Recommendations on reporting requirements for flavonoids in research¹⁻³

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ABSTRACT
Numerous observational and intervention-based human studies support the notion of a beneficial role for dietary flavonoids in human health. Despite these studies, it is not yet possible to make dietary recommendations with regard to the types and amounts of flavonoids to be consumed. The inherent diversity of flavonoid structure, chemistry, and natural distribution in foods lends itself to errors in reporting the types and/or amounts of flavonoids consumed, as well as incomplete recognition of requirements for intervention studies that aim to assess their benefits in a clinical setting. A need exists for guidelines that facilitate the design and reporting of flavonoid research. With a focus on clinical studies, this article 1) outlines limitations commonly encountered in the field of flavonoid research, including the inconsistent use of nomenclature, inappropriate analytic methods, inconsistent use of existing flavonoid databases, and the lack of full consideration in the design of test materials for intervention trials, and 2) provides guidance for future studies with a focus on clinical intervention trials. Adoption of this guidance will facilitate more accurate and interpretable research that will support the development of dietary recommendations regarding the intake of flavonoids. Am J Clin Nutr 2015;101:1113–25.

Keywords: bioactives, guidance, intervention, polyphenols, supplements

INTRODUCTION
Flavonoids, a subclass of polyphenols, represent a diverse group of plant constituents that are widely distributed in nature and present in a variety of plant-based foods common in the human diet. In the past few decades, the potential benefits of dietary flavonoids to human health have become the subject of intensive investigation. Numerous associations between flavonoid intake (total and subclasses) and human health have been made by using publicly available flavonoid databases (1–5). They include the relations between intake of specific flavonoids or flavonoid-containing foods and the prevalence or incidence of conditions such as stroke (6–8), cardiovascular disease (8, 9), cognitive dysfunction (10, 11), and cancer (12, 13). In many cases, an inverse association was identified between disease risk and the intake of flavonoids or flavonoid-rich foods, particularly for cardiovascular disease risk. In addition to epidemiologic studies, numerous preclinical and clinical studies conducted over the past 2 decades have demonstrated a range of potential health benefits for flavonoids (14–18). Taken together, accumulating evidence exists in support of the notion that diets rich in specific flavonoids may play a role not only in promoting health but also in preventing and mitigating the consequences of a range of chronic degenerative diseases.

In moving from research findings to dietary guidance, the evidence base is built from a combination of studies, including in vitro and animal studies, human intervention trials, and epidemiologic analyses. Although each of these types of studies is important in contributing to the body of literature, the results of human intervention trials, with consideration of the epidemiologic evidence, generally form the basis for consensus science statements, recommendations of dietary guidelines, and authorization of health claims. Despite scientific progress made over the past decades, several critical issues in the design and reporting of studies continue to limit progress in leveraging flavonoid research findings into meaningful recommendations for consumers. These issues include 1) inconsistent use of terminology/nomenclature in description of flavonoids; 2) incomplete/inappropriate application of analytic methods, making determination of food content and dietary intake levels...
challenging; 3) limited data and/or description of flavonoid test materials used in dietary intervention trials; and 4) challenges with application of appropriate methods for assessment of relevant flavonoid bioavailability and metabolite formation in biological tissues that can provide key insights into food and clinical markers/outcomes. In consideration of these limitations, the objective of this article is to outline key considerations for the design and reporting of flavonoid research, with specific focus on consistency in reporting and analytics for use of materials in dietary intervention trials.

SUMMARY OF EXISTING GUIDELINES RELEVANT TO FLAVONOIND RESEARCH

Several position papers and guidelines for research on the evaluation of health benefits of foods have addressed research considerations and legal requirements for health claims. These include guidance statements and documents on assessment of safety and efficacy of functional foods (19–21), probiotics (22, 23), and, to a very limited extent, flavonoids (24). In 2011, the International Life Sciences Institute (ILSI) Europe Functional Foods Task Force commissioned an expert panel to evaluate relevant research studies investigating health benefits of foods to identify strengths and weaknesses of reported designs, methods, and reporting (19). These findings were summarized by using the structure recommended by the Consolidated Standards of Reporting Trials checklist for medical trials (25). Although application of guidelines such as these, including the Consolidated Standards of Reporting Trials checklist, is key to advancing flavonoid research, many unique challenges exist to working with flavonoids and flavonoid-rich foods. For example, material, analytic, and study design challenges have complicated our ability to translate clinical findings to practical guidance for consumers. In 2009, the NIH sponsored a workshop to address common research challenges in the design and evaluation of intervention studies on soy protein/isoflavones, with the main intent of improving the quality of soy studies (24). This remains one of the few attempts at providing flavonoid-specific clinical guidance. Although promising, this guidance was specific to soy-based products and did not address broader issues affecting the translation of flavonoid research. Additional guidance that is more applicable to flavonoid research in general is described below.

KEY CONSIDERATIONS IN DESIGN, CONDUCT, AND REPORTING OF RESEARCH ON FLAVONOIDS

Guidance for flavonoid identification and test material characterization

As researchers attempt to evaluate the potential impact of consuming high flavonoid–containing foods and/or extract-based dietary supplements, an appropriate level of characterization to document the diversity of flavonoid content and forms is required. Flavonoid characterization, whether for a single isolated compound or part of a complex food or extract, must consider aspects of source documentation and accurate reporting of the type and extent of processing along with the flavonoid profile. Proposed guidelines for material characterization, flavonoid identification, and reporting are given below.

Identification of flavonoid-based source material by using taxonomic nomenclature

This step should be applied to any food product or extract used in an intervention study, even when only a single isolated component is examined. Taxonomic nomenclature and description of flavonoid material source must be provided to provide authentication of material. When possible, information on the geographic origin of the material, as well as any details on growing conditions or material processing that may affect flavonoid content, such as harvest date and postharvest processes applied (e.g., fresh preparation, fermentation, acidification, and type and extent of thermal processing such as roasting or heating) or other specific processing methods before use, should be clearly documented. These processes have been reported to have significant affect flavonoid content, composition, and overall bioavailability (26–31) and thus ultimately may have an impact on the efficacy of the flavonoids present. If materials are purchased or donated from a commercial supplier, appropriate information should be provided regarding botanical source, lot information (when available), presence of excipients, and extent to which any further processing has been done for use in a study (e.g., via incorporation into a food matrix, encapsulation, or extraction).

Reporting flavonoid composition by using established and specific nomenclature

Broad terms such as phenolics, polyphenolics, phytonutrients, antioxidants, and even the term flavonoid should be avoided when reporting information on specific flavonoid components. Although these terms are perhaps acceptable for general classification, they are too broad and do not provide the appropriate subclassification required. Standardized flavonoid nomenclature, as described previously (32–34) and summarized in Figure 1 and Table 1, should be adopted for routine use in studies.

Detailed chemical composition of test material used, including specific flavonoid components

A summary of best practices for material definition and quality has been adapted from product quality guidance by the National Center for Complementary and Alternative Medicine (now the National Center for Complementary and Integrative Medicine) (35). The chemical composition of food, extracts, or flavonoid test materials should include accurate flavonoid compositional detail and information on the specific flavonoid chemical form present (e.g., specific glycoside or aglycone form). Methods used to analyze flavonoid constituents should be correctly referenced and described. In studies in which commercial products are used,
flavonoid content should be verified. Although flavonoid composition is a primary quality target, in cases of complex products, proximate compositional data (carbohydrates, fats, protein, and minerals), as well as the characterization of other bioactives such as alkaloids, glucosinolates, phenolics, methylxanthines, and terpenes, should also be assayed by using validated methods and reported because these components in food can have their own biological activity or function or, as part of the product matrix, may have an impact on the absorption/metabolism of the flavonoid components (34–37). Finally, compositional information of all batches of materials used in a study should be included to illustrate variability through a study period.

**TABLE 1**

<table>
<thead>
<tr>
<th>Flavonoid subclass¹</th>
<th>Common dietary forms²</th>
<th>Common dietary sources for flavonoid subclass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple flavonoid subclasses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavan-3-ol</td>
<td>(+)-catechin</td>
<td>Tea</td>
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<tr>
<td></td>
<td>(−)-epicatechin</td>
<td>Cocoa and cocoa products</td>
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<tr>
<td></td>
<td>(−)-epigallocatechin-3-gallate</td>
<td>Grape and grape products</td>
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<tr>
<td>Flavanone</td>
<td>Hesperetin (hesperetin-7-rutinoside)</td>
<td>Apples and apple products</td>
</tr>
<tr>
<td>Flavone</td>
<td>Apigenin (apigenin 7-apioglucoside)</td>
<td>Parsley</td>
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<tr>
<td></td>
<td>Luteolin (luteolin-7-digluco side)</td>
<td>Celery seed</td>
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<tr>
<td>Isoflavone</td>
<td>Genistein (genistein-7-glucoside)</td>
<td>Soybeans</td>
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<td></td>
<td>Daidzein (daidzein-7-glucoside)</td>
<td>Soy-based foods</td>
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<td></td>
<td>Glycitein (glycitein-7-glucoside)</td>
<td>Legumes</td>
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<tr>
<td>Flavonol</td>
<td>Quercetin (quercetin-3-rhamnoside and quercetin-3-glucoside)</td>
<td>Tea</td>
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<td></td>
<td>Isorhamnetin (isorhamnetin-3-rutinoside)</td>
<td>Onions</td>
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<tr>
<td></td>
<td>Kaempferol (kaempferol-3-glucoside)</td>
<td>Apple products</td>
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<tr>
<td>Anthocyanin</td>
<td>Cyanidin (cyanidin-3-glucoside)</td>
<td>Cranberries</td>
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<td></td>
<td>Delphinidin (delphinidin-3-glucoside)</td>
<td>Raspberries</td>
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<tr>
<td></td>
<td>Malvidin (malvidin-3-glucoside)</td>
<td>Blackberries</td>
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<tr>
<td>Complex flavonoid subclasses</td>
<td></td>
<td></td>
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<tr>
<td>Condensed tannins³</td>
<td>Procyanidins</td>
<td>Cocoa and cocoa products</td>
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<td></td>
<td>Prodelphinidins</td>
<td>Stone fruit (apples and pears)</td>
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<tr>
<td></td>
<td>Propelargonidin</td>
<td>Grape and grape products</td>
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<td>Strawberries</td>
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<td>Cranberries</td>
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<td>Nut skins</td>
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<td>Barley</td>
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<td>Legumes</td>
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<td>Derived tannins</td>
<td>Thearubigins</td>
<td>Fermented teas (black and oolong)</td>
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<td></td>
<td>Theabrownins</td>
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<tr>
<td></td>
<td>Theaflavins</td>
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</tbody>
</table>

¹Flavonoid subclasses noted by their aglycone designation.
²Example of common glycoside forms found in food noted in parentheses.
³Condensed tannins consist of 15 subclasses; the 3 most common in the human diet are noted.

**Detailed data on flavonoid administration and relevant information on stability of test materials**

Factors including light, temperature, and pH affect the stability of flavonoids (38). Thus, it is also important to provide details on how flavonoid materials were stored and administered for use in a study. Because study participants may be asked to individually prepare and consume products to be tested over an extended time period (i.e., weeks to months), it is important to consider the stability of the flavonoids and material in use, not only through the duration of the trial but also through any preparation or process (e.g., heating or cooking) that may be applied to the product before consumption. Although it is impossible to control for all possible scenarios in longer term human studies, logical controls should be put in place to minimize deterioration, including the provision of detailed instructions on product use and preparation, by using appropriate packaging and providing instructions on proper test material storage to minimize degradation over time. To document any evident deterioration in the flavonoid content of test materials over time, regular analytic assessments of test materials (all batches) used in a study should be conducted at regular intervals from the beginning to the end of the study period. Acceptance criteria for test materials should be pre-established and used to determine the acceptance/rejection of materials used in a study, at production, and through the course of a study.
Flavonoid analysis: identification and quantification

Given the diverse chemistry of flavonoids, liquid chromatography (LC)\(^4\) and mass spectrometry (MS) have become the most commonly employed method in flavonoid identification and quantification. Recent advances in LC-MS methods have made identification of flavonoids more common practice (39–42). However, these methods cannot provide absolute structural confirmation of all individual flavonoid species, including physiologically relevant metabolites. That can be achieved through rigorous application of nuclear magnetic resonance spectroscopy. Characterization efforts are also limited by the lack of commercially available standards to accurately confirm and quantify the thousands of flavonoid species reported in nature and their metabolites in biological tissues, thus further complicating analysis (43). Although some standards have become available, limited variety and quantities and high cost have continued to challenge quantitative efforts. Therefore, despite the availability of appropriate analytic techniques, the quantification of all but the common flavonoids remains inconsistent and challenging.

Specific analytic method

At present, no fully validated methods for quantification of all flavonoid forms in plant materials and foods exist, and consensus on such methods is lacking. Existing methods are generally not comprehensive and tend to fall into 3 categories that target specific flavonoid subclasses or different foods. In the first approach, a select set of commercially available flavonoid standards can be used to quantify key glycosylated flavonoid forms in foods or preparations (44, 45). In the second approach, flavonoids are hydrolyzed and quantified as aglycones (46, 47). In the third approach, an available flavonoid standard is used as a reference standard for a family of flavonoids (48–50). All of these methods require chromatographic separation of conjugated glycosides or aglycones before quantification.

The first approach is not comprehensive and requires an a priori decision as to which flavonoids are most significant. This approach is also dependent on the commercial availability of standards or internal standard approaches. The major shortcoming of this approach is that the selected flavonoids may vary significantly between studies, making comparisons challenging. The second approach is analytically simpler and more appropriate for broad quantitation but sacrifices information regarding specific flavonoid-glycoside composition (46, 47). This approach leverages the wide availability of commercially available flavonoid aglycone standards. However, optimum hydrolysis conditions may vary significantly between samples and flavonoid species, leading to artifact formation and contributing to analytic variability (45). Most important, as a result of the process, quantitative data for specific glycosides are lost with the adoption of this approach, making it challenging to distinguish between what is reported and what is actually present in foods. For example, although quercetin is often reported as a common flavonoid in food, quercetin naturally exists as a variety of glycosides such as rutin and quercitrin (51–53). Therefore, although useful for targeted quantitative results, this approach is not recommended in isolation, considering the lack of insight into the full flavonoid profile.

A third approach, using single or multiple reference standards, is the most reasonable but has not been practiced on a large scale (48–50). This approach either assumes a similar analytic response for each flavonoid or requires the determination of a relative response factor for each flavonoid. Thus, after chromatographic separation, calibration with a single reference standard can be used to quantify the remaining flavonoids. Large-scale use of this approach requires the following features: quantitative or reproducible extraction, LC with MS detection for putative identification of the flavonoids, UV detection for quantification, addition of a suitable compound to serve as a reference standard, and measurement of integrated peak absorbances for each identified flavonoid and the reference standard. The concentration of each compound can then be determined from calibration for the reference standard by using relative response factors. This approach is applicable to flavonoids, phenolic compounds, all secondary metabolites, and metabolomic studies.

The reference standard approach was recently suggested for the quantification of flavan-3-ols in teas and in a wide range of phenolic compounds in foods (54). Caffeine was used as the reference standard for tea. Rutin was used for quantifying hydroxycinnamic acid derivatives and flavonol and flavone glycosides (55), as well as gallic acid for flavanols, flavanones, isoflavones, proanthocyanidins, and hydrolysable tannins (54). Accuracy for all compounds was determined to have a relative SD of 13%. To put this in perspective, the biological variation of flavonoids in foods was reported with a relative SD of 160% in one major study (56). Whereas an accuracy of ±27% (at the 95% confidence level) may be considered marginal for most analytic methods, it would be acceptable for >5000 flavonoids in cases where authentic standards may not exist. Considering the strengths and limitations of these specific approaches, and because no single method is recognized to quantitate all flavonoid forms in test foods, methods should be selected that, at a minimum, provide quantitative data for major flavonoids present before initiating further clinical or preclinical studies.

Nonspecific analytic method

The complexity of flavonoid profiles, a lack of commercially available standards, and the need to have a “total” value appropriate for setting dosing and usage levels have led to the use of nonspecific colorimetric methods or antioxidant capacity assays as an estimate of the total phenolic content or antioxidant activity content, without separation of individual phenolic/flavonoid species. Examples include the Folin-Ciocalteu method (57), which is commonly used for total phenolic determinations, whereas the oxygen radical absorbance capacity (58), diphenylpicrylhydrazyl (59), and ferric reducing ability of plasma (60) methods are commonly used methods to determine antioxidants. Two additional colorimetric methods, the pH differential and p-dimethylaminocinnamaldehyde assays (61, 62), measure total monomeric anthocyanins. It is generally acknowledged that these in vitro assessments are physiologically irrelevant (63, 64) and lack analytic specificity (i.e., any reducing agent will appear to be a flavonoid). These methods cannot be used to establish...
Correlations between individual flavonoids, or groups of flavonoids, and health outcomes. Appropriately, the USDA removed its oxygen radical absorbance capacity database for selected foods, citing “mounting evidence that the values indicating antioxidant capacity have no relevance to the effects of specific bioactive compounds, including polyphenols on human health” (65). These methods are only appropriate for general quality assurance under highly constrained conditions.

Considerations for improved accuracy of research assessment and reporting of flavonoid intake

Over the past 2 decades, there have been shifts in how flavonoid intake is assessed. In the landmark Dutch study by Hertog et al. (66), the first to report an association between dietary flavonoid intake and reduced risk of coronary artery disease, flavonoid intake was reported as the sum of only flavonols and flavones from select foods and beverages typically consumed by the Dutch population. In recent years, publicly available flavonoid databases have enabled a more comprehensive and evolving picture of intakes to emerge. However, only recently has there been a more consistent use of correct flavonoid terminology (67–69), creating the opportunity for easier cross-study comparisons in the future. Some of the key considerations of the proper application of these databases in epidemiologic and clinical studies are outlined in the following sections.

Composition databases

In recent years, databases describing flavonoid components have emerged and continue to be expanded and refined. The USDA has made available 3 databases characterizing the flavonoid content of many foods, including one for several flavonoids (1), one for proanthocyanidins (2), and one for isoflavones (3). In Europe, there is the European Food Information Resource EuroFIR-BASIS (4). The French National Institute for Agricultural Research’s Phenol-Explorer (5), launched in 2010, de-

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The USDA is updating the proanthocyanidin database (2), which will not contain “total monomers.” Finally, it is important to recognize that these databases are still growing; thus, gaps still exist in these available databases with regard to the range of foods captured. Specifically, these databases do not represent the true variability expected in foods due to such factors as seasonality, brands (finished products), varietals (differences in foodstuffs), and food processing/preparation and product age (shelf life) and therefore do not replace analytically assessment of test materials in cases of experimental studies. These are the expected limitations of databases, and although they are not readily overcome, recognizing these limitations in the context of a study is important to the overall interpretation of the results of a study or studies.

Dietary assessment

When investigators conduct meta-analyses of epidemiologic studies, consideration should be given to both the method used for the assessment of dietary flavonoid intake and which food composition databases were employed. Robust studies should include multiple food-frequency questionnaires (FFQs), preferably fit for the purpose of ascertaining flavonoid intakes, and better methods for ensuring that intake estimates are reflective of the population over the longer term (e.g., 24-h recalls and food diaries). With regard to reporting flavonoid intake, it is recommended that investigators begin to adopt the general classification schemes for flavonoids as outlined in Table 1. Studies applying proper and detailed classification of various flavonoid classes in foods, such as those derived from databases, and clear articulation of what constitutes “total flavonoid intake” can be better grouped and evaluated, thus providing a more complete picture of dietary flavonoid intakes and association with health endpoints.

It is also critical that the complexity of determining dietary intake of flavonoids be taken into account by rigorously reporting details of food sources identified in their studies (i.e., listing the top sources of each subclass) and, when possible, reporting on the potential variations of flavonoid content found in key food sources (due to such things as processing, cooking, season, regional differences, etc.). Reporting not only the intakes of the distinct flavonoid subclasses but also the primary dietary sources for each subclass within the particular study may allow investigators to better compare findings across studies. Given the diversity of diets across the globe, as well as changes in dietary patterns across decades, the provision of such details may be useful in the identification of commonalities as well as critical differences across epidemiologic and experimental studies.
Finally, most FFQs are designed to capture the food components from those foods providing most of the dietary energy and are not always optimized for capturing intake measures of nonessential food components, such as flavonoids. Although targeted FFQs have demonstrated usefulness in targeted assessments of select flavonoids, including isoflavones (70), flavonones (71), and flavan-3-ols (72), information on the exact food or brand of product consumed, or method of preparation, is usually not documented. Although difficult to obtain in broad population studies, these factors can have a significant impact on intake estimates for flavonoids. For example, there are known variations in the flavonoid content of fruit cultivars or varietals (e.g., red vs. white grapes), as well as variations by product form and preparation (e.g., milk vs. dark chocolate, ready-to-drink vs. brewed tea) (1, 34, 73). Caution should therefore be exercised in using database-derived flavonoid values for general food classes across the board without some additional consideration of potential variation. When possible, intake estimates generated by FFQs and databases should be augmented by additional application of specific methods to provide more robust estimates of flavonoid composition for key food sources and/or measures of relevant markers of consumption (described below).

**Specific considerations for design and reporting of dietary intervention trials**

A primary goal of intervention trials is to eventually translate the findings to public health recommendations for free-living individuals. During the development of flavonoid intervention trials, it is suggested that clinical investigators consult with food technologists, analytic chemists, dietitians, behaviorists, biostatisticians, and others to foster opportunities for translation on trial completion. In clinical trials, intention-to-treat analysis is preferable for the primary analysis because any other analysis may be biased and lose the validity advantages of a randomized study for causal inference. In addition to the above-mentioned issues, other considerations should be taken into account when designing trials.

**Consideration of background flavonoid intake in the context of dietary intervention**

When planning and executing a flavonoid-specific dietary intervention, it is critical to evaluate the background diet for flavonoid content to understand results in the context of total dietary flavonoid intake and study outcomes. The capture of dietary background through an initial dietary assessment is relevant because it is critical to know whether the planned flavonoid-based intervention contains a flavonoid intake level sufficiently above the expected background intake, thereby confirming that the intervention itself is sufficiently higher than what can be achieved through the diet. Furthermore, during the trial itself, it may be necessary to consider altering, managing, and/or monitoring the intake level of the specific flavonoid under investigation. Depending on the question being investigated and the duration of the trial, investigators have the following options: 1) providing all foods to study participants throughout the study period to ensure low/controlled flavonoid intake, 2) recommending a low flavonoid–containing diet throughout the study, and/or 3) using a washout diet to ensure dietary flavonoid content is within a specified range before study initiation for acute or short-term studies. In addition, assessment of background dietary intake may provide a basis for the stratification of study data or may even be used as the basis for subject randomization or exclusion of study participants. For example, a study investigator may want to exclude subjects for whom the intake of dietary flavonoids is highly atypical relative to normative patterns of consumption in the specified population, such as vegans or strict vegetarians, for whom flavonoid intake may be very high, or individuals for whom average intake of fruit and vegetables may be much lower than the population average. Researchers may also consider stratification by risk level of outcomes being studied or avoid studying a healthy population because flavonoids may be expected to have minimal or no impact on markers of disease in healthy populations.

Finally, dietary patterns can be influenced by seasonality and even by participation in the trial itself. For long-term intervention trials, it may be necessary to periodically monitor and record dietary flavonoid intake by using a dietary survey to ensure that dietary patterns, including habitual flavonoid intakes, remain relatively unchanged throughout the study period.

**Development and application of an appropriate control**

As described previously, adequate characterization of the test material is a vital component to all research examining the health effects of flavonoids. However, with regard to intervention trials, another critical consideration is the nature of the control substance to which the flavonoid test material is being compared. In aiming for the highest research standards for a control product, it is important that it is not only well matched but also in a form that is reflective of what could be expected to be consumed in the context of a diet. Thus, an ideal control would be a material or product devoid of the components of interest, yet indistinguishable in taste, color, and composition from the test material. These considerations in construction of the control are critical, particularly if one aims to translate findings to specific flavonoid-containing foods and not solely to an isolated compound. Appropriate and careful analytic characterization and construction of both active and compositionally matched test materials remain critical, because a true causal link can only be established with an appropriate control. Although ideal controls have, to date, not often been fully realized in the field of flavonoid research, several noteworthy examples have been published for well-matched control products for cocoa/chocolate (31), tea (74, 75), purple grape juice (76), and cranberry (77).

The National Center for Complementary and Alternative Medicine (now National Center for Complementary and Integrative Medicine) guidelines also address construction of a matching control (37), and the development of standard reference food materials by federal and industrial groups is encouraged to provide appropriate controls for animal or human intervention trials. Development of these improved compositionally and taste-matched control materials will also serve to enable the blinding of participants and investigators in dietary and feeding intervention trials. To the extent possible, blinding must be achieved because it minimizes the likelihood of bias, which is critical to the integrity of the trial.

**Considerations for measuring markers of intake and compliance of flavonoid-based test materials**

Where possible, flavonoid-based intervention studies should include robust measurements of flavonoid and flavonoid...
metabolites in biological fluids such as plasma and/or urine as primary markers that can be leveraged to assess compliance, estimate absorption/bioavailability, and potentially provide a link between specific flavonoid forms in the test material with clinical biomarkers. This is not trivial and is complicated by variability of absorption and the degree and extent of flavonoid metabolism that exhibits variability from subject to subject and from tissue to tissue. The LC-MS method has been broadly applied for the characterization and quantification of structurally related flavonoid metabolites. Similar to the situation described for specific analysis of flavonoids in foods, these methods, although powerful, suffer from a lack of standardization and the limited availability of authentic metabolite standards. Although efforts to generate synthetic and semisynthetic routes to relevant flavonoid metabolites exist, these have been limited to select flavonoid classes such as the flavan-3-ols (78, 79).

Assessment of flavonoid bioavailability through measurement of pharmacokinetic behavior after acute consumption of a test material will provide clinically relevant parameters for individual flavonoids and their structurally related metabolites, including C_{max} (maximum plasma concentration), T_{max} (time of maximum plasma concentration), t_{1/2} (plasma half-life), and incremental AUC (area under the plasma pharmacokinetic curve). Examples of well-designed and reported studies on absorption and metabolism of flavonoids have been previously reviewed (34, 80–83). These studies can serve as a basis for comparison when assessment of flavonoid test materials is being considered. However, to broaden relevance to clinical investigations that rely on long-term administration of flavonoid test materials or diets, one must consider the potential for differences in food matrix effects (34–37) and adaptation in absorption of flavonoids during the duration of the trial (84).

In cases where assessment of flavonoid metabolites and their bioavailability is not possible, characteristic markers may be substituted as surrogates to serve as measures of compliance. For example, theobromine might be used as a marker of chocolate/cocoa intake, and 4-O-methyl gallic acid, a metabolite of gallic acid, has been used for tea (85). Another approach for compliance is the use of para-aminobenzoic acid (86, 87) or lithium (88) incorporated into the test material as an “internal” standard. However, it is important to note that there are advantages and disadvantages to markers such as para-aminobenzoic acid, including its contribution to phenolic and phenolic metabolites in the urine that may be captured in a nonspecific method. Knowing the appropriate levels of markers of the test substance achieved in blood facilitates better comparison of studies from different research groups.

Training participants on how to consume test products and properly record consumption is also critical. As appropriate, it may be necessary to specify preparation of the intervention (e.g., cooking, heating, and incorporation into other foods) to minimize potential loss of the flavonoids once introduced to the participant. Furthermore, because the specific flavonoids being investigated may be generally found in other foods, proper consideration of how to advise subjects on the consumption of these foods is needed to minimize significant fluctuations or between-subject variations in background intake, as well as to avoid total restriction of these foods, which could potentially affect overall diet quality. Subject adherence may be a much greater issue and one that can be difficult to determine and ensure. Providing adequate instructions regarding preparation and intake should help increase compliance.

Consumption frequency during the intervention must also be considered. The half-life of many absorbed flavonoids in the circulation is short (typically <6–8 h) (89–91), and thus the metabolic effect of single or multiple daily intake occasions might be different. For example, the benefits of tea or wine consumption among consumers, as shown in epidemiologic studies, may be due, in part, to spreading consumption throughout the day (i.e., smaller individual intake amounts spread across multiple eating occasions in a day). Thus, the amount and pattern of consumption used in an intervention study should, when possible, seek to match a typical dietary pattern of intake. For example, a trial providing only tea- or grape-derived flavonoid supplements or products consumed once daily (often preferred to maximize compliance in a study) cannot be assumed to reflect the pattern of consumption (and subsequent metabolism) that may exist among free-living individuals, particularly if the amount consumed in a single occasion totals what would be consumed in a day. Therefore, because of the expected variances in consumption patterns, consideration should be given to the provision of instructions that specify the timing of consumption during the intervention and whether to consume the test material with or without other foods. This context should also be considered when interpreting and attempting to translate findings to a free-living population.

GENERAL CONSIDERATION FOR PRECLINICAL AND MECHANISTIC STUDIES

Although the focus of this guidance is on improving the research and reporting of human studies involving flavonoids, it is important to acknowledge ways in which basic research in animal and in vitro models can be enhanced to better align preclinical and mechanism-of-action studies with dietary intervention studies. Better research spanning across the spectrum of evidence will aid in achieving the ultimate goal of establishing clear dietary recommendations regarding the intake of flavonoids to support and improve human health.

Consideration for animal studies

Animal studies are helpful tools to begin understanding the effects of diets or food components on living organisms by providing insights into disease processes, mechanisms of action, and even possible toxicologic indicators to estimate upper safe limits and potential adverse effects. To achieve this, it is critical to document and understand the similarities and differences in flavonoid bioavailability and metabolism between species because animal studies will be appropriate for the evaluation of efficacy and safety only if there are strong metabolic equivalencies between the animal model and humans. It is also important to consider when to time the intervention in the life cycle in animal studies to best test the human exposure in question. In addition, flavonoid intake levels and duration should be relevant and, when possible, set to achieve similar blood concentrations and/or urinary profiles to those found after short-term and/or long-term consumption by humans. Reporting results of animal trials should include a detailed definition of experimental materials and composition (as previously described for human
**TABLE 2**
Summary of recommendations for designing, implementing, and reporting human clinical and epidemiologic studies on flavonoids

<table>
<thead>
<tr>
<th>Study element</th>
<th>Study type</th>
<th>Recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Background considerations</strong></td>
<td>Clinical studies</td>
<td>Consider information on product structure, characterization, stability, and analytic methods that were used to determine product integrity and the foods or compounds that were fed in previous studies. When evaluating prior studies, consider the date of the study, analytic methods used, source and quality of test products, flavonoid databases used, participant characteristics, background diet, interventions, and method to assess adherence.</td>
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<tr>
<td><strong>Methods</strong></td>
<td></td>
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<tr>
<td>Participants</td>
<td>Clinical studies</td>
<td>Clearly state study eligibility criteria. For intervention studies, consider the flavonoid content of background diets and avoid any major deviations from usual intakes if they are to represent habitual diets. Consider exclusion of subjects with highly unusual dietary patterns, either extremely low flavonoid intakes that are likely to reflect a poor overall diet or very high intakes (e.g., vegans, vegetarians, and those consuming some Asian diets whose background flavonoid intakes may be quite high), eating and lifestyle patterns that involve consumption of other foods, dietary supplements (e.g., red clover, hawthorn berry, conventional multivitamin/mineral supplements with added plant extracts or flavonoids, e.g., EGCG, etc), or certain medications (e.g., prescriptions, over-the-counter items, other phytochemicals, and other prescription drugs such as antibiotics) that have known effects on the gut flora or flavonoid metabolism or that possess similar mechanisms of action (e.g., coumeastes).</td>
</tr>
<tr>
<td>Interventions</td>
<td>Epidemiologic studies</td>
<td>State eligibility and exclusion criteria.</td>
</tr>
<tr>
<td>Test material or product</td>
<td>Clinical studies</td>
<td>For acute studies in which efficacy of a specific compound is being examined, a washout period (minimum 3 d) and removal or reduction of specific foods or compounds from the diet are feasible approaches. For longer intervention trials to determine physiologic effects of a flavonoid-containing food, flavonoid-enriched extract, or specific flavonoid compound or mixture, a washout period and the removal or reduction of foods may not be practical. For these situations, it is important that the background diet (including habitual flavonoid intake) of the subjects is known and monitored to evaluate dietary consistency.</td>
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<tr>
<td>Characteristics</td>
<td></td>
<td>Product/test material name. Use appropriate and accurate scientific nomenclature and terminology to describe test materials/products and diets. When no pharmacopeial monograph exists for a study ingredient or in cases in which the ingredient does not conform to the existing monograph, provide specifications that include all of the same tests as those in the monograph.</td>
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(Continued)
TABLE 2 (Continued)

<table>
<thead>
<tr>
<th>Study element</th>
<th>Study type</th>
<th>Recommendations</th>
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<tr>
<td>7) Consider both the biochemical and physical properties that are important in choosing an intervention product/test material and the control substance.</td>
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<td>8) Ensure that test and control materials have been produced under appropriate food-safe conditions. Obtain the specifications and Certificate of Analysis if available to show compliance with the specifications for purity and content from the supplier/manufacturer or other supporting manufacturer information, relating to the batches of the test material/product to be used in the study.</td>
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<td>9) Ensure that microbiologic testing is sufficient to meet requirements and that the product meets relevant food safety standards and does not contain contaminants such as toxic elements, mycotoxins, and adulterants. Any pesticide residues or heavy metals should be well within recognized safety standards.</td>
<td>Controls or placebos Clinical studies</td>
<td>Choose appropriate control product. Match the control to the test substance as possible on composition, taste, and appearance. If a placebo is used, provide relevant information on its composition.</td>
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<tr>
<td>Intervention regimen Clinical studies</td>
<td>Design. Characterize the intervention test materials or products chemically and physically and describe the study populations and experimental conditions in detail. The gold standard for making causal inferences between bioactive food components and health outcomes is the randomized, double-blind, controlled clinical trial and is recommended when such inferences are desired.</td>
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<td>Determine the conditions of administration for the flavonoid-containing test material in the study. Describe the amount of the test material/product that will be fed during the intervention, how the product is to be prepared and consumed, and the feeding schedule. Choose the conditions for feeding based on the clearance rate of active constituents or their metabolism and by whether the matrix in which the test constituent is administered (e.g., in or with food, or as a supplement) influences clearance (thus possibly having an impact on the frequency and timing of administration). Consider the bioavailability, dissolution, disintegration, and release characteristics of the flavonoids and other active constituents, if the information is available.</td>
<td>Adherence to the intervention Clinical studies</td>
<td>Consider how the dietary assessment and analytic methods in the study being planned compare with those used in previous clinical studies. Instruct participants on how to use the test material/product, how to use assessment tools, and how to report use and adherence. Assess flavonoid metabolites in target tissues and biologic fluids (optimally in both blood and urine), as appropriate, to enhance study and nonstudy exposure assessment. When collecting biologic fluids, consider the time lapse after flavonoid intake, taking into account the relevant half-life of structurally related metabolites.</td>
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<tr>
<td>Outcomes Clinical studies</td>
<td>Assess safety, which includes identifying, monitoring, and reporting adverse events. In particular, consider possible effects of high intakes of flavonoids after acute, as well as chronic, longer-term flavonoid feeding. Predetermine and report relevant study endpoint(s). Determine whether the endpoint is relevant to the timing and duration of intervention with the test material/product. Endpoints should be prespecified and be of health significance but, at minimum, always relevant to the hypothesis being tested. Consider including relevant intermediate markers known to reflect the biological pathway(s) of interest, as well as ultimate health outcomes. When possible, determine absorption or bioavailability as part of each intervention study to assist in interpretation of variability of results. Consider the influence of gut microbiota on bioavailability and metabolism of flavonoids or their foods, as well as the effects of flavonoids on gut microbiota. Consider gene-gene, gene-diet, and diet-diet interactions. When possible, take these and related biomarkers into account.</td>
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</table>
Sample size Clinical and epidemiologic studies
Consider variability in response to the intervention when determining sample size and expected effect size (e.g., equal producers and nonproducers). When possible, power the study to analyze predefined subgroups separately. Consider the sample size needed to understand gene-flavonoid interactions if this is of interest. When reporting if a study is null, it is helpful to conduct a post hoc power calculation to ascertain whether the study was simply underpowered or if it were adequately powered and still had no effect.

Statistical methods Clinical and epidemiologic studies
Prespecify comparisons to be made and endpoints, including covariates. When possible, analyze health outcomes in relation to specific concentrations of serum, plasma, or urinary flavonoids because intake/bioavailability may be directly related to efficacy. It is important to separate out and account for confounding effects.

Reporting results Clinical and epidemiologic studies
Use the CONSORT (24) statement recommendations for designing and reporting randomized clinical trials, the STROBE (95) and other relevant statements for observational studies, and the PRISMA (96) and QUOROM (97) recommendations for meta-analyses. In reporting, address potential confounding factors such as the flavonoid profile of the intervention products, flavonoid intake, background diet, and participant characteristics (e.g., genotypes).

Investigators Clinical and epidemiologic studies
Assemble a multidisciplinary investigative team that includes sufficient expertise in study design, analytic chemical methods and statistical issues, clinical nutrition (to design appropriate and acceptable interventions), food science (for product composition and integrity and expertise related to the specific compounds of interest), epidemiology (for assessment of exposures), behavioral science (for maximizing protocol adherence), and clinical medicine (for knowledge of health conditions and of chronic disease risk factors being studied).

In vitro studies
In vitro studies are also important tools for understanding mechanisms and for early hypothesis setting. They are often the only feasible approach for determining the effect of food components on specific enzymes or enzyme pathways, cell signaling, and gene expression. However, several critical considerations must be taken into account when conducting and reporting the evidence from in vitro studies on flavonoids. As previously described, flavonoids are extensively metabolized on ingestion (18, 19, 29), and in recent years, there has been growing appreciation for the biotransformations that are facilitated by the microbiota within the lower gut (82–84). Therefore, in vitro studies of pure compounds or mixtures of nonbiologically relevant metabolites will not reflect the form of the components existing in the circulation or intracellularly and therefore will not reflect real physiology. To the extent possible, application of biologically relevant metabolites, as described by select investigations (92–94), should be used for mechanistic in vitro studies. In addition, many in vitro studies use flavonoid amounts that are not physiologically achievable through typically dietary (oral) consumption. For example, the circulating concentrations of many flavonoids have been reported in the range of 1–5 μmol/L, whereas researchers have employed concentrations of 50–100 μmol/L in culture (18, 29). Proper in vitro studies should take into account both metabolism and potential modification by gut microbiota, by using known metabolites or employing simulations of metabolism. Furthermore, these studies should use physiologically relevant amounts of the compounds. Proper reporting should include these facts so that the results can be put into physiologic context.

CONCLUSIONS
In summary, inconsistent reporting and design of studies for the investigation of flavonoids in both epidemiologic and intervention trials have significantly hampered the ability to develop clear dietary recommendations for the intake of flavonoids to support or promote human health. A need for greater harmonization has led to these recommendations for designing, implementing, and reporting flavonoid studies (summarized in Table 2). These recommendations are based on the Consolidated Standards of Reporting Trials statement for reporting randomized double-blind clinical trials, as well as principles identified by the NIH for the standardization of studies on soy (18, 27). Key considerations for flavonoid research include consistency in reporting according to standardized flavonoid nomenclature, improved characterization of the specific identity and quantity of constituents in test materials, development of appropriate controls for the flavonoid materials being investigated, and consideration of how the flavonoid-based intervention should be included in a background diet that is unlikely devoid of flavonoids. Admittedly, flavonoid research reporting today often does not meet these standards. The standards are goals toward which investigators in the field must continue to strive.
2 provides general recommendations that apply flavonoid studies broadly, this does not preclude additional considerations that may be necessary to investigate the different flavonoid subclasses. Considering differences in biological responses, as well as in the mechanisms of action involved, individualization of these recommendations may be needed and appropriate for specific studies.

Recommendations in this article are particularly focused on how human dietary intervention studies are conducted, reported, and are reviewed in the flavonoid field. The clinical implications of adopting these recommendations include better assessment of the true relation between the consumption of specific flavonoids or flavonoid-containing foods and health, as well as greater understanding of the levels at which benefits may be demonstrated. These recommendations will help to guide future studies and reporting in flavonoid research and lay the foundation for the evaluation of other nonessential bioactive food components where similar principles apply. These efforts should lead to the generation of a more cohesive body of scientific evidence that can be evaluated to provide evidence-based conclusions on the potential health effects of flavonoids and advice on their use.

Finally, it is hoped that these guidelines will foster broader scientific collaborations between industry, academia, and the public sector and help facilitate the translation of research into practice. In addition to becoming familiar with the guidelines by reading this report, there is a need to incorporate the guidelines into graduate food science and nutrition curricula, as well as educational workshops at conferences, to increase awareness of these issues on the part of journal reviewers and editorial boards when reviewing articles.

Eleven experts in the field reviewed this article and agreed to serve as content reviewers and drafters of the final content. All authors have read and approved the final manuscript. The authors declare no conflict of interest. The final content was drafted and is now employed by The Sugar Association. JMH has no disclosures. CLK-U is employed by Mars Inc. The authors were not compensated for the writing of the manuscript and shared responsibility for the final content. All authors have read and approved the final manuscript. The final content is now employed by The Sugar Association. JMH has no disclosures.

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