# Kinetics Method for the Quantitation of Anthocyanidins, Flavonols, and Flavones in Foods

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Flavonoids are important dietary constituents owing to their health-promoting properties. As a result, simplified analytic techniques are required for the population of databases with food values so that associations between dietary intake and disease risk/incidence can be established. The current research provides a simplified sample preparation procedure for the accurate estimation of food anthocyanidins, flavones, and flavonols as aglycons. Traditionally, flavonoid aglycons have been formed by acidic hydrolysis. However, some flavonoid aglycons are slowly degraded by acid. A procedure has been developed whereby anthocyanidins and flavonols are deglycosylated with HCl in 50% aqueous methanol and the resulting aglycons subsequently quantified by application of pseudo-first-order kinetics to their degradation. Flavones are also deglycosolated under similar conditions but, at appropriate temperatures, their aglycons are stable in acid, so kinetics were not required for the quantitation of this subclass of flavonoids. Catechins and flavanones were rapidly degraded under the hydrolytic conditions used in these studies.

**Keywords:** *Anthocyanidin; anthocyanin; extraction; flavonoid; flavone; flavonol; HPLC; hydrolysis; kinetics; aglycons*

## INTRODUCTION

Flavonoids are important constituents of the human diet. Their reported benefits to human health include their antioxidant activities (*1*), metal chelation (*2*), stimulation of the immune system (*3*), prevention of tyrosine nitration (*4*), and antiproliferative, antiallergic, antibacterial, anticarcinogenic, antiviral, and anti-inflammatory effects (*3*). There are five subclasses of common monomeric food flavonoids (*5*): anthocyanidins, catechins, flavanones, flavones, and flavonols (Figure 1). Naturally occurring flavonoids, with the exception of catechins, are glycosylated at positions C3, C7, and C4′ (*5*). Carbohydrates commonly found include glucose, galactose, rhamnose, xylose, neohesperidose, and rutinose. Catechins (flavan-3-ols) are generally not glycosylated in commonly consumed foods. However, some are gallated, which can be easily separated on high-performance liquid chromatographic systems (HPLC) and therefore require only extraction from the food matrix prior to analysis.

Generally, flavonoids within a single subclass are characterized and measured without removal of the glycosides (*6*, *7*), for example, anthocyanins in raspberry (*8*), catechins in tea infusions (*9*), flavanone glycosides in orange and grapefruit juice concentrates (*10*), flavone glycosides in the botanical *Matricaria chamomilla* (Roman chamomile) (*11*), and the flavonol glycoside rutin from buckwheat (*12*). In the analysis of the prominent flavonoids in the food supply, this approach would require many different analytical systems or, if a single system could be developed, chromatograms would be produced with a large number of peaks, for which there is a substantial lack of commercially available standards for identity, validation, and quantification. Our approach is to measure food flavonoids as aglycons, which are the type of data currently required by health professionals to develop associations between dietary intake of flavonoids and health status.

Anthocyanins and anthocyanidins are usually extracted with HCl, ranging from 0.1 to 1.0 M (*13*, *14*), and then sometimes hydrolyzed to anthocyanidins in 2−6 M HCl (*15*, *16*). Anthocyanin pigments also have been extracted from fruits with several organic solvents and hydrolyzed to anthocyanidins in 2 M HCl followed by solid phase extraction (SPE) prior to HPLC analysis (*17*, *18*).

Hertog and colleagues (*19*) reported a method for the production and extraction of aglycons of flavonols and flavones via acidic hydrolysis in several fruits and vegetables. This method and several modifications have been used for the hydrolysis of these flavonoid subclasses in a wide variety of foods and beverages (*20*−*22*). With exceptions including catechin compounds and delphinidin (*23*), these hydrolyses are generally limited to the anthoxanthins: flavanones, flavones, and flavonols (*24*). Reporting on flavonoids and phenolic acids in Finnish berries, Hakkinen and colleagues (*22*) noted that using a variation of the Hertog method (*19*), catechin analysis was impossible and the extraction and

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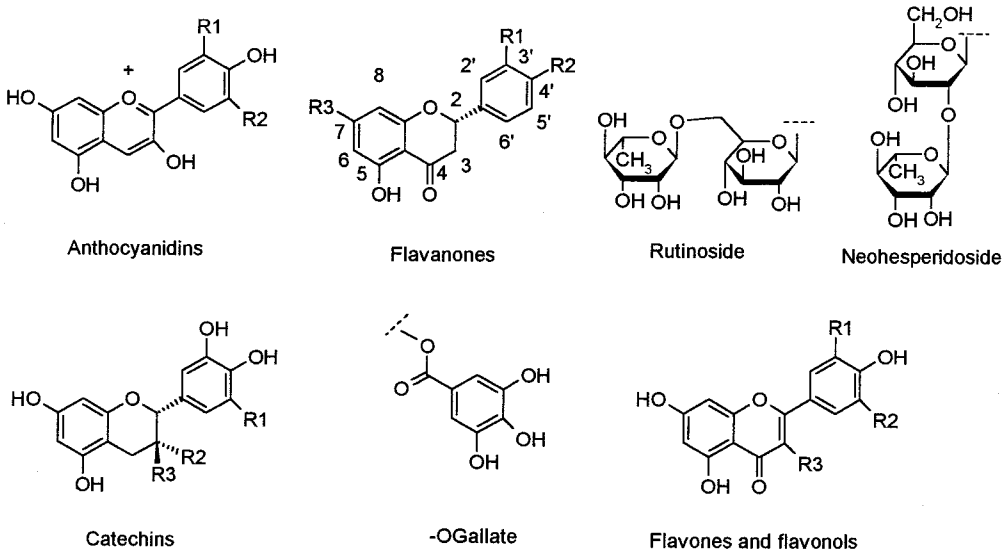


**Figure 1.** Aglycons of flavonoids and four flavanone glycosides common in foods, with the numbering system shown on the flavanones. Anthocyanidins: cyanidin, R1 = OH, R2 = H; delphinidin, R1 = R2 = OH; malvidin, R1 = R2 = OMe; pelargonidin, R1 = R2 = H; peonidin, R1 = OMe, R2 = H. Flavanones: hesperetin, R1 = R3 = OH, R2 = OMe; hesperidin, R1 = OH, R2 = OMe, R3 = *O*-*β*-rutinoside; naringenin, Rl = H, R2 = R3 = OH; naringin, R1 = H, R2 = OH, R3 = *O*-*β*-neohesperidoside; narirutin, R1 = H, R2 = OH, R3 = *O*-*β*-rutinoside; neohesperidin, R1 = OH, R2 = OMe, R3 = *O*-*β*-neohesperidoside. Catechins: (+)-catechin (Cat), R1 = R2 = H, R3 = OH; (−)-epicatechin (EC), R1 = R3 = H, R2 = OH; (−)-epicatechin-3-gallate (ECG), R1 = R3 = H, R2 = *O*-gallate; (−)-epigallocatechin (EGC), R1 = R2 = OH, R3 = H; (−)-epigallocatchin-3-gallate (EGCG), R1 = OH, R2 = *O*-gallate, R3 = H. Flavones: apigenin, R1 = R2 = R3 = H; luteolin, R1 = OH, R2 = R3 = H. Flavonols: kaempferol, R1 = R2 = H, R3 = OH; myricetin, R1 = R2 = R3 = OH; quercetin, R1 = R3 = OH, R2 = H.

hydrolysis procedure was insufficiently sensitive for anthocyanin analysis.

We have developed a system of separation and quantitation of the nearly 20 aglycons representing all five subclasses of simple flavonoids common in food (*25*). This system requires a sample preparation procedure that will form flavonoid aglycons in a manner that they can be accurately quantified in the original food matrix. We report here such a method for anthocyanidins and flavonols. Both subclasses are extracted by acidic aqueous methanol, yet the acid degrades the flavonoids slowly via pseudo-first-order kinetics, which is used to extrapolate to zero-time and estimate concentrations. Owing to the stability of flavone aglycons to the acid concentrations and temperatures employed, this subclass of flavonoids also can be quantified from the same sample digests.

## MATERIALS AND METHODS

(+)-Rutin and spectrophotometric grade trifluoroacetic acid (TFA) were purchased from Aldrich Chemical (Milwaukee, WI). *tert*-Butylhydroquinone (TBHQ) was purchased from Eastman Chemical Products, Inc. (Kingsport, TN). Apigenin, callistephin chloride, cyanidin chloride, delphinidin chloride, malvidin chloride, naringin, pelargonidin chloride, and peonidin chloride were purchased from Indofine Chemical Co. (Somerville, NJ). Quercetin was purchased from Sigma (St. Louis, MO). Sulfuric acid, hydrochloric acid (HCl), and HPLC grade acetonitrile ($CH_3CN$) and methanol (MeOH) were purchased from Fisher Chemical (Fair Lawn, NJ). HPLC grade water (18 MΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., New Bedford, MA).

**Apparatus.** An Omni mixer homogenizer (Omni International, Waterbury, CT) was used to prepare berries for lyophilization. The lyophilizer was made by Virtis Research Equipment (Gardiner, NY) and was connected to a Welch duoseal vacuum pump, model 1402 (Sargent-Welch Scientific Equipment, Skokie, IL). The HPLC system and solvent parameters were previously reported (*25*).

**Sample Preparation, Standards, and Calculations.** Freeze-dried onion and dehydrated flaked parsley leaf were ground in a Thomas-Wiley intermediate mill (Arthur H. Thomas Co., Philadelphia, PA) through a 20 mesh screen. Frozen berries, purchased from a local supermarket, were thawed, homogenized, and lyophilized.

The food extractions were modified, following the method reported by Hertog et al. (*19*). A carefully measured freeze-dried sample (0.5−5 g) was refluxed under nitrogen for up to 5 h in 50 mL of various concentrations of HCl in 50% aqueous MeOH in which 0.4 g/L TBHQ was dissolved. The mixture was purged with helium before starting and the vapor temperature of the hydrolysis mixture was maintained at 75 °C, except where noted. Anthocyanidin chloride standards (~50% of unspiked concentrations) were added for spiked samples. Aliquots for analysis were taken at 0.5 h intervals from 0.5 to 5 h. After cooling, each aliquot was sonicated for 5 min and then filtered through a 0.2 $\mu$m Anotop syringe filter (Whatman International Ltd., Maidstone, U.K.) for HPLC analysis.

Standard solutions for HPLC calibration were made as previously reported (*25*). Plots and calculations for standard calibrations and kinetics parameters were conducted on Microsoft Excel 7.0. Statistical comparisons were based on two-tailed *t* tests (*26*).

## RESULTS

**Optimization of Hydrolytic Conditions for Formation of Anthocyanidins.** In an attempt to move away from the harsh conditions of mineral acids, a wide variety of commercially available glucosidases and other hydrolytic enzymes were tested on several pure anthocyanins with negative results. In addition, aqueous and methanolic TFA as well as dilute aqueous sulfuric acid was also tested unsuccessfully on representative anthocyanins, naringin and rutin. Hertog was successful in the formation of flavone and flavonol aglycons from foods with 1.2 M HCl in 50% aqueous methanol (*19*). We tested several concentrations of HCl, which bracketed 1.2 M in 50% aqueous methanol (0.6−2.4 M) with variable results. In general, HCl concentrations of <1.8

**Table 1. Kinetics Characteristics and Calculated Flavonoid Content of Selected Foods[a,b]**

| food and aglycon | −slope × $10^1$ | $k_{obs}$ × $10^5$ ($s^{-1}$) | *y*-intercept × $10^1$ | $C_1$ (mg/kg) | $C_0$ (mg/kg) |
|---|---|---|---|---|---|
| blackberry,[c] cyanidin | 1.21 ± 0.10 | 3.37 ± 0.28 | 3.68 ± 0.28 | 5287 ± 405 | 7645 ± 699 |
| blueberry,[d] cyanidin | 2.72 ± 0.11 | 7.55 ± 0.32 | 5.34 ± 0.38 | 90 ± 6 | 154 ± 16 |
| blueberry, delphinidin | 3.72 ± 0.13 | 10.3 ± 0.37 | 7.30 ± 0.30 | 249 ± 19 | 517 ± 50 |
| blueberry, malvidin | 3.92 ± 0.12 | 10.9 ± 0.03 | 7.82 ± 0.41 | 1085 ± 60 | 2374 ± 225 |
| strawberry,[e] pelargonidin | 2.02 ± 0.14 | 5.60 ± 0. 40 | 5.93 ± 0.43 | 1175 ± 229 | 2138 ± 506 |
| onion,[e,f] quercetin | 2.79 ± 0.27 | 7.74 ± 0.75 | 2.04 ± 0.23 | 2249 ± 65 | 2758 ± 17 |

[a] All runs done in triplicate unless otherwise noted. [b] All values expressed as mean ± 1 SD. [c] $t_1$ = 3.0 h. [d] $t_1$ = 2.0 h. [e] Runs done in duplicate. [f] $t_1$ = 1.0 h.

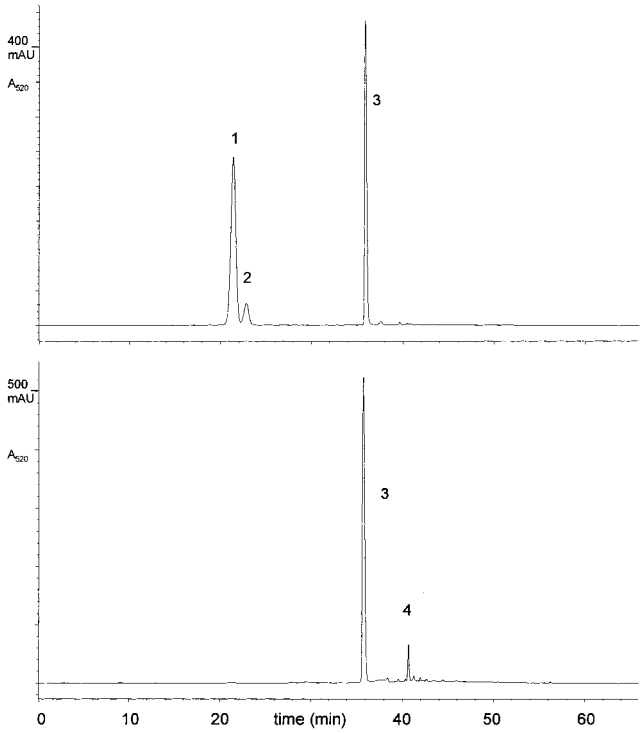


**Figure 2.** Chromatograms at 520 nm of 5 g of blackberry extracted in 1.8 M HCl/50% aqueous MeOH at 0.5 h (top) and 5.0 h (bottom). Peaks 1 and 2 are putatively cyanidin glycosides; peak 3 is cyanidin; peak 4 is unknown.

M were unsatisfactory because complete hydrolysis of the glycosides from anthocyanins in food samples required ≥4 h, and at low concentrations of acid, some foods formed unmanageable gelatinous masses.

Results of the extraction, hydrolysis, and chromatography of a sample of freeze-dried blackberries with 1.8 M HCl in 50% aqueous methanol at 0.5 and 5 h are shown in Figure 2. At the early time period, peaks corresponding to cyanidin and, putatively, cyanidin glycosides were observed, whereas at 5 h a peak analogous to cyanidin (retention time and spectral agreement) was the major component of the chromatogram. A time course of disappearance of putative cyanidin glycosides and production of cyanidin in the presence of 1.8 and 2.4 M HCl from samples of a blackberry preparation is shown in Figure 3. It is important to note that the maximum amount of cyanidin appeared prior to the disappearance of detectable levels of cyanidin glycosides. It should also be observed that cyanidin is gradually degraded during the hydrolysis process and that, as expected, 2.4 M HCl hydrolyzed cyanidin glycosides and degraded cyanidin more rapidly than 1.8 M HCl. The shapes of degradation curves were similar for the hydrolysis of glycosides of other prominent anthocyanidins (delphinidin, malvidin, and pelargonidin) in several berries (blueberry, blackberry, and

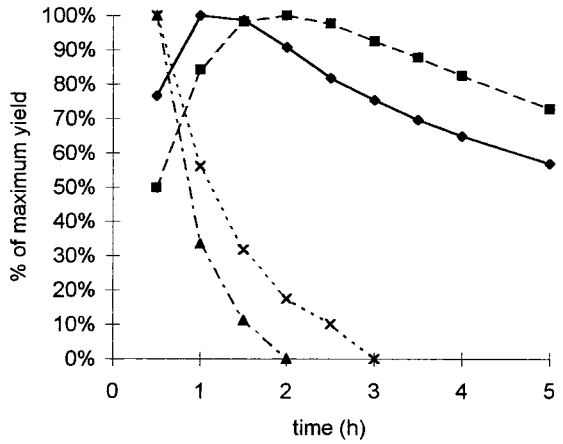


**Figure 3.** Cyanidin and cyanidin glycosides in blackberry in 2.4 and 1.8 M HCl/50% aqueous MeOH extractions, each the average of three trials of 5 g of lyophilized blackberry extracted in 50 mL of solvent: (◆) 2.4 M HCl, cyanidin; (■) 1.8 M HCl, cyanidin; (▲) 2.4 M HCl, glycosides; (×) 1.8 M HCl, glycosides. See text for details. The area of all glycoside peaks is summed (Figure 2) and reported as a single entity.

strawberry) to those for cyanidin in blueberries (data not shown). However, the time during the hydrolysis at which anthocyanidin glycosides could no longer be detected was dependent upon the specific anthocyanidin being hydrolyzed and the food matrix (Table 1). In this study 1.8 M HCl in 50% aqueous methanol was chosen as the hydrolysis conditions under which additional studies would be conducted because glycosides of common food anthocyanidins, flavones, and flavonols were undetectable at 3 h, and the fit of lines to kinetics data (anthocyanidins and flavonols) was somewhat better than for 2.4 M HC (see Kinetics Treatment of Hydrolytic Data).

**Kinetics Treatment of Hydrolytic Data.** The general negative slope and the curvilinear nature of the hydrolysis curves for cyanidin in blueberries (Figure 3) suggested anthocyanidin levels could be corrected based on equations fit to the kinetics of the degradation. The concentrations of cyanidin for the samples shown in Figure 3 were plotted logarithmically (Figure 4), with the *y*-axis as the natural log of the ratio of the cyanidin concentrations at each time point to that at $t_1$, the first sampling time when there was no longer detectable glycoside (2 h for 2.4 M HCl and 3 h for 1.8 M HCl). Assuming pseudo-first-order (*27*, *28*) kinetics

$$\ln(C_t/C_0) = -kt \quad (1)$$

$C_0$ extrapolation of the least-squares fit line to the *y*-axis ($t_0$) allowed calculation of $C_0$ (Table 1) by

$$C_0 = C_1 \exp(b) \quad (2)$$

where $C_0$ is the calculated initial concentration of the

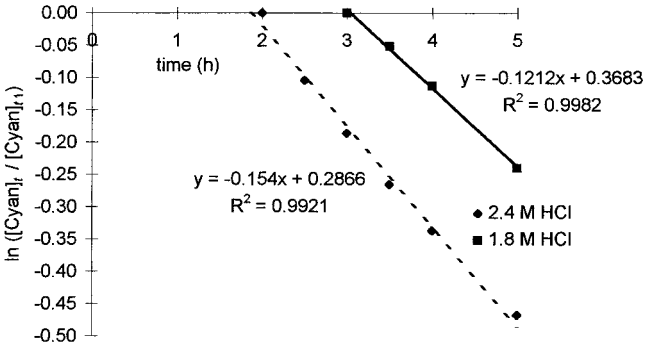


**Figure 4.** Kinetics plots of cyanidin in blackberry produced in 2.4 and 1.8 M HCl/50% aqueous MeOH extractions, each the average of three trials of 5 g of lyophilized blackberry extracted in 50 mL of solvent. $t_1$ = 3.0 h (1.8 M HCl/50% aqueous MeOH), 2.0 h (2.4 M HCl/50% aqueous MeOH). See text for details.

aglycon and $C_1$ is the concentration of the aglycon at the time at which undetectable glycoside(s) first occur ($t_1$). The variable $b$ is the $y$-intercept of the line used to determine $k_{obs}$, that is, the least-squares fit connecting the points in a graph of $\ln(C_t/C_1)$ versus time.

The high coefficients of determination ($R^2$; 1.0 is the maximum value) for the least-squares fit lines in Figure 4 indicate that pseudo-first-order kinetics is a good fit for the degradation of cyanidin during the extraction of anthocyanidins from blackberries. Also, the larger negative slope of the line fit to data from the 2.4 M HCl extraction, compared to the slope from the 1.8 M HCl hydrolysis, indicates that degradation of cyanidin is slightly more rapid in the higher concentration acid. Pseudo-first-order kinetics plots for the concentration of cyanidin and other anthocyanidins from several foods during acid hydrolysis had coefficients of determination ($R^2$) that were similar to those tabulated in Figure 4 (data not shown). Kinetics data are given in Table 1 for prominent anthocyanidins produced during the hydrolysis (1.8 M HCl) of several foods.

Analytical values for several anthocyanidins in a few commonly consumed berries at the time glycosides were first undetectable during the hydrolysis ($C_1$) as well as calculated values for the same anthocyanidins at $C_0$ are given in Table 1. Comparison of these two values shows that considerable degradation of anthocyanidins occurs during the hydrolysis procedure even though glycosides are still present. With the exception of pelargonidin in strawberries, the coefficient of variation for the measurement of anthocyanidins in berries was 9−10%. Part of this error may be due to the method of calculation employed (extrapolation of ln data and subsequent conversion to linear data), which tends to amplify small variations in analytical data. Another source of error may be the relatively long time (several hours) samples in the presence of 1.8 M HCl might wait for HPLC analysis (each HPLC run is 65 min). However, repeated analysis of the same vials with an 18 h interval at room temperature resulted in a <10% loss of anthocyanidins.

**Acid Hydrolysis of Glycosides of Other Flavonoid Subclasses.** The flavonol quercetin is the prominent flavonoid aglycon after hydrolysis of onion. The yields of quercetin during a 5 h hydrolysis from a freeze-dried onion preparation employing 1.8 M HCl in 50% aqueous methanol are shown in Figure 5. These data suggest that glycosides of quercetin are cleaved during the first hour of hydrolysis. As a result, data points for the kinetics were taken between 1 and 5 h

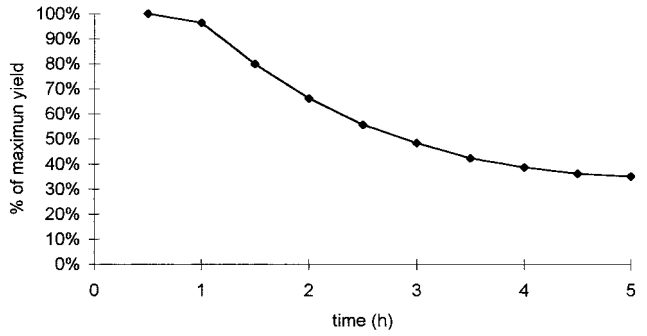


**Figure 5.** Percent maximum yield of quercetin for the average of two trials of onion, each extracted as 0.5 g of lyophilized onion in 50 mL of 1.8 M HCl/50% aqueous MeOH. See text for details.

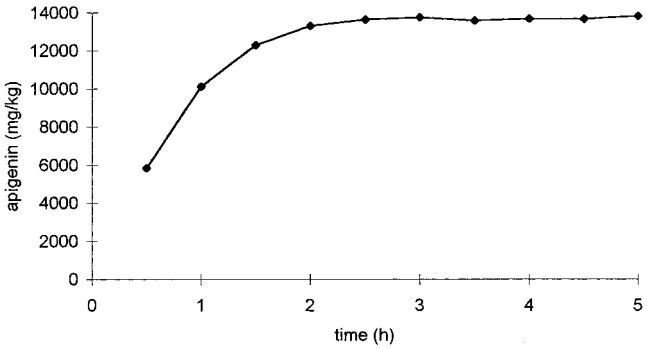


**Figure 6.** Yield of apigenin from parsley, average of two trials, each extracted as 0.5 g of in 50 mL of 1.8 M HCl/50% aqueous MeOH. See text for details.

and the resulting data are given in Table 1. Coefficients of determination for the least-squares fit lines of the hydrolysis of quercetin were all >0.97 (data not shown). The average calculated value for quercetin in this sample of onions was 2758 mg/kg of dry weight (Table 1).

Apigenin, a flavone, is the flavonoid aglycon in greatest abundance after hydrolysis of parsley. Its concentration first increased (0.5−2 h, during hydrolysis of glycosides) and subsequently remained constant during the 5 h hydrolysis in 1.8 M HCl/50% aqeuous MeOH at 75 °C (Figure 6). Hydrolysis was judged complete at ∼3 h with the inability to detect rhoifolin (apigenin-7-$\beta$-neohesperidoside), a prominent parsley flavone glycoside. However, when the hydrolysis temperature was increased to 85 °C, the pattern of apigenin concentration was similar to that of cyanidin shown in Figure 3 (data not shown). The increased stability of apigenin over anthocyanidins and quercetin is presumably due to the resonance introduced by the conjugated system in C2−C3−C4 and the 4-oxo group (Figure 1) as well as the lack of the hydroxyl group on C3.

Flavanones and catechins, two additional subclasses of flavonoids, are present in commonly consumed foods. Citrus foods are abundant in flavanones, whereas tea, red grapes, and red wine are prominent sources of catechins. When aliquots of orange juice concentrate were subjected to hydrolysis in 1.8 M HCl/50% aqueous MeOH, flavanone aglycons were destroyed nearly as fast as the flavanone glycosides were hydrolyzed (data not shown). Relative to catechins, when aliquots of foods rich in these flavanoids (red grapes) were subjected to hydrolysis in 1.8 M HCl/50% aqueous MeOH, all chromatographic peaks corresponding to identifiable flavan-3-ols were absent. Accurate measurement of these two

subclasses of flavonoids in foods requires a separate extraction, in the absence of hydrolytic conditions, followed by sample purification and chromatography. The four prominent food flavanone glycosides as well as their aglycons, hesperetin and naringenin, and the commonly occurring food catechins were separated with the chromatography system employed (*25*) and quantified with commercially available standards (*9*, *10*).

## DISCUSSION

The proposed sample extraction and preparation procedure is intended for the generation of quantitative data on the anthocyanidin, flavone, and flavonol contents of foods as aglycons. The total aglycon content of each flavonoid is the type of data required by food composition databases, which will permit epidemiologists and other health professionals to draw associations between dietary intake of these minor food constituents and the health status of human beings. There are no certified reference materials for flavonoids in food matrices, which makes validation of new analytical methods difficult. Therefore, we attempted to establish the accuracy of the proposed procedure with several alternate approaches.

First, addition of "spikes" (methods of standards addition) of anthocyanidins and an anthocyanin (callistiephin) to the hydrolysis mixture of several berries resulted in extremely low recoveries of anthocyanidins, whereas addition of quercetin spikes resulted in recoveries >95%. These observations suggest that hydrolysis of anthocyanins and release of anthocyanidins from the food matrix is a complex process and that the method of standards addition is an inappropriate validation procedure for this subclass of flavonoids.

Second, an aliquot of freeze-dried blackberries, which contains a relatively simple anthocyanin profile, was extracted in the absence of acid (*29*) and the resulting sample subjected to HPLC (chromatogram similar to Figure 4, top). The putative anthocyanins were quantified as keracyanin (a commercially available standard) and then converted to concentrations of cyanidin. The resulting 7883 mg/kg cyanidin equivalents was similar ($P > 0.05$) to the calculated value for the proposed kinetics method of 7645 ± 699 mg/kg (Table 1).

Finally, results for quercetin and apigenin employing the proposed method were compared with data obtained using a commonly accepted single time point hydrolysis procedure, for example, 2 h of extraction in 1.2 M HCl/50% aqueous MeOH after the method of Hertog et al. (*19*). The value obtained for quercetin in onions with this method was 3078 mg/kg, which is slightly but significantly ($P < 0.05$) higher than the concentration derived by the kinetics method of 2758 mg/kg. Values for apigenin in parsley were similar for the two procedures: 13.2 g/kg from the Hertog procedure and 13.3 g/kg from the proposed kinetics method with data taken at the 2 h of hydrolysis (75 °C) time point.

In general, these observations suggest that accurate data can be generated for the anthocyanidin and flavonol content of foods, as aglycons, employing the proposed method of hydrolysis and extraction followed by extrapolation of kinetics data to calculate initial concentrations. In addition, due to the stability of flavones, accurate data for this subclass of flavonoids can also be generated using the proposed procedures as long as samples for quantification are taken after glycosides have been cleaved (~2 h).

## ABBREVIATIONS USED

$C_0$, concentration at $t_0$; $C_1$, concentration at $t_1$; $CH_3CN$, acetonitrile; HCl, hydrochloric acid; $H_2O$, water; HPLC, high-performance liquid chromatography; $k_{obs}$, rate of aglycon degradation; MeOH, methanol; $R^2$, coefficient of determination; SPE, solid-phase extraction; $t_0$, time zero; $t_1$, first sampling interval in which glycosides are no longer present; TBHQ, *tert*-butylhydroquinone; TFA, trifluoroacetic acid.

## ACKNOWLEDGMENT

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