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Part F. Advances in evaluation and validity of the dietary intake of specific food components including nutrients and non-nutrients

Measuring flavonoid intake: need for advanced tools

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Abstract

Objective: Flavonoids are phytochemicals with potentially beneficial biological effects that are poorly characterised in existing tables of food composition.

Design: To describe new techniques for analysis, absorption, informatics and dietary assessment that are important in measuring the flavonoid content in foods and in developing a flavonoid food composition database.

Setting: Data on chemical analyses of the major flavonoid compounds that exist in the food science literature are being located, collated and compiled into a preliminary flavonoid food composition database.

Results: The analytical process begins with preparation of the food for flavonoid analysis using techniques to disrupt the food matrix, alcoholic extraction, and enzymatic or acidic hydrolysis to remove sugars. Separation is usually accomplished using high-performance liquid chromatography. Flavonoids are identified by diode array spectrometry, mass spectrometry or nuclear magnetic resonance spectroscopy. Quantification usually employs comparison of the sample with standards, if available, using the area under the curve of the chromatogram to estimate quantity. Absorption studies are useful since flavonoids vary in their absorption. Finally, information management technologies (informatics) are used to translate flavonoid data information into food composition databases. This process involves identification of foods containing the compounds of interest, collection and organisation of sources of existing analytical data, assignment of quality scores or aggregation of acceptable data for each component and food, calculation of appropriate statistics, assignment of food codes and verification, and finalisation. The resulting food flavonoid database can be used with state-of-the-art dietary assessment methods to develop estimates of flavonoid intakes in foods and to correlate these with estimates of disease risk.

Conclusions: A provisional flavonoid database, including at least two components from each of six classes of flavonoids, should be completed in 2002.

Keywords
Food composition
Dietary intake
Flavonoids
Dietary assessment
Food tables

Flavonoids are phytochemicals with potentially beneficial biological effects that are poorly characterised in existing tables of food composition. Three basic questions must be answered in developing a food flavonoid database. First, what substances should be considered and why? Figure 1 shows the chemical structures of the six most common classes of flavonoids in foods. They are of interest because of their possible connections with human health or disease and their organoleptic attributes (for example, the colour and taste of teas and wines).

Second, which foods need to be included in the database? One approach to answering this question is to reason from what is known about food consumption. For most individuals, a limited number of foods contribute most of their caloric intake. For example, out of 10 000 or more foods and ingredients eaten in the United States, the 'rule of 1000' usually applies; that is, about 1000 foods and ingredients account for over 85% of dietary intake[1]. By analogy, it is assumed that this is also true for flavonoids. Therefore, if the flavonoid content of these foods is known, estimates of intakes of flavonoids in most diets can be made. The National Food and Nutrient Analysis Program (NFNAP) is currently collating and evaluating existing data for scientific quality, identifying key foods and critical nutrients for sampling and analysis, and designing and implementing a nationally based sampling survey of 1000 commonly eaten foods in the United States. These foods will be the initial targets for developing a food flavonoid database.

Third, a decision that must be made is which flavonoid

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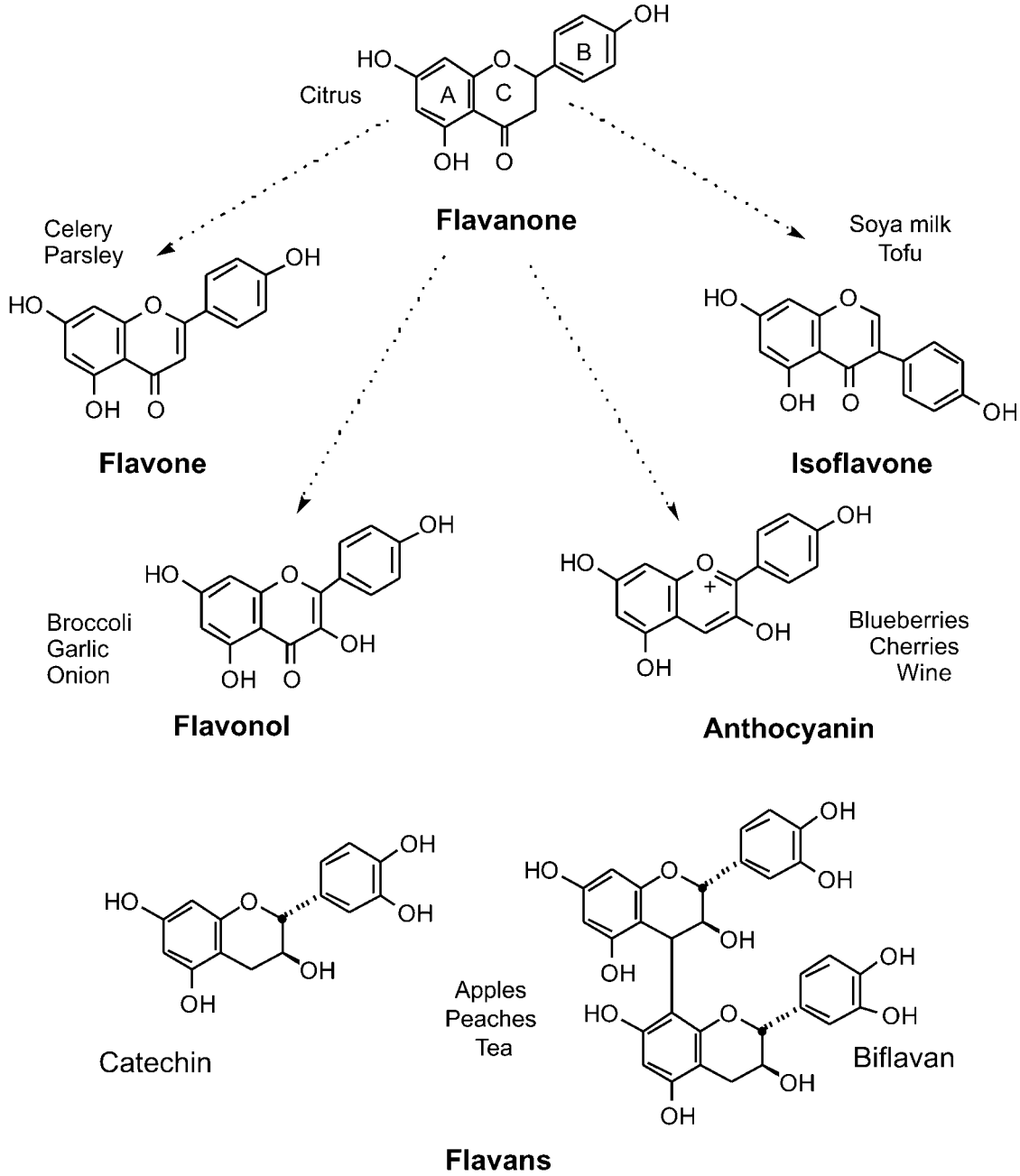

Fig. 1 Flavonoid structures and occurrence. The propanoid structure consists of two fused rings. The first ring (A) is aromatic, the second ring (C) is an oxygen-containing heterocyclic ring attached by a carbon–carbon bond to a third aromatic ring (B). These flavonoids differ from one another in their structure at ring C. The flavans include biflavans, catechins, proanthocyanidins and tannins. The biflavan shown here is a procyanidin. (The flavonoids found in citrus rinds are called bioflavonoids.) Arrows indicate biosynthetic path

classes and compounds should be included. Depending on the chemical classification used, there are between 11 and 26 classes of flavonoids and over 4000 compounds. Six classes and 20–30 compounds are especially common in foods. These are of particular interest from the health and sensory standpoints. Table 1 describes some of the most common compounds in each of these flavonoid classes in American diets. These are the first priority for building the database.

Advanced tools needed

In order to measure intakes of flavonoids, new techniques for analysis, absorption, informatics and dietary assessment must be applied appropriately. This paper describes why state-of-the-art methods in each of these four areas are needed to develop valid and reliable measures of intake.

The flavonoids in tea are used as an example to illustrate

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Table 1 Flavonoid classes

Class	Colour	Examples	Comments
Anthocyanins	Blue, red, violet	Cyanidin, delphinidin, peonidin, pelargonidin	Predominant in fruits and flowers and were among the first flavonoids to be isolated – from flowering plants as their individual names indicate. They are often used as food dyes
Flavans (monoflavans, biflavans, triflavans)	Colourless	Catechins (epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate), procyanidins, theaflavins, thearubigins	Found in fruits and teas (green and black). Biflavans are commonly found in fruits, hops, nuts and in beverages made from them, including cocoa and tea. The astringent taste of teas, beer, fruits, fruit juices and wines is mainly due to their biflavan content. Some flavans are coloured such as theaflavins (orange) and thearubigins (brown)
Flavanones	Colourless to very pale yellow	Hesperidin, naringin, narirutin, neohesperidin	Found almost exclusively in citrus fruits. Hesperidin is also found in cumin and peppermint. Naringin and neohesperidin have bitter tastes
Flavones	Pale yellow	Hydroxyflavones (apigenin, diosmetin, luteolin, neodiosmin), methoxyflavones (nobiletin, sinensetin, tangeretin)	Most prominent in cereals, herbs and vegetables. Small amounts are in tea. They are the yellow pigments of some flowers. The most common compounds are apigenin and luteolin. Nobiletin, sinensetin and tangeretin (citrus flavones) have a bitter taste. Neodiosmin, another citrus flavone, reduces the bitterness of limonin, naringin, caffeine, quinine and saccharin
Flavonols	Pale yellow	Isorhamnetin, kaempferol, myricetin, quercetin	Ubiquitous but predominant in vegetables and fruits. Quercetin is the most common of all flavonoids. Small amounts are in tea
Isoflavonoids	Colourless	Daidzein, genistein	Found almost exclusively in legumes, particularly soyabean

current problems and processes. Tea, *Camellia sinensis*, is a plant that contains many complex flavonoid compounds. The flavans contribute many of the monomers present such as catechins (catechin, epicatechin, epicatechin-3-gallate and epigallocatechin-3-gallate). Complex dimers (theaflavins) and polymeric compounds containing catechins (thearubigins) also exist. Tea also contains lesser amounts of some flavonols, including quercetin and kaempferol, and some flavones, such as apigenin.

Tea is processed before it is consumed. The chemistry of the flavonoids is affected dramatically by these processing techniques. Because the individual compounds, rather than the entire class of flavonoids, are involved in most of the health effects that are postulated, the type of tea consumed may make a difference. Green tea is treated to prevent fermentation, although a slight amount of fermentation occurs. Oolong tea is subjected to some natural enzymatic fermentation. Black tea undergoes a great deal of enzymatic fermentation. As green tea is processed, the catechins form dimers (theaflavins) and polymers (thearubigins). Thus green tea has more catechins, especially epigallocatechin gallate, and black tea has more theaflavins and thearubigins. Another form of tea is popular in parts of Asia but not in the USA. It is called Pu'er and produced by wet fermentation with bacteria, analogous to a composting process. It produces a different profile of compounds.

Analytical procedures for food flavonoids

Advances in analytical chemistry must be applied to develop valid quantitative estimates of food flavonoids. Until recently robust methods that were easy to set up and replicate for food flavonoid analysis were lacking, and consequently flavonoid analyses were not done routinely[2].

Analytical procedures for determining food flavonoids all involve *preparation of the food sample* in the same form in which it is likely to be eaten. For example, for tea, a water infusion is prepared since that is the way tea is brewed for consumption. Water extraction probably liberates 85% of the flavonoids present in the tea. In the older tea literature and even in some present studies, methanol extracts of the leaves were used, liberating 100% of the flavonoids. However, methanol extraction did not ensure that the compounds analysed were those consumed by humans in brewed tea. The next analytic step is to *disrupt the food or beverage matrix*. The analyte is then subjected to *alcoholic extraction* with methanol or ethanol to remove the flavonoids. *Acid or enzymatic*

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hydrolysis is used to remove sugars from the flavonoids. Since new evidence suggests that acid hydrolysis destroys some of the flavonoids (thus causing their underestimation), enzymatic hydrolysis is used whenever it is possible.*

The flavonoid compounds must then be separated, identified and quantified. For *separation*, the best analytical method at present is high-performance liquid chromatography (HPLC), which separates compounds by their differential preference for the solvent or the column. HPLC requires a smaller sample and is more complete and precise than other methods. Other methods tend to underestimate the flavonoids present. If the separation technique employed is faulty and other components co-elute with or contaminate the sample, flavonoids can be overestimated.

Analytical values for tea and many other foods include older, less precise methods, such as gas chromatography, column chromatography, thin layer chromatography (TLC) and paper chromatography; in decreasing order of precision. The problem with gas chromatography is that gas is the solvent and therefore the flavonoid compounds to be measured must be either volatile at relatively low temperature or derivatised to be made volatile. In column chromatography, solvent and alumina or silica are placed in a column, the compounds to be studied are added at the top, and then solvent moves through the column. With column chromatography larger amounts of the compound can be analysed, and it is easier to recover the compounds of interest, than with gas chromatography. In TLC the compound is put on a plastic or glass plate that is coated with silica or alumina, and then the plate is placed in a container with solvent, which is allowed to run up the plate. The compounds to be characterised separate out by their affinity for the silica/alumina or the solvent. The compounds are then visualised by ultraviolet light and recovered. TLC is still used today for rapid analysis of compounds and to determine the solvent systems that are needed for HPLC. Paper chromatography, the earliest form of chromatography to be developed, is highly imprecise and is rarely used today to separate flavonoids.

Identification of the various flavonoid compounds is most accurate with mass spectrometry (MS), nuclear magnetic resonance spectroscopy (NMR) or diode array spectrometry. MS breaks the compound and provides both its molecular weight and a characteristic fragmentation spectrum. NMR identifies the molecule by characteristic patterns of hydrogen and carbon relationships. Both MS and NMR are very precise and sensitive. Diode array spectrometry measures the ultraviolet spectrum simultaneously over several wavelengths at 10^{-9} to 10^{-12} g compound. In the older literature, other less sensitive techniques for identifying flavonoids were also used, including ultraviolet spectrometry at 10^{-6} to 10^{-8} g and optical density (refractive index) at 10^{-3} to 10^{-5} g. These measurements require more sample than state-of-the-art measurements today.

Quantification of the amounts of flavonoid present is done by comparing the area under the curve of the chromatogram of the sample from diode array with the area under the curve of the chromatogram of the standard. However, for some flavonoids, standards are not readily available. This means that it is possible to identify but not accurately quantify the compound. For most of the monomeric flavonoids and dimers (catechins and theaflavins) in tea, standards are available. However, standards are not available for thearubigins. Older and less precise analytical tools for quantification include ultraviolet light alone and absorbance as determined by spectrophotometry or calculation of the refractive index. Both of these methods require larger amounts of sample.

Absorption

Advances in physiology are also needed since absorption of most phytochemicals, including the flavonoids, is not 100% in humans[3,4]. Absorption depends on the flavonoid class. There are surprisingly few data available on the absorption of most flavonoids, other than the isoflavonoids, in foods. Current information suggests that absorption is approximately 4% for the anthocyanins, 3–5% for the catechins, 7–24% for the flavanones, unknown for the flavones, 17–52% for the flavonols and perhaps 9–35% for the isoflavonoids. For many compounds absorption depends on the presence of sugars. Other compounds may also require the presence of specific enzymes and bacteria in the digestive tract. The speed with which the compounds are absorbed also varies. At present there is so little information available on flavonoids that food flavonoid databases cannot be adjusted for absorption.

Informatics

Informatics is a term referring to the development of computerised food composition databases that includes appropriate checks of data quality. Better information systems (informatics) are sorely needed to translate analytical and physiological information into forms that can be used for construction of flavonoid food composition tables, dietary assessment and planning. The process is described in depth elsewhere[5], and is summarised in Fig. 2. Tea, *C. sinensis*, is an example of how the process works in practice, and illustrates why interdisciplinary dialogue is so essential in developing sound food flavonoid databases.

Using a search engine database, we were able to identify 6177 citations for flavonoids and 4691 citations for

*Hydrolysis of sugars reduces the number of standards required for quantification (and identification).

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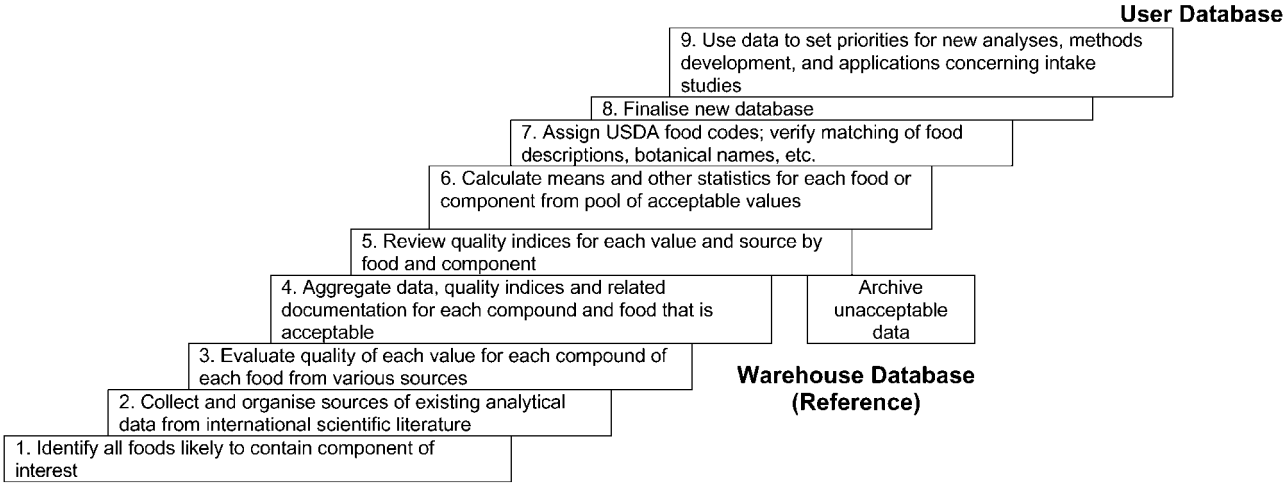

Fig. 2 Schematic view of the development of a flavonoid food composition database[5]

tea. We then crossed the terms for tea with flavonoids, and 594 citations in the literature emerged. The citations were evaluated by one of us (J.P.) and 108 were judged to be relevant. Approximately 91 articles were obtained. Data from the relevant food composition analytical articles were then aggregated and placed in a warehouse database. Articles were evaluated with criteria developed by United States Department of Agriculture (USDA) workers and used successfully in developing a carotenoid database. The evaluation system rates each analysis on analytical method, analytical quality control, number of samples, sample handling and sampling plan. Articles with acceptable data are then included in the database. Means and other statistics are calculated for each flavonoid. Data are harmonised: USDA food codes, botanical names (e.g. *C. sinensis*) and chemical names (e.g. theaflavin, thearubigin, etc.) are checked. Experts in the complex chemistry of tea then review the data. In our work, a government, private and voluntary sector panel is carrying out the review.

Dietary assessment

The tools for dietary assessment currently available do not include complete food composition databases for all of the flavonoid classes, but they need to in the future. Rough guesses about food flavonoid compounds today can be made if the foods consumed are known, but this is only a qualitative solution[6]. Lack of information on the flavonoid content of foods may cause associations between flavonoids and various chronic diseases to be overlooked in epidemiological studies. The current inadequacy of food flavonoid databases is the reason for the widely different estimates of flavonoid intakes that currently appear in the literature. Compare, for example, Kuhnau and Hertog *et al.*'s flavonoid intake estimates[7,8]. Kuhnau's is based on five classes of flavonoids, about 100 flavonoid glycosides, excluding the isoflavonoids (which would increase estimates) and including values for sugars (which would increase estimates). It is expressed in terms of quercitrin equivalents[7]. In contrast, Hertog *et al.*'s estimates are much lower, as might be expected, since they are based on only three flavonols and two flavones

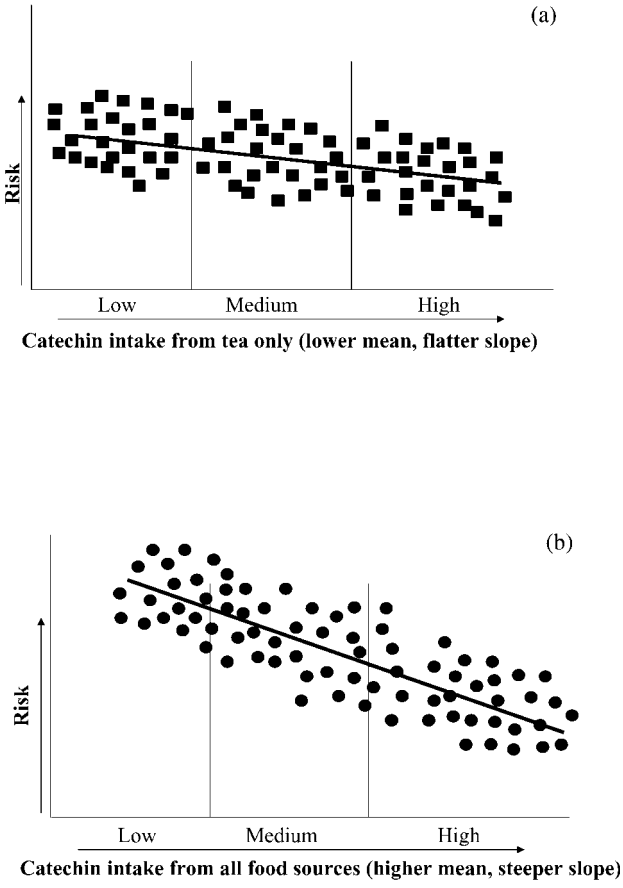

Fig. 3 Associations between dietary intakes of catechins and disease risk: (a) catechins assessed only from tea, (b) catechins assessed from all foods

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without sugars[8]. Not only were there differences in the flavonoid classes, but in specific flavonoid compounds measured and the completeness of the data. All of these factors may have obscured true differences in intakes in these recent studies[7,8].

Complete food flavonoid databases are sorely needed. Unless virtually all the dietary intake of a specific flavonoid compound is confined to one food, accurate estimates of flavonoid intakes cannot be developed from a single food. The flavonoids in tea are widely available in other foods as well, so for them the entire diet needs to be assessed. Figures 3a and 3b illustrate this point. Note how in Fig. 3a, if only the catechin content of tea were known and flavonoid intakes were calculated on that basis, the associations between risk and intakes would be low because catechins are widely distributed in many foods, and misclassification would be high. In contrast, when catechin intakes are estimated more precisely using more complete tables of food composition with the catechin content of all foods, misclassification is very much lessened, and relationships, if any, with disease risk are much more dramatic (Fig. 3b). As our knowledge of flavonoid–disease relationships becomes clearer, perhaps it shall soon be possible to specify recommended intake levels. However, much additional work is needed before evidence-based recommendations will be possible.

Conclusions

Slow but steady progress is being made in developing and applying food flavonoid values to studies of human health. Over the long run, sampling plans for obtaining representative samples of foods at the appropriate degree of processing must be developed and put into action. Also, the number of chemical analyses for food flavonoids needs to be increased. All flavonoid classes need to be assayed using state-of-the-art methods such as HPLC separation systems, quantification tools such as standards, and identification of compounds using MS. More studies on flavonoid absorption and biomarkers of flavonoid intake are also needed[9]. Over the short run, some provisional food flavonoid databases and software updates can be developed by culling and evaluating existing data for all the classes of flavonoids. Such databases may be sufficiently accurate to be useful in epidemiological and physiological studies. However, food composition tables are only as good as the analytical information they contain. The only way to increase analytical information is to do more appropriate analyses. At present most of the values that are available come from European laboratories. In the future it is hoped that more studies of foods will be done in the United States. If such studies are carried out, taken together with more basic mechanistic and clinical studies, flavonoid–health relationships will soon be clarified.

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