* in the target tissue to establish whether they can conceivably contribute to, or account for, different effects *in vivo*. Therefore, they allow increasing the comprehension of their health beneficial effects rather than the concentrations determined in more common biological matrices.

Practical recommendations to CBA tissue distribution

To evaluate the tissue distribution of CBAs we propose the method described by Serra et al. (2012) in which the metabolism and distribution of phenolic compounds were examined by UPLC–MS/MS after an acute intake of a phenolic extract analyzing different target tissues (heart, brain, liver, kidney, spleen, testicle and thymus) 1, 2 and 4 h after ingestion using Wistar rats. The rats were kept under fasting condition for between 16 and 17 h with access to tap water and after this time, a single dose of test food (3 g extract/kg of body weight) dispersed in water was administered by intragastric gavage. Similarly, the metabolism and tissue distribution studies may be performed after a long-term consumption of the CBAs of interest. We propose the experiment described by Serra et al. (2013) to determine plasma bioavailability and disposition in different tissues (liver, muscle, brown and white adipose tissue) in rats after a long-term consumption of three doses of grape seed phenolic extract (GSPE) for 21 days in order to determine whether there is a dose–response relationship. After 1 week of adaptation, the animals were trained to lick condensed milk (1 ml), which is used as a vehicle for administering GSPE. After this period, the animals were randomly divided into four groups, including the control group. Each group was treated with 5, 25 or 50 mg GSPE/kg body weight per day dispersed in condensed milk. The control group was treated with condensed milk. GSPE was administered every day at 09.00 h for 21 days. On day 21, 5 h after the GSPE treatment, rats were anesthetized with ketamine/xylazine and killed by exsanguination from the abdominal aorta using syringes, with heparin as the anticoagulant. Plasma was obtained by centrifugation and stored at −80 °C until analysis. In both experiments (acute intake and long-term consumption), different tissues were excised from the rats, frozen immediately in liquid N_2 and stored at −80 °C until CBAs metabolites analysis.

Human intervention studies: pharmacokinetic and pharmacodynamic studies

After the *in vitro* and *in vivo* (animal experimentation) studies and having clear evidence of the planned hypothesis, the last step in all the nutritional research should be a human intervention study, as this is the only manner to assess the real importance of foods or bioactive components in relation to their health benefits (Lund, 2003). Human studies could be classified into intervention studies and observational epidemiological studies. In nutrition research, an intervention study is based on applying a nutritional intervention under controlled conditions, and measuring the biological outcome. Subsequently, intervention studies can be divided into double blind/single blind/(non-blinded) and randomized/(non-randomized) controlled trials. Commonly, nutritional studies in human may examine the biological effect of a nutrient or food commodities or the effect of a specific type of diet (such as the

Mediterranean, western-style or low-fat dietary patterns). Besides, observational epidemiological studies are based on the ability of the epidemiological instruments to measure the habitual dietary intake and are divided into descriptive (correlational, case-report series, cross sectional) and analytical experimentation (case–control, cohort), and meta-analyses (Mortensen et al., 2008).

Although human studies represent the final objective of nutritional research, on occasions the effect of nutrition on health and disease cannot be fully explained through these. Human clinical trials often only provide information about endpoints of interest and in some cases the period of intervention is short, unable to assess changes in the variables studied. Moreover, the studied phenomenon is generally quantified by the measurement of related chemical biomarkers. Environmental factors, lifestyle, difficult organization, ethical considerations and poor compliance by human subjects hinder the interpretation of the results even more. In addition to all these considerations, the lack of knowledge about how the CBAs act at the molecular level and their role in the physiological processes represents the reason why alternative model systems (such as *in vitro* and animal models) are widely used in nutritional research (Mortensen et al., 2008).

Over recent decades, once the bioactive molecule had been identified, the next step was to isolate the molecule from the food or chemical synthesis, the potential bioactivity of which was then evaluated *in vitro*. Nonetheless, structural modifications of the bioactive molecules occur during food digestion and metabolism and the absorption, metabolism and tissue distribution can be influenced by the food matrix composition. Thus, the new tendency is to know the possible modifications that the CBAs can undergo during food processing and establish the postconsumption pharmacokinetics and metabolism of the food that include the CBAs. This way, the physiological concentration of metabolites can be established and the relevant identified structures assayed by *in vitro* or *in vivo* studies to evaluate their bioactivity at physiological levels.

Based on the intense metabolism of some CBAs, mainly phenolic compounds, it is very important to identify properly the circulating metabolites in order to have a better understanding of the fate of the parent compounds. Only when the circulating forms and the pharmacokinetics of CBA are known, a more complete picture related to bioavailability and possible correlation to bioefficacy can be obtained. For drugs, this is a requirement when performing bioavailability studies and this type of approach can be translated to nutrition research too. So, as it is established by the EFSA, *studies on bioavailability represent a key issue that should be addressed in the application to substantiate the health claim* (EFSA, 2011a). However, any guidance referring to the design and conduct of these studies is provided.

Ideally, as a first step, a feasibility study should be performed in a small group of humans as a *proof-of-concept* to explore the effect of the dose and the food matrix composition on the pharmacokinetic parameters. This first step can be crucial in the design of a functional food or beverage as it allows selecting the optimal dose and food matrix through which the CBA will be administered. So previous to the interventional efficacy study it is essential to acquire knowledge of the factors affecting CBA bioavailability. After the dose–response study, a long-term interventional trial is needed to proof the human efficacy of the CBA of interest. At this point, it is crucial to complement the outcome measure(s) used to assess the claimed effect in humans with quantitative information regarding the compliance biomarkers. It has proved extremely difficult to quantitatively establish the benefit afforded by CBAs due to the limited understanding regarding the extent of absorption and metabolic fate of individual CBAs from particular foods. Therefore, the determination of appropriate biological biomarkers measured in both blood and urine is essential for making accurate estimates of CBA intake and further relates them with the observed effects.

In this section, our efforts will be directed to provide guidance in the selection of the most appropriate designs to provide comprehensive knowledge regarding CBA human bioavailability, with special emphasis in polyphenols related with their intense metabolism, when presenting an application for authorization of a health claim. For this, we will explore and analyze the most recent human bioavailability studies that have been well designed and conducted and thus they can be considered as models to follow.

Postprandial study: dose–exposure evaluation

A dose–response study is a feasibility study that should be performed in a small group of subjects (10–15) as a *proof-of-concept* to explore the effect of the dose and the food matrix composition on the CBA pharmacokinetic parameters. In this first stage of the CBA bioavailability assessment, the main objective is to evaluate the relationship between the dose level and the pharmacokinetic parameters of the studied metabolites (such as the t_{max}, $t_{1/2}$, C_{max} and C_{min}, and the AUC in plasma samples). The results may also provide useful information in determining dose levels at which the minimum concentration for therapeutic effect will be achieved. Although the metabolic pathways to which CBAs and mainly most polyphenols are subjected are similar, pharmacokinetic profiles differ markedly among the different classes of CBAs. Therefore, it is a vital factor to consider the kinetic profile of a CBA metabolite or metabolites and their appearance in a specific biofluid (complete blood, plasma, serum or urine).

Regarding the design of an acute intake study, it is important to bear in mind that the individual subjects may differ very widely in the pharmacokinetic parameters. Consequently, an appropriate design should identify, estimate and isolate the inter-subject variability in data analysis. In this sense, the most accepted design is the complete randomized and crossover design (Patel et al., 2010). Regarding the collection of biological samples, many studies to date have analyzed plasma polyphenol levels 1–6 h postintake. Such sampling will provide good data regarding the CBA metabolites of small-intestinal origin, mainly in polyphenol bioavailability studies. However, in the majority of cases, such a time course will not provide information regarding metabolic products formed in the large intestine. In this case, multiple blood sampling over a 24 and even 48–72 h period should be considered, mainly in the case of phenol catabolism studies based on the colonic catabolites formed after 72 h incubation in *in vitro* model (Mosele et al., 2014). The time–response relationship between intake and appearance of the metabolite biomarker in plasma will also be dependent on the elimination rate of the metabolite (urine). So, urinary excretion of polyphenol metabolites for example should also be included in such human studies as it is generally consistent with plasma kinetic data. Twenty-four hours urine sampling offers additional advantages over plasma measurements, mainly because it allows for total polyphenol absorption to be more accurately assessed. Urine sampling is particularly useful for polyphenols with short half-lives, where plasma measurements may fail to monitor even acute intake. The biggest advantage of analyzing 24 h urinary output is that it is quantitative and will provide a measure of the total output of polyphenol metabolites over a 24 h period. Twenty four hours urinary data will also provide a better index of intake, as one may monitor the total concentrations of both small- and large-intestinal metabolites, without the need for multiple blood collections (Zamora-Ros et al., 2012).

Postprandial studies can be also very useful to establish the effects of food matrix composition on CBAs bioavailability. The food source is likely to have a large impact on the dose–response relationship between intake and appearance of a specific metabolite. This is in part due to the fact that the food matrix may greatly influence absorption of polyphenols from the gastrointestinal tract and partly because polyphenols exist in a variety of chemical forms depending on the food. Therefore, data obtained from postprandial studies considering the form (i.e. the type of food) in which the polyphenol is ingested can contribute to knowledge of the factors affecting polyphenols bioavailability and can be of benefit in future in the design of functional foods and beverages.

One of the selected works as a model of postprandial study design is the one performed by Hollands et al. (2013). They compared the bioavailability and dose–response of epicatechin from whole apple and an epicatechin-rich extract. The study consisted on a randomized crossover trial in which the acute pharmacokinetic parameters were assessed in 14 subjects who consumed drinks containing 70 and 140 mg epicatechin from an apple extract, and an apple puree containing 70 mg epicatechin. This study allowed to analyze the differences observed between the administration of epicatechins through a phenolic extract or through its natural matrix (apples). Multiple blood samples were taken until 24 h and also urine from 24 h. Results showed that C_{max} for plasma epicatechin after ingestion of apple puree was significantly lower than the equivalent dose fed as a flavored beverage, and the 24 h urine excretion was also significantly lower than the low-dose drink. To explain this reduced bioavailability of apple epicatechin in the presence of the whole apple matrix, authors hypothesize that it could be in part due to the other polyphenols present in the whole apple that could compete with epicatechin for enterocyte phase-II conjugating enzymes and efflux transporters. The dose effect showed that epicatechin bioavailability was >2-fold higher after ingestion of the 140 mg epicatechin drink compared to the 70 mg epicatechin drink, indicating that the dose normalized bioavailability of epicatechin from a flavanol-rich apple extract increased at higher doses.

Renouf et al. (2013) also performed a well designed dose–response study in which they investigated the human bioavailability (plasma appearance) of tea catechins after drinking three doses of infused green tea (the way the beverage is normally drunk by consumers) in a randomized cross-over design with 12 healthy subjects. The doses chosen remained within a nutritionally relevant range and covered most of the likely tea consumption dosages. The AUC increased between the small and medium dose of ingested green tea but not between the medium and the high dose. Thus, they did not observe a linear response between plasma appearance of tea epigallocatechins and the ingested dose. This saturation or plateauing at the highest dose suggests that some of the absorption mechanisms involved may be active and saturate. The overall pattern for the sum of catechins did not reflect the fate of individual catechin. While (–)-epigallocatechin and 4-*O*-Me-epigallocatechin showed saturation in plasma between the medium and high green tea doses, (–)-epigallocatechin gallate and (–)-epicatechin did not ''saturate'' and increased proportionally with the ingested dose demonstrating that green tea polyphenol absorption and plasma appearance should not be discussed as a combination, but each catechin and metabolite must be understood and described individually. In accordance with this study, Rubió et al. (2012) also performed a dose–response study in which three different functional phenol-enriched virgin olive oils with a phenolic content of 250, 500 and 750 mg total phenols/kg olive oil were administered in a randomized, cross-over study with 12 healthy volunteers. The pharmacokinetics parameters showed an increasing trend but not linear for hydroxytyrosol sulfate and hydroxytyrosol acetate sulfate, the main olive oil phenolic metabolites quantified in plasma. So, authors also hypothesized that some sort of saturation of the intestinal transporters or conjugating enzymes could occur at higher doses. In this study, the effect of the phenol enrichment on the sensory acceptability of olive oils was also studied as the phenolic compounds of olive oils increase the bitter taste. This aspect should also be considered in these studies as is the principal barrier for the acceptance of a functional food and can provide very useful information in the design of the functional food or beverage.

In another study based on a randomized, double-blind, cross-over design (Schroeter et al., 2006), two powdered cocoa drinks mixed in 300 ml of water with a high or low flavanol content (917 mg and 37 mg of total flavanols, respectively) based on the epicatechin content, and pure (−)-epicatechin were administered to 16 subjects. Apart from analyzing the pharmacokinetic parameters, this study also demonstrated that the maximum effect on endothelial function (impaired flow-mediated vasodilation, FMD) coincides with peak plasma levels of epicatechin metabolites, observing significant univariate correlations between flavanol metabolites and FMD. Authors also identified circulating epicatechin and epicatechin-7-*O*-glucuronide as predictors for a modulation of vascular function. In the case of pure (−)-epicatechin administration, the magnitude and kinetics of changes in vascular function were similar to the effects observed after ingestion of high-favanol cocoa drink. Thus, this study provide compelling evidence that the flavanol compound (−)-epicatechin could mediate, at least in part, the beneficial vascular effects associated with the consumption of flavanol-rich cocoa in humans.

A novel and interesting way to enhance the bioavailability of polyphenols and alter their interaction with the food matrix may by via encapsulation and many postprandial studies are being performed in order to study its effect on bioavailability modulation. Encapsulation may be used for product stabilization, to enhance bioavailability, or to achieve controlled polyphenol release during digestion (de Vos, 2010). For enhanced bioavailability, encapsulation strategies that aim to improve polyphenol solubility, thereby minimizing interactions with other dietary ingredients, and to achieve targeted gut release have been sought. In particular, enhancing the solubility of poorly soluble polyphenols such as resveratrol or curcumin may be an interesting strategy. Regarding this kind of studies, Vitaglione et al. (2012) performed a well-conducted study that could be taken as a model. The study consisted on a double-blind randomized crossover study with 10 healthy subjects in which they evaluated the bioavailability of curcumin from different types of bread containing free curcumin and microencapsulated curcumin in a cellulose derivative coating. Curcuminoid bioavailability was assessed over 24 h determining blood, urine and fecal concentration of metabolites. The AUC was approximately seven times higher for encapsulated curcumin than for free curcumin in bread, possibly due to protection from degradation in the gut into phenolic acids.

In another study, Vitaglione et al. (2013) evaluated the human bioavailability of cocoa flavanols and phenolic acids from a cocoa-nut cream (CC) and from CC enriched with a 1.5% (w/w) cocoa polyphenol extract in free form or encapsulated with a gastric-resistant high-amylose maize starch. Previous to the study, in order to establish what was the maximum enrichment of the creams achievable with the polyphenol-rich ingredients, a sensory analysis of 10 cream prototypes was performed. This kind of sensory test can be very useful as a previous step to select the final products to be used in the bioavailability studies. For the postprandial study, 12 healthy volunteers participated in a

randomized cross-over protocol, and blood, urine a fecal samples were collected over 24 h. Results showed that parental cocoa flavanols are absorbed by CCs in a dose-dependent manner and phenolic acids are the major metabolites in the short term, being at a concentration ratio of 50:1 v. (epi)-catechin. Encapsulation of cocoa polyphenols caused a reduced 24 h bioavailability of these compounds. However, encapsulation effectively masked bitter taste and allowed delivering of flavanol monomers into the gut and the successive metabolism by local microflora. This study demonstrate that the analysis of fecal metabolites provide very important information regarding polyphenol pharmacokinetics confirming the major role of gut microflora on phenolic acids formation that could be the bioactive structures at colon level.

Long-term efficacy studies associated to compliance biomarkers

In nutritional interventional trials, an accurate and objective measure of dietary intake of phenolic compounds is mandatory. The fulfillment of the criteria defined by Spencer et al. (2008) denotes the usefulness of the compliance biomarkers of polyphenols provided, which guarantees a successful dietary intervention. Thus, the criteria that need to be satisfied are listed below.

Criteria 1. Quantification of the biomarker of interest should be qualitatively and quantitatively robust (Spencer et al., 2008). Sensitive and specific techniques should be used to accurately quantify the biomarker of interest in the biofluid, which has been appropriately collected and stored to ensure minimum biomarker degradation. The circulating concentrations of both native and metabolic forms of polyphenols are in the nanomolar to low micromolar range and normally represent only a very small proportion of the ingested dose. Furthermore, the metabolic forms that predominate are difficult to characterize and/or quantify due to a lack of suitable standards percentage of the amount consumed. Consequently, the most appropriate approach is the application of HPLC–tandem mass spectrometry (MS/MS) technology, which provides a useful tool for the positive identification of compounds in biological fluids. This technique is likely to aid the identification and quantification of polyphenols and their metabolites in biological fluids such as plasma and urine. Finally, consideration should also be given to the stability of polyphenol metabolites during biological sample storage and during sample extraction.

Criteria 2. Concentrations of the biomarker in the biofluid of interest should be sensitive to changes in intake of the dietary component of interest (Spencer et al., 2008). Bioavailability and/or pharmacokinetic studies have shown that plasma metabolite concentrations achieved following consumption of polyphenols vary greatly according to the nature of the polyphenol, and in particular the nature of the food source. Therefore, quantitative information regarding the relationship between dose administered and plasma concentrations is required. Moreover, there appears to be considerable inter-individual variation in the metabolic response to a given dose of a particular polyphenol-rich food. This is observed particularly in the case of metabolites produced by colonic microflora (Rechner et al., 2004). However, even for polyphenols for which absorption is not dependent on bacterial metabolism, there is considerable inter-individual variation (Lotito & Frei, 2006). Therefore, it is important to bear in mind that the dietary polyphenol–biomarker relationship may be dependent on inter-individual genetic variability as well as on the food source.

Criteria 3. The biomarker should be specific to the dietary component of interest, i.e. variation in its concentration should be due to changes in the intake of the dietary component of interest only (Spencer et al., 2008). This means that such a metabolite should only appear in human plasma or urine following the intake of a specific polyphenol and should not be formed as a product of the metabolism of any other compound either consumed or produced endogenously. Unfortunately, the ingestion of a number of polyphenols often leads to the formation of common metabolites in the circulation (Rubió et al., 2014b; Valls et al., 2010). Therefore, further research is required in order to expand our current knowledge regarding the biotransformation of the different classes of polyphenols and to assess the usefulness of such metabolites as biomarkers of intake.

Balzer et al. (2008) has been selected as a model of successful dietary intervention with an appropriate study of bioavailability. This study was one of the human intervention studies that substantiated the accepted health claim related to cocoa flavanols and maintenance of normal endothelium-dependent vasodilation (EFSA, 2012), and therefore, it can be considered as a model to follow. The study consisted on an efficacy study performed after a dose-finding study, with 41 diabetic subjects with previous history of coronary artery disease (CAD) that were randomized to receive 75 or 966 mg of cocoa flavanols per day divided in three doses for 30 days. Endothelium-dependent flow-mediated dilation (ED-FMD), which was the primary outcome of the study, was assessed at baseline and on days 8 and 30 of the study, both after an overnight fast and two hours after consumption of the test cocoa drinks assigned. Sample-size calculations, based on the feasibility study, indicated that 20 patients in each group were needed to detect an effect size of 0.9% with a 5% two-sided significance and 80% power. In the high-flavanol dose group, baseline ED-FMD values significantly increased on days 8 and 30, whereas no significant changes were reported in the low flavanol group. The acute effects on FMD (2 h after cocoa ingestion) in the treatment group were of similar effect size at study entry and after 8 and 30 days of cocoa intake, whereas no acute effects were observed in the low flavanol group. Fasting plasma concentrations of flavanol metabolites significantly increased on day 8 and on day 30 from baseline. The EFSA Panel considered that this study showed an effect of cocoa flavanols on fasting ED-FMD, at doses of 966 mg/day consumed for 30 days in diabetic subjects with CAD, and that the effect is already observed after 7 days.

Conclusions

The effect exerted by the food matrix composition during the digestion process may modify important parameters, such as the stability of the food CBAs during digestion and their release from the food, modulating their bioavailability. Thus, prior to carrying out human clinical trial, *in vitro* models are very useful techniques for mimicking biological situations and allowing the study of the food matrix effect. The next step is *in vivo* preclinical trials using experimental animals, generally rats or mice, which might be very useful to study the tissue distribution of CBAs as support to the mechanistic studies. Nonetheless, the response obtained by *in vitro* models is not always directly extrapolated to *in vivo* systems. So, according to the technical guidance for the preparation and presentation of an application for authorization of a health claim draw up by the EFSA, *studies realized in animal or other model systems cannot substitute human data* (EFSA, 2011a). To better-defined human intervention studies aimed to assessing physiological endpoints linked to disease, research regarding the bioavailability of CBAs is required if a complete application for authorization of a health claim wants to be presented. Particularly, it is necessary to perform postprandial human studies to assess the effects of the dose and the food matrices on absorption, as a previous step in the design of the final functional food or beverage that will be tested in the

interventional efficacy study. The postprandial studies may be very useful also to explore different approaches to improve the bioavailability of the CBAs such as the encapsulation. In addition, long-term, randomized, controlled, dietary intervention trials with appropriate CBA compliance biomarkers are warranted in order to assess the full and unequivocal role that a CBA or a characterized combination of CBAs play in preventing chronic human disease. If the biomarker/s used to assess the claimed effect in humans is complemented with well designed and conducted pharmacokinetic studies, these studies may ultimately be used to present an appropriate application for authorization of a health claim.

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