Levels of Flavan-3-ols in French Wines

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French wines are abundant sources of phenolic compounds. The content of several catechins, i.e., (+)-catechin, (−)-epicatechin, dimers B1, B2, B3, and B4, trimers C1, and trimer 2 (T2), of 160 French wines was determined by HPLC with UV detection. Red wines ($n = 95$) were found to have high levels of catechins, ranging from 32.8 to 209.8 mg/L (mean concentration 114.5 mg/L) for (+)-catechin, from 22.1 to 130.7 mg/L (mean concentration 75.7 mg/L) for (−)-epicatechin, from 7.8 to 39.1 mg/L (mean concentration 25.4 mg/L) for B1, from 18.3 to 93 mg/L (mean concentration 47.4 mg/L) for B2, from 21.4 to 215.6 mg/L (mean concentration 119.6 mg/L) for B3, from 20.2 to 107.2 mg/L (mean concentration 81.9 mg/l) for B4, from 8.6 to 36.9 mg/L (mean concentration 26.3 mg/L) for C1, and from 26.7 to 79.3 mg/L (mean concentration 67.1 mg/L) for T2. White and rosé wines ($n = 57$ and $n = 8$) were found to have low levels of (+)-catechin (mean concentrations 9.8 and 10.6 mg/L, respectively) and (−)-epicatechin (mean concentrations 5.3 and 6.5 mg/L, respectively). These data provide a basis for the epidemiological evaluation of catechin intake by the consumption of French wine.

Keywords: *Wine; antioxidant; flavonoid; (+)-catechin; (−)-epicatechin; procyanidin dimers; procyanidin trimers*

INTRODUCTION

The phenolic compounds present in plants are important constituents of vegetal cells and are associated with a physiological protective role against bacteria and virus. They can also have an important role in the regulation of fruit maturation. The phenolic compounds in grapes and wines are important in that they contribute to the following properties: color, astringency, and bitterness (Singleton and Esau, 1969; Robichaud and Noble, 1990), oxidation reactions (Oszmianski and Sapis, 1989; Cheynier and Ricardo Da Silva, 1991), interactions with proteins (Ho et al., 1985; Mehansho et al., 1987; Powers et al., 1988; Ricardo Da Silva et al., 1991), and aging behavior of wines (Haslam, 1980). Recently, the flavonoid group has received considerable attention owing to the antioxidant, antimutagenic, and anticarcinogenic properties of these compounds (Kato et al., 1983; Huang et al., 1983; Verma et al., 1988; Kondo et al., 1994; Clifford et al., 1996). Flavonoids consist mainly of anthocyanins, flavonols, flavones, isoflavones, and flavanols. Dietary flavonoids originating from fruit and vegetables appear to reduce the incidence of coronary heart disease (Knekt et al., 1996; Hertog et al., 1993). Flavonoids in wine have been shown to strongly inhibit low-density lipoprotein oxidation (Frankel et al., 1993; Furhman et al., 1995) both in vitro and in vivo and to reduce platelet aggregation. Flavan-3-ol (catechins) are some of the most widely occuring flavonoids, and the most important sources of these compounds in the diet, at least in the Mediterranean region, are grapes and wine. Flavan-3-ol monomeric units, (+)-catechin and (−)-epicatechin, as well as several oligomers, were separated and identified (Bourzeix et al., 1986; Romeyer et al., 1986; Boukharta et al., 1988; Ricardo Da Silva, 1992). Proanthocyanidins are oligomers and polymers of poly(hydroxyflavan-3-ol) units, which are (+)-catechin and (−)-epicatechin in the case of procyanidins. The simplest procyanidins are dimeric forms, and the most common of these are the four C4→C8-linked dimers (B1, B2, B3, B4). Procyanidin trimer C1 and trimer 2 are also C4→C8-linked forms. These procyanidin dimers and trimers are sometimes accompanied by lower concentrations of the corresponding C4→C6 isomers. The part of the grape containing the highest concentration of catechins is the seed, from where they are extracted during the wine-making process. Different authors have studied the concentration of catechins in wine with particular emphasis on influencing factors such as the wine-making process (temperature of fermentation, duration of maceration of pomace) or the use of different grape varieties (Bourzeix et al., 1986; Archier et al., 1992). Several studies have also been concerned with the concentration of catechins in wine from localized regions (Waterhouse and Teissedre, 1997). The aim of the present study was to quantify eight major catechins (Figure 1): (+)-catechin, (−)-epicatechin, dimers B1, B2, B3, B4, trimers C1, and trimer 2 in 160 commercial wines taken from all regions of France and so to provide a profile of the concentration of catechins in French wines. The influence of the production year on the occurrence of catechins in these wines was also studied.

EXPERIMENTAL PROCEDURES

Standards. (+)-Catechin and (−)-epicatechin were obtained from Aldrich (St. Quentin Fallavier, France). Procyanidin dimers B1, B2, B3, and B4 and procyanidin trimer C1 and trimer 2 were obtained from grape seeds as detailed below.

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10.1021/jf9810564 CCC: $18.00
Published on Web 09/28/1999

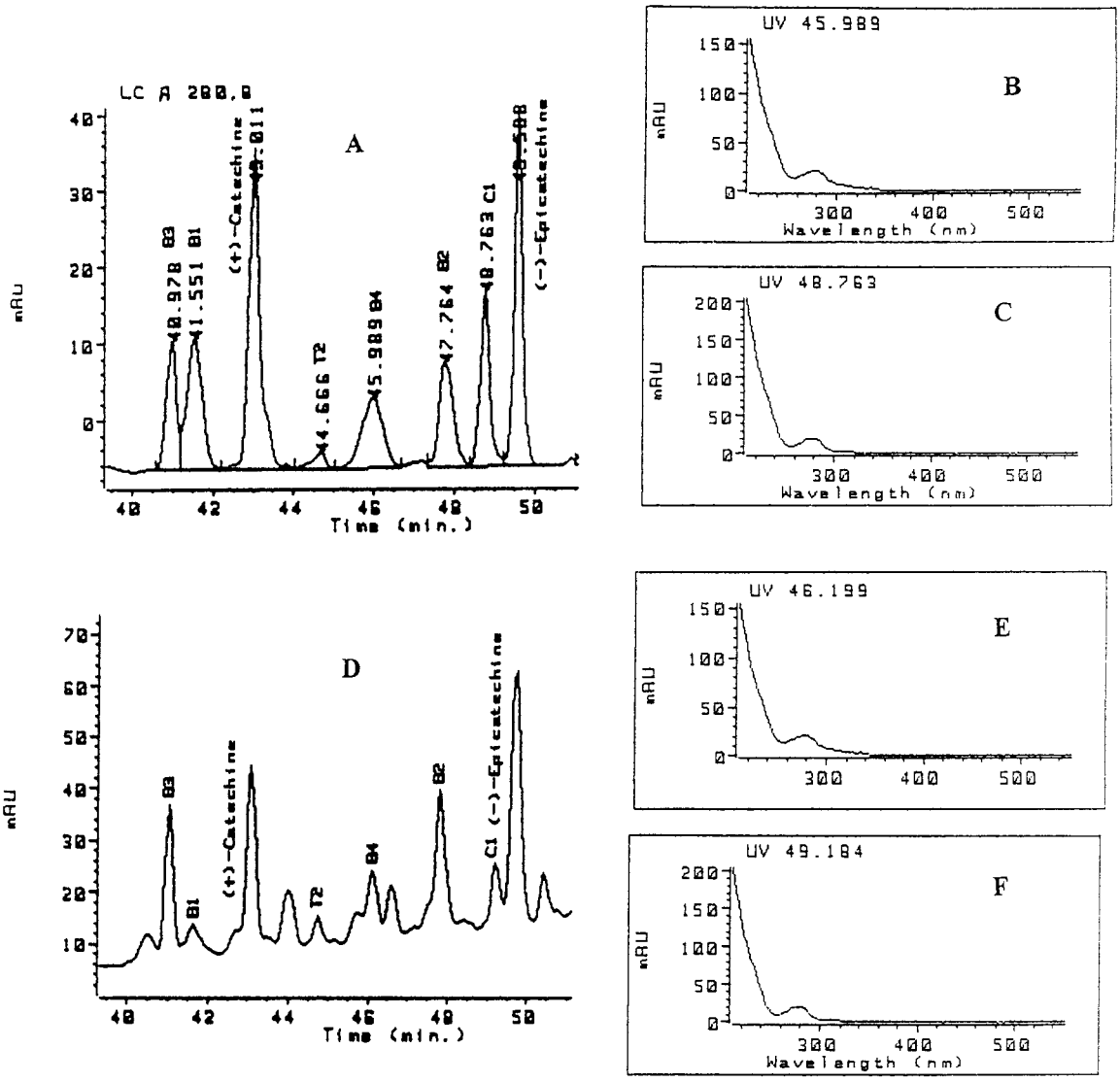

Figure 1. HPLC/UV analysis of a red wine monitored at 280 nm: (A) chromatogram of the purified compounds; (B) UV spectrum of the purified B4 dimer: (C) UV spectrum of the purified C1 trimer; (D) chromatogram of a red wine and identification of the catechins and procyanidins; (E) UV spectrum of B4 dimer identified in wine; (F) UV spectrum of C1 trimer identified in wine.

Wine Samples. A total of 160 samples of different French wines in commercial bottles were studied. The wine samples analyzed were from all viticultural areas of France; they were of different vintage years and included 95 red wines, 57 white wines, and 8 rosé wines. All the wines analyzed are widely and frequently consumed in France.

Extraction and Isolation of Crude Procyanidins. Grape seeds (*Vitis vinifera*; 150 g) were extracted with methanol as described by Bourzeix et al. (1986) and by Weinges and Piretti (1971). The extract (3 mL, 300 mg) was separated on Fractogel TSK HW-40(s)(25−40 μm)(450 × 25 mm i.d.) with methanol as eluant, using an ISCO (Lincoln, NE) Model UA-5 absorbance detector set at 280 nm, a peristaltic Miniplus2 pump (Gilson Inc., Middleton, WI), and an ISCO 328 fraction collector. Ten fractions containing procyanidins were collected. This procedure has been recently detailed by Teissedre et al. (1996).

Isolation of Purified Procyanidins. Semipreparative HPLC was performed with a Waters (Milford, MA) 510 pump a U6K injector, and a Hewlett-Packard (Palo Alto, CA) Model 1050 UV−vis detector set at 280 nm. The column was a Waters RCM Novapak C18 (25 × 100 mm, 4 μm particle size). Elution was carried out by a linear gradient of 0−500 mL/L of methanol with the solvent described below at 2 mL/min.

TLC Analysis. Silica plates (DC Alufolien-Kieselgel 60, 0.2 mm thick, Merck, EM Separation Technology, Gibbstown, NJ), were developed with toluene/acetone/formic acid (3:3:1 v/v/v) as described by Lea et al. (1979). The plates were visualized by spraying with a solution of vanillin (100 g/L) in concentrated HCl.

HPLC Analysis. *With UV Detection*. A Hewlett-Packard Model 1090 with three low-pressure pumps and a diode array UV detector coupled to a Hewlett-Packard Chem Station was used for solvent delivery and detection. A Hewlett-Packard column packed with Nucleosil 100 C18 (250 × 4 mm, 5 μm particle size) was used for the stationary phase with a flow of 0.7 mL/min. The solvents used for separation (Lamuela-Raventos et al., 1994) were as follows: solvent A, 50 mM dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid; solvent B, 20% A with 80% acetonitrile; solvent C, 0.2 M orthophosphoric acid adjusted with ammonia to pH 1.5. Elution was performed with the follwing gradient: 0−5 min, 100% solvent A; 5−15 min, from 0 to 4% solvent B; 15−25 min, from 4 to 8% B; 25.1 min, 8% B and 92% C; 25.1−45 min, from 8 to 20% B; 45−50 min, from 20 to 30% B; 50−55 min, from 30 to 40% B; 55−60 min, from 40 to 80% B. The temperature was maintained at 25 °C.

External standard curves were prepared for each compound. The calibration curves were established by plotting the area of peaks against different concentrations of pure compound. Five replicates were made for five different concentrations. Limit of quantification (LOQ) was defined as the amount of

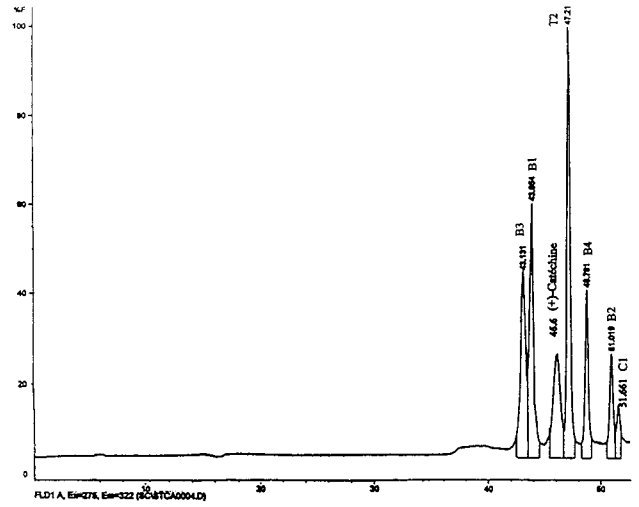

Figure 2. HPLC−fluorimetric analysis of procyanidins of a pool of purified standards (excitation, 275 nm; emission, 322 nm).

flavanols resulting in a peak area of 10 times the standard deviation of the baseline noise.

Wine samples were filtered (Millipore), and 20 μL portions were injected into the HPLC system. Chromatograms of wines were monitored at 280 nm. The compounds tested were identified by comparison of the retention times. In addition, peak identity and purity were confirmed using a photodiode array detector to record UV spectra of the flavanols in samples on line.

To study the reproducibility, the same red, rosé, and white wine samples were analyzed on five different days.

With Fluorimetric Detection. To comfirm HPLC−UV analysis, we used an HPLC−fluorimetry method for determination of procyanidin dimers and trimers in wines as described by Carando et al. (1999). Procyanidins B1, B2, B3, B4, C1, and T2 were determined with optima in excitation and emission wavelengths with a 1064 Hewlett-Packard spectrofluorimeter. Fluorescence properties of the compounds were determined individually. The six compounds were found to have the same optima in excitation and emission wavelengths: 275 and 322 nm, respectively.

A Hewlett-Packard Model 1100 with two low-pressure pumps and a 1064 Hewlett-Packard fluorimetric detector coupled to an Hewlett-Packard Chem Station were used for solvent delivery and detection. A Hewlett-Packard column packed with Nucleosil 100 C18 (250 × 4 mm, 5 μm particle size) was used for the stationary phase. The eluents were a solution of acetic acid (20 mM; solvent A) and methanol (solvent B), both filtered through 0.45 μm Millipore filters. Elution was performed with the following gradient: 0−5 min, 98% solvent A; 5−12 min, from 98 to 93% solvent A; 12−18 min, from 93 to 89% A; 18−28 min, from 89 to 85% A; 28−38 min, from 85 to 70% A; 38−48 min, from 70 to 60% A; 48−55 min, from 60 to 50% A. The flow rate was 0.5 mL min^{-1}, and the injection volume of the diluted wine was 20 μL.

The maximum excitation and emission wavelengths in a methanolic solution as well as in different methanol−20 mM acetic acid mixtures (the composition of procyanidin gradients measured in the spectrofluorimeter cell) were 275 and 322 nm.

Limits of quantification ranged from 12 to 16 μg/L. The use of a fluorimetric detector decreases the threshold of measurable concentration of procyanidins in comparisons of UV characteristics. Purified procyanidins were first chromatographed to determine the retention of each compound. Chromatograms of the procyanidins monitored by fluorescence are reported in Figure 2. Catechin was also chromatographed to avoid confusion in the identification of each compound. Addition of procyanidin standards to samples, HPLC separation, and good resolution of the peak without interference permitted the identification of procyanidin dimers B1, B3, and B4 and procyanidin trimers C1 and T2 in wine.

RESULTS AND DISCUSSION

Procyanidin Identity. The identities of the purified procyanidins were confirmed by HPLC−UV and HPLC−fluorimetry comparisons with certified compounds purchased by Lea as already described (Teissedre et al., 1996).

Chromatography. Chromatograms of pure standards and of red wine monitored in UV at 280 nm are shown in Figure 1. All compounds tested were elued between 40 and 50 min and separated with good resolution. The comparison of the retention times between pure standard and wine compounds and the comparison of the UV spectra permitted us to identify each compound. Figure 2 shows a small difference of the retention times (comparison between chromatograms of the known standards and chomatogram of the red wine) for B4 dimer and C1 trimer. The information given by the respective UV spectra and peak purity permitted us to confirm the identity of the two compounds (Figure 1).

Calibration and Analytical Characteristics. The calibration plot was obtained using solutions ranging from LOQ to 200 mg/L prepared from a working solution of 250 mg/L. The equations for the calibration plot are reported in Table 1. The reproducibility of replicate measurements for a given sample was good: the CV values obtained for the flavanols of red wine samples were less than 2.7%, and the CV values for rosé and white wine samples were less than 6%.

Flavanol Concentrations. It is well-known that the phenolic content in wine is related to the wine-making technique and aging process. Red wines for which the process of extraction from pomace is optimized had the hightest mean concentration of catechins ((+)-catechin, (−)-epicatechin, procyanidin dimers B1, B2, B3, and B4, and procyanidin trimers C1 and T2): 557.9 mg/L. White varieties, which are made by separation of pulp grape juice from pomace by direct pressure, and rosé varieties, which are also made by rapid separation, had lower mean concentrations of catechins (15.2 and 17.1 mg/L, respectively).

All the compounds studied were present in each of the 95 red wines analyzed. Mean concentrations of each compound are reported in Table 1. The mean concentration of monomers ((+)-catechin, (−)-epicatechin) was found to be 190.2 mg/L. (+)-Catechin content ranged from 32.8 to 209.8 mg/L and (−)-epicatechin from 22.1 to 130.7 mg/L. According to previous data (Roggero et al., 1993; Waterhouse et al., 1997), (+)-catechin was the predominant monomeric form (114.5 mg/L) and the occurrence of (−)-epicatechin was less important (75.7 mg/L). Procyanidin dimers (B1, B2, B3, B4) were found to be present at mean concentrations of 274.3 mg/L, with higher mean concentrations for B3 (119.6 mg/L) and B4 (81.9 mg/L) than for B2 (47.4 mg/L) and B1 (25.4 mg/L). Large variations were found: the B3 content ranged from 21.4 to 215.6 mg/L, B2 from 18.3 to 93 mg/L, B1 from 7.8 to 39.1 mg/L, and B4 from 20.2 to 107.2 mg/L. These variations were already observed by Bourzeix et al. (1986). Procyanidin trimers (C1, T2) were found at a mean concentration of 93.4 mg/L, with a mean concentration of 67.1 mg/L for C1 (from 26.7 to 79.3 mg/L) and 26.3 mg/L for T2 (from 8.6 to 38.5 mg/L).

White wines (n = 57) and rosé wines (n = 8) were found to contain lower concentrations of monomers (15.1

Table 1. Retention Times, Linear Regression, Limits of Quantifications, and Levels of Flavan-3-ols in Red, White, and Rosé Wines[a]

compd	rt (min)	linear regression $y = ax + b$ (r^2)	LOQ (mg/L)	mean concn (mg/L) red wine ($n = 95$)	white wine ($n = 57$) mean ± sd	rosé wine ($n = 8$) (CV %)
(+)-Cat.	43.01	y = 0.145x − 0.32 (0.99)	1.1	114.5 ± 30.5 (26.6)	9.8 ± 1.7 (17.0)	10.6 ± 2.9 (27.4)
(−)-Epi.	49.58	$y = 0.146x - 0.65$ (0.99)	1.3	75.5 ± 22.1 (29.2)	5.3 ± 1.2 (22.1)	6.5 ± 2.4 (36.5)
B1	41.55	$y = 0.141x - 1.03$ (0.99)	1.6	119.6 ± 25.7 (21.5)		
B2	47.76	$y = 0.117x + 0.29$ (0.99)	1.4	47.4 ± 16.6 (33.9)		
B3	40.97	$y = 0.116x - 2.66$ (0.99)	1.7	25.4 ± 5.9 (22.8)		
B4	45.98	$y = 0.135x - 0.85$ (0.99)	1.8	81.9 ± 13.0 (15.7)		
C1	48.76	$y = 0.125x - 1.50$ (0.99)	1.4	26.3 ± 6.1 (23.3)		
T2	44.66	$y = 0.202x - 0.88$ (0.99)	1.4	67.1 ± 9.8 (14.6)		
total				557.7	15.1	17.1

[a] Legend: rt = retention time; n = number of wines; LOQ = limit of quantification; CV = coefficient of variance; sd = standard deviation.

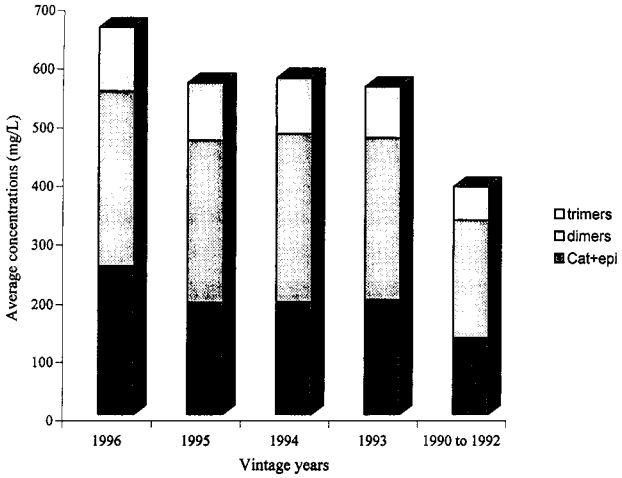

Figure 3. Average levels of catechins, procyanidin dimers, and procyanidin trimers in red wines, by vintage.

and 17.1 mg/L, respectively) than red wines. The results for each monomeric compound are shown in Table 1. Procyanidin dimers and procyanidin trimers were at concentrations too low to be quantified with the present methodology, and for further studies using UV detection, preconcentration of white and rosé varieties is required before injection into the HPLC system. The comparison of high levels of catechins for red wines and low levels for white and rosé wines show that the wine-making technique is the most influencing factor for their extraction from grape seeds.

Another influencing factor reported by different authors is the evolution of these compounds by storing of wine bottles for several years. We have therefore analyzed the influence of this parameter for the red wines. Among the 95 red wines analyzed, 5 were 1996 vintage, 34 were 1995 vintage, 36 were 1994 vintage, 14 were 1993 vintage, and 6 were 1990−1992 vintage. Catechin levels were higher for the more recent wines (1996 vintage) than for the 1990−1992 vintage (Figure 3), but no important variation was observed from the 1993 to the 1995 vintages on the basis of the concentration of the monomers, procyanidin dimers, and procyanidin trimers, respectively. Decreasing levels of catechins are actually explained to be the result of condensation reactions with anthocyanins to form the so-called condensed tannins.

Because of the large number of influencing factors, the final concentration of catechins is quite variable in red wine. For red wines, it appeared that there was a large variation in monomer, dimer, and trimer concentrations (Figure 4). (+)-Catechin concentration ranged from 32.8 to 209.8 mg/L, but 73% of the 95 red wines analyzed contained greater than 100 mg of catechin/L and only 11.6% contained less than 50 mg/L. For (−)-epicatechin, 86.3% of the wines analyzed contained more

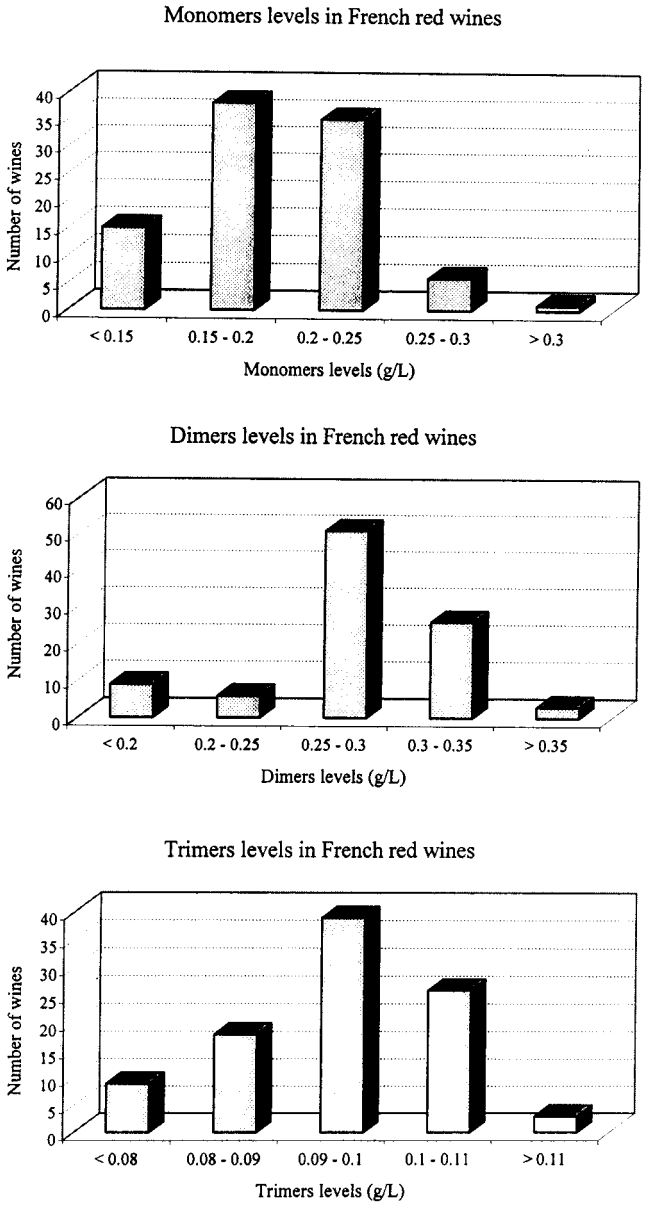

Figure 4. Occurrence of catechins, procyanidin dimers, and procyanidin trimers in wine.

than 50 mg/L. A large variability was also observed for procyanidin dimers. B3 dimer ranged from 21.4 to 215.6 mg/L and B4 from 20.2 to 107.2 mg/L; nevertheless, 84.2% of the wines were between 100 mg/L and 150 mg of B1/L, and 62.1% were between 80 mg/L and 90 mg of B4/L. Procyanidin dimers B2 and B1 were found to have lower mean concentrations than procyanidin dimers B1 and B4: 87.3% of the wines were between 25 and 75 mg/L for B2, and 62.1% were between 20 and 30 mg/L for B3. A histogram of the incidence of procyanidin trimer is also reported in Figure 4. It was observed that 60% of the wines contained between 20 and 30 mg/L of C1. The variability of occurrence of the procyanidin trimer 2 is also important, but 57.8% of the wine contained between 65 and 75 mg/L. All white and rosé wines contained less than 14 mg/L for (+)-catechin and less than 10 mg/L for (−)-epicatechin.

Estimation of Catechin Intake from Wine. Recently, epidemiological studies have shown that the consumption of wine correlates with reduced coronary heart disease mortality (Gronbaek et al., 1995), and Renaud et al. (1992) proposed that phenolic compounds in wine may be responsible for the so-called French Paradox. (+)-Catechin and (−)-epicatechin, some of the most abundant monomeric phenolic compounds found in wine, have also been shown to strongly inhibit low-density lipoprotein oxidation in vitro. Procyanidin dimers and procyanidin trimers have been shown also to have considerable antioxidant activity (Teissedre et al., 1996). In recent years, the consumption of French wine has dropped from 305 mL/day (Darret et al., 1986) to 180 mL/day (Boulet et al., 1995). On the basis of these data, the current daily intake of catechins (monomers, procyanidin dimers B1, B2, B3, and B4, procyanidin trimers C1 and T2) can be estimated to be respectively 100 mg/day per red wine consumer, to 2.7 mg/day per white wine consumer, to 3 mg/day per rosé wine consumer. Therefore, consumption of a moderate amount of red wine is likely to provide a greater intake of (+)-catechin, (−)-epicatechin, and oligomers than the consumption of white and rosé wines. Nevertheless, over a long time period, wine drinkers are likely to consume all of the three types of wine, and the daily intake of catechins can then be estimated on the basis of the 160 wines analyzed to be 60 mg/day per person. These data provide a basis for epidemiological evaluation of catechin intake by wine consumption for the French population, but further studies are necessary to characterize the intake levels of the numerous other phenolic compounds present in wine.

Wine is an abundant source of catechins. Nevertheless, the behavior of these compounds in the body is not clearly established. At present, only (+)-catechin has been determined in human plasma after wine consumption (Waterhouse et al., 1996). Further studies are necessary to determine the behavior of the other compounds and to determine the in vivo antioxidant activity of catechins.

ACKNOWLEDGMENT

We are grateful to Marry Kelly for assistance with the translation and to Francis Gasc for glassware technical assistance.

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Received for review September 21, 1998. Revised manuscript received July 27, 1999. Accepted July 27, 1999.

JF9810564