Analysis of Flavanols in Foods: What Methods are Required to Enable Meaningful Health Recommendations?

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Abstract: Flavanols and their related oligomeric compounds, the procyanidins, have received increased attention during the past decade due to their reported health benefits. On the basis of compelling data published during the past decade demonstrating that the consumption of certain flavanol-rich foods can improve markers of cardiovascular health, additional clinical, and epidemiological research is clearly warranted to establish appropriate public health recommendations. However, recommendations on the consumption of these foods appropriate for use by health professionals can only be made on the basis of clinical investigations that accurately identify and quantify—through proper analytical measurement systems—the flavanols in the foods used in these investigations. This manuscript provides an overview of the strengths, weaknesses, and limitations of commonly used analytical methods to characterize the content of flavanols in foods. Two nonspecific measurements widely used by investigators, the Folin-Ciocalteu assay and the Oxygen Radical Absorbance Capacity (ORAC) measurement, are discussed in this context, as is the use of various high-performance liquid chromatography methods that provide more specific data related to the content of flavanols in foods. A comparison of the data obtained from these analytical methods to those of the more rigorous high-performance liquid chromatography analyses demonstrates that these nonspecific methods are ill-suited for providing unequivocal data necessary to evaluate the importance of dietary flavanols in the context of improving cardiovascular health. Meaningful dietary recommendations for the consumption of flavanol-rich foods will only be made possible by additional well-designed clinical and epidemiological studies enabled by detailed compositional data obtained through use of appropriate analytical methods.

Key Words: antioxidant, cardiovascular, database, flavanol, flavonoid, food composition, methods of analysis, polyphenol, proanthocyanidin, procyanidin, Folin-Ciocalteu, oxygen radical absorbance capacity, high-performance liquid chromatography

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Cardiovascular diseases (CVDs) are the primary cause of death worldwide.[1] Diet is well recognized as an important factor in the development of CVD, and interest pertaining to the preventive and therapeutic potential of specific foods in the context of cardiovascular health is increasing.[2–4] During the past decade, plant food-derived flavanols, including the flavanol-based oligmeric compounds known as procyanidins, have received increased attention. (From this point on, the term "flavanols" will encompass both the flavanol monomers, epicatechin and catechin, as well as their related oligomeric forms such as dimers, trimers, etc. examples depicted in Fig. 3). Flavanols derived from certain cocoas have been systematically studied during this period, and evidence from several laboratories demonstrates that the short-term consumption of flavanol-rich cocoa-based products can improve important markers of cardiovascular health.[5–9] Other plant foods common in the diet also contain a variety of flavanol subclasses[10] and research exists suggesting certain of these foods, including grapes, green tea, and apples, may also confer cardiovascular health benefits.[11]

The promising results reported by several investigators demonstrating that the consumption of certain flavanol-rich foods can improve markers of cardiovascular health warrant additional efforts to validate efficacy and establish appropriate public health recommendations. The quality of these efforts will depend first on the analytical methodologies used to identify and accurately quantify these food components. Given the natural diversity of flavanols in foods, and the inherent complexity of food matrices, numerous challenges must be addressed to ensure that accurate analytical data is obtained. Unfortunately, many of the studies published to date suggesting that consumption of flavanol-rich foods may provide cardiovascular benefits have relied on methods that insufficiently characterize the flavanol content of the foods under investigation.[12–16] Often, data are simply derived from compositional databases and nonspecific assays that measure total phenolics content, for example, Folin method, or antioxidant capacity, for example, oxygen radical absorbance capacity (ORAC). Although these approaches are rapid and relatively easy, they do not provide the level of compositional detail available through use of more specific methods. Establishment of meaningful dietary recommendations can only be possible through the execution of "gold standard" dietary

intervention trials, and it is only through the use of methods that more specifically characterize a food's flavanol composition that trials of high quality can be conducted.

The aim of this manuscript is to provide an overview of the chemistry of flavanols with a specific emphasis on the strengths, weaknesses, and limitations of the analytical methods commonly used to characterize the flavanols content of foods. In addition, important recent advances will be highlighted that should further enhance the ability of researchers to accurately characterize the flavanols content in foods. Through a comparison of these methods, it becomes obvious that the more rigorous measurements that provide detailed compositional information are necessary for studies aimed at evaluating the potential cardioprotective effects of flavanol-rich foods.

CHEMICAL STRUCTURE AND DIVERSITY

Flavonoids are a large subclass of a much broader class of compounds known as polyphenols. Polyphenols are a structurally diverse group of compounds that occur widely throughout the plant kingdom—sometimes also referred to as "phenolics"—due to the presence of at least one phenol substructure (a hydroxyl group on an aromatic ring—C_6). Figure 1 depicts a classification based on the number of phenol subunits. An amazing diversity of flavonoids exist, with over 5000 individual flavonoids having been identified to date.[17,18] The absolute structure of these flavonoids can vary dramatically; however, all plant flavonoids share a common 15-carbon structural backbone designated as C_6-C_3-C_6. Figure 2 provides the generic structure of the most common flavonoids found in foods. Differences in the structure of the heterocyclic C ring result in distinct classes of flavonoids, including flavanols, flavanones, flavones, isoflavonols, and anthocyanidins. In nature, many flavonoids exist as glycosides (carbohydrate adducts on different hydroxyl groups), which add significantly to the complexity of characterization.

Flavanols (highlighted in Fig. 2) are one particular class of flavanoids for which potentially important cardiovascular health benefits have been reported. Flavanols, different types and of varying levels, are present in many types of finished food products, including certain red wines, grape juices, teas, cocoas, and chocolates.[19–21] In these foods, flavanols can exist as monomers, such as epicatechin, or in oligomeric forms referred to as procyanidins or more broadly, proanthocyanidins. These flavanol-based oligomers are linked primarily through carbon-carbon bonds from the 4 position of one flavanol subunit to the 8 position (C4→C8) of another, and to a lesser extent through a C4→C6 linkage. Figure 3 demonstrates, with a few examples, the variety of structural skeletons. Additional variety stems from the number and position of the hydroxyl groups on the aromatic (C_6) rings (eg, epigallocatechin). Flavanol-based oligomers having single carbon-carbon linkages between subunits are referred to as B-types. Less common are those with A-type linkages, which consist of an additional ether bond between C2→O7. Commonly, the molecular weight of flavanol-based oligomers is expressed as their degree of polymerization,[18] and individual oligomers are commonly referred to as dimers, trimers, etc. Procyanidins are a specific subclass of these flavanol-based oligomers, and represent the largest class of proanthocyanidins (Fig. 1). The flavanol subunits that comprise the procyanidins consist only of epicatechin and catechin.

CARDIOVASCULAR HEALTH AND DIETARY INTAKE OF FLAVANOLS

CVDs are the leading cause of death in Western Europe, the United States and other countries in the so-called developed world, accounting for nearly one-third of total deaths world-wide.[22] In the United States alone, preliminary mortality data from 2003 indicate that 37% of all deaths in the United States were due to CVD, and that 58% of the deaths in that year identified CVD as an underlying or contributing cause of death.[1] In addition to these mortality figures, the economic costs of CVD are also staggering. It is estimated that in 2006, the direct and indirect cost of CVD will exceed 400 billion dollars in the United States alone. During the past 3 decades, understanding of the pathophysiology of CVD and the factors, contributing to the disease process, has grown considerably. As a result of these advances, there is a growing appreciation that while prescription, and even some over-the-counter medications, may mitigate risk factors associated with CVD, dietary, and other lifestyle factors continue to be important in the prevention, management, and, potentially, treatment of CVD.

Epidemiological studies suggest that diets rich in plant foods offer protection against a number of diseases including those of cardiovascular origin.[4,23–25] Traditionally, the macronutrient, vitamins, and essential minerals content of these foods were thought to be responsible for their putative health benefits; however, epidemiological research during the past 2 decades increasingly supports the concept that certain flavonoids in these foods may play an important role in conferring the cardiovascular benefits observed. Among the most popularized studies published was that of Renaud and de Lorgeril[26] in 1992 which postulated that the lower occurrence of heart attacks in the French, a population with a higher intake of saturated fat, was associated with the daily consumption of red wine (often referred to as the "French Paradox"). Although, it was originally postulated that the alcohol was the cardioprotective component, data reported by Frankel et al[27,28] were among the first to suggest that the phenolic constituents of red wine could also be protective.

Since these initial investigations, additional studies with foods rich in flavonoids (and phenolics in general) continue to suggest that these natural constituents have a potential role in promoting cardiovascular health.[4,29] As a result of systematic dietary intervention trials with

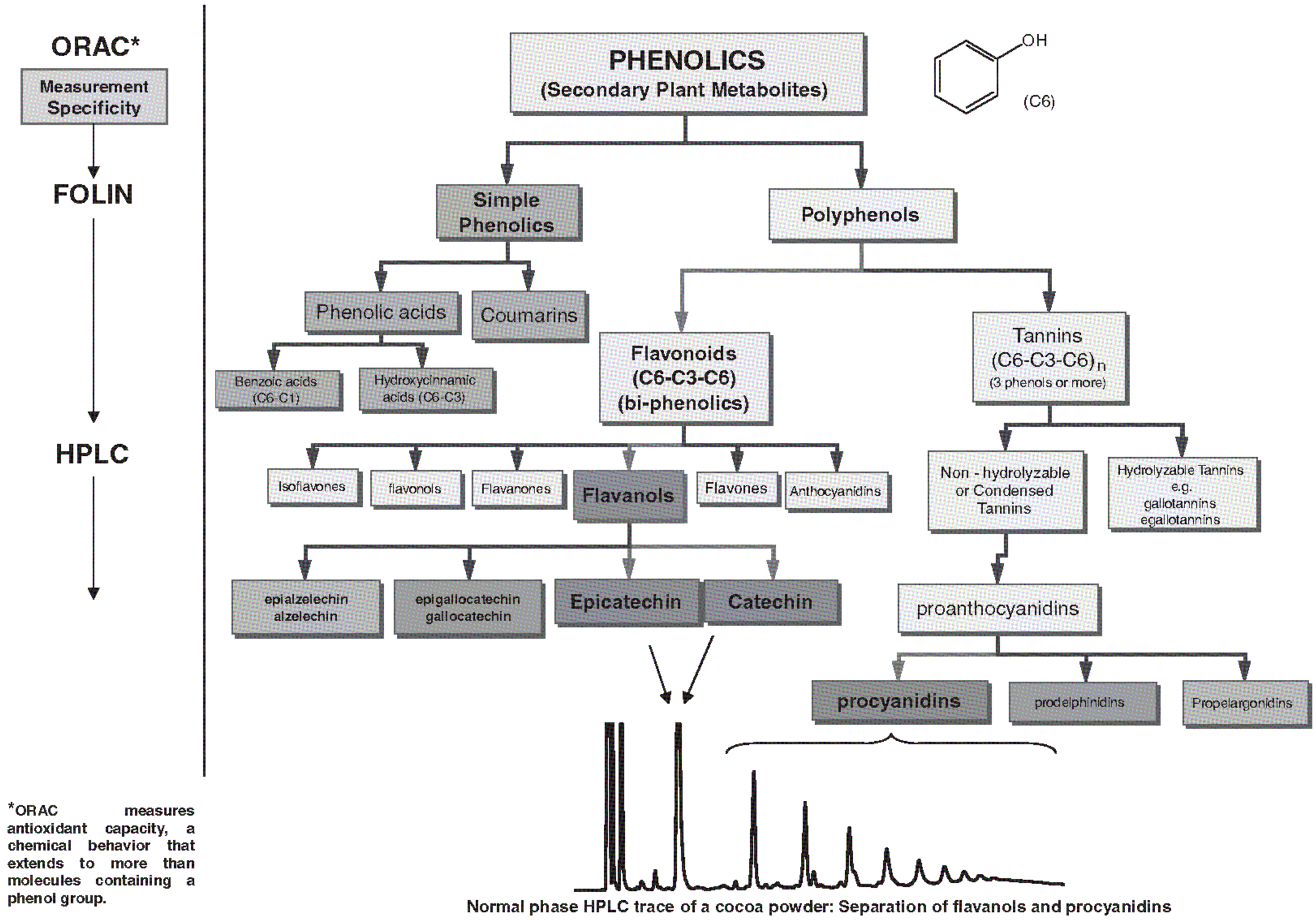

FIGURE 1. Flowchart depicting classification scheme for phenolics, according to the number of phenol subunits (1, 2, ≥ 3) and the hierarchy of common flavonoids monomers and polymers. The specificity of common analytical measurement tools is given (left-hand side). An HPLC trace for cocoa powder demonstrating the separation of flavanols and procyanidins is provided as an example.

flavanol-rich cocoa products, investigating both mechanisms and functional effects, the compelling hypothesis that certain flavanol-rich foods may have the potential to improve key aspects of health has emerged. These investigations, conducted with specific cocoas having a well-characterized flavanol content, have demonstrated that the consumption of these flavanol-rich cocoa products can improve important markers related to cardiovascular health including endothelial function, nitric oxide synthesis, and platelet function.[6–8,30,31] Additional data supporting these findings and, in some cases, extending vascular targets to include blood pressure, have also emerged from studies using less rigorously characterized foods—in the context of flavanols—as intervention vehicles.[11–13,32] Although together these studies further contribute to the growing excitement around flavanol-rich foods and their potential applications in public health, larger carefully designed dietary intervention trials are still needed to conclusively validate the impact that certain, or perhaps all, flavanol-rich foods may have on cardiovascular-associated mortality and morbidity (Fig. 4). Although often overlooked, the accurate measurement of the flavanols is a critical aspect of validating the results from these dietary interventions.

ANALYTICAL MEASUREMENT SYSTEMS: ESTABLISHING THE LINK BETWEEN FLAVANOLS AND CARDIOVASCULAR HEALTH

Underpinning the studies required to establish the relationship between the consumption of flavanol-rich foods and cardiovascular health is the need to have specific flavanol composition data of the foods employed in these studies. Without this information, meaningful studies cannot be conducted that establish the causal links necessary to enable the development of clear dietary and public health recommendations. A variety of analytical methods are available which can provide information of varying quality regarding the flavanol content of foods; each measurement system has advantages and limitations

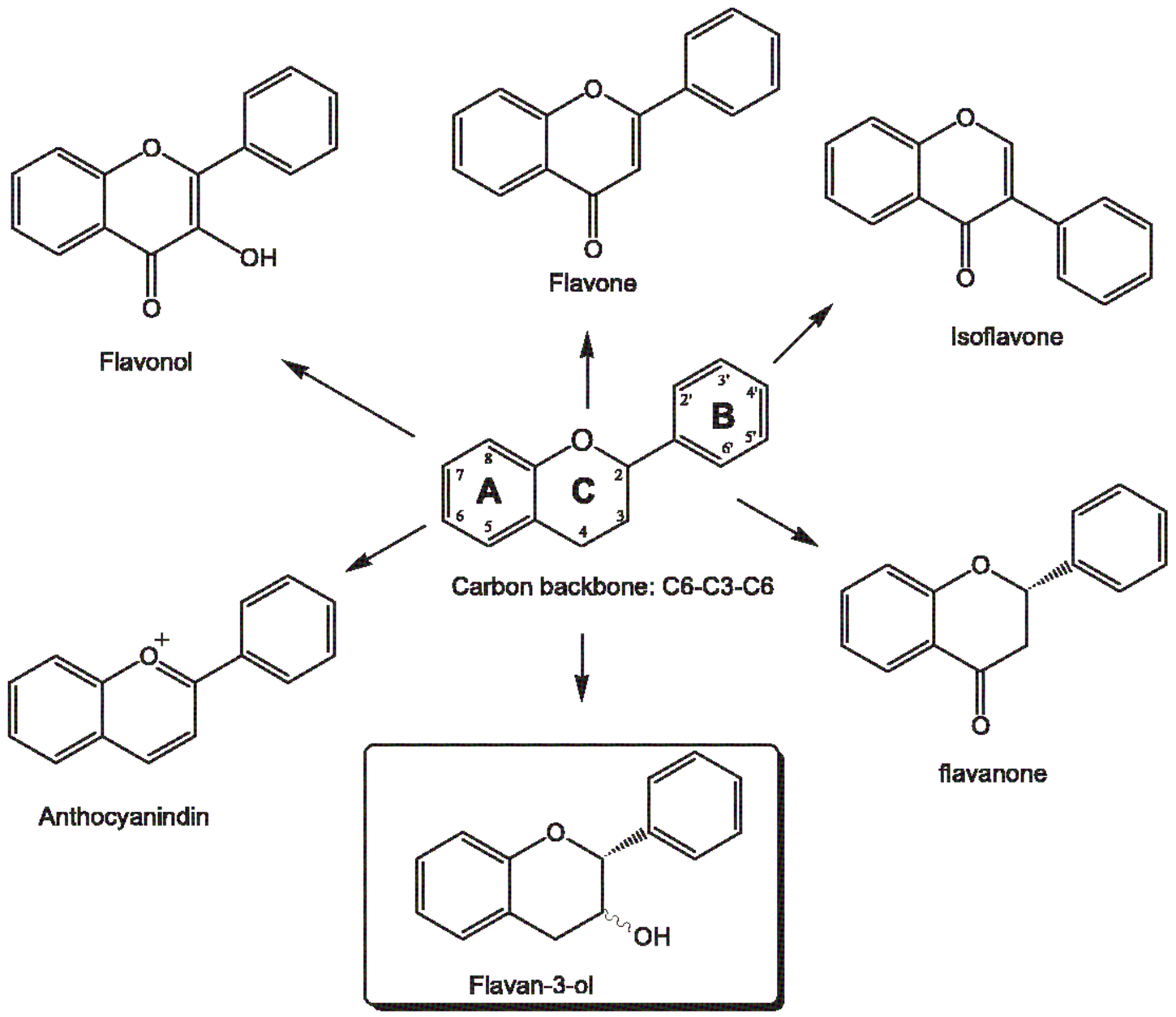

FIGURE 2. The generic 3 ring (A, B, and C) carbon backbone structures (C_6-C_3-C_6) of the major flavonoid subclasses found in foods (flavone, isoflavone, flavonol, anthocyanidin, flavanone, and flavanol) are depicted. The flavanol generic structure is highlighted.

in its use. It is important to understand these distinct measurement systems and the information they are capable of providing because the quality of these measurements plays a critical role in determining the strength of the causal link between these natural food constituents and potential cardiovascular health benefits.

Folin-Ciocalteu (FC), ORAC, and HPLC

Owing to the complexity and diversity of these naturally occurring phenolic plant components, quantitative analysis, isolation and structural determination of flavanols is a difficult—seemingly overwhelming—task. Different measurement systems exist, each of which exploit different chemical behaviors and/or variations in chemical structure.[33] These assays vary widely in their performance, specificity, and required instrumental sophistication. Not all available tools will be described in this review; however, the applicability of popular assays such as FC, ORAC, and the common chromatographic technique, high performance liquid chromatography (HPLC), will be discussed. The former 2 are nonspecific measurement systems, and measure the total chemical behavior of a solution. In contrast, HPLC is more specific in that it separates and provides the potential for more comprehensive identification and quantification of individual flavanols in foods.

Because the chemistry of a phenolic functionality is distinct, it is exploited for nonspecific measurement systems.[34] In addition, molecules containing a phenolic substructure can behave as antioxidants. This antioxidant behavior can be partly attributed to the chemical behavior of the phenol moiety (ie, a hydroxyl group on the aromatic ring—shown in Fig. 1) through formation of stabililzed phenoxyl radicals. Although these molecules can also demonstrate favorable redox-potentials and have radical scavenging activity (due in part to the phenolic moiety), these latter 2 behaviors are not exclusive to molecules containing a phenol moiety (eg, carotenoids can also behave as antioxidants).

The FC assay—also referred to as a colorimetric measurement system—is an assay that produces colored complexes that are easily detected spectrophotometrically with high sensitivity.[35] Fundamentally, the FC assay measures the reducing capacity of phenolic components present in a basic solution environment. However, it is not specific to flavanols as it can detect a wide variety of other hydroxyl containing species, including other flavonoids, phenolic acids, amino acids, ascorbic acid, and reducing sugars, that commonly occur in food matrices. It is of great utility for screening of plant materials for phenolics and as a way to estimate gross phenolic content. Used in the appropriate context, the FC assay is a robust and convenient measurement system. It is, most likely, this long history of convenience that has led to such wide acceptance and, in some countries, increasing efforts to use FC to support polyphenol content claims for certain products. Indeed, there is currently activity targeting FC for adoption as a standard method by official organizations such as the AOAC International.[36] Unfortunately, the "acceptance" of this generalized, nonspecific measurement tool in place of more rigorous methods capable of providing more accurate compositional data will likely

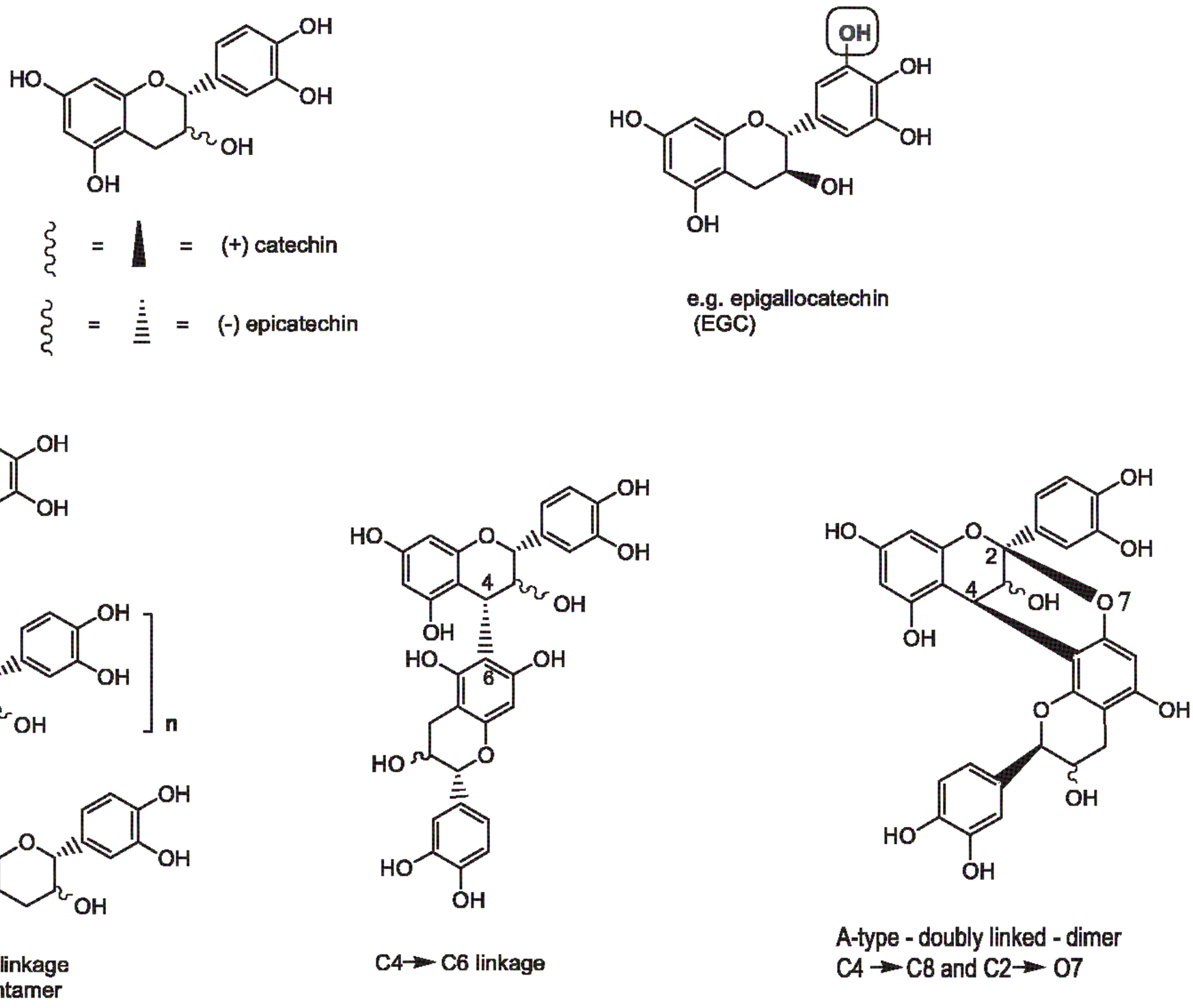

FIGURE 3. Examples of common structural features of procyanidins: Monomer, pentamer, and two dimers are shown. Note linkage differences of B-type and A-type dimers. Epigallocatechin is an example of a structural variation of a flavanol: additional hydroxyl group highlighted in a box.

undermine the efforts aimed at establishing a firm link between the consumption of flavanol-rich foods and cardiovascular health benefits.

Another measurement system that has gained more recent notoriety is the ORAC. It is also currently being formally standardized through a multilaboratories validation process for the AOAC International. It is one of several measurement systems referred to as antioxidant capacity assays which have been reviewed elsewhere.[37] It measures, through the addition of a molecular probe, the inhibition of a radical reaction but also the degree of inhibition (rate and completion of quenching reaction).[38,39] The ORAC tends to be the preferred method to determine antioxidant capacity in foods and is considered an indicator for potential biologic activity although the link between antioxidant capacity and protective health effects has yet to be clearly established.[40] And is, in fact, increasingly controversial due to the lack of effects seen in large randomized clinical trials investigating vitamin E and other putative antioxidant nutrients in cardiovascular health.[41–44] This antioxidant assay is neither specific for flavanols nor for phenolic containing molecules (Fig. 1); rather, it is instead a measure for a chemical behavior—antioxidant behavior (ie, it is a measure for molecules that can quench or retard radical reactions).

HPLC is a chromatographic technique that physically separates molecules based on interactions with various packing materials (phase) in a column, and is often coupled to various modes of detection, including ultraviolet-visible (UV-vis) absorption, fluoresence detection (FLD), and/or mass spectrometic detection (MS). UV-vis detection is a readily available mode of detection; however, it is not specific for flavanols relative to other polyphenolic compounds. In contrast, FLD offers increased sensitivity and selectivity for flavanols. MS detection can assist with identification through mass and cleaving patterns of the components.[45] HPLC/MS is currently used primarily as a qualitative tool due to lack of necessary analytical standard materials.

One of the more commonly used HPLC methods for flavanols is normal phase (NP-HPLC) separation. These NP-HPLC methods rely on hydrogen bonding interactions between these compounds and the silica hydroxyl groups of the column's stationary phase.[46] Under appropriate conditions (mobile phase, stationary

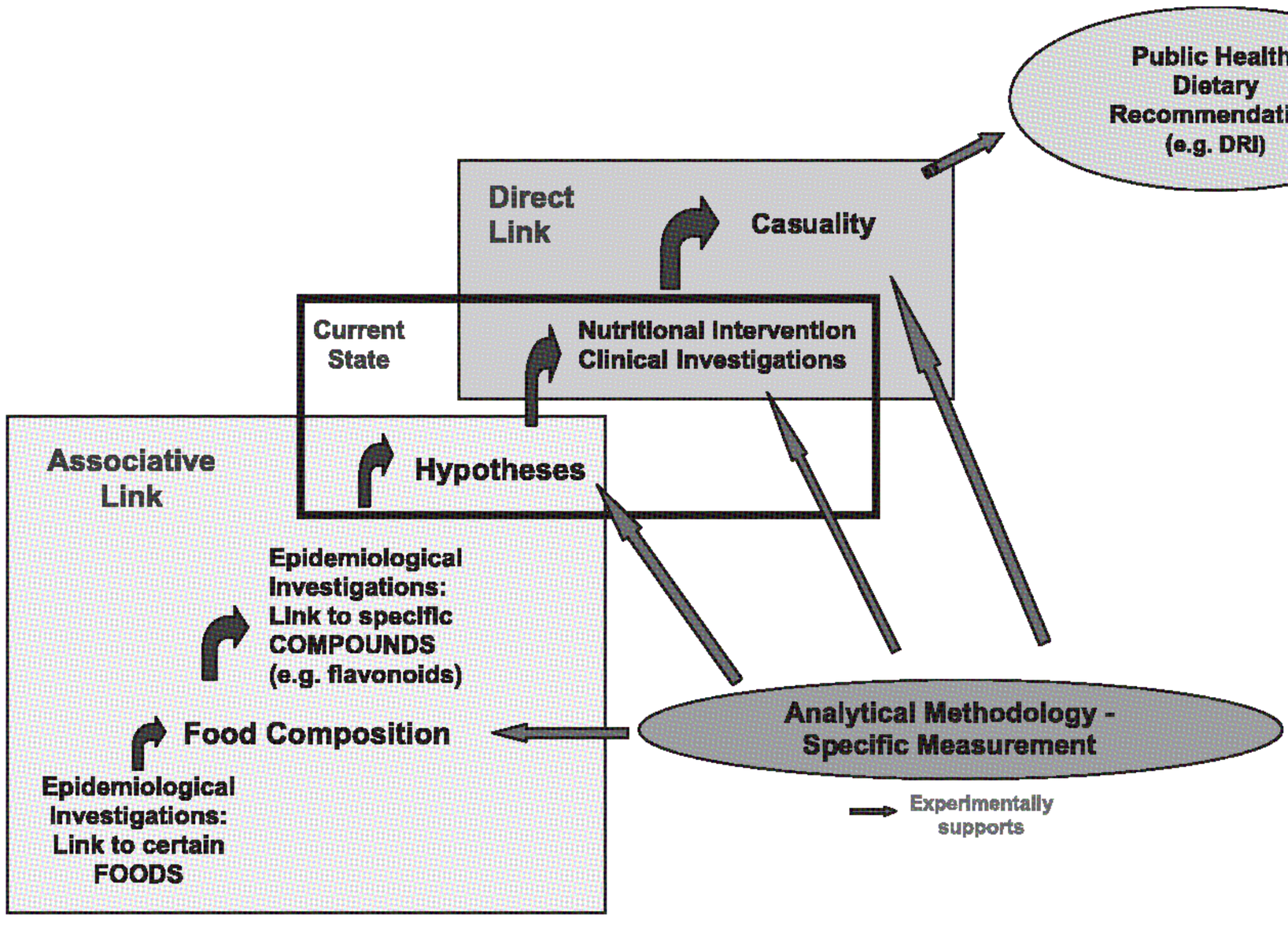

FIGURE 4. Schematic describing the research approaches employed to establish the link between dietary intake of flavanol-rich foods and protective health effects ultimately leading to public health recommendations. Highlighting the importance of analytical measurement systems throughout the process.

phase, and detection technique), flavanols can be separated as individual molecules or by degree of polymerization.[45,47,48] NP-HPLC-FLD is commonly employed as quantitative measurement system for flavanols.[10] Thus, in contrast to the FC and ORAC methods described above which offer a single nonspecific value and no substantive compositional information, this NP-HPLC approach can provide detailed information on the flavanol content of foods (refer to the section below for more details).

Recent advances in HPLC methodology have introduced simpler and more robust chromatographic approach for separating larger molecular weight flavanols, that is, the procyanidins.[49] Importantly, this new separation approach has been extended to the preparative scale, thus providing a robust method to isolate a wide range of pure flavanols thus opening the door for the generation of standards to further improve analytical measurements, and the provision of characterized materials for biologic investigations.

Comparison of Analytical Methods

As detailed above, the FC assay is *not* a method specific for flavanols as it readily detects other hydroxyl containing species present in the food. Hence, when employed as a surrogate indicator for flavanols, due to its nonspecific nature, it often generates values greater than the actual content of these compounds in a given food. A comparison of FC data obtained for milk chocolate, dark chocolate, and cocoa powder to that obtained through a quantitative HPLC demonstrates clearly this discrepancy in precision. The FC assay provides polyphenol data that is consistently 2 to 4 times greater than that obtained from the HPLC method, which measured flavanols specifically. Figure 5 graphically represents this deviation in specificity. As depicted in Figure 1, the FC measures a total phenolic count and more, whereas appropriate HPLC methods can quantify a specific class of flavonoids. At this time, to strengthen the link between observed cardiovascular health benefits to flavanol-rich foods, measurement systems that provide this more accurate and comprehensive level of compositional information are required.

Like the FC method, ORAC measurements suffer from their nonspecificity. ORAC measures the antioxidant capacity of a solution and is not directly a measure of compounds containing phenolic substructures. Examining the ORAC values versus those obtained via HPLC for the same food samples exemplifies these incongruencies. Antioxidant capacity (ORAC) values for apples and certain dark chocolates are reported to be 0.2 versus 13.1 (mmol Trolox equivalents), respectively, whereas values of 106 mg versus 170 mg of flavanols (per 100 g food sample) are reported.[50,51] Another example is with flavanol-rich red wine. Although it is reported to have less flavanols (22 mg/100 g) than apples (106 mg/100 g), it gives a higher ORAC value (0.7 mmol Trolox equivalents for red wine vs. 0.2 mmol Trolox equivalents for apples).

Although it is commonly stated that flavanols are antioxidants, clinical studies have failed to consistently demonstrate these effects in vivo.[52–54] In addition, clinical

evidence obtained from dietary intervention trials has demonstrated that the vascular benefits of flavanol-rich foods stretch well beyond their in vitro antioxidant behavior.[8,9,55,56] When evaluating the potential for food components such as flavanols to function as antioxidants in vivo, it is important to recognize the fact that these compounds are subject to significant structural modifications upon absorption. The metabolic fate of many flavanols can involve methylation, sulphation, as well as glucuronidation.[57] These compounds also undergo degradation into simpler phenolics, producing molecules with far different chemical properties, and thus, potentially far different biologic effects than that of the native, nontransformed species. Given that methylation and glucuronidation incapacitates the formation of stabilizing phenoxyl radicals, dramatically reducing the "antioxidant capacity" of these molecules,[58] the value of measurement tools such as ORAC in studies aimed at linking food constituents such as flavanols to health should be considered with extreme caution.

HPLC is a technique that requires more sophisticated and costly instrumentation relative to FC and ORAC, has broad applicability and, under the correct experimental conditions, is much more specific for flavanols (Fig. 1). Although HPLC is a measurement system with a vast improvement in distinguishing flavanols from all phenolic or antioxidant species, difficulties still exist. In the analyses of foods deemed cardioprotective—due to their flavanol content—is the fact that different foods contain different flavanol profiles (ie, structurally different molecules). Figure 6 depicts the normal phase separation (with FLD) of specific cocoa, tea, and apple products that are flavanol-rich.[45,59] Clearly, each trace indicates a different flavanol profile associated with each food. Indeed, the specific flavanols in cocoa and tea are quite different—information not obtained when simply reporting either a content value or an antioxidant capacity value. Also, tea contains the flavanols epigallocatechin gallate, epicatechin gallate that are not present in cocoa (Fig. 6). These differences are a concern in that the flavanols in foods reported as "flavanol-rich foods" are actually structurally different and can potentially evoke different biologic responses. In addition, under the HPLC conditions used to generate the data shown in Figure 6, catechin (C) and epicatechin (E) present in cocoa are not distinguishable.

CONCLUSIONS

Increasingly compelling evidence suggests that certain flavanol-rich foods have the potential to improve cardiovascular health, and therefore contribute another public health tool in the fight to reduce the widespread morbidity and mortality from cardiovascular health complications. Additional clinical and epidemiological research is needed to provide unambiguous data necessary to underpin dietary recommendations regarding flavanol-rich foods. In this context, it is essential that investigators employ appropriate analytical methodologies that accurately characterize and quantify the major flavanols present in the flavanol-rich foods under investigation to ensure high-quality data relevant to assessing the health impact of consuming these foods. Although convenient, assays such as FC and ORAC measure a general chemical behavior that are not specific to the flavanol content of foods. Use of these assays cannot accurately quantify the flavanol content of foods and beverages; therefore, the results of investigations in which these nonspecific measurement tools have been used must be interpreted with caution, as they are not capable of linking the putative flavanol components of these foods to cardiovascular health. It should therefore

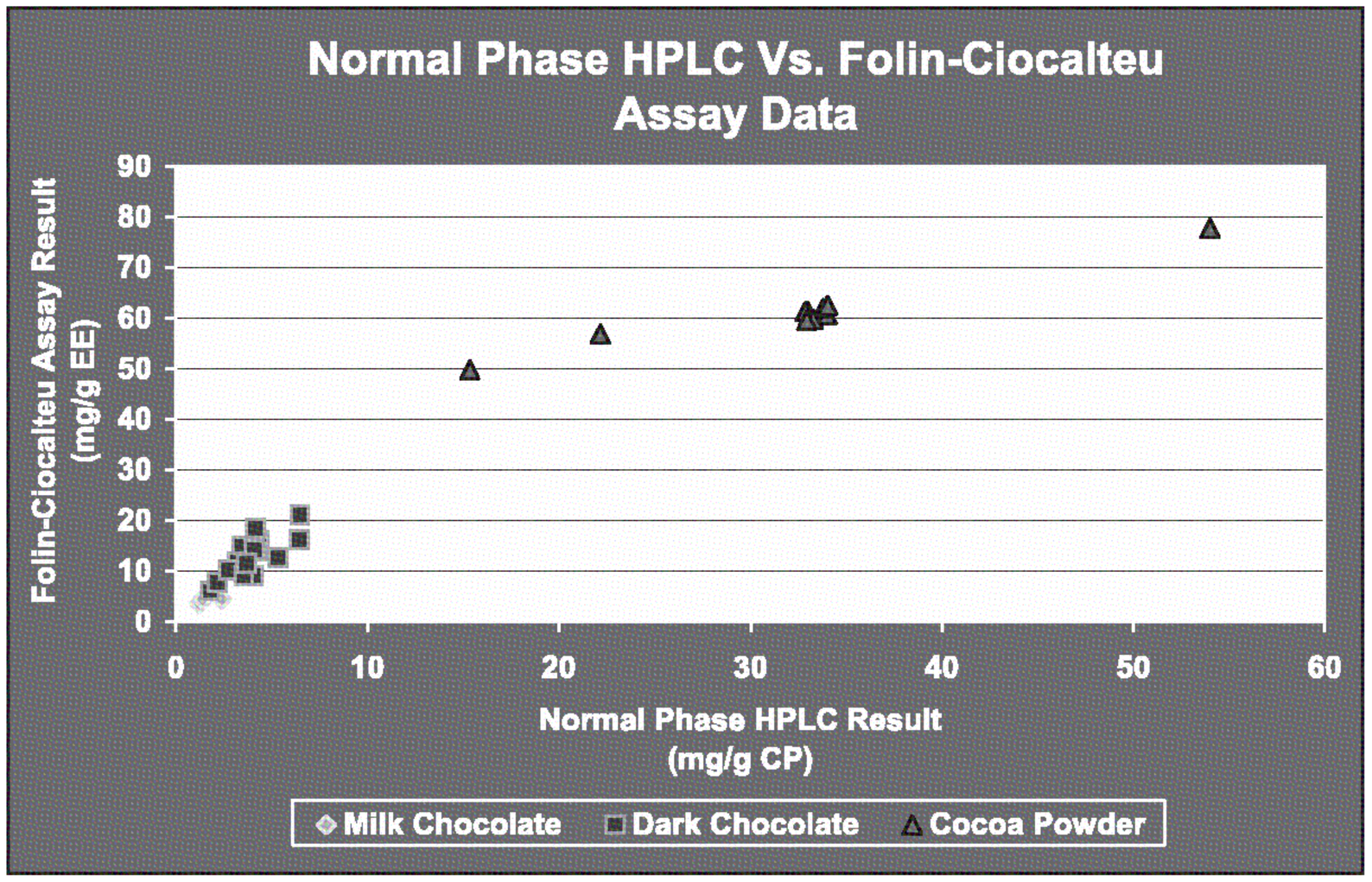

FIGURE 5. Comparison between HPLC and FC assay data for milk chocolate, dark chocolate, cocoa powder (mg/g EE indicates epicatechin equivalents per gram of sample). The data show that FC is on average 2 to 4 times greater than values obtained using HPLC.

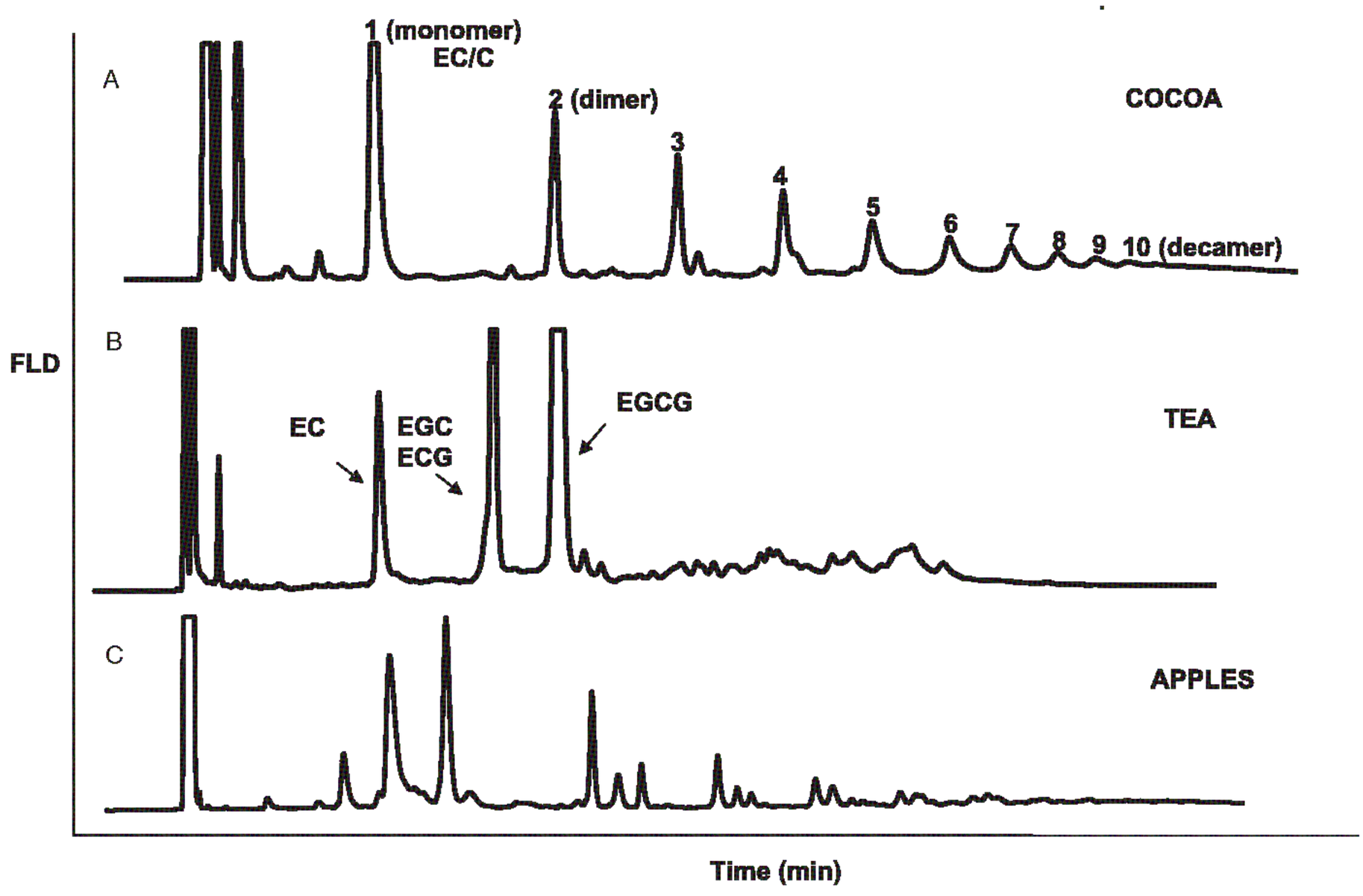

FIGURE 6. Normal phase HPLC traces—with *fluorescence detection* (FLD)—for (A) cocoa powder; (B) tea, and (C) apples, demonstrating the differences between flavanols and proanthocyanidins content of three flavanol-rich foods.

be clear that flavanol content or health claims on products on the basis of these assays should be viewed with skepticism.

Analytical methods capable of providing high-quality information about the specific flavanol content of foods are not necessarily convenient, and can yield unreliable data in the hands of inexperienced investigators. It is therefore recommended that a multidisciplinary approach, combining analytical, medical, and nutrition science communities, is pursued in the design and execution of gold standard dietary intervention trials. This type of collaboration will ensure the flavanol-rich foods under investigation are appropriately characterized and adequate controls are implemented in these studies. Approaching future investigations with appropriate compositional analysis rigor will make it possible to establish a clear, causal link between the consumption of these flavanol-rich foods and cardiovascular health, and also make possible the development of meaningful dietary recommendations by health professionals for implementation to address critical public health needs.

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