Optimization of a Quantitative Method for the Determination of Catechins in Fruits and Legumes

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Catechins are found in many foods of plant origin. In vivo and in vitro studies have shown their potential health effects. The quantification of catechins has been optimized in three model foods: apples, black grapes, and canned kidney beans. Catechins [(+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate] were separated with gradient reversed phase HPLC and quantified by UV (270 nm) or fluorescence (280/310 nm excitation/emission) detection in series. Type (ethanol, methanol, or acetone) and concentration (40−100% in water) of extraction solvent influenced catechin yield, whereas extraction time (10−60 min) did not. Adequate extraction was attained with 60−100% methanol for apples and grapes and with 40−80% methanol for beans. Recovery (>94%), within-run repeatability (1−5%), between-run reproducibility (3−9%), and detection limits (0.1−3.9 mg/kg of fresh apple or 0.01−0.29 μg/mL extract) were satisfactory. With this method 40 solid food samples a day can be analyzed, without the need for sample cleanup.

Keywords: *Catechins; fruits; legumes; extraction; analytical techniques; fluorescence*

INTRODUCTION

Catechins, or flavanols, are polyphenolic compounds of the flavonoid type, which are present in tea (Khokhar et al., 1997), wine (Goldberg et al., 1996; Soleas et al., 1997), fruits (Macheix et al., 1990; Risch and Herrmann, 1988), and legumes (Hanefeld and Herrmann, 1976). The five major catechins are (+)-catechin, (−)-epicatechin, (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), and (−)-epigallocatechin gallate (EGCg) (Figure 1). Catechins are potentially beneficial to human health: they are strong antioxidants, anticarcinogens, anti-inflammatory agents, and inhibitors of platelet aggregation in in vivo or in vitro studies (Cook and Samman, 1996; Katiyar and Mukhtar, 1996; Kohlmeier et al., 1997). There is some epidemiological evidence that the consumption of tea, a rich source of catechins, could reduce the risk for certain cancers (Blot et al., 1996; Kohlmeier et al., 1997) and for coronary heart disease mortality and stroke (Tijburg et al., 1997). However, not all studies have shown a protective effect of tea consumption. Epidemiological studies that take into account not only tea but all catechin-containing foods could possibly clarify the relation between catechin intake and disease. However, reliable quantitative data on the catechin content of foods are lacking, particularly for EGC, EGCg, and ECg, which are the major catechins in tea. EGCg has been suggested as an important compound in the prevention of cancer (Jankun et al., 1997).

A number of protocols for the quantitative determination of catechins are available. However, these are mostly developed for liquids such as tea infusions (Bailey et al., 1991; Goto et al., 1996; Khokhar et al., 1997; Kuhr and Engelhardt, 1991), wine (Goldberg et al., 1996; Ricardo da Silva et al., 1990), or fruit juices (Suárez Vallés et al., 1994; Suárez et al., 1996). Analytical methods for catechins in solid foods generally focus on identification of new derivatives or polymeric catechins (proanthocyanidins) and are not designed for quantification. In addition, they are lengthy and require considerable sample cleanup. Determination of compounds in solid foods requires extraction from the sample matrix prior to injection into the HPLC system. Thus far, extraction methods for catechins have not been systematically examined.

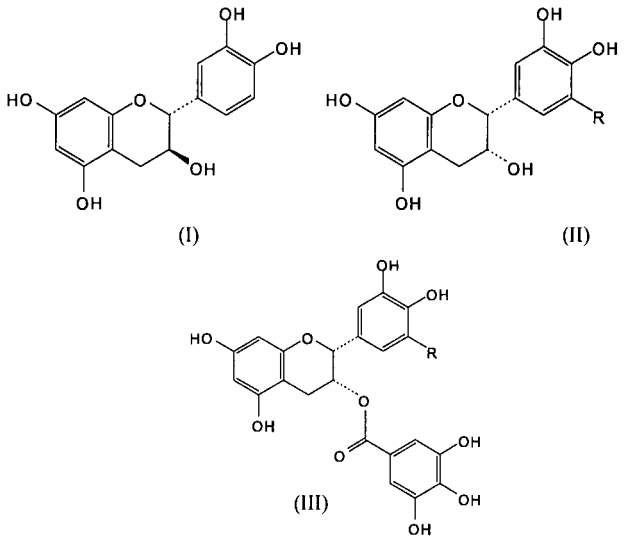

Figure 1. Chemical structures of catechins: (**I**) (+)-catechin; (**II**) R = H (−)-epicatechin, R = OH (−)-epigallocatechin; (**III**) R = H (−)-epicatechin gallate, R = OH (−)-epigallocatechin gallate.

In this paper we present a quantitative method for the determination of (+)-catechin, (−)-epicatechin, EGC,

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ECg, and EGCg in solid foods, which can be applied to analyze a large number of samples efficiently. We studied fluorescence detection because it has better specificity than UV detection, which may reduce sample cleanup and thus analysis time. Fluorescence detection has been used as a selective and sensitive method to measure (+)-catechin in plasma (Ho et al., 1995). Furthermore, we optimized the extraction procedure using three model foods: apples, black grapes, and canned beans.

EXPERIMENTAL PROCEDURES

Chromatography. The HPLC equipment consisted of a Gilson 234 autoinjector (Gilson, Villiers-le-Bel, France), which injected 10 μL of sample onto an Inertsil ODS-2 column (150 × 4.6 mm, 5 μm; GL Sciences Inc., Tokyo, Japan) protected by an Opti-Guard PR C18 Violet A guard column (Optimize Technologies Inc., Oregon City, OR), both placed in a column oven (Jones Chromatography 7971 column heater, Lakewood, CO) set at 30 °C. Merck Hitachi L6000A and L6200 pumps (Hitachi Ltd., Tokyo, Japan) were used to create a gradient with a flow rate of 1.0 mL min^{-1}. Choice of column type and gradient had been optimized before for catechins in tea (Khokhar et al., 1997). However, small adaptations had to be made for the analysis of fruits and legumes, to prevent matrix compounds from accumulating on the column. The solvents used for separation were 5% acetonitrile (eluent A) and 25% acetonitrile (eluent B) in phosphate buffer (0.025 M, pH 2.4). The gradient was as follows: 0−5 min, 10% B; 5−20 min, linear gradient from 10 to 80% B; 20−22 min, linear gradient from 80 to 90% B; 22−25 min, isocratic at 90% B; 25−28 min, linear return to 10% B; 28−37 min, isocratic at 10% B to re-equilibrate. The monitoring of the effluent was done by a Merck Hitachi F1050 fluorescence spectrophotometer (280 nm excitation, 310 nm emission wavelengths) and a Kratos (Kratos Analytical Instruments, Ramsey, NJ) Spectroflow 783 UV detector (270 nm), which were connected in series. Fluorescence spectra of the pure compounds were measured with a Perkin-Elmer (Beaconsfield, England) LS 50 B luminescence spectrometer.

Standards. Pure standards of (+)-catechin (purity >98%, catalog no. C1251), (−)-epicatechin (crystalline, E1753), and EGC (purity >98%, E3768) were obtained from Sigma (St. Louis, MO). ECg and EGCg (purity of both >95%) were kindly donated by Alan Davies (Unilever Research Colworth Laboratory, U.K.). Calibration solutions were freshly prepared for each series of analyses in 90% methanol from stock solutions that were kept at 4 °C. Stock solutions contained 1 mg of catechins/mL of methanol. The stability of the stock solutions was followed spectrophotometrically every other day for 1 week; no deterioration occurred. Calibration curves were constructed by linear regression of the peak area against concentration of the calibration solution [(+)-catechin and (−)-epicatechin, 2−10 μg/mL; ECg and EGCg, 5−15 μg/mL; EGC, 10−30 μg/mL]. All calibration curves were linear when forced through the origin, with correlation coefficients close to unity: 1.000 for (+)-catechin, (−)-epicatechin, and ECg; 0.996 for EGC; and 0.999 for EGCg.

Limits of detection were determined by injecting 10 μL of a standard mixture. The limit of detection was defined as the amount of catechin that resulted in a peak height 3 times the standard deviation of the baseline noise.

Peaks were identified by comparing their retention times with the retention times of pure standards. A diode array detector (HP 1040A upgraded version, Hewlett-Packard, Palo Alto, CA) was used to confirm peak identity and to check peak purity.

Sample Preparation. Jonagold apples and canned kidney beans were purchased at a local supermarket in February 1997, and black grapes were purchased in June 1997. The apples were quartered, and the core was removed. The apple segments, including skin, were immediately chopped under liquid nitrogen and stored at −20 °C. Freeze-drying was started the same day. The black grapes were washed and dried carefully, cut in halves, frozen under liquid nitrogen, and freeze-dried immediately. The kidney beans were allowed to leak out, washed with running water, frozen at −80 °C for 24 h, and freeze-dried. After freeze-drying, the samples were ground to a powder and stored at −20 °C until analyzed.

Extraction. *Procedure.* Approximately 0.5 g of freeze-dried sample was mixed with 25 mL of 90% v/v methanol/water for apple and grapes and with 25 mL of 70% v/v methanol/water for beans. The extract was shaken in a mechanical shaker (250 rpm) for 60 min at room temperature. After extraction, the volume was made up to 50 mL with the same solvent, filtered over a 0.45 μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI), and injected without further processing.

Optimization. For apples and beans, the following extraction conditions were optimized: type of extraction solvent, concentration of extraction solvent, extraction time, and amount of sample. Methanol concentration was the only variable studied for black grapes. Three extraction solvents were tested: ethanol, methanol, and acetone, in concentrations that varied from 40 to 100% solvent in water. Results are expressed as a percentage of the maximum yield obtained with one of these solvents for each component in each product. Samples were extracted in a mechanical shaker (250 rpm) at room temperature for 10−60 min. The sample amount (0.25, 0.50, 0.75, or 1.00 g) in 25 mL of extraction solvent was varied to check whether the solubility of catechins was a limiting factor.

Recovery and Stability. Recovery was calculated by comparing catechin levels in apples with those in apples spiked with a known amount of standard compound. Standards were added immediately following the addition of extraction solvent, at 50 and 100% of the original level of the apples. Whenever the compound was not present in apples, a well detectable amount of the standard was added.

The stability of standard solutions was studied because sample extracts could stay in the autoinjector at room temperature for up to 24 h during routine analyses. The peak areas of the first standard mixture in a run were compared with the peak areas of the last standard mixture in the same run. Data from five runs were pooled, and a two-sided paired *t* test was used for statistical analysis.

To test the effect of freeze-drying, we compared the catechin content determined in fresh apples with that in freeze-dried apples. Four apples were quartered; two pieces of each apple were freeze-dried as described under Sample Preparation. The remaining two pieces were ground in a Waring Blendor (model 8011G, 1 L, Waring Commercial, New Hartford, CT) under methanol for 2 min and subsequently placed in an ultrasonic bath at room temperature for 5 min. Antecedent testing had shown that for freeze-dried samples 5 min of ultrasonification gave yields similar to those obtained after 60 min of mechanical shaking. The water content of the fresh apple extract totaled ∼18%. Prior to injection, the extract was filtered over a 0.45 μm Acrodisc filter. There were no indications that freeze-drying notably affected the catechin content of apples: compared with fresh apples, we observed a 0.3% lower (+)-catechin level and a 2.4% higher (−)-epicatechin level in freeze-dried apples.

Repeatability and Reproducibility. Within-run repeatability of the method was assessed in apples and black grapes by analyzing 10 samples of each food in 1 day. Five duplicate analyses were carried out on separate days within a period of 3 months to determine between-run reproducibility.

RESULTS AND DISCUSSION

Detection and Chromatography. Fluorescence detection at 280 nm excitation and 310 nm emission wavelength is a specific and sensitive method to determine (+)-catechin and (−)-epicatechin in freeze-dried food samples. Detection limits of (+)-catechin and (−)-

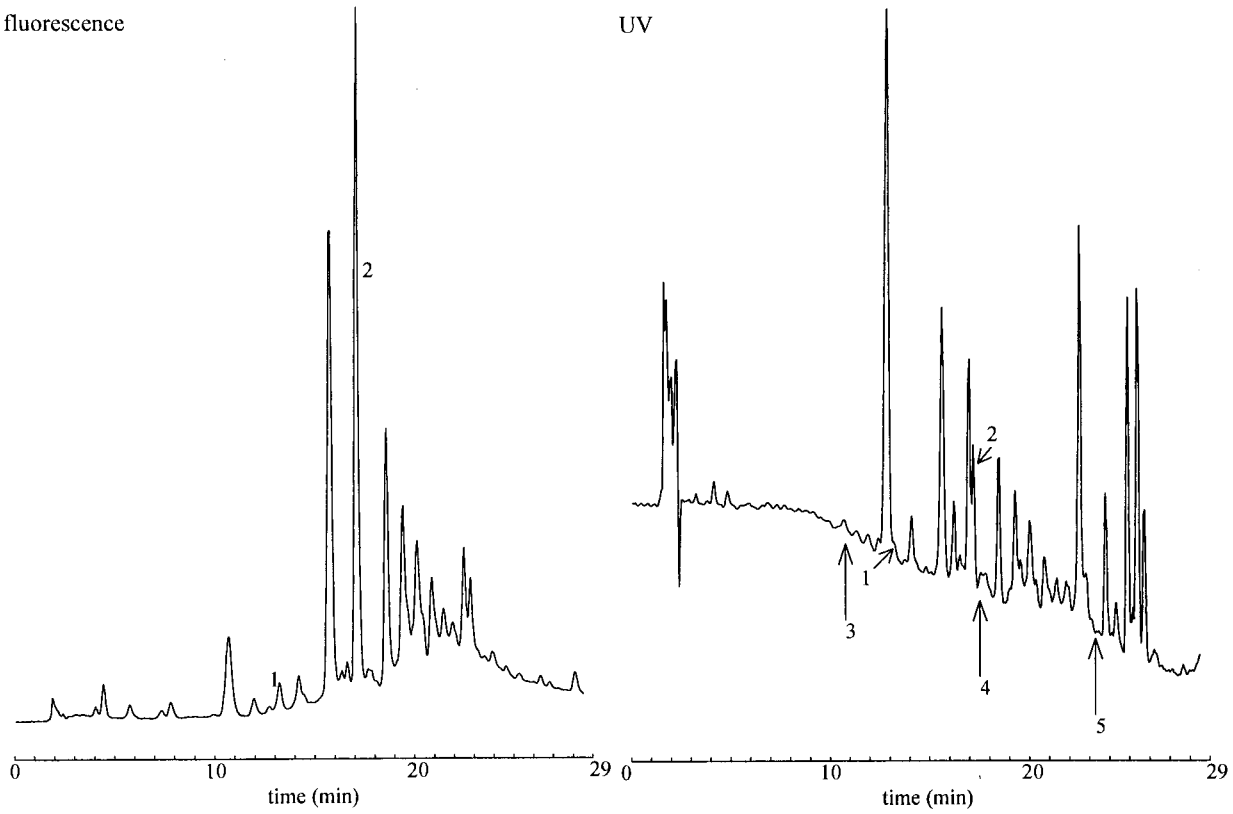

Figure 2. Typical chromatograms of an apple extract with UV (270 nm) and fluorescence (280 nm excitation, 310 nm emission) detection (acetonitrile/phosphate buffer gradient, pH 2.4, flow rate = 1.0 mL min^{-1}). Peaks: (1) (+)-catechin; (2) (−)-epicatechin; (3) EGC; (4) EGCg; (5) ECg. Compounds **3**−**5** are not present in apples. Their peak positions, based on retention times of the pure standards, are indicated in the chromatogram.

Table 1. Detection Limits of Catechins

	fluorescence		UV	
	standard (μg/mL)	apple[a] (mg/kg of fresh wt)	standard (μg/mL)	apple[a] (mg/kg of fresh wt)
(+)-catechin	0.008	0.1	0.13	1.8
(−)-epicatechin	0.007	0.1	0.09	1.2
EGC			0.29	3.9
EGCg			0.03	0.4
ECg			0.03	0.4

[a] Detection limits in apple are calculated from the standard data as follows: (value in μg/mL/sample wt) × 50 mL × (fraction dry wt); sample weight is 0.5 g, fraction dry weight is 0.136.

epicatechin improved >10-fold compared to UV detection (Table 1), and peak separation and specificity were better when fluorescence detection was used (Figure 2). Comparable detection limits have been reported for the fluorescence detection of (+)-catechin in plasma (0.02 μg/mL limit of quantitation, no definition provided) (Ho et al., 1995) and for electrochemical detection of (+)-catechin (0.002 μg/mL) and (−)-epicatechin (0.005 μg/mL) (Kermasha et al., 1995). Lower detection limits have been reported using GC/MS (Luthria et al., 1997) or HPLC after derivatization of catechins with 4-dimethylaminocinnamaldehyde (DMACA) (Treutter, 1989). However, the purpose of this study was not to maximize the sensitivity of the method. We intended to develop a method that can efficiently analyze a variety of food samples with a broad range in levels of catechins. Trace quantities of catechins in a food are irrelevant because of their small contribution to dietary intake. As we will show later in this paper, catechin levels may range from 4 to 226 mg/kg of fresh weight.

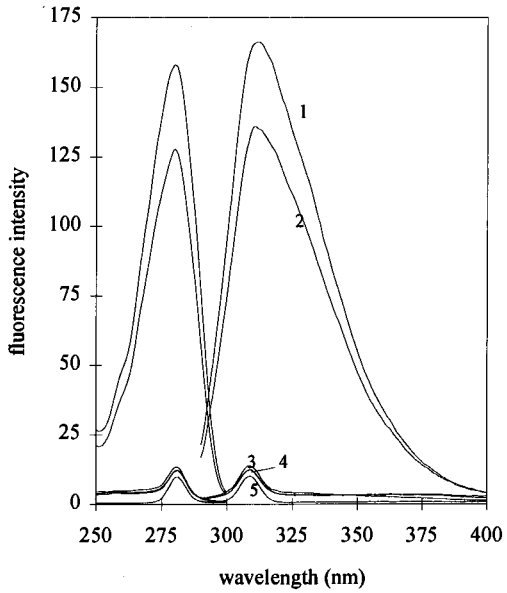

Figure 3. Fluorescence excitation (curves on the left side) and emission (curves on the right side) spectra of 1 μg/mL (+)-catechin (1), (−)-epicatechin (2), EGC (3), ECg (4), and EGCg (4) in eluent and of eluent only (5).

EGC, EGCg, and ECg showed little native fluorescence (Figure 3). Attempts to improve the fluorescence of EGC, EGCg, and ECg, by changing the eluent composition or by complexation of the catechins with borate, were not successful (data not shown). Therefore, we monitored (+)-catechin and (−)-epicatechin with fluorescence detection and EGC, EGCg, and ECg with UV detection at 270 nm, the wavelength that corre-

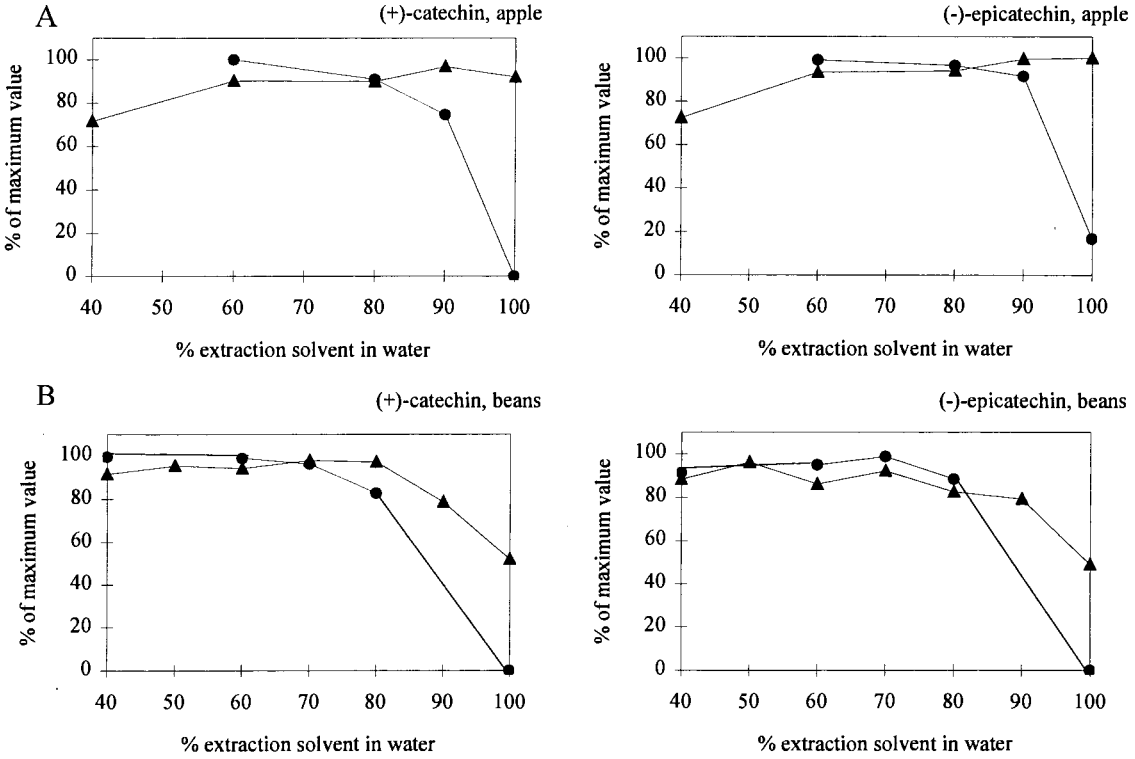

Figure 4. (+)-Catechin and (−)-epicatechin yield in apples (A) and beans (B), expressed as a percentage of the maximum yield, using 40−100% (v/v) methanol (▲) or acetone (●) in water as an extraction solvent (each data point represents the average of duplicate analyses).

sponds to an absorption maximum for EGC, the compound with the lowest UV absorbance. The detectors were connected in series so that analysis time was not affected. Fluorescence detection was the preferred method for (+)-catechin and (−)-epicatechin because it increased specificity: these two compounds elute in an area of the chromatogram where many unknown peaks elute (Figure 2). Monitoring (+)-catechin and (−)-epicatechin with UV would necessitate sample cleanup. UV limits of detection of EGCg and ECg (both 0.03 μg/mL) were only ∼4 times higher than those for (+)-catechin and (−)-epicatechin with fluorescence detection. EGC had the highest limit of detection: 0.3 μg/mL (Table 1). Lower detection limits have been reported in studies with a coulochem electrode array detection system (EGC, 1.5 ng/mL; EGCg, 0.5 ng/mL) (Lee et al., 1995) and with chemiluminescence detection (EGCg, 0.9 ng/mL) (Nakagawa and Miyazawa, 1997). These systems are as yet, however, not suitable for routine analyses.

Extraction. Both type and concentration of the extraction solvent (Figures 4 and 5) affected the yield of catechins. Adequate extraction was achieved with 60−100% methanol for apples and grapes and with 40−80% methanol for beans. The extraction solvents chosen were 90% methanol for apples and grapes and 70% methanol for beans. Chromatogram peak shape was adequate when methanol or acetone was used, but extraction with ethanol caused severe peak tailing. Therefore, the ethanol data are not shown in the figures. Extractions with either methanol or acetone gave similar maximum catechin yields. Because methanol is more agreeable to work with than acetone, we continued our experiments with methanol as an extraction solvent. When the percentage of methanol in the extraction solvent was reduced to 40%, catechin yield

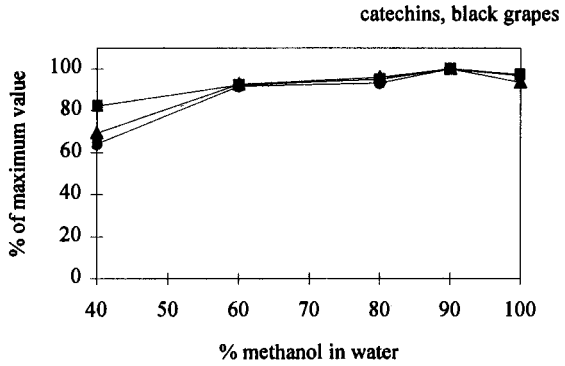

Figure 5. (+)-Catechin (■), (−)-epicatechin (▲), and (−)-epicatechin gallate (●) yield in black grapes, expressed as a percentage of the maximum yield, using 40−100% (v/v) methanol in water as an extraction solvent (each data point represents the average of duplicate analyses).

in apples and grapes decreased to ∼70% of the maximum value. In contrast, beans showed no yield reduction with decreasing methanol concentrations, but the yield reduced to 50% of the maximum value when 100% methanol was used. This difference in extraction behavior between canned beans, on the one hand, and grapes and apples, on the other hand, may be explained by the action of polyphenol oxidase. Polyphenol oxidase is an enzyme that is widely distributed in plants and which catalyzes the oxidation and polymerization of catechins to brown pigments when cells are ruptured (Whitaker and Lee, 1995). Methanol reduces the polyphenol oxidase activity (Tome et al., 1978). It is therefore possible that extraction with low methanol solvents does not completely inactivate polyphenol oxidase in fresh fruits, which results in reduced catechin yields. We observed browning in the low methanol apple and grape extracts, which supports this hypoth-

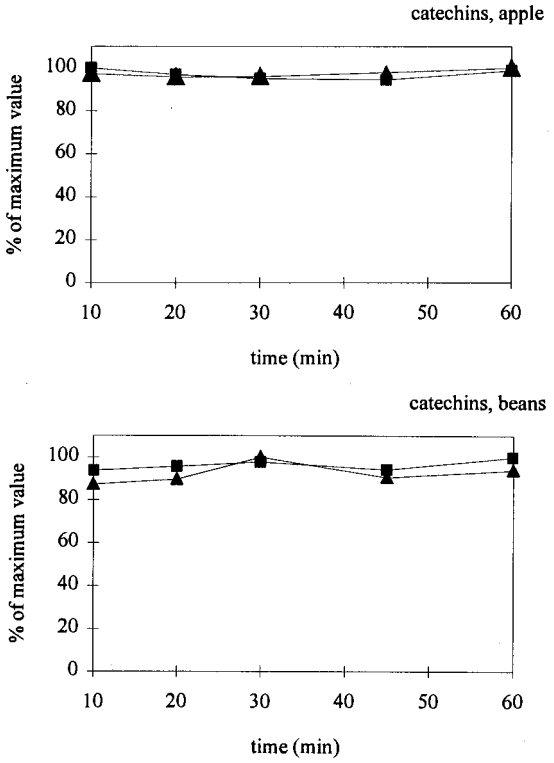

Figure 6. Influence of extraction time on (+)-catechin (■) and (−)-epicatechin (▲) yield in apples and beans, expressed as a percentage of the maximum yield (each data point represents the average of duplicate analyses). Apples were extracted in 90% methanol, beans in 70% methanol.

esis. Canned beans have undergone a heat treatment, which denatures all polyphenol oxidase. Prolonging the extraction time from 10 to 60 min (Figure 6) or increasing the amount of sample from 0.25 to 1.00 g (data not shown) did not affect extraction efficiency.

When optimum extraction conditions were applied, Jonagold apples contained 68.0 mg of (−)-epicatechin/kg of fresh edible weight and 3.8 mg of (+)-catechin/kg of fresh edible weight. Risch and Herrmann (1988) reported values in the same order of magnitude for Jonagold apples: 29 mg of (−)-epicatechin and 2 mg of (+)-catechin per kilogram of fresh weight. Growing conditions, maturity, and storage period of the fruits in the latter study may be somewhat different from those in our study and may therefore account for the differences in catechin contents. Maturity stage appears to be the most important contributor to differences within varieties (Amiot et al., 1992; Burda et al., 1990; McRae et al., 1990). We took particular care to process mature fruits only. In addition, differences in analytical methods may have contributed to some extent. Risch and Herrmann (1988) did not provide data on the recovery of their method.

In black grapes we detected ECg (84.4 mg/kg of fresh weight) in addition to high levels of (+)-catechin (225.5 mg/kg of fresh weight) and (−)-epicatechin (190.3 mg/kg of fresh weight). The same compounds were detected in qualitative studies in grape seeds (Santos-Buelga et al., 1995) and skins (Escribano-Bailón et al., 1995). Despite the vast amount of literature on catechins in wines, no quantitative data on whole black grapes were found.

Canned kidney beans contained mostly (+)-catechin (16.5 mg/kg of fresh weight) and some (−)-epicatechin (7.2 mg/kg of fresh weight). None of the other compounds were detected in beans. Hanefeld and Herrmann (1976) reported the presence of (+)-catechin and (−)-epicatechin in several varieties of beans but did not quantify them. Other studies are less reliable because simple colorimetric methods were used to determine the total polyphenol content of beans (Deshpande and Cheryan, 1987; Laurena et al., 1994). These data clearly cannot be compared with ours.

Recovery and Stability. Recovery of catechins added prior to the extraction procedure approximated 100% for all compounds, ranging from 92% for (+)-catechin to 105% for EGCg (Table 2). These data are in accordance with recoveries of (+)-catechin and (−)-epicatechin reported elsewhere in apple must (Suárez Vallés et al., 1994; Suárez et al., 1996) and wine (Goldberg et al., 1996; Ricardo da Silva et al., 1990) and of (−)-epicatechin in cocoa beans (92%) (Kim and Keeney, 1983). To our knowledge, there are no recovery studies available for EGC, ECg, and EGCg in food samples. Lee et al. (1995) studied EGC, (−)-epicatechin, and EGCg in human plasma and urine. Recoveries from plasma (EGC, 88%; (−)-epicatechin, 85%; EGCg, 82%) were lower than those from urine (92, 95, and 87%, respectively). Another study reported a similar recovery for EGCg (84%) from rat plasma (Nakagawa and Miyazawa, 1997). Plasma requires considerable cleanup before analysis, which may have caused the reduced recovery compared to our method in food samples.

The last standard mixture in a run tended to have slightly lower peak areas than the first mixture for (−)-epicatechin (3.1% decrease, $p = 0.01$), (+)-catechin (3.7% decrease, $p = 0.06$), EGC (8.1% decrease, $p = 0.06$), and EGCg (5.2% decrease, $p = 0.08$) but not for ECg (0.1% increase, $p = 0.96$). On average, there were 29 samples between the two standards (~18 h). It is unlikely that the small decreases in standard areas have seriously affected the quantitative analysis of our samples, also because standard peak areas were averaged before the catechin content of food samples was calculated.

Repeatability and Reproducibility. Within-run repeatability of the method was excellent. Coefficients of variation (CV) ranged from 1.3% for the determination of (−)-epicatechin in apples to 5.4% for ECg in

Table 2. Recovery of Catechins Added to Apples and CVs of the Analysis of Catechins from Apples and Black Grapes

	recovery[a] (%)		CV (%)			
			apples		black grapes	
	50% addition	100% addition	within-run[c]	between-run[d]	within-run[c]	between-run[d]
(+)-catechin	96	92	2.5	9.0	2.6	3.4
(−)-epicatechin	100	94	1.3	4.4	3.4	8.2
ECg[b]	98	98			5.4	5.0
EGCg[b]	96	105				
EGC[b]		101				

[a] Average of duplicate analyses. [b] Recoveries were calculated by adding a well detectable amount; EGC was added at only one level. [c] $n = 10$. [d] n = five duplicate analyses.

grapes (Table 2). Comparable CVs are reported by other authors in wine (Goldberg et al., 1996; Ricardo da Silva et al., 1990) and plasma (Ho et al., 1995). Lee and et al. (1995) found a slightly higher intraday variation for (−)-epicatechin in plasma (8.2%) and urine (7.8%).

Between-run reproducibility varied between 3.4% for (+)-catechin in black grapes and 9.0% for (+)-catechin in apples (Table 2). Literature values for the between-run CVs in plasma are 2.1−5.2% for (+)-catechin (Ho et al., 1995) and 10.4% for (−)-epicatechin (Lee et al., 1995). The lower (+)-catechin content of apples compared to grapes causes the relatively large CV in apple.

In this paper, we describe a simple method for the determination of (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate in fruits and legumes, without the need for sample cleanup. The relatively short HPLC run of 37 min and the extremely simple extraction method make it possible to run ∼40 samples a day. Recovery, repeatability, and reproducibility are comparable to or better than currently available methods. Sensitivity is adequate and allows the determination of a wide range of catechin levels in one run.

ABBREVIATIONS USED

CV, coefficient of variation; EGC, (−)-epigallocatechin; EGCg, (−)-epigallocatechin gallate; ECg, (−)-epicatechin gallate.

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