Analysis of Flavanols in Beverages by High-Performance Liquid Chromatography with Chemical Reaction Detection

S. de Pascual-Teresa,[†] D. Treutter,[‡] J. C. Rivas-Gonzalo,[†] and C. Santos-Buelga*[,†]

Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno s/n, E-37007 Salamanca, Spain, and Lehrstuhl für Obstbau, Technische Universität München, D-85350 Freising-Weihenstephan, Germany

A screening method, which allows the simple and rapid determination of the flavanolic composition of plant and food extracts, has been developed. The method is based on a postcolumn chemical reaction with *p*-dimethylaminocinnamaldehyde with detection at 640 nm after HPLC separation and measurement of the UV absorbance at 280 nm in a diode array detector. This approach allows one not only to obtain the flavanol profiles of the samples but also to characterize the compounds present in the chromatograms through their A_{640}/A_{280} absorbance ratio combined with the retention time and the UV spectrum. The advantages of this procedure are shown by applying it to the study of the flavanol composition of some beverages, including red wine, beer, apple cider, and sour cherry and blackthorn fruit liqueurs.

Keywords: *Flavan-3-ols; procyanidins; prodelphinidins; beverages; wine; beer; cider*

INTRODUCTION

Flavan-3-ols are of particular interest to nutritionists as they have been shown to possess a bioactive potential [reviewed by Treutter (1996)]. Moreover, those flavan-3-ols present in foodstuffs have an additional organoleptic and technological interest, as they have influence on organoleptic characteristics such as astringency and also, indirectly, color. They can be the cause of instability and turbidity in beverages as well (McMurrough et al., 1996). However, our knowledge of their occurrence in foods and beverages is fairly poor, because there are no methods selective enough for their analysis.

The variety of flavanols that can occur in food products is large, and their analysis creates different challenges. When the analysis is carried out by HPLC with UV detection, as is usual, interferences with other substances may exist, particularly with phenolic compounds, present in higher amounts and/or with higher extinction coefficients. Furthermore, in samples that possess a complex proanthocyanidin composition, it is difficult to obtain satisfactory separations in a single run; for example, no good separations of prodelphinidins are obtained using the HPLC methods ordinarily applied for procyanidins. Another challenge is the accurate identification of substances in the chromatograms, which usually requires their previous isolation, as no standards are commercially available. Such an isolation is not always easy to achieve when one takes into account the quantities in which the proanthocyanidins are usually present in foods and beverages and the difficulty of their separation and characterization.

The technique of HPLC coupled to a chemical reaction detection (HPLC-CRD), first described by Treutter (1989) for the analysis of flavanols and later optimized by Treutter et al. (1994a), can assist in overcoming some of these obstacles. This technique is based on the ability of aldehydes to act as electrophiles and condense with activated aromatic rings in strongly acidic media (Delcour et al., 1985). The reagent used, *p*-dimethylaminocinnamaldehyde (DMACA), gives colored adducts with flavanols (Figure 1) showing maximum absorption

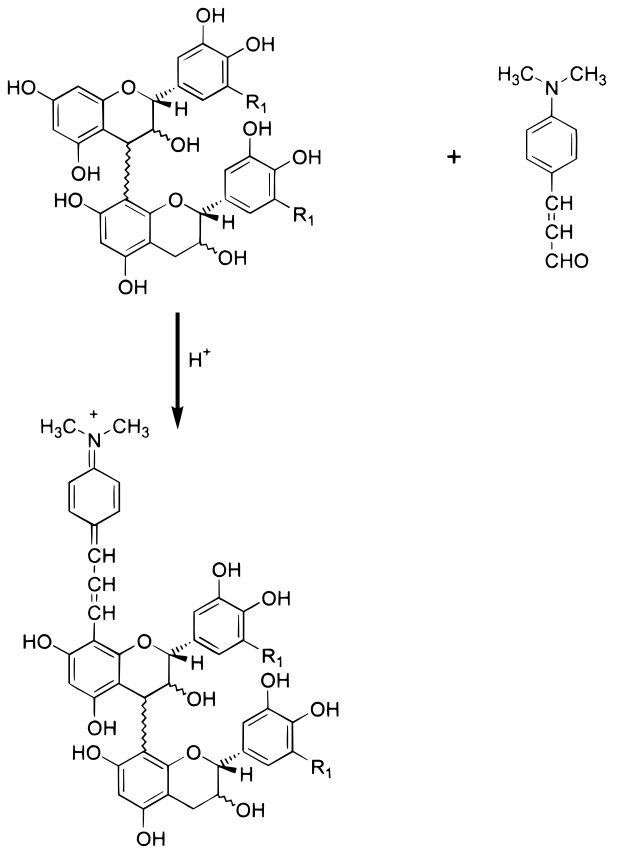

Figure 1. Reaction between flavanols and DMACA in acidic media (R_1 = H in procyanidins; R_1 = OH in prodelphinidins).

* Author to whom correspondence should be addressed (fax +34 23 294515; e-mail csb@gugu.usal.es).

[†] Universidad de Salamanca.

[‡] Technische Universität München.

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between 632 and 640 nm, thus preventing the interference of other colored compounds that might be present in the same extracts, such as anthocyanins. The reagent shows a high specificity for flavanols (Treutter et al., 1994b). This is why, in the detection carried out at 640 nm after reaction, only peaks corresponding to these compounds are seen in the chromatograms, thus overcoming separation problems. In addition to its selectivity, the derivatization method increases the sensitivity, when compared with the detection at 280 nm (Treutter et al., 1994a,b), allowing the detection of substances present in low concentrations. Finally, another interesting characteristic of this technique is that information about the chemical structure of the compounds can be obtained from the ratio of their peak areas at 640 and 280 nm (Santos-Buelga and Treutter, 1995; Treutter et al., 1994a). This characteristic is very useful for the identification of substances in chromatograms, especially when this information is combined with their chromatographic behavior and the UV absorbance spectra measured prior reaction with a diode array detector.

In this paper, the advantages of the HPLC-CRD technique for the analysis of the flavanol composition of some beverages are shown. This kind of product has the additional advantage of being suitable for direct injection into the chromatographic system, avoiding in this manner problems inherent to the extraction and/or preparation of samples. Prior to the analysis, a range of flavanols was previously isolated from different plant sources, with the aim of obtaining data about their chromatographic and spectral characteristics, which further facilitated the identification of peaks in the chromatograms of the samples. Likewise, a new HPLC method was previously optimized, to get the separation of procyanidins and prodelphinidins in the same run.

MATERIALS AND METHODS

Preparation of Flavan-3-ol Standards. Flavan-3-ols were isolated from different plant sources. Monomeric catechins were isolated from green tea, prodelphinidins from lentils, and procyanidins from grape seeds (Escribano-Bailón et al., 1992), almonds (de Pascual-Teresa et al., 1998), and horse chestnut shells (Santos-Buelga et al., 1995). For the isolation of compounds, methanol extracts from the different plant materials were fractionated on a Sephadex LH-20 column (30 × 2.5 cm). The composition of the resulting fractions obtained was checked by TLC and HPLC. For TLC, both silica gel and cellulose have been employed using toluene/acetone/formic acid, 3:6:1, and formic acid (10%), respectively, as mobile phases (Thompson et al., 1972). When necessary, the compounds were later purified by semipreparative HPLC, as described by de Pascual-Teresa et al. (1998). The identification of the substances isolated was carried out by using selective cleavages (Escribano-Bailón et al., 1992) and LC/MS (de Pascual-Teresa et al., 1998). The following compounds were obtained: catechin (C), epicatechin (EC), gallocatechin (GC), epigallocatechin (EGC), and epigallocatechin 3-*O*-gallate; dimers GC-(4,8)-GC, GC-(4,6)-GC, GC-(4,8)-C, GC-(4,6)-C, C-(4,8)-C (B3), EC-(4,8)-C (B1), C-(4,8)-EC (B4), EC-(4,8)-EC (B2), EC-(4,6)-C (B7), and EC-(4,6)-EC (B5); trimers GC-(4,8)-GC-(4,8)-C, EC-(4,8)-EC-(4,8)-C, EC-(4,8)-EC-(4,8)-EC (C1) and EC-(4,6)-EC-(4,6)-EC; and tetramers EC-(4,8)-EC-(4,8)-EC-(4,8)-C and EC-(4,8)-EC-(4,8)-EC-(4,8)-EC.

HPLC Analysis. A gradient forming system Waters 600 E apparatus coupled to an autosampler Waters 717 plus was used. The separation was performed on a Spherisorb ODS2 column (150 × 46 mm i.d.), particle size 3 μm, with the following solvents: water (solvent A), methanol (solvent B), 4.5% aqueous formic acid (solvent C), and a mixture of 4.5% aqueous formic acid/methanol, 90:10 (solvent D). The gradient was as follows: 0−10 min, 100% A to 100% C; 10−20 min, 0−15% D in C; 20−30 min, 15% D in C, isocratically; 30−40 min, 15−35% D in C; 40−45 min, 35% D in C, isocratically; 45−60 min, 35−45% D in C; 60−75 min, 45−100% D in C; 75−175 min, 0−50% B in D; 175−180 min, 50−80% B in D. Flow rate was 0.5 mL/min.

A double on-line detection was carried out, first at 280 nm in a diode array detector (Hewlett-Packard 1040A) coupled to an HP79994A data treatment station and then at 640 nm after derivatization with DMACA reagent (1% DMACA in 1.5 M sulfuric acid in methanol) in a Milton Roy Spectromonitor 3100, coupled to a PC equipped with HP-ChemStation software. The reagent was added by a Kontron 320 pump with stainless steal pump heads at a flow rate of 0.5 mL/min. The reactor was a knitted Teflon tube (0.5 mm i.d.) with 9 m in length resulting in a reaction time of 2.5 min.

With the aim of testing the applicability of the method, actual samples of different kinds of beverages were analyzed: red wine, lager beer (from Spain and Germany), Bavarian wheat beer, apple cider, and sour cherry and blackthorn fruit liqueurs. Carbon dioxide was eliminated under vacuum (when necessary), and the samples were directly injected into the HPLC system after filtration through a 0.45 μm filter. Each sample was injected, at least, three times, obtaining always coincident peak profiles.

RESULTS AND DISCUSSION

HPLC Separation and Peak Identification. To interpret the results obtained by the HPLC-CRD technique, a good chromatographic separation of substances should exist. If not, neither adequate peak integration nor good peak spectra can be obtained. Prodelphinidins elute very early in reversed-phase chromatography, and they are not well separated with the gradients commonly used for procyanidins. For this reason a new HPLC gradient was developed that allows the separation of prodelphinidins, catechin-based procyanidins, and epicatechin derivatives in the same run. Prior to the injection of the sample, the column is thoroughly stabilized with water, and then a step gradient is established between 4.5% aqueous formic acid and a mixture of methanol/4.5% aqueous formic acid (10:90, v/v), providing a slow and modulated increase in the percentage of methanol. Although acetonitrile might provide a better chromatographic separation of flavanols, in this system, the use of methanol is necessary, because the derivatization reaction is inhibited by the first but favored in alcoholic media (Treutter, 1988). Prior to the reaction with DMACA, another detection was made in a diode array detector to obtain the UV absorbance spectra of the peaks. To test the gradient, a mixture of 20 flavanols, including monomers, procyanidins, and prodelphinidins, obtained from several plant sources, was injected in the system. The results are shown in Table 1.

It has been previously shown that the CRD/UV absorbance ratio (A_{640}/A_{280}) of the flavanols is structure-related (Treutter et al., 1994a; Santos-Buelga and Treutter, 1995). Thus, in the same experimental conditions (solvent composition, flow rate, and reactor length), the different flavanols show characteristic CRD/UV ratios, which can be used as a criterion for their identification. The ratios obtained in the experimental conditions of the optimized technique for the flavanols analyzed are in Table 1. With the aim of allowing a better interpretation of the results obtained in different chromatographic runs, the ratios are also expressed in terms of percentage referred to EGC (100%), which possesses the highest ratio of all the flavanols consid-

Table 1. Data Obtained for Different Flavanols with the Optimized HPLC-CRD Method

peak no.	flavan-3-ol	t_R[a] (min)	λ_{max}[b] (nm)	ratio A_{640}/A_{280}	ratio rel to EGC[c]
1	GC-(4,8)-GC	26.5	271	4.62	26.89
2	(+)-gallocatechin (GC)	29.7	268−270	13.75	80.03
3	GC-(4,8)-GC-(4,8)-C	30.6	275−277	2.17	12.63
4	GC-(4,8)-C	32.8	277	2.23	12.98
5	GC-(4,6)-C	39.3	277	2.52	14.67
6	C0(4,8)-C (B3)	46.2	279	1.38	8.03
7	EC-(4,8)-C (B1)	49.0	279	1.89	11.00
8	(+)-catechin (C)	51.5	279	3.31	19.27
9	EC-(4,8)-EC-(4,8)-C	59.8	279	1.52	8.86
10	EC-(4,8)-EC-(4,8)-EC-(4,8)-C	60.2	279	1.19	6.93
11	(−)-epigallocatechin (EGC)	63.2	269	17.18	100
12	C-(4,8)-EC (B4)	68.0	279	1.71	9.97
13	EC-(4,8)-EC (B2)	95.0	279	2.37	13.80
14	(−)-epicatechin (EC)	98.4	279	4.40	25.61
15	EC-(4,6)-C (B7)	105.9	279	1.86	13.08
16	EC-(4,8)-EC-(4,8)-EC (C1)	108.7	279	1.36	7.92
17	EC-(4,8)-EC-(4,8)-EC-(4,8)-EC	112.0	279	1.18	6.84
18	epicatechin 3-*O*-gallate (ECG)	119.5	279	1.05	6.11
19	EC-(4,6)-EC (B5)	130.0	279	2.76	16.07
20	EC-(4,6)-EC-(4,6)-EC	136.0	279	1.54	8.96

[a] t_R corresponding to the HPLC chromatogram obtained at 640 nm after reaction with DMACA. [b] λ_{max} refers to the maximum absorbance of the peak spectra obtained with the diode array detector prior to the reaction with the DMACA reagent. [c] The relative ratio of each peak *i* was calculated from their peak area (*A*) as [*A*(*i*, 640)/*A*(EGC, 640)] × [*A*(EGC, 280)/*A*(*i*,280)] × 100.

ered. It can be seen that gallocatechins and prodelphinidins show higher ratios than the equivalent catechins and procyanidins, the compounds with (C4−C6) linkages have higher ratios than the corresponding ones with (C4−C8) linkages, and within a correlative series of compounds, the ratio decreases as the degree of polymerization increases. When the composition of the sample is not very complex and the peaks of the flavanols are well-defined in the chromatogram recorded at 280 nm, the CRD/UV absorbance ratios are highly reproducible. Thus, the information obtained from them, combined with the retention times and the UV spectrum, allows the identification of a wide variety of flavanols in a rapid and simple way. In more complex samples, some flavanols may coelute with other compounds and, even though they are specifically recorded after the derivatization reaction, it is not possible to obtain their UV spectra or calculate their absorbance ratios accurately. When this happens, a fractionation of the sample (e.g., in Sephadex LH-20 or Fractogel TSK-HW40) is necessary to eliminate interferences, so as to obtain suitable UV spectrum and absorbance ratios.

Sample Analysis. In Figure 2, chromatograms at 280 and 640 nm corresponding to a sample of a two-year-old commercial Spanish red wine from grapes of *Vitis vinifera* var. Tempranillo are shown. A simpler chromatographic profile, showing only peaks corresponding to flavanols, is obtained at 640 nm after the chemical reaction with DMACA. In addition to this selectivity, which overcomes separation problems, the derivatization also increases the sensitivity, when compared to the detection at 280 nm. The flavanol profile of this sample is characterized by the presence of both procyanidins and prodelphinidins. C, EC, and the procyanidin dimers B1−B4 and trimers C1 and EC-(4,8)-EC-(4,8)-C, already described in wine (Ricardo da

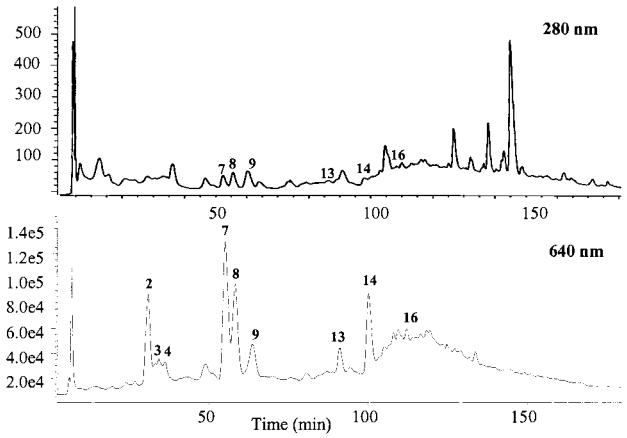

Figure 2. HPLC chromatograms of a red wine sample: (upper part) chromatogram corresponding to the first detection at 280 nm; (lower part) chromatogram at 640 nm, after chemical reaction with DMACA. Numbering of the peaks is the same as in Table 1.

Silva et al., 1990, 1992), have been identified together with GC and the prodelphinidin dimer GC-(4,8)-C and trimer GC-(4,8)-GC-(4,8)-C, which have not been reported in wines up to now. All of these compounds are hardly detected at 280 nm, because they are overlapped by other substances present in greater amounts or with higher extinction coefficients. The presence of a hump in the ending part of the chromatogram is attributed to compounds with a higher degree of polymerization, as well as to the possible presence of products resulting from the oxidation and/or condensation of flavanols (derived tannins) that can also react with DMACA. The almost complete absence of galloyl derivatives, characteristic of grape seeds (Ricardo da Silva et al., 1992; Escribano-Bailón et al., 1992), and the presence of prodelphinidins, characteristic of grape skins (Escribano-Bailón et al., 1995; Souquet et al., 1996), suggest that the latter are the most important source for the cession of flavanols to wine.

Figure 3 shows the chromatograms (at 640 nm) obtained for different beverages. No important differences exist between the flavanol profiles from samples of lager beer from Spain (Figure 3A) and Germany (Figure 3B). The major peaks correspond to the monomers GC, C, and EC and dimers GC-(4,8)-C and procyanidin B3, confirming the results of McMurrough et al. (1994). Flavanols were hardly detected in the Bavarian wheat beer (Weizenbier). The flavanol composition of cider (Figure 3C) is characterized by the presence of epicatechin-based derivatives (EC, B2, B5, C1), although some catechin and procyanidin B1 are also observed. This profile is in agreement with those previously reported for cider (Lea and Arnold, 1978; Lea and Timberlake, 1974) and apple (Mayr et al., 1995). Guyot et al. (1997) reported the presence of procyanidins of an average degree of polymerization up to 12.5 in apple skin and pulp. However, no polymers have been detected in the cider samples analyzed, as demonstrated by the absence of a hump or peaks in the final part of the chromatogram. The flavanol profiles of the two liqueurs analyzed (Figure 3D,E) are very similar to each other and also close to that of the cider. This is in agreement with the fact that all of them are obtained from fruits of Rosaceae, whose composition seems to be characterized by the presence of epicatechin-based flavanols [see Treutter (1996) and references cited therein].

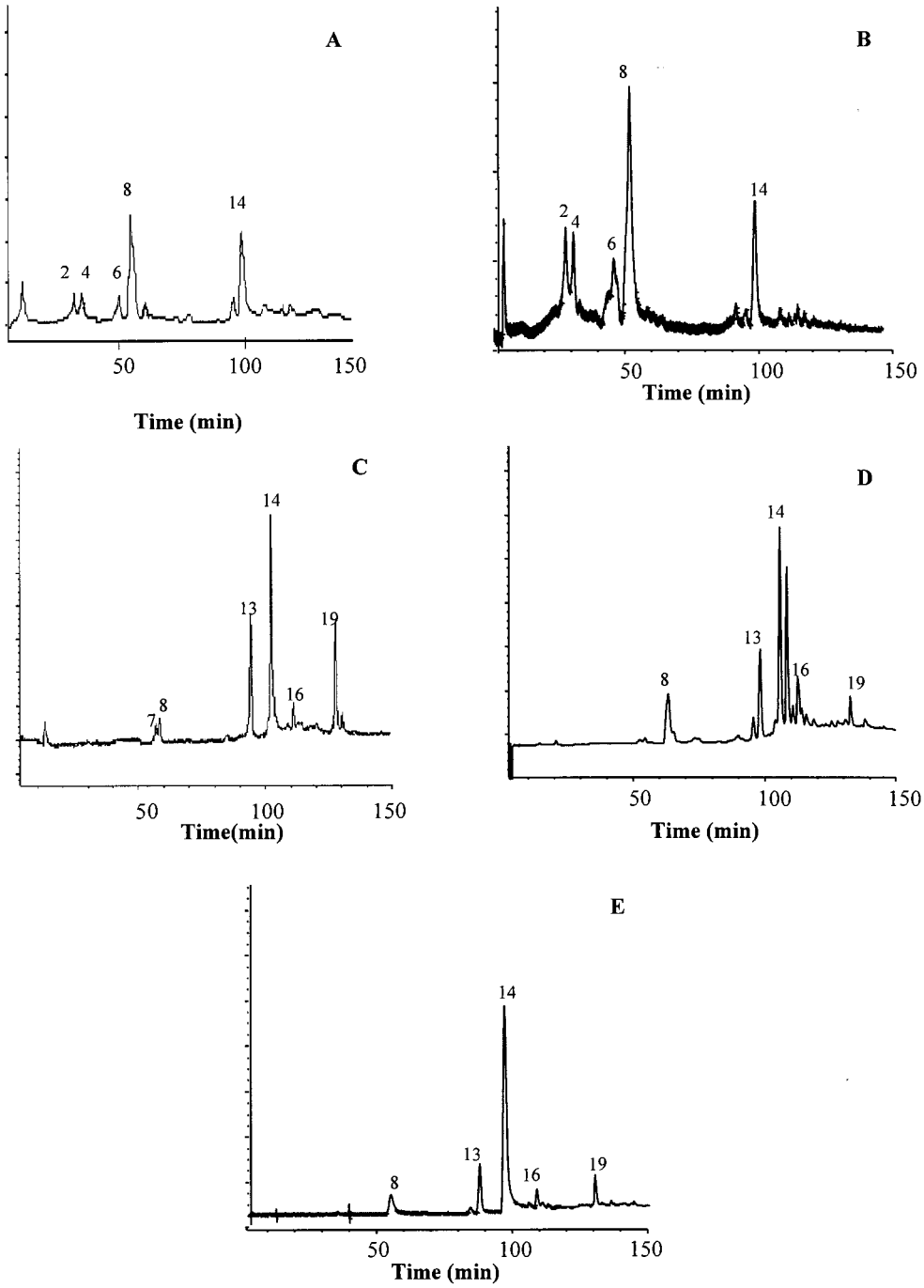

Figure 3. HPLC chromatograms recorded at 640 nm, after chemical reaction with DMACA of different beverage samples: Spanish lager beer (A); German lager beer (B); cider (C); blackthorn liqueur (D); cherry liqueur (E).

These examples show the interest of the methodology set up to characterize the flavanol composition in beverages or plant extracts in a rapid and simple way. Until now, isolation, purification, and later identification of the compounds by hydrolysis, MS, or NMR were usually required. Furthermore, the HPLC-CRD method allows the detection of proanthocyanidins that may be present in low amounts or difficult to isolate due to the complex composition of the sample. The method can be especially useful for the detection of gallocatechins, because they have low extinction coefficients at 280 nm and, therefore, are hardly detected in the HPLC chromatograms after UV detection. The fact that the beverages can be injected directly in the chromatographic system makes it possible to apply this technique to the quantitative analysis, because problems arising from the extraction step are avoided. Nevertheless, for the quantification of flavanols, previous calibration of the method using adequate standards would be necessary. For this purpose, the use of monomeric catechins (the only commercially available) as reference compounds would lead to erroneous estimations, because the distinct flavanols have different extinction coefficients and reactivity with DMACA, depending on their degree of polymerization and stereochemistry. Thus, for a more accurate calibration, compounds with different structural characteristics should be used, making necessary their previous isolation from suitable natural sources. It is clear that it is not possible to get individual standards of each flavanol that could be

found in any sample and, therefore, selected substances will have to be chosen as references for the quantification of the different groups of flavan-3-ols (e.g., monomers, dimers, trimers, oligomers, and polymers).

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