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Review

Measuring the intake of dietary bioactives: Pitfalls and how to avoid them

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ABSTRACT

Bioactives are food constituents that, while not essential to human life, can affect health. Thus, there is increased interest in developing dietary recommendations for bioactives. Such recommendations require detailed information about the long-term association between habitual intake and health at population scale, and these can only be provided by large-scale observational studies. Nutritional epidemiology relies on the accurate estimation of intake, but currently used methods, commonly based on a 2-step process involving self-reports and food composition tables, are fraught with significant challenges and are unable to estimate the systemic presence of bioactives. Intake assessments based on nutritional biomarkers can provide an advanced alternative, but there are a number of pitfalls that need to be addressed in order to obtain reliable data on intake. Using flavan-3-ols as a case study, we highlight here key challenges and how they may be avoided. Taken together, we believe that the approaches outlined in this review can be applied to a wide range of food constituents, and doing so will improve assessments of the dietary intake of bioactives.

1. Introduction

Bioactives are food constituents that are not considered essential to human life and procreation, but that can affect human health. With an increased focus to base dietary recommendations not only on the prevention of deficiencies but also the prevention of chronic diseases (Yetley et al., 2017), there is a growing interest in the development of dietary recommendations for these compounds (The National Academies of Sciences et al., 2017). Research into the physiological effect of these compounds relies on nutritional epidemiology. It is the only method that can provide information about the long-term association between habitual intake and health at population scale. However, in order to investigate such an association, it is crucial to be able to measure dietary intake – and as we pointed out previously (Kuhnle, 2018), this is a process fraught with significant challenges and complexity.

Intake of bioactives is usually estimated using a two-step process, in which both steps carry a considerable scope for bias, inadequate specificity and precision, and thus error:

1. Food intake is usually estimated using self-reported dietary data. The limitations of these methods have been discussed in great detail previously (Subar et al., 2015) and are mainly due to recall bias and reporting affected by social and other biases. In addition to these limitations, food-frequency questionnaires (FFQs), commonly used in many epidemiology studies, add another source of uncertainty by often combining foods with vastly different bioactive content (Kuhnle, 2018).
2. Bioactive intake has to be calculated from food intake data and food composition databases (FCDB). While it is well known that food intake data are subject to considerable variability, food composition data are generally not questioned. However, as we have shown and discussed previously, the bioactive content in the same food can vary considerably and apart from few exceptions (e.g. the Phenol Explorer (Rothwell et al., 2013) or the nitrate content database (Blekkenhorst et al., 2017), Fig. 1), most databases provide only information about the average content of a given constituent of foods. Any estimate of bioactive intake is therefore based on average and not actual food composition. Furthermore, it is the systemic presence of bioactives that is key to physiological effects in the human body, and not the mere consumption. Systemic presence is not only affected by the amount consumed, but also by other factors such as food preparation methods, the gut microbiome and other individual determinants of absorption and the pharmacokinetics of specific bioactive. This is not accounted for by this approach.

These shortcomings are generally acknowledged in the *limitations*

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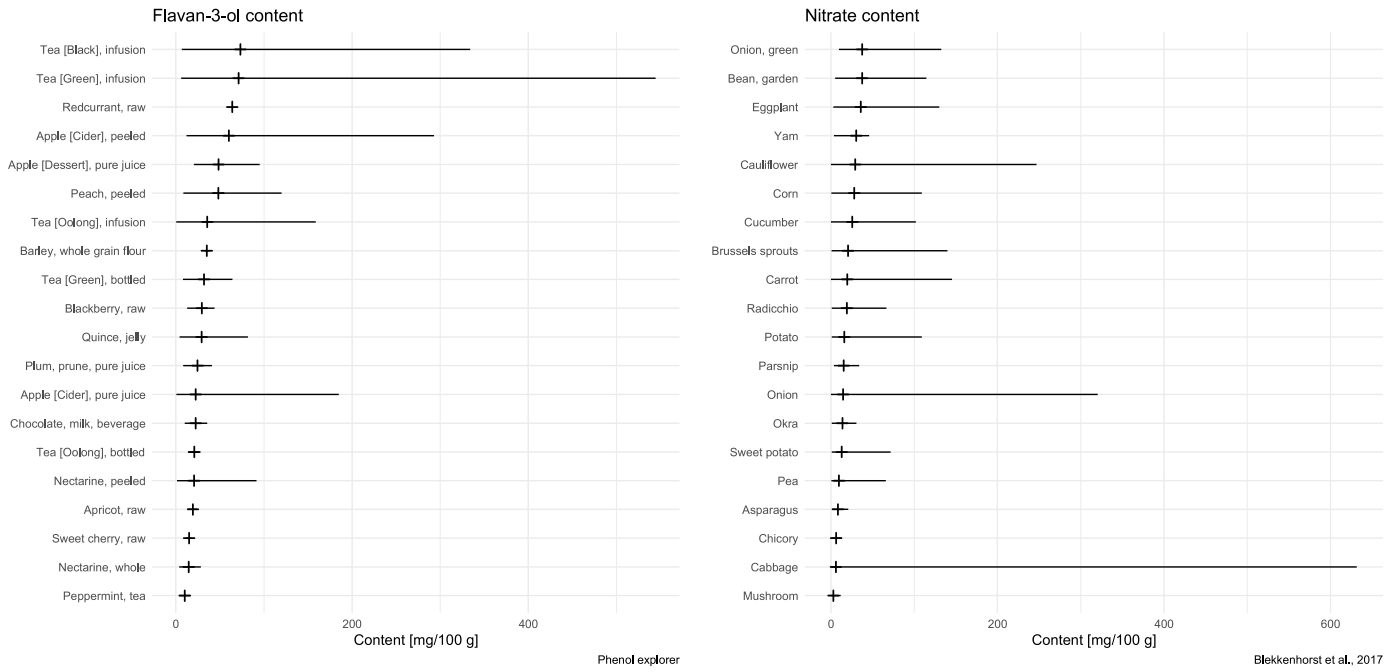

Fig. 1. Food content of two common bioactives, flavan-3-ols and nitrate. The figure shows mean content as commonly used in nutrition research and the range of reported intake.Data from Phenol Explorer (Rothwell et al., 2013) and the nitrate content database (Blekkenhorst et al., 2017).

section of the discussion, but have rarely resulted in further investigation. The impact of these shortcomings can be considerable, and it is likely that they result in significant bias. For example, the mean flavan-3-ol content of a cup of tea is 130 mg, and this value is used in most nutrition studies. In reality, based on published food composition data, a cup might contain as little as 10 mg or as much as 600 mg depending on a range of factors that are not captured by the usual dietary assessment methods. This is a considerable margin of error, which is likely to increase when taking the effect of the dietary matrix on bioavailability and nutrient-nutrient interactions into consideration.

The consequence of this is that estimated bioactive intake becomes simply a proxy for specific food intake, but not necessarily related to actual bioactive intake. An estimated high intake of flavan-3-ols, for example, is therefore more likely a marker of high tea and pome fruit intake – but does not necessarily reflect high actual flavan-3-ol intake. Likewise, a high anthocyanidin intake is simply a marker of berry intake – but not of actual anthocyanidins. This has far-reaching consequences, as associations, or a lack thereof, between intake and health are wrongly attributed to bioactives and not foods.

An alternative to this approach are nutritional biomarkers. These rely on the systemic presence of a bioactive and are therefore not affected by differences in food composition and misreporting. However, biomarkers also need to be carefully developed and validated.

2. Nutritional biomarkers

Nutritional biomarkers are often seen as an objective measure of dietary intake that is not affected by the limitations inherent to intake assessments based on self-reported methods. Biomarkers of bioactive intake are usually based on the presence of these compounds or their metabolites in blood, urine or other biospecimens. This is affected by the specific absorption, distribution, metabolism and excretion of the bioactive, and their use therefore requires careful validation. There are two main aspects of the pharmacokinetic of candidate biomarkers that are important to evaluate:

1) Many bioactives are extensively metabolised after intake by enzymes encoded in the human genome as well as part of the gut microbiome. This results foremost in the fact that bioactives systemically exist in the human body commonly in chemical forms very distinct from those as present in plants and foods, a fact frequently overlooked in investigations of the mechanisms of action based on cell culture system *in vitro*. Such metabolism-induced changes in chemical structure also lead to differences in the biological half-life and other pharmacokinetic properties, and thus represent a significant driver for intra- and inter-individual variability in the systemic presence of bioactives in humans.
2) The extensive metabolism of bioactives leads to a convergence of metabolic pathways. Several metabolites, especially those derived from the gut microbiome, have several bioactives as potential

Box 1
Validation criteria for nutritional biomarkers

1. Biomarkers need to be predictive of intake, *i.e.* there needs to be a causal relationship between intake and biomarker, including a dose-dependent response. This relationship needs to be robust and reproducible.
2. Biomarkers need to be specific for the compound of interest
3. Analytical methods for the analysis of biomarkers need to be robust and validated.
4. The biomarker needs to be reproducible and applicable at scale.

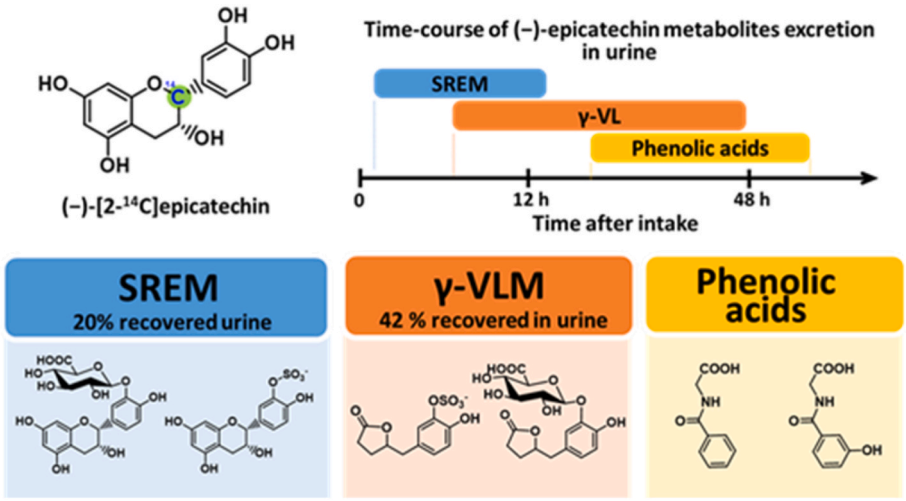

Fig. 2. Metabolites of flavan-3-ols (Ottaviani et al., 2016).

precursors, and this can result in a loss of specificity of candidate biomarkers.

Various evaluation criteria for the evaluation and accreditation of biomarkers have been proposed, and the most important aspects of these criteria are summarized in Box 1. These evaluation criteria provide a clear guideline for establishing the suitability of biomarker intake. However, like with any other process, there are a number of pitfalls that need to be addressed in order to obtain reliable data on intake. Using the example of the development of biomarkers of flavan-3-ol intake, this work will describe the different steps in the process of identifying and evaluating biomarkers of intake for bioactives while highlighting some common pitfalls and suggested solutions.

2.1. *Identification of candidate biomarkers of bioactive intake*

The selection of suitable candidate biomarkers of bioactives intake is crucial for the development of a reliable and robust nutritional biomarker. New candidate biomarkers can be identified by either using a data-driven- or a causality-driven approach. The former approach relies on multivariate techniques to identify suitable biomarkers. An example of this consists in the identification of erythronic acid for sucrose intake (Beckmann et al., 2016). The advantage of data-driven approaches for the development of biomarkers is that the search for candidate biomarkers is conducted already on the biospecimen of choice and that this approach requires very little *a priori* information about the metabolism of individual bioactive compounds. Conversely, this approach may result in the identification of compounds not directly derived from the metabolome of the bioactive, but from bystanders, e.g. contaminants, present in foods consumed. Thus, a data-driven approach to discover candidate biomarkers will require subsequent work to establish causality between the selected compounds as candidate biomarker and the bioactive in question.

In contrast to data-driven methods, the causality-driven approach uses detailed information about the metabolism of the bioactive under investigation. Information on half-life and route of elimination enable the identification of candidate biomarkers that are more suitable for acute (e.g. short plasma half-life) or chronic (long plasma half-life) intake as well as the preferred biospecimen for analysis. This approach allows establishing a causal relationship between bioactive intake and the candidate biomarker, thus contributing to address the first point of the biomarker validation criteria, i.e. establishing causality (Box 1). Overall, data-driven approaches provide a much better characterisation of strength and weaknesses as valuable insights are already provided into the origin of metabolites, inter-individual variability and intake-response relationship. Pharmacokinetic studies with isotopically labelled bioactives provides the greatest advantages for identifying candidate biomarkers. They provide detailed information on the entire metabolism of a bioactive and provide key information about half-life of metabolites and excretion pathways. This information are essential for the selection of candidate biomarkers. For example, a study based on (−)-[2–^{14}C]-epicatechin (Ottaviani et al., 2016) provided detailed pharmacokinetic information and allowed the identification of two distinct groups of metabolites suitable as biomarkers of intake (Fig. 2):

- **Structurally related (−)-epicatechin metabolites (SREM):** approximately 20% of consumed (−)-epicatechin are metabolised as SREMs (Ottaviani et al., 2019a). These compounds have a relatively short plasma half-life (approximately 2 h) and are therefore suitable to measure acute intake or frequent long-term intake when steady state is reached.
- **5-(3,4-dihydroxyphenyl)-γ-Valerolactone metabolites (gVLM):** colonic microbial ring fission metabolites, which make up approximately 40% of metabolites formed. gVLM have a considerably longer plasma half-life (4–8 h) that SREMs and are therefore more suitable to estimate longer term intake.

2.2. *Gut microbiome-derived catabolites as candidate biomarkers for bioactives*

The gut microbiome presents a vast set of enzymes with the potential of catalysing a plethora of different chemical reactions and thus transform dietary compounds, drugs and other xenobiotics (Koppel et al., 2017). This introduces a considerable amount of variability in the formation of metabolites that needs to be investigated in the biomarker evaluation process. In the case of polyphenolic bioactives, the gut microbiome transforms these compounds and affects the bioavailability of polyphenols. More than 70% of (−)-epicatechin is absorbed as metabolites derived from the gut microbiome (Ottaviani et al., 2016). Similarly, cyanidin-3-glucoside is mostly present in circulation and urine in the form of gut metabolites (Kroon et al., 2013). These gut metabolites often have a much longer plasma half-life than structurally-related metabolites and are therefore better candidate biomarkers to assess longer term intake. While abundance and increased half-life are desirable characteristics of gut metabolites, there are also drawbacks in the use of gut metabolites. The functional composition of the gut microbiome shows a very high inter-individual variability (Lozupone et al., 2012), and this will affect the suitability of biomarkers developed from microbial metabolites. One of the most extreme example is equol, which would be a prominent candidate biomarker for

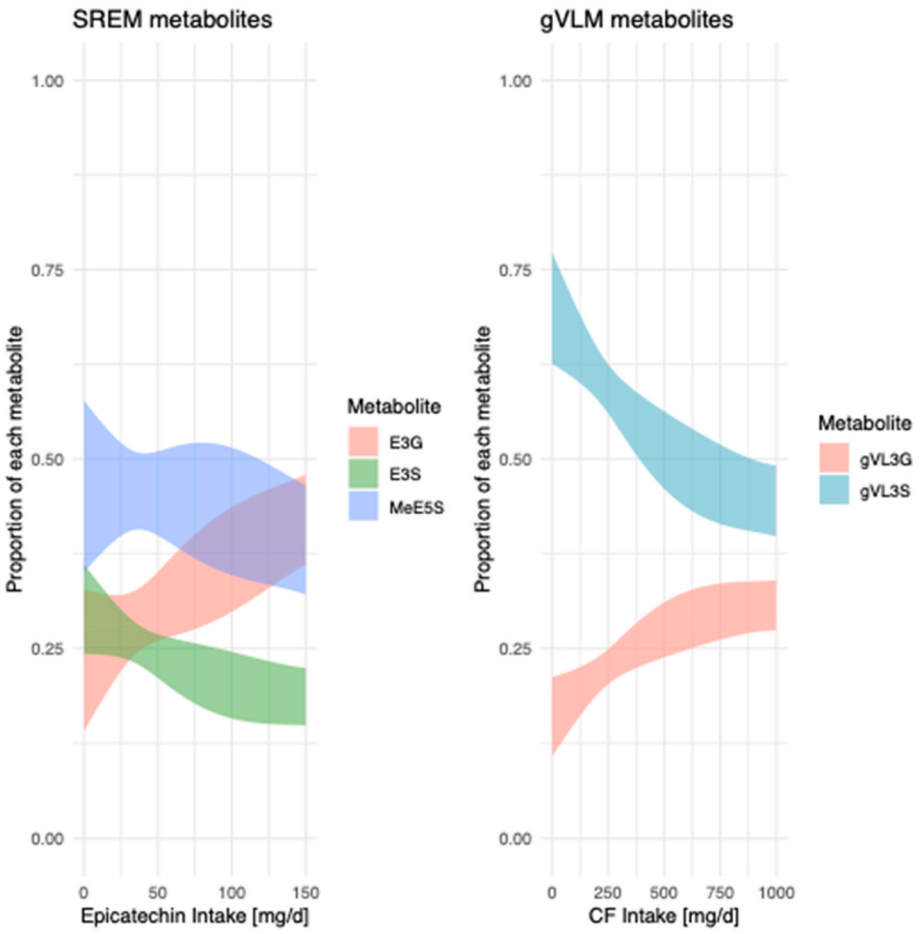

Fig. 3. Intake-dependent relative contribution of individual metabolites on total biomarker (E3S: (−)-epicatechin-3′-sulphate; E3G: (−)-epicatechin-3′-glucuronide; MeE5S: 3′-O-methyl-(−)-epicatechin-5-sulphate; gVL3S: 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulphate; gVL3G: 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide) (Ottaviani et al., 2018a, 2019a).

daidzein intake in a population of equol producers – but would not be suitable for the general public, where equol producer status is not necessarily known (Setchell and Cole, 2006). Enterolignans however, which are also gut metabolites, have much less variability and are commonly used as biomarkers of intake (Rowland et al., 2000). There is a dearth of data and it is crucial to establish the variability of gut metabolites to support the validity for their use as suitable biomarkers of intake. Despite these limitations, gut metabolites can and have been used successfully as biomarkers of intake, for example hippuric acid as biomarker of flavonoid intake (Penczynski et al., 2017), enterolactone as a biomarker of lignan intake (Kilkkinen et al., 2003) and gVLM as biomarker of flavanol intake (Ottaviani et al., 2020).

2.3. Validation against actual intake

The validation of nutritional biomarkers requires establishing a dose response relationship between intake and biomarker. It is therefore important to get reliable information on both, dietary intake and biomarker. Accurate data on actual intake of bioactives can only be obtained from an analysis of the actual foods consumed or by providing known amounts of the bioactive as isolated compounds. Self-report data on food intake is not sufficient, as food composition tables can only provide information about the average food content but not the content of the actual food consumed. As outlined above, this content is affected by a wide range of factors such as cultivar, growing conditions, processing and storage – even apples harvested at the same time from the self-same tree vary considerably in their composition (Wilkinson and Perring, 1961). This means that is not possible to validate biomarkers with self-reported dietary data, as these not only rely on food composition data, but on dietary data that are subject to considerable bias. Consequently, only weak correlations between biomarker and diet-derived data has been shown by us intake (Ottaviani et al., 2020) and others (Almanza-Aguilera et al., 2021).

2.4. Single compound or multiple metabolites

The extensive metabolism of bioactives and inter-individual variability can affect the performance of biomarkers based on a single metabolite. For example, the ratio of sulphonated and glucuronidated metabolites depends on the amount consumed due to different capacities in these enzyme systems (Koster et al., 1981) and is also affected by polymorphisms of SULT and UGT (Lampe, 2009). Therefore, the combination of a set of metabolites can significantly improve performance by improving robustness of biomarkers across a wide range of intake. For instance, the intake of increasing amounts of (−)-epicatechin and other flavanols resulted in significant differences in the profile of sulphated and glucuronidated SREM and gVLM (Fig. 3). It is therefore important to investigate sulphated and glucuronidated metabolites in order to allow the biomarker to be suitable for a wide range of intakes irrespective or UGT and SULT genotype.

2.5. Specificity of nutritional biomarkers

The specificity of nutritional biomarkers is an often neglected yet crucial aspect of biomarker development. Investigating the specificity of a candidate biomarker usually requires a good understanding of

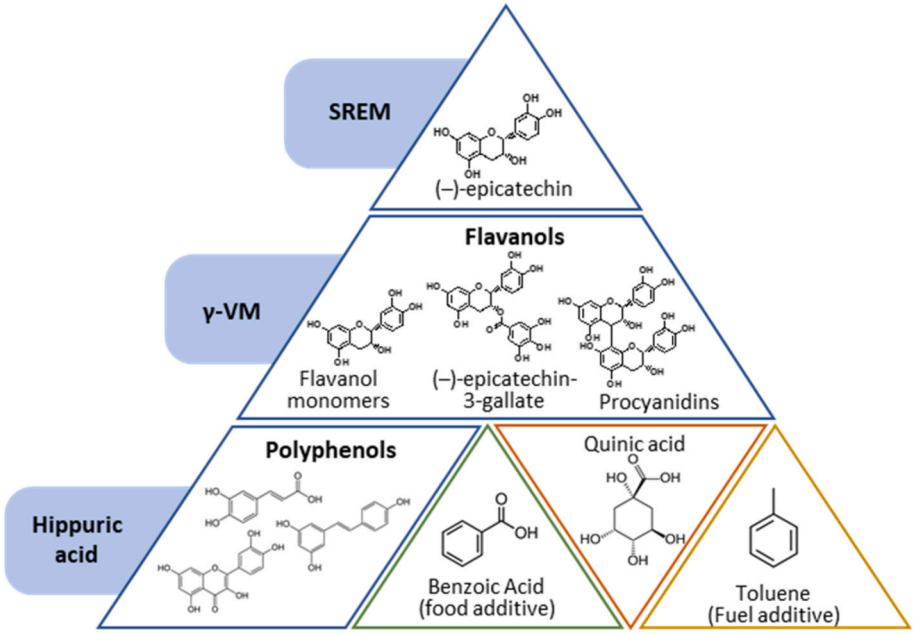

Fig. 4. Specificity of nutritional biomarkers. Structurally-related (−)-epicatechin metabolites (SREMs) can only derive from (−)-epicatechin, whereas γ-valerolactone metabolites (γ-VM) and hippuric acid can have considerably more precursors.

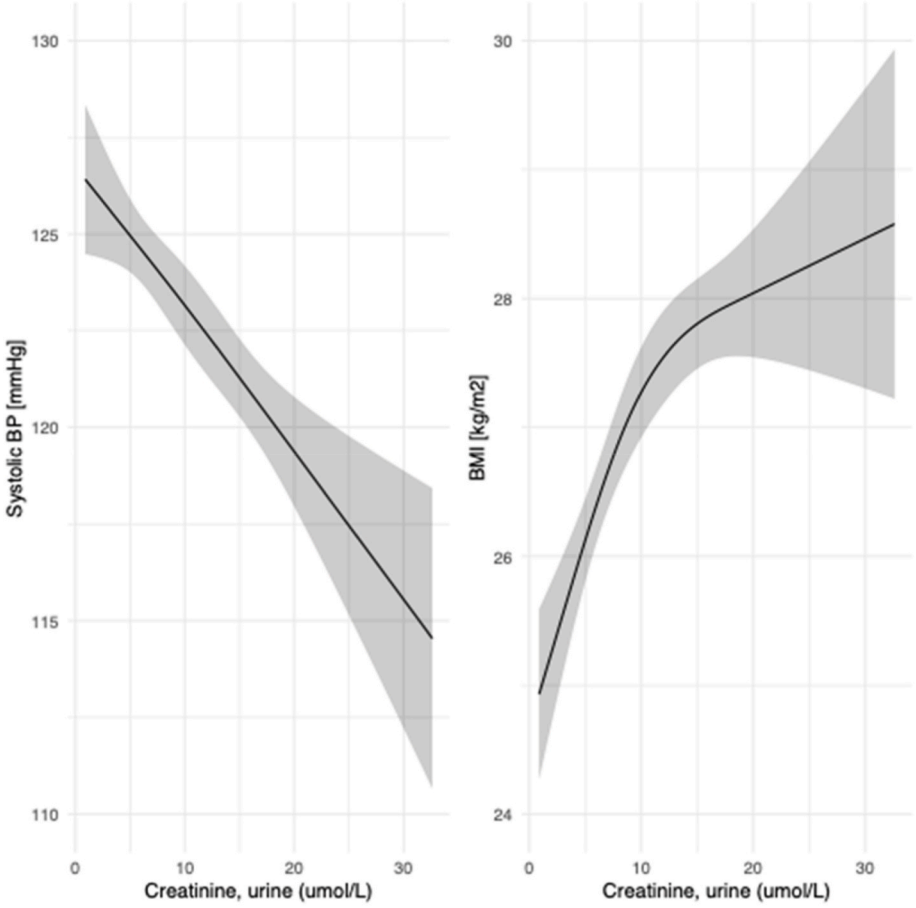

Fig. 5. Association between urinary creatinine and systolic blood pressure (left) and BMI (right) in 3025 participants of NHANES 2017. Figure shows regression analyses (with restricted cubic splines) with urinary creatinine as independent and blood pressure or BMI as dependent variable. Shaded areas are 95% confidence interval.

potential metabolic precursors and carefully controlled dietary intervention studies. There is a paucity of data for many bioactives apart from polyphenols. While structurally-related metabolites, such as SREMs for (−)-epicatechin, are highly specific, colonic metabolites often have several precursors and are therefore not necessarily *predictive* of intake. For example for polyphenols, ring fission metabolites, such as phenolic acids, can have even more potential precursor compounds and are thus only suitable as biomarkers for larger compound classes.

The complexity of the functional microbiome represents therefore a major pitfall when using colonic metabolites as candidate biomarkers. A particular example is hippuric acid, a polyphenol metabolite resulting from the glycination of benzoic acid. Benzoic acid is formed by the gut microbiome following the consumption of polyphenols, and has thus been proposed as a biomarker of total polyphenol intake and indirectly of fruit and vegetable intake (Krupp et al., 2012). However, polyphenols are not the only compounds that result in the formation of hippuric acid and it therefore lacks specificity (Fig. 4). An important contributor to intestinal benzoic acid is dietary benzoic acid, as it is used as food additive and while the average intake in European adults is lower (375 mg/d in the EU (EFSA Panel on Food Additives Nutrient Sources, 2016) than that of polyphenols (500–1800 mg/d (Zamora-Ros et al., 2016), the amount is sufficiently high to interfere with the use of hippuric acid as a biomarker (Christiani et al., 2000). Benzoic acid is also the result of gut microbiome metabolism of quinic acid, aromatic amino acids and a product of phase I metabolism of toluene (Lees et al., 2013). In fact, hippuric acid has been used as a marker of exposure to toluene-added fuels (Kawai et al., 2008).

2.6. Biospecimen selection and the challenges of using spot urine samples

A nutritional biomarker is always the combination of the metabolites selected and the biospecimen in which they are determined, that is a biomarker needs to be seen as *metabolite(s) in a specific biospecimen*. The selection of the appropriate biospecimen is therefore a crucial part of biomarker development and validation. 24h urine is the standard specimen for urinary biomarker as they provide information on total excretion within 24h. These data are essential for *recovery biomarkers,* which provide an estimate of absolute dietary intake. However, 24h urine samples are not always available as they are cumbersome to collect and process. Instead, many studies use spot urine samples consisting of a single void. The advantage of these samples is that they can be easily collected and processed, but they do not provide any direct information of total 24h excretion. In contrast to 24h urine, where total absolute excretion is the parameter of interest, spot urine samples can only provide information on biomarker concentration. This concentration is affected by various factors, in particular ADME and urine volume. ADME can be addressed by the careful selection of various metabolites, but it is more difficult to adjust for urine volume.

A method commonly used in nutrition research, and previously in toxicology for exposure assessment, is the adjustment by urinary creatinine. This adjustment is based on the assumption that creatinine excretion is constant and therefore a suitable correction. However, this assumption is not correct and urinary excretion of creatinine varies according to age, sex, BMI as well health condition such as kidney disease and hypertension (Boeniger et al., 1993; Bruce et al., 1990; Jain, 2017). Due to these limitations, the use of creatinine to adjust for urine volume and dilution is depreciated for toxicological exposure assessments (Barr et al., 2005; O'Brien et al., 2016). In nutrition research, this limitation has become apparent when investigating the association between sugar intake and obesity: the strong association between urinary creatinine and BMI attenuated the observed associations between biomarker and intake almost completely (Bingham et al., 2007). There is also a strong association with blood pressure (Fig. 5), resulting in a potential attenuation or intensification of associations.

An alternative to urinary creatinine is specific gravity, the relative density of urine compared to water (Vij and Howell, 1998). Creatinine and specific gravity can both be used as long as they are not associated with endpoints or exposure (Muscat et al., 2011), and this approach has been used successfully in nutritional epidemiology (Bingham et al., 2007; Kuhnle et al., 2015; Kuhnle, 2021).

Table 1
Common analytical strategies used for the quantification of polyphenol metabolites, including the quantification of polyphenol metabolites as biomarkers of intake.

	Enzymatic hydrolysis	Surrogate standards
Method description	Sulphatases and glcuronidase enzymes are used to hydrolyse sulphated and glucuronidated metabolites of polyphenol metabolites into the parent compound. Parent compound is then measured via LC coupled with different detection methods.	Polyphenol metabolites are resolved and detected via LCMS. In the absence of authentic standards, the corresponding unmetabolized polyphenol is used as a surrogate standard for all metabolites of that polyphenol (e.g. epicatechin as a reference standard to quantify epicatechin-sulphates)
Limitations affecting accuracy and precision	Incomplete hydrolysis, particularly of sulphated metabolites. Stability of polyphenol and polyphenol metabolites is affected during hydrolysis	Different ionization between sulphated/ glucuronidated polyphenol and its corresponding unmetabolized form used as surrogate standard
Limitations affecting robustness	Variation in enzyme activity depending on the origin and lot number	Differences in ionization of analytes between MS instruments and LC conditions

2.7. Analytical considerations

Various analytical strategies have been used for the quantification of polyphenols metabolites over the years, and these have been also applied for the quantification of polyphenols metabolites as biomarkers of intake. However, significant limitations were described for some of these methods, particularly those based on enzymatic hydrolysis and use of surrogate analytical standards (Actis-Goretta et al., 2012; Gu et al., 2005; Ottaviani et al., 2012, 2018c; Saha et al., 2012). A summary of these shortcomings is presented in Table 1. When considering the large number of samples derived from epidemiological studies and other large-scale studies, consistency in the performance of analytical methods is essential to assure validity of findings and to enable comparisons between study cohorts. Thus, methods on enzymatic hydrolysis and surrogate standards should be avoided when applied in the context of quantification of polyphenol metabolites as biomarkers of intake.

More recently, the use of hyphenated MS methodologies – LC-MS – with authentic standards for polyphenol metabolites has become the method of preference for the quantification of polyphenol metabolites (de Ferrars et al., 2014; Dominguez-Fernandez et al., 2021; Mena et al., 2019) and polyphenol metabolites as biomarkers intake (Fong et al., 2021; Ottaviani et al., 2018b, 2019b). This has been enabled by the increasing availability of different chemistry platforms for the *de novo* chemical synthesis of polyphenol metabolites to be used as analytical standards (Curti et al., 2015; Gonzalez-Manzano et al., 2009; Mena et al., 2019; Mull et al., 2012; Romanov-Michailidis et al., 2012; Sanchez-Patan et al., 2011; Sharma et al., 2010; Zhang et al., 2013a, 2013b). This has also made isotopically-labelled internal standards available, which allow for a better control of matrix-specific effects in the ionization of analytes in LC-MS. The matrix effect can affect quantification and remains a major source of error in polyphenol metabolite quantification (Panuwet et al., 2016). While LC-MS methodologies are arguably the preferred technology for the analysis of nutritional biomarkers, this technology may still represent a hurdle for the transfer and wide implementation of these methodologies, especially when using

Box 2
Pitfalls when using biomarkers – and how to avoid them

Candidate biomarkers with very short half-life or high inter-individual variability	Pharmacokinetic parameters of biomarkers are important to allow the selection of suitable metabolites. They can help identify metabolites with particularly long half-life or lower inter-individual variability.
Avoiding colonic metabolites because of variability of gut microbiome	Colonic metabolites can be very useful as biomarkers as they often have a much longer plasma half-life, but they require careful evaluation regarding their inter-individual variability
Unreliable validation data	Biomarkers have to be evaluated and validated against actual intake – not estimated intake based on food composition data.
Dose-dependent metabolism and inter-individual variability	Metabolism depends on a range of factors, including the amount consumed and differences in genotype. A combination of mutilple metabolites can therefore improve outcomes over a larger range of intake
Lack of specificity	Many candidate-biomarkers have more than one pre-cursor, not just colonic metabolites. It is therefore important to establish specificity of any new biomarker.
Selection of appropriate specimen and adjusting for dilution	While 24h urine is ideal, spot urine samples are often more feasible. Adjusting spot urine samples for dilution by creatinine can introduce considerable bias that might attenuate (or exaggerate) associations.
Unreliable analytical methods	Analytical methods should be based on the use of LC-MS with authentic standards. Further development should seek translating methods into moreaccessible analytical platforms

biomarkers during field work or in clinical settings. Thus, translation and adaptation of LC-MS methods to other analytical platforms should be pursued to increase accessibility. Examples of these methodologies for polyphenol biomarker intake already exist, such as the case of colorimetric reagents for the quantification of hippuric acid (Tomokuni and Ogata, 1972; Umberger and Fiorese, 1963). In addition, immunological detection and quantification approaches have been developed for the quantification of biomarkers of lignans and isoflavonoid intake (Adlercreutz et al., 1998; Shinkaruk et al., 2014; Uehara et al., 2000). Ultimately, these advancements will also make possible the adaptation of current technologies into the use-context of personal sensors and devices that could represent valuable tools for developing personalized recommendations for polyphenol intake.

3. Conclusion

Nutritional biomarkers, as molecular tools to assess dietary intake, are crucial for nutrition research. As their use can address and overcome current challenges and shortcomings in meaningfully establishing and interpreting key associations between human health and nutrition. They are the only reliable instrument to estimate intake of bioactive food constituents and therefore have an important role when establishing the health effect of bioactives. The main application of nutritional biomarkers are observational cohort studies, where obtaining reliable estimates of intake at population-relevant scales is crucial. However, applications can go far beyond this: nutritional biomarkers can be used to assess compliance in clinical studies or to stratify participants in clinical trials according to intake. They are also crucial for personalized nutrition, as accurate dietary advice can only be provided with a known baseline and adequate and objective feedback loops. It is therefore important that biomarkers are evaluated and validated to ensure that their application results in reliable, meaningful, and actionable insights. Our findings from developing and evaluating nutritional biomarkers of flavan-3-ol intake represent a useful case study in this context, have utility beyond phenolic compounds, and are applicable to food constituents, generally (Box 2).

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Declaration of competing interest

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