Contribution of the diverse experimental models to unravelling the biological scope of dietary (poly)phenols

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Abstract

The health benefits associated with (poly)phenols need to be supported by robust and insightful information on their biological effects. The use of *in vitro*, *ex vivo*, and *in vivo* models is crucial to demonstrate functionalities in specific targets. In this regard, bioaccessibility, bioavailability, and tissue/organ distribution need to be fully understood and established. In addition, the structure–function relationships, concerning both descriptive and mechanistic information, between specific compounds and therapeutic objectives, need to be supported by results obtained from *in vivo* studies. Nevertheless, these studies are not always possible or have some limitations, particularly concerning the mechanistic information explaining the health benefits provided that should be covered with complementary experimental models. Based on these premises, this review aims to overview the contribution of the separate experimental approaches to gain insights into the bioaccessibility, bioavailability, and bioactivity of (poly)phenols. To achieve this objective, recent evidence available on the linkage of healthy/functional foods with the incidence of non-communicable pathologies is presented. The different experimental approaches provide complementary information that allows advances to be applied to the knowledge gained on the functional properties and mechanistic facts responsible for the health attributions of polyphenols.

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INTRODUCTION

The consumption of fruits and vegetables has been related to health benefits triggered by bioactive nutrients and phytochemicals. Within the latter group, (poly)phenols have been extensively explored regarding their chemical diversity and biological power.[1] These molecules can be found in specific quantitative profiles in the diverse plant-based foods, critical to the scope as health promoters.[2] The healthy attributions of (poly)phenols are associated with the radical scavenging capacity that has turned them into the largest group of dietary antioxidants that also control signalling pathways that maintain the redox and metabolic homeostasis of cells.[3] More recently, (poly)phenols have been demonstrated as having powerful anti-inflammatory, anti-tumoral, and anti-microbial effects, thus contributing to the prognosis of different diseases.[4-7]

This association is relevant because of the increase in the incidence of non-communicable disorders (diabetes, obesity, cancer, cardiovascular diseases, and neurological disorders) experienced in the last decades.[8-11] The epidemiological indicators on these processes show diabetes, cancer, and cardiovascular diseases as responsible for up to 29.1 million deaths per year.[12-15] Even more important, the incidence of these pathologies is expected to increase in the next decades and, proportionally, the costs for the health systems that nowadays account altogether for almost 5 trillion USD per year.[9,10,16,17]

Although for almost all these diseases the aetiology remains uncertain, the role of genetics and environmental factors is broadly accepted, with dietary patterns appearing as a critical factor.[8,9,18-20] This preventive capacity is attributed to non-nutrient bioactive phytochemicals that contribute to fine-tuning the sugars and fatty acids metabolism, besides the balanced nutritional composition of plant-based foods.[21-26] The bioactivities displayed by these food components not only enhance the control of the mechanisms responsible for diseases but also prevent the development of comorbidities.[27-30] The strategy to increase the knowledge about the positive contribution of plant-based foods in the diets, especially with regard to (poly)phenols, is focusing the efforts of research in the field of food science and technology.[31,32]

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The biological properties of (poly)phenols have been demonstrated in studies that have noticed them as potential molecules to combat different types of cancer,[33-35] and their healthy attributions to cardiovascular[6] and neurological health.[36,37] However, it should be considered that the health-promoting effect of these compounds can be overshadowed by a reduced bioavailability due to low intestinal absorption, rapid biotransformation, and the activity of gut microbiota.[38-40]

The description of these biological traits has boosted the demand for this type of product by consumers, encouraging the application of plant-based sources of these compounds in the agro-food, nutraceutical, and cosmetic industries by developing new products.[2,41-43] Taking advantage of these bioactivities could be achieved by the fortification of foods, but it has to be borne in mind that changes in organoleptic properties, oxidative stability, and shelf-life have to fit the consumers' expectations.[44-47]

Despite this emerging trend, the approval of healthy claims under the label of 'functional foods', 'health foods', or 'nutraceuticals' requires a diversity of *in vitro* and *in vivo* studies to provide evidence on the benefits, functionality, and mechanisms of action (Fig. 1).[44,48-53] Although unravelling the biological function continues to be a cornerstone of the development of new functional foods or ingredients, in addition to specific bioactivities, the current challenges involve the effect of gastrointestinal digestion and the absorption of bioactive compounds because these processes condition the concentration of phytochemicals in cells and, thereby, the bioactivity. Hence, this review summarizes the most relevant findings described in recent years concerning the relationship between food components and molecular pathways related to relevant pathophysiological conditions. An overview of the specific contribution of the diverse experimental models to sort out existing gaps of knowledge on bioaccessibility and bioavailability of (poly)phenols are addressed.

BIOAVAILABILITY OF (POLY)PHENOLS: A KEY ISSUE TO EXPLAIN THEIR BIOACTIVITY

Bioaccessibility (the proportion of a food constituent that releases from the matrix during digestion and remains available for absorption[38,54,55]), intestinal absorption, and metabolism define bioavailability (the proportion of an ingested compound that reaches the systemic circulation and is spread through the organism).[56] All these processes are key for the bioactivity of (poly)phenols, since they are linked to the final concentration in cells.[38,57-59] This association suggests the necessity of characterizing the profile of the raw foods and the diversity formed as a consequence of gastrointestinal digestion and metabolism,[56] which are responsible for the health benefits attributed to them.[56] Also, it should be considered that they are conditioned by inter-individual variability (genetic background, gut microbiota profile, dietary pattern, or physical activity, among other factors) (Table 1).[57,69,70]

The digestive extraction of food components starts in the oral cavity, due to mastication and the activity of salivary amylase, which break down the food structure.[70,71] Then, the food matrix is degraded in the stomach because of the acidic environment and enzymatic activity of gastric pepsin, jointly to peristaltic motion.[72] This process causes the hydrolysis of some phenolics, namely phenolic acids, anthocyanins, and tannins.[72,73] After gastric digestion, non-absorbed (poly)phenols pass into the small intestine, where the process continues under alkaline conditions with the aid of specific enzymes (alkaline phosphatase and pancreatin).[73] When the pH changes from acidic to neutral, only 5–10% of (poly)phenols present in the food matrix are absorbed.[72] As the pH rises, bile salts and pancreatic enzymes promote the digestion of apolar compounds and the formation of water-soluble micelles, decreasing bioavailability.[73] During the intestinal phase, most phenolic compounds suffer hydrolysis reactions catalysed by lactase-phlorizin hydrolase or cytosolic β-glucosidase, giving rise to aglycones that are absorbed by active or facilitated transport.[74]

A relevant factor influencing the bioaccessibility and bioavailability of (poly)phenols and derived bioactive compounds is the metabolism of the intestinal microbiota.[75] Microbial derivatives are synthesized from molecules not absorbed in the small intestine, refluxed into the intestinal lumen from epithelial cells, or participating in the enterohepatic circulation (e.g. urolithins).[72] For instance, the glycosylated forms of phenolics (e.g. linked to a rhamnose moiety) that cannot be absorbed in the small intestine reach the colon and are hydrolysed by the microbiota metabolism (α-rhamnosidase activity). This is important, because microbial derivatives could show higher bioactivity than the original compounds (e.g. equol or dihydro-resveratrol),[73] augmenting the biological scope of the phenolic fraction of foods.[76]
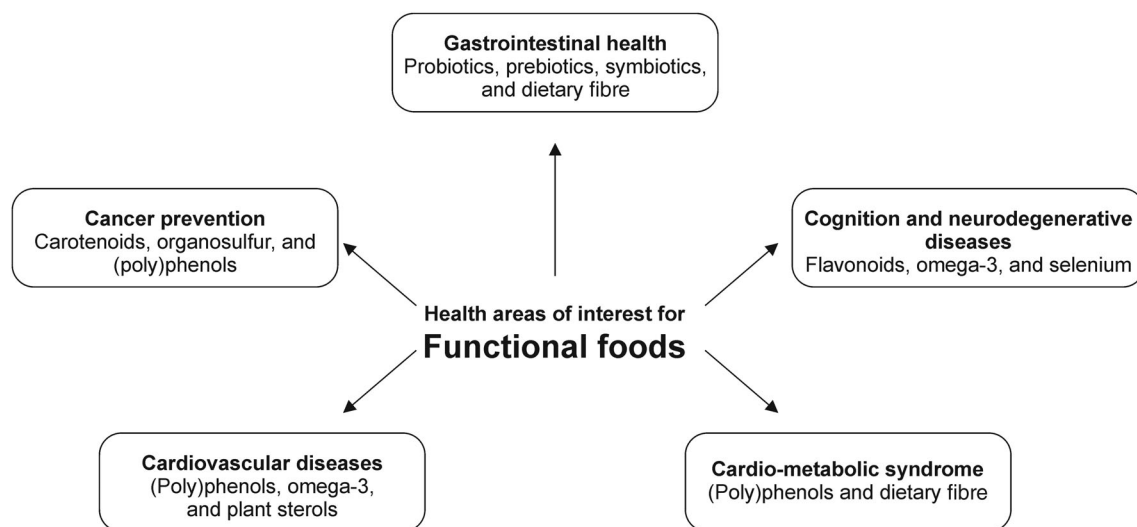

Figure 1. Benefits for health and prevention of pathological conditions attributed to diverse components of functional foods.

Table 1. Factors conditioning bioaccessibility of dietary (poly)phenols

Factor			Ref.
Phenolics-related factors	Chemical structure	Chemical structure solubility bonds with sugars (glycosides), methyl groups, etc. stereo-configuration	60
	Interaction with other compounds	Bonds with proteins (e.g. albumin) or with (poly)phenols with a similar mechanism of absorption	61,62
Food-related factors	Food processing	Thermal treatments (freeze-dry, cooking, transport, storage, and culinary methods)	63,64
	Food interaction	Food intrinsic factors (physiological age, ripeness, absence of diseases) and food matrix effects (presence of positive or negative effectors of absorption; e.g. fat and fibre)	65
Host-related factors	Dietary intake	Differences between countries and seasons quantity and frequency of exposure, single or multiple doses	66
	Absorption and metabolism	Intestinal factors (e.g. enzyme activity intestinal transit time colonic microbiota); systemic factors (e.g. gender and age disorders and/or pathologies genetics physiological condition)	66
Other factors	Distribution and food content	Exclusivity in some foods (e.g. soy isoflavones, flavanones in citrus); ubiquity (e.g. quercetin)	67
	External factors	Environmental conditions (e.g. photoperiod, temperature, humidity, abiotic stress)	68

The absorption of bioaccessible (poly)phenols initiates in the gastric mucosa for specific classes of phenolic compounds (e.g. anthocyanins),[77-79] whereas most phenolics are absorbed in the small intestine (Fig. 2).[80,81] The most relevant property of (poly)phenols influencing gastrointestinal absorption is the presence of esterifications that provide high molecular weight compounds and differential hydro-/lipophylicity.[82] The degree of glycosylation influences the effectiveness of intestinal absorption, as demonstrated regarding glycoside derivatives of quercetin (52%), whose absorption rate is higher than that of the
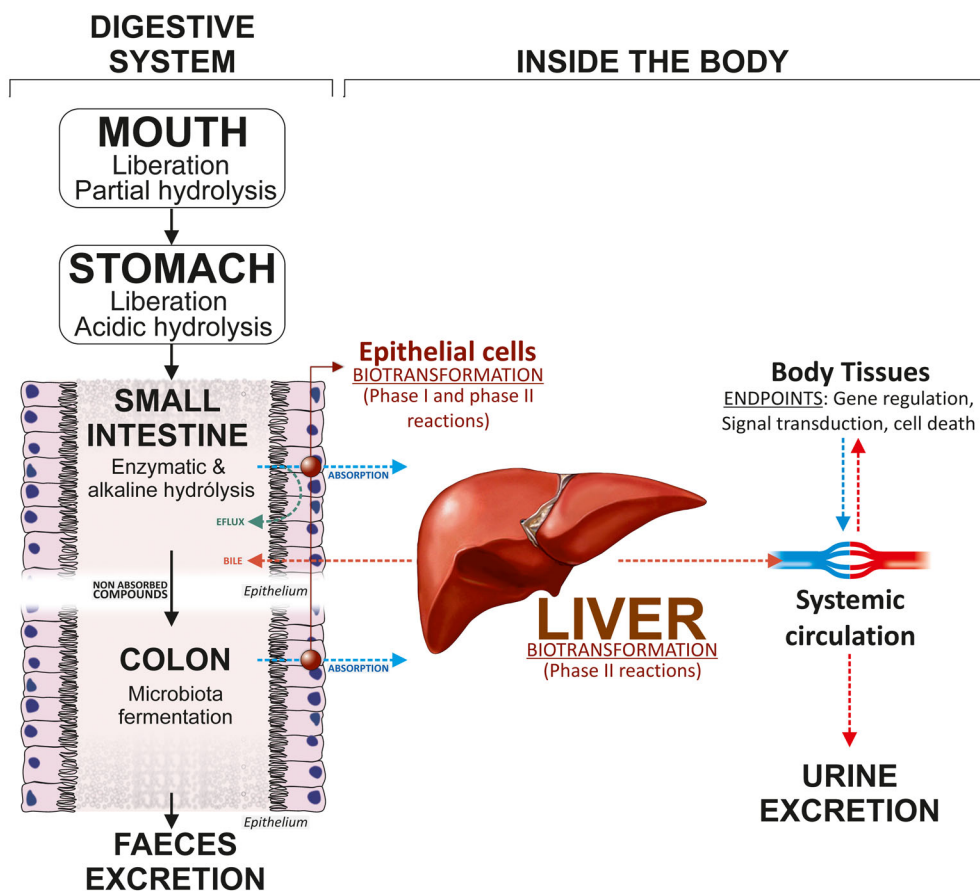

Figure 2. The journey of food bioactive compounds from food through the human body.

aglycone form (24%).[83,84] Only 5–10% of the total (poly)phenols ingested are absorbed in the small intestine. The remaining 90–95%, representing compounds not absorbed or refluxed in the intestinal lumen from epithelial cells or the bile (Fig. 2), reaches the colonic sections of the intestine, where they are metabolized by the resident microbiota via hydrolysis, dihydroxylation, demethylation, and/or decarboxylation reactions[38,85] to produce compounds of lower molecular weight that can be absorbed more easily.[86] However, this percentage varies depending on the criteria used for identifying the amount of compounds absorbed and spread over the body tissues. Some workers consider that only those compounds maintaining the chemical form described in foods should be used for calculating the bioavailability rate,[87] whereas others also account for the phase I, phase II, and microbial derivatives.[88] The latter criterion gives rise to percentages of absorption of up to 60–70%.[88] This is not a negligible decision, because (poly)phenol derivatives are formed to neutralize and speed up the excretion of xenogeneic compounds and potentially harmful compounds, and these reactions would lower their biological capacity.[75]

Concerning the mechanisms of absorption, most (poly)phenols are absorbed by passive diffusion, characteristic of low molecular weight phenolic compounds such as phenolic acids, catechin, and epicatechin, or with the participation of transporters present in the enterocytes' membrane, such as P-glycoprotein, sodium-glucose cotransporters (SGLT1) or glucose transporter 2, in the case of some glucosides like quercetin-3,4′-O-glucoside.[55,70,78,89,90] The participation of these mechanisms is dependent on the structural features of the phenolic compounds, such as molecular weight, lipophilic features, stereochemistry, and/or the presence of reactive groups capable of bonding hydrogen.[70]

Once absorbed, (poly)phenols and their microbial derivatives are metabolized via an array of enzymatic and non-enzymatic reactions. Phase I reactions (hydrolysis, oxidation, and reduction) modulate the bioactivity, increasing, decreasing, or cancelling the biological relevance of (poly)phenols as a result of the change of their chemical structure.[70] The phase II reactions involve the addition of chemical radicals to the phenolic structure (glucuronidation, methylation, and/or sulfation).[38,70] The metabolism of the dietary (poly)phenols critically influences their distribution over the different tissues and cell types, as well as their excretion rate since there is a direct impact on the compounds' polarity (Fig. 3).[40,58]

As a side effect of the metabolic transformation of (poly)phenols, the resulting new molecules constitute molecular tools that turn the biological systems more efficient by providing enhanced capacity to respond to the physiological requirements of cells.[38] However, nowadays, there is an information gap on the biological relevance of the (poly)phenolic derivatives formed after ingestion,[91,92] being needed further research on the biological properties of phase I and II derivatives, and microbial metabolites to set up the most useful concept of bioavailability of (poly)phenols to make predictions (Fig. 3).[38] In this concern, for instance, caffeic acid, a natural phenolic compound and a common degradation product of more complex (poly)phenols,[93] is of special relevance since it is associated with an array of biological attributions related to health, such as a protective effect against angiopathy and type II diabetes mellitus[94] or reduction in body weight, due to regulation of the gut microbiota.[95] Another metabolite of interest is 3,4-dihydroxyphenylacetic acid, a colonic metabolite of flavonoids that prevents or delays the damage caused by glucotoxicity[96] or protects against pancreatic β-cells dysfunction.[97]

Although bioavailability can be assessed complementarily by applying in vivo, ex vivo, and in vitro models, only in vivo assays provide real and accurate bioactivity outcomes for specific compounds, given the compensatory mechanisms available in complex organisms that are not accurately represented by in vitro and ex vivo models. This fact dulls the scope of the findings retrieved from the diverse methodologies,[98] with an integrative perspective that helps to make sense of the complex information available being required.

LIMITATIONS OF THE DIVERSE EXPERIMENTAL MODELS FOR ASSESSING BIOAVAILABILITY

Preclinical and clinical models are confined to a limited capacity to assess the effect of gastrointestinal digestion and absorption on the bioavailability of phytochemicals, and the mechanisms responsible for their biological attributions (Fig. 4). As a result, a diverse number of ex vivo and in vitro models have been developed to obtain mechanistic outcomes and overcome the ethics limits related to in vivo investigations.

Experimental models of interest to research on bioavailability

The information available on the bioaccessibility and/or bioavailability of phenolic compounds is increasing day by day due to methodological advances regarding experimental designs and models that provide complementary information and the use of less-invasive approaches.[99] In this regard, bioaccessibility is mostly studied by resorting to in vitro models that provide accurate information on the concentration of food compounds available for intestinal absorption. On the other hand, bioavailability is studied by both in vitro (monolayer and multilayer models of the intestinal barrier) and in vivo studies that resort to acute and chronic interventions to measure the concentration of phenolics in plasma (pharmacokinetic) and urine (bioavailability) (Fig. 4).[98]

Thus, although in vivo models allow the development of holistic studies on the health effects of the compounds present in foods and are useful to monitor the physiological regulation and feedback factors, they are difficult and expensive studies to carry out and are ethically questionable.[100] In addition, the results expected from in vivo research are weighed down by the high inter-individual variability, which along with the limited number of experimental groups does not lead to retrieving significant evidence. In this sense, although the results obtained so far using in vivo models are essential to get recognized by the European Food Safety Authority, in vitro methodologies appear as a tool that helps to obtain comprehensive characterizations of the biological interest of bioactive compounds. Beyond just the scientific consideration, in vitro models are faster and more cost-effective alternatives, although the results obtained by resorting to these methodologies cannot be directly extrapolated to in vivo systems due to gaps in the capacity to reproduce the dynamic environment of the digestive system and the chance to monitor regulatory capacities (Fig. 4).[58]

In vitro and ex vivo models for assessing bioaccessibility and bioavailability

The objective of in vitro models in the frame of studying the impact of gastrointestinal physiology on the bioavailability of phytochemicals is to study changes in the quantitative profile of the compounds ingested, resulting from the digestion and spread over the organism.[101] Currently, two types of in vitro digestion models have been developed: first, models mirroring in vitro the
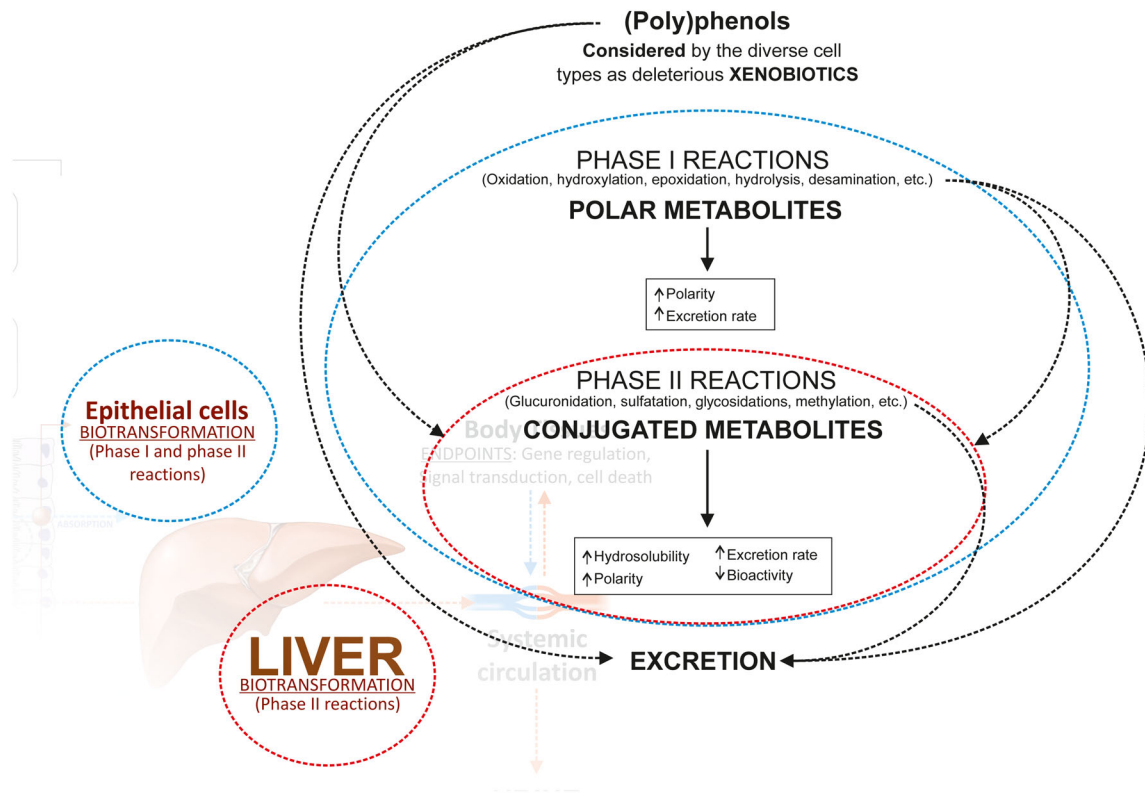
Figure 3. Metabolizations included in phase I and II reactions and their effects on the excretability and bioactivity of the compounds.
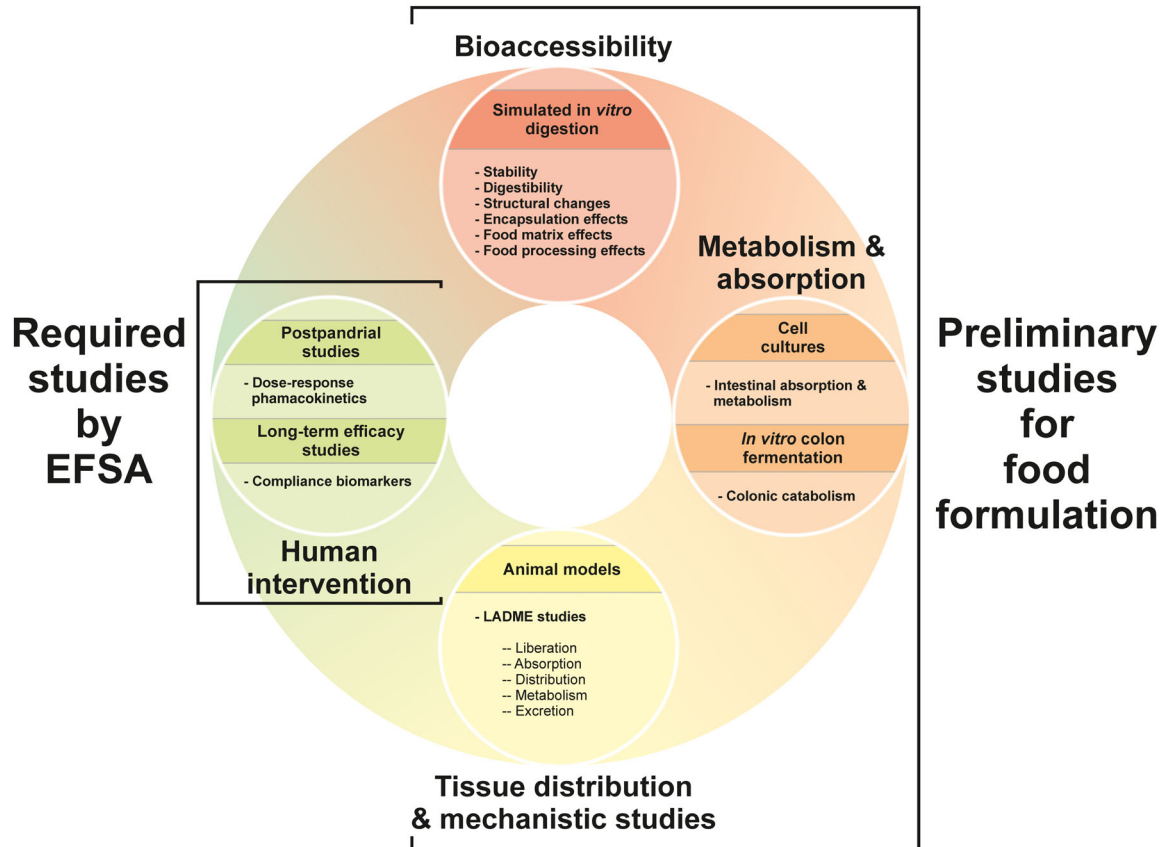

Figure 4. Experimental workflow to perform nutrikinetic studies with food bioactive compounds. EFSA, European Food Safety Authority.

functioning of the small intestine that have been promoted as very useful to study bioaccessibility and digestibility, structural changes, and release of food constituents under gastrointestinal conditions[102]; and second, the colonic fermentation models that are applied to study the relevance of the colonic metabolism for generating bioavailable compounds contributing to the biological effects of foods and the impact of dietary components on the gut microbiota (prebiotics).[103]

Concerning digestion models, two different subsets (static and dynamic) can be differentiated. *In vitro* static digestion models are simple, cheap, easy to build and handle, and allow permanently monitoring of the physicochemical processes during digestion. Moreover, they allow the kinetic study of the release of bio-components from food matrices and nanoparticles.[56] Static models reproduce the digestion conditions of independent stages of the gastrointestinal digestion process or all the stages as a whole integration of the sequence of successive digestions.[104] However, the analysis of the different digestion phases by the passage from one compartment to another is a discontinuous process, and hence these models do not allow the faithful mimicking of the mechanical forces involved. To enhance the capacity of the static *in vitro* models, dynamic *in vitro* alternatives have been used.[56] In these models, the physical and biochemical parameters of the digestion can be controlled, allowing the taking of samples from different 'digestive compartments' and simulating the digestive conditions regarding the entire process in a continuous workflow.[105] Also, dynamic models reproduce specific conditions affecting groups of the population, namely infants, adults, and elderly people, and/or a variety of pathophysiological conditions.[105] The main limitations identified regarding dynamic *in vitro* digestion models are their sophistication, high cost, handling difficulty, and greater time of analysis. Despite these constraints, these alternatives are considered a valuable option to study the relationship between gastrointestinal physiology and the food components.[56]

The use of *in vitro* digestion models is useful to shed light on the bioaccessibility of phenolic compounds. In this regard, related to static models, Lingua *et al.* showed the effect of digestion on the phenolic profile and the antioxidant capacity of grapes and red wines, consisting of three sequential and independent steps: the digestive process in the mouth, stomach (gastric), and small intestine (duodenal) digestion.[106] This research proved that mouth and stomach digestion phases favour the bioaccessibility of (poly)phenols, whereas intestinal digestion decreases the bioaccessible fraction, mainly due to the alkaline pH of the digestive fluid. However, despite this diminution, both matrices still retain antioxidant capacity, mainly due to the stability of anthocyanins (the most relevant compounds) at low pH.[106] Moreover, Czubinski *et al.* demonstrated how the digestion process increases the bioaccessibility of (poly)phenolic in defatted lupin seeds.[104] In this case, the simulation of gastric processes *in vitro* resulted in qualitatively altered phenolic compound profiles of lupin seeds, and approximately 80% of phenolic compounds released during the *in vitro* digestion.[104]

Regarding the colonic fermentation models, several alternatives have been described to date, including 'batch', the simplest model used to perform fermentation studies and substrate digestion assessments, and a continuous fermentation model used to assess long-term studies, owing to its capacity to simulate diverse *in vivo* conditions upon a continuous workflow.[107] The major disadvantage of these studies is the absence of host functionality, limiting the capacity to simulate the intestinal motility, bile secretion, pH, and absorption capacity, among other traits. This drawback was suppressed, to some extent, by the development of artificial digestive systems that can simulate the different human digestive functions.[108]

These models are very useful, as the colonic microbiota plays an important role in the bioavailability of phenolic compounds. In this sense, Sun *et al.* described the metabolism of *Tetrastigma hemsleyanum* roots using the colonic fermentation model and demonstrated that colonic microorganisms can decompose the (poly)phenols present in this plant, as the total phenolic and total flavonoid contents were reduced by 61% and 76% respectively.[109] Moreover, a recent study carried out by Vázquez-Rodríguez *et al.* showed the potential of *Silvetia compressa* to improve human gut microbiota by the use of this model.[110]

Once the effect of the gastrointestinal digestion on the release and stability of bioactive phytochemicals present in dietary plant-based foods available to be absorbed at the intestinal level is clarified, cell cultures represent an interesting predictive tool to study the capacity of bioactive compounds to cross the intestinal barrier.[111] In this sense, it allows assessment of the transport efficiency across a simulated intestinal barrier and the metabolism of absorbed compounds using human or animal tissue to clarify the behaviour of specific alive cell types under controlled environmental conditions.[102] However, some drawbacks of *in vitro* approaches have been identified, among which are the preservation of the tissue structures (including different layers of cell types interacting with each one to another), obtaining adequate cell differentiation, the inability of cells to proliferate, their rapid degradation, and the requirement of technical or specific culturing conditions.[111] Following the successful development of these models, some of the different strategies or techniques nowadays include the use of cells isolated from the small intestine or the colon, cell lines from normal tissues, normal cell lines transfected with regulatory genes, and tumour-derived cell lines.[111] In the case of tumour-derived cells, the Caco-2 (human colorectal adenocarcinoma cells) cell line is widely used in nutritional modelling to simulate the characteristics and processes that take place in the non-pathologic adult intestine.[112] Also, the HepG2 (human hepatoma cells) cell line and primary hepatocytes are used to evaluate hepatic biotransformations, toxicity, and pharmacology of xenogeneic compounds, including phytochemicals of plant-based foods origin.[112]

In this sense, Gonçalves *et al.* used Caco-2 cells to study the bioaccessibility and bioavailability of sweet cherries phenolics describing the close relationship between both parameters.[113] For its part, Sun *et al.* used HepG2 cells to study the bioaccessibility of flavonoids, phenolic acids, and carotenoids of citrus fruits.[114]

Other types of experimental models contributing significantly to the study of the digestive behaviour of food components are the *ex vivo* ones. These models are an intermediate step between the *in vitro* and *in vivo* options and involve a whole/part of living tissues with functional environments to cells found *in vivo* that will be cultured *in vitro* and analysed accordingly.[115] The *ex vivo* research conditions allow experiments in cells or tissues of organisms under more controlled conditions than those possible resorting to preclinical and clinical *in vivo* models, as the natural environment is modified, and experiments or measurements can be established that would not be possible or ethical otherwise in living subjects.[115]

As an example of the application of *ex vivo* models, Huyut *et al.* studied the inhibitory effects of some phenolic compounds on the activities of carbonic anhydrase and suggested the consumption of

(poly)phenols as a treatment against antiglaucoma or as a diuretic,[116] and Sartini *et al.* demonstrated that green tea extract increases the antibacterial activity of amoxicillin.[117]

Preclinical and clinical *in vivo* *models of digestion and bioavailability*

Despite the constant evolution of *in vitro* models and the improvement of their capacity to simulate the physiological events occurring *in vivo*, replacement of the use of experimental animals, or directly the avoidance of *in vivo* models, even if existing *in vitro* alternatives, currently, *in vivo* studies still cannot be replaced as easy. In this sense, animal (preclinical) models continue to be widely used to fill the gap between *in vitro* studies and clinical or nutritional interventions in humans.[101]

The main advantage of using experimental animals, relative to clinical models, is that preclinical studies can be established at almost any stage of their life cycle, from *in utero* exposure to the animal's death, and can also include intergenerational studies when the experimental hypothesis is consistent enough. Moreover, the variability that is dependent on environmental factors is minimized by using animals with known genetic and health conditions, also so-called 'defined animals'.[108]

Most nutritional studies related to the metabolism of bioactive compounds from food are developed with the use of rat and mice models, owing to their similarity to human physiology regarding the digestive processes and metabolism, from absorption to excretion, their low cost, and easy reproduction and handling. The use of rodents allows the determination of the concentration of bioactive compounds and their metabolites in the diverse tissues and to set up biological targets attributable to the compounds under study, *in vivo*.[108]

The use of animal models has allowed advancing through bioavailability and bioactivity of phenolic compounds. In this regard, Agulló *et al.* demonstrated the analgesic power of maqui berry (anthocyanins), and the synergism of this berry and citrus (flavanones) in this activity.[118,119] Moreover, the use of animal models has allowed us to learn more about the distribution of the different phenolics in the organism, and it has been possible to suggest that the neuroprotective activity of anthocyanins could be due to their ability to cross the blood–brain barrier.[120]

The 'last' step in nutritional research is represented by human interventions that allow establishing the actual effect of foods or their bioactive components on the incidence and/or severity of diverse pathologies. These studies also help to understand the different absorption mechanisms involved in the bioavailability of (poly)phenols and to identify the metabolic profile of the compounds absorbed.[86]

Human studies can be divided into nutritional intervention and observational epidemiological studies, depending on the objective. In the field of nutrition, the former is focused on the design of a nutritional intervention under controlled conditions and monitoring the biological result from diverse perspectives (bioavailability or biological attributions).[108] These studies can be divided into single, double, or non-blinded interventions, depending respectively on whether the treatment is hidden for the subject, for the subject and the observer, or if it is not hidden.[108] Moreover, an additional classification allows for dissecting interventional studies into randomized or non-randomized controlled trials, reliant on whether or not subjects have been randomized to receive the intervention. Concerning observational epidemiological studies, these are aimed at measuring the variables defined in the study, without intervention by the researcher. Based on the specific objective, observational epidemiological studies are divided into descriptive (correlational, case-report series, and cross-sectional), analytical experimentation (cases–control and cohorts), and meta-analyses, depending on the capacity of the epidemiological instruments to measure the habitual dietary intake.[108]

It is important to note that the biological effect of the bioactive compounds of foods cannot be fully explained by resorting to the use of human studies since, frequently, these provide information of the end of the process of interest by measuring specific biochemical markers.[100] On the other hand, the intervention period is frequently too short to evaluate changes in the variables studied. In addition, these models are affected by constraints that make the results difficult to understand, such as inter-individual variations, environmental factors, lifestyle, complex organization, ethical considerations, poor compliance by subjects, or dropout of subjects.[108] These aspects, along with the lack of information about the activity of phytochemicals at the molecular level and their role in different processes, tip the balance towards the choice of other models.[101] Also, the methodology of these trials includes the consumption of high doses of the compound or food after a wash-out period, which entails differences from the results that could be obtained after the intake of normal food quantities.[86]

To overwhelm these constraints, it is required to implement some actions. A dose–response study, also known as a postprandial study or acute intake study, should be performed in the frame of a small experimental group as a test to enhance the knowledge about the factors that would affect the bioavailability of target bioactive compounds, as well as their pharmacokinetics.[108] This approach allows the choice of biomarkers of the different phenolic compounds to be addressed. Moreover, this type of study would be critical for the design of new functional foods or beverages, for selecting the optimal dose required to be administered, as well as for a better food matrix for achieving optimal results regarding the bioaccessibility of the compounds of interest.[108]

As an example of what can be studied through postprandial interventions, Mecha *et al.* evaluated both the bioavailability of phenolic compounds from common beans and the interindividual differences, observing an increase in the concentration of the compounds studied in urine and plasma after ingestion.[121] In addition, this preliminary study allowed Agulló and co-workers to identify the metabolites coming from anthocyanins and flavanones mainly after the intake of a maqui–citrus-based beverage, and thus demonstrate that these compounds are bioavailable.[122-124]

Once the dose–response study has been completed, to retrieve evidence on the efficiency of bioactive compounds present in foods, long-term or chronic interventional trials should be performed. The prolonged intake of the food or bioactive compound of interest with time will allow evaluation of the later modification of markers, such as parameters related to inflammation or oxidative stress and long-term hormonal response,[108] which are of special relevance because of the close relationship of these functionalities to (poly)phenols. Thereby, both acute and chronic studies are important and should be considered complementary for gaining insights into the bioavailability mechanisms. Therefore, the best option would be to carry out both studies in a complementary fashion.

In this sense, Curtis *et al.* studied the capacity of blueberries to improve biomarkers of cardiometabolic function in volunteers with metabolic syndrome in a 6 months intervention study and demonstrated that these berries improved endothelial function and systemic arterial stiffness and attenuated cyclic guanosine monophosphate concentrations.[125] Another study, carried out

by Farràs *et al.* during 3 weeks, showed that phenol-enriched olive oils improve high-density lipoprotein antioxidant content in hypercholesterolemic subjects.[126]

Besides the complementarity of human intervention studies with additional experimental approaches to obtain further evidence on the effect on the health of foods, the development of successful long-term studies is confined to the use of optimal biomarkers of bioavailability and bioactivity. In this regard, Spencer *et al.* proposed a list of the most suitable biomarkers to set up an efficient dietary intervention,[127] as follows:

1. *Quantification of the biomarker of interest should be qualitatively and quantitatively robust.* Sensitive and specific techniques should be used to quantify the biomarker of interest in the biofluids previously collected and stored appropriately to avoid the degradation of biomarkers. Thereby, the application of high-performance liquid chromatography–mass spectrometry technology is highly recommended, as the concentration of the bioactive compounds circulating is quite low.

2. *Concentrations of the biomarker in the biofluid of interest should be sensitive to changes in the intake of the dietary component of interest.* It is important to know that interindividual variability and food source affect the relationship between dietary (poly)phenols and changes in the quantitative profile of specific biomarkers.

3. *The biomarker should be specific to the dietary component of interest; that is, variation in its concentration should be due to changes in the intake of the dietary source of the component of interest.* Under ideal conditions, the metabolite should only appear in human biofluids after the intake of a specific (poly)phenol; for instance, excluding the possibility that it is formed after the ingestion of another food or endogenously. Nevertheless, several metabolites in circulation are common for the diverse (poly)phenols.

4. *The interpretation of dietary intake–biomarker relationships will require a clear understanding of the impact of physiological factors and whole diet composition on the kinetics of absorption, metabolism, and excretion of the putative biomarker.*

Regarding biological samples, plasma and urine are the most frequently matrices analysed for assessing bioavailability, pharmacokinetics, and total absorption in the frame of in preclinical and clinical studies to establish the compounds of interest, although this does not exclude the analysis of other tissues to address the organic distribution of the target bioactive compounds. For plasma, many studies analyse samples between 1 and 6 h after the food intake, which provides information about phenolic metabolites.[127] However, the time frame will not provide information about metabolic products that originated in the large intestine, as a result of the microbiota metabolism, where multiple blood sampling over a 24–72 h period should be considered.[127] In this sense, Mosele *et al.*, in their phenol catabolism studies, demonstrated the identity of the colonic catabolites formed after 72 h incubation using an *in vitro* model.[128] In addition, the relationship between the intake and the presence of the metabolites in plasma is dependent on the excretion rate of the metabolite.[127] Sampling urine excreted 24 h after food intake provides information of the total (poly)phenol absorption, as the total concentration of both intestines is included, without the requirement for multiple blood collections.[129] Therefore, the analysis of urinary samples should be included together with the plasma ones to retrieve complementary information from the human studies.

At present, the effects of the dose, background diet, food matrix, age, genetics, and lifestyle on the metabolism and bioavailability

of phenolic compounds are not fully understood, which makes it difficult to compare the outcomes reported as a result of different feeding studies.[130]

In the frame of dietary interventions, to unravel the behaviour of the bioactive compounds present in the food matrix in terms of bioavailability and pharmacokinetics, to identify appropriately the circulating metabolites is a cornerstone to obtaining comprehensive results. In this regard, retrieving quality data on the quantitative profile of circulating molecules derived from the dietary intervention, along with the knowledge of their pharmacokinetics, provides very valuable information on the bioavailability and possible correlation to bioefficacy of the compounds of interest.[108] This is even more important because of the extreme complexity of establishing associations between the biological benefits attributed to a given food and the bioactive compounds responsible for such events, due to the limited information available about their absorption and metabolism. Owing to this difficulty, the identification of suitable biomarkers, measured in biofluids, is essential to set up the relationship between the bioactive compounds ingested and the observed biological effects.[108]

Based on these premises, anthocyanins are metabolized forming glucuronic-, sulfo-, or methyl-derivatives in the proximal gastrointestinal tract, as occurs with other flavonoids, although low concentrations of them have been identified in their intact form in biofluids. Nevertheless, the anthocyanins absorbed are most frequently found as lower molecular weight metabolites, synthesized as a result of the chemical and microbial degradation of anthocyanins.[24,80,131] Exemplifying the results of these coloured flavonoids metabolism, the possible pathways for the production of the diverse phenolic catabolites were exposed by Kay *et al.*[130] According to their study, microbial and mammalian enzymes, as well as pH-mediated degradation, could be involved in the biotransformation of cyanidin into caffeic acid. Later on, conjugation reactions, such as methylation, glucuronidation, or sulfation, are carried out by mammalian enzymes, despite dehydroxylation and demethoxylation reactions that are mediated by the metabolic enzymes of the gut microbiota. However, microbial and mammalian enzymes can mediate demethylation and hydrogenation reactions.[130]

C6–C3 catabolites are converted to C6–C1 ones by two α-oxidation reactions mediated by mammalian and/or microbial enzymes, although C6–C3 catabolites may suffer β-oxidation and progress directly to the C6–C1 ones.[130] Moreover, C6–C2 catabolites arise independently, due to α-oxidation reactions or through the intact flavonoid by an independent pathway mediated by the intestinal microbiota.[130] However, the multiple points of absorption of the resulting metabolites do not allow gaining deeper knowledge on the pathways and metabolism affecting phenolic compounds.[130] To sum up, it is necessary to monitor all the metabolites commented herein to have a certain idea of the bioavailability of anthocyanins. Thereby, these compounds will be part of the set of metabolites responsible for the expected biological activity of anthocyanins.

On the other hand, for flavanones, the hydroxylation of flavanone-*O*-glucosides occurs by the action of cytosolic β-glucosidase and/or lactase-phlorizin hydrolase enzymes of the small intestine.[130] The resulting aglycone is subjected to phase II metabolism before entering the circulatory system in the epithelial cells of the intestine.[130] However, most flavanone-*O*-rutinosides pass to the distal gastrointestinal tract, and the colonic microbiota releases the aglycones that, after being subjected to phase II metabolism in hepatocytes and/or colonocytes, pass to the circulatory system.[130] In this regard, the

potential catabolic routes of naringenin and hesperetin, as examples for further flavanones, after the comparison of the results obtained *in vitro* and *in vivo* by Pereira-Caro and co-workers[132,133] were proposed by Kay *et al. Eubacterium limosum* and members of the *Enterobacter* and *Escherichia* genera, with *O*-demethylase and hydrolase activity, are colonic bacteria potentially involved in the transformations proposed by Kay *et al.*[130] Moreover, as the A- and B-ring configuration patterns of many flavanones are analogous to anthocyanins, common catabolites of both can be detected; namely ferulic, hydroxybenzoic, hippuric, and phenylacetic acids, among others.[130]

CONCLUSIONS

As referred to during this review, various *in vitro* and *in vivo* (preclinical and clinical approaches) have been developed for testing the effects of foods on health and unravelling the molecular mechanisms responsible for such benefits. Concern over the use of animals in scientific research has, however, encouraged laboratories to develop alternatives to *in vivo* testing, including a broad diversity of *in vitro* assays for all stages and traits that need to be monitored (bioaccessibility, bioavailability, and bioactivity regarding an array of pathophysiological conditions). Indeed, the advances achieved over recent years regarding the diversity of experimental models has entailed and improved knowledge on both descriptive information on the health benefits that could be foreseen from healthy and functional foods and the molecular mechanisms for such results. Now and shortly, the challenges to be overcome would be incorporating new tools such as computational modelling and algorithms to the humongous amount of data obtained from the available powerful resources on chemical, metabolic, and molecular analyses. This would allow the performance of integrated analysis of the most relevant and urgent outcomes, towards obtaining useful information on combinations of foods, food ingredients, and pathophysiological targets, to take advantage of the functionalities identified and validated, for designing more targeted interventions at the nutritional and clinical practices.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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