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Anthocyanin Stability and Recovery: Implications for the Analysis of Clinical and Experimental Samples

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The proportion of ingested anthocyanins to reach the systemic circulation is reported to be a small percentage of their ingested dose. This may be due to physiochemical degradation in vivo or following routine sample treatment. Therefore, this study aimed to quantitatively investigate the effect of anthocyanin structure on their stability under simulated (in vitro) physiological conditions and to assess their degradation and recovery following routine preanalytical sample extraction and storage. It was demonstrated that B-ring hydroxylation mediated the degradation of anthocyanins to their phenolic acid and aldehyde constituents, successful anthocyanin extraction is dependent on both sample preparation technique and anthocyanin structure, and anthocyanins are stable through multiple freeze−thaw cycles. These data indicate that significant portions of ingested anthocyanins are likely to degrade to phenolic acids and aldehyde in vivo. Consequently, these compounds should be the target of future bioavailability and bioactivity studies to establish the true occurrence and impact of anthocyanins on human health.

KEYWORDS: Degradation; anthocyanin; phenolic acids; flavonoid; stability

INTRODUCTION

Many plant species with red to purple pigments owe their hue to natural polyphenolic compounds known as anthocyanins. Over the past decade, interest in anthocyanins has grown considerably, as evidence of their beneficial effects on health continues to increase, particularly regarding their role in reducing cardiovascular disease risk (*1*−*3*), cancer (*4*, *5*), and obesity (*6*).

In plants, anthocyanins are almost exclusively found as glycosides of the aglycone form (anthocyanidins) (**Figure 1**), where three common structures, cyanidin, delphinidin, and pelargonidin, exist, differing only in the number and position of hydroxyl groups on their B-ring. Anthocyanins may exist in at least four different pH-dependent structural isoforms, namely, flavylium anion, hemiketals, quinoid bases, and chalcones, arising at pH 1−3, 4−5, 6−8, and 7−8, respectively (*7*). At more alkaline pH values, anthocyanins have consistently been shown to degrade to their constituent phenolic acids, where delphinidin, cyanidin, and pelargonidin degrade to form gallic acid, protocatechuic acid, and 4-hydroxybenzoic acid, respectively (*8*, *9*) (**Figure 1**). In addition, it is known that all species of anthocyanins degrade to a common phenolic aldehyde, with the most frequently reported constituent being phloroglucinol aldehyde (*10*, *11*).

Since the 1970s, many studies have investigated the occurrence and utility of anthocyanins in plants and in food production, while research concerning their potential health-related benefits has become prominent only since the late 1990s. It has since been established that anthocyanins are poorly available to the systemic circulation (*12*−*14*). We postulate that this is a result of their reported instability, as it has been shown that, under certain conditions [e.g., food processing (*15*)], anthocyanins are subject to significant degradation (*8*, *16*, *17*). Therefore, it is paramount that the chemical stability of anthocyanins is assessed under physiological and experimental conditions to determine the contribution of degradation to their reported bioavailability. While a great deal of studies have reported on aspects of anthocyanin stability, there is no single investigation fully describing their recovery and degradation following the routine extraction, preanalytical preparation, and storage of clinically and experimentally derived samples. Thus, the aim of this study was to assess the degradation and recovery of selected anthocyanins (aglycones and monoglucosides), with mono-, di-, and trihydroxylated B rings, under simulated (in vitro) physiological conditions (i.e., in vitro models designed to simulate aspects of in vivo conditions) and routine experimental conditions.

MATERIALS AND METHODS

Standards and Reagents. Cyanidin-3-glucoside (kuromanin chloride), delphinidin-3-glucoside (myrtillin chloride), pelargonidin-3-glucoside (callistephin chloride), cyanidin chloride, delphinidin chloride, pelargonidin chloride, and 4-hydroxybenzoic acid were purchased from Extrasynthese (Genay, France) and dissolved in dimethyl sulfoxide (DMSO). Protocatechuic acid, gallic acid, *trans*-cinnamic acid, 4-hydroxycinnamic acid, and phloroglucinol aldehyde were purchased from Sigma-Adlrich (United Kingdom) and dissolved in DMSO. Phenol-free Dulbecco's modified Eagle's medium (DMEM) and bovine calf serum were purchased from Invitrogen (United Kingdom). All water used was 18 MΩ/cm Milli-Q water, and solvents were of high-performance liquid chromatography (HPLC) grade.

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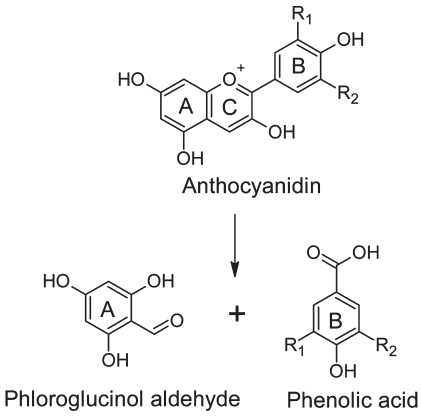

Anthocyanidin	Phenolic acid	R_1	R_2
Pelargonidin	4-Hydroxybenzoic acid	H	H
Cyanidin	Protocatechuic acid	OH	H
Delphinidin	Gallic acid	OH	OH

Figure 1. Structures of anthocyanidins and their respective phenolic acid and aldehyde constituents.

Influence of B-Ring Hydroxylation on the Rate of Anthocyanin Degradation and the Formation of Phenolic Acids. To assess the effect of B-ring hydroxylation on anthocyanin degradation and the formation of phenolic acid and aldehyde constituents under physiological conditions, pelargonidin-3-glucoside, cyanidin-3-glucoside, delphinidin-3-glucoside, pelargonidin, cyanidin, and delphinidin were individually made up to 150 μM in preincubated physiological buffer (10 mM Na/K phosphate, pH 7.4) or water (titrated to pH 7 with the dropwise addition of dilute NaOH) and incubated at 37 °C (the final DMSO concentration was 1.5%). After 0, 2, 6, 12, and 24 h, 150 μL aliquots were acidified with HCl (2% final concentration) and injected onto an HPLC-diode array detection (DAD) system. Control samples consisted of acidified (2% HCl) buffer matrices that were filtered and then spiked with anthocyanins and anthocyanidins to a final concentration of 150 μM. An automated method was utilized to further assess the rate of anthocyanin degradation in the buffer matrix, where samples were incubated in an HPLC autosampler at 37 °C with injections at 0, 2, 4, 8, and 12 h, using the same conditions as above.

Anthocyanin Recovery Following Routine Preanalytical Treatments. *Sample Preparation*. To determine the recovery of anthocyanins with different B-ring hydroxyl moieties under common experimental conditions, 0.5 M Na/K phosphate buffer, fetal calf serum (Invitrogen), and cell culture media (phenol-free DMEM) were acidified to pH ~2 with the dropwise addition of HCl and spiked to a final concentration of 25 μg/mL with either pelargonidin-3-glucoside, cyanidin-3-glucoside, or delphinidin-3-glucoside. The final serum and cell culture medium concentrations were 99.5% in a 1 mL volume. In addition, to establish the recovery of anthocyanin degradation products, protocatechuic acid, 4-hydroxybenzoic acid, gallic acid, and phloroglucinol aldehyde were spiked to 100 μM in buffer and serum samples. Recoveries were established following filtration, centrifugation, and solid-phase extraction (SPE) (as outlined below) and were quantified using HPLC-DAD.

Filtration. Sample preparations (1 mL) were filtered through 17 mm Cronus PVDF 0.45 μm syringe filters (SMI-Labhut Ltd., Maisemore, United Kingdom). The subsequent filtrates were stored at −80 °C until HPLC analysis. Control samples consisted of prefiltered, acidified, buffer matrices, spiked to 25 μg/mL with either pelargonidin-3-glucoside, cyanidin-3-glucoside, or delphinidin-3-glucoside, respectively (1 mL final volume), and exposed to the same thermal conditions as the extracted samples.

Centrifugation. Sample preparations were centrifuged at 10000 rpm for 20 min, and the supernatants were stored at −80 °C until HPLC analysis. Sample controls consisted of spiked buffer preparations, exposed to the same thermal conditions as the extracted samples, but not exposed to centrifugation.

SPE Method 1. SPE was adapted from previously reviewed methods (*18*). Briefly, Strata-X 6 mL/500 mg SPE cartridges (Phenomenex, Macclesfield, United Kingdom) were preconditioned with 1 column volume (6 mL) of methanol followed by 1 column volume of 0.5% HCl in water. Prior to extraction, samples and controls were spiked with 25 μg/mL *trans*-cinnamic acid as an internal standard. The recovery of the internal standard was established to be 94 ± 3%. SPE cartridges were loaded with 1 mL of 0.5% HCl followed by 1 mL of spiked sample preparation. Sample tubes were further rinsed with 1 mL of 0.5% HCl and loaded onto the SPE cartridges. Samples were eluted using a peristaltic pump at approximately 1 drop/s and washed with 2 column volumes of 0.5% HCl under a stream of nitrogen. Extraction cartridges were then loaded with 3 mL of 0.5% HCl in methanol and allowed to soak for 10 min. Samples were eluted with 7 mL of 0.5% HCl in methanol, and the eluate was evaporated to dryness on a rotary evaporator, redissolved in 1 mL of initial HPLC mobile phase (details below), and stored at −80 °C until HPLC analysis. Sample controls consisted of spiked buffer preparations, exposed to the same thermal conditions as the extracted samples, but not subjected to SPE.

SPE Method 2 (Minimalized Evaporation Method). SPE was performed as previously stated, with the following alterations. Prior to extraction, samples and controls were spiked to 25 μg/mL with 4-hydroxycinamic acid as an internal standard. Samples were eluted with 8 mL of acidified methanol, and the eluate was evaporated to approximately 500 μL on a rotary evaporator. Sample eluates were spiked to 25 μL with 1 mg/mL *trans*-cinnamic acid as a second internal standard. Sample volumes were adjusted to 1 mL with 0.5% HCl. Actual sample volumes were calculated from the two internal standard concentrations determined by HPLC-DAD.

Anthocyanin Freeze−Thaw Stability. To determine the storage stability of various anthocyanins, acidified buffer solutions (10 mM Na/K phosphate buffer in 2% HCl) were spiked with 150 μM delphinidin-3-glucoside, cyanidin-3-glucoside, pelargonidin-3-glucoside, delphinidin, cyanidin, or pelargonidin (individually). Samples were immediately injected onto an HPLC system and subsequently frozen at −80 °C. Samples were reanalyzed sequentially for six freeze−thaw cycles. One freeze−thaw cycle consisted of a 24 h storage at −80 °C followed by a 20 min defrost at ambient room temperature prior to HPLC analysis.

HPLC-DAD-MS Conditions. HPLC analysis was performed on an Agilent 1100 series HPLC equipped with a DAD and a 4 μm, 250 mm × 4.6 mm Synergi Max-RP reverse phase column (Phenomenex) with a SecurityGuard guard cartridge (Phenomenex AJO-6074) for samples from anthocyanin recovery and freeze−thaw experiments and a 4 μm, 250 mm × 4.6 mm Synergi Polar-RP (polar-N-capped) reverse-phase column (Phenomenex) with a SecurityGuard guard cartridge (Phenomenex AJO-6074) for samples from anthocyanin degradation experiments. The column temperature was set at 37 °C with an injection volume of 50 μL and mobile phases consisting of 5% formic acid (v/v) in water (solvent A) and 5% formic acid (v/v) in acetonitrile (solvent B). For anthocyanin recovery and freeze−thaw stability experiments, the solvent gradient consisted of 2.5% B at 0 min, 25% B at 15 min, 45% B at 20 min, and 100% B at 25−30 min at a flow rate of 1 mL/min. For anthocyanin degradation experiments, the solvent gradient consisted of 0% B at 0 min, 0.5% B at 5 min, 8% B at 20 min, 8% B at 28 min, 10% B at 39 min, 13% B at 40 min, 20% B at 44 min, 25% B at 50 min, 50% B at 65 min, and 100% B at 75−80 min at a flow rate of 1 mL/min. The absorbance was recorded at 520, 360, and 280 nm for both HPLC methods.

LC-MS Conditions. Following HPLC-DAD analysis, a subset of samples were then injected onto an HPLC-electrospray ionization (ESI)-MS system for structural elucidation, using an Agilent 1100 series HPLC-DAD coupled to an Agilent 1100 series LC/MSD SL single quadrupole mass spectrometer. ESI was performed in full scan mode (mass range, 100−1000 Da) with the following spray chamber conditions: drying gas flow of 13 L/min; nebulizer pressure of 50 Psi; and drying gas temperature of 350 °C. Positive mode ionization was used to analyze all anthocyanin species at a capillary voltage of 4000 V and a fragmentor setting of 100. Negative mode ionization was used to analyze phenolic acids, aldehyde, and anthocyanin intermediates at a capillary voltage of −3000 V and a fragmentor setting of 70. HPLC conditions were as stated above

with the following alterations. Levels of formic acid in the mobile phases were reduced to prevent ion suppression. Solvent A consisted of 0.1% formic acid in water, and solvent B consisted of 0.1% formic acid in acetonitrile. Anthocyanins, phenolic acids, and PGA were identified by retention times, optical density ratios ($\lambda_{440}/\lambda_{max}$ for anthocyanins and $\lambda_{280}/\lambda_{max}$ for phenolic acids), and UV/mass spectra in comparison to analytical standards and previously reported data (*19*−*21*). Quantification was by HPLC-DAD using standard curves generated from pure analytical standards with R^2 values greater than 0.98.

Statistical Analysis. Differences within anthocyanin species were evaluated by one-way analysis of variance (ANOVA) with Tukey posthoc tests (Windows SPSS, version 15) on concentration data derived from standard curves. Significance was determined at $P < 0.05$. To evaluate significant differences between species, all recovery and degradation data were transformed to percent of control values and analyzed using one-way ANOVA with Tukey posthoc tests. Differences between anthocyanin degradation within water and buffer matrices were analyzed by independent Student's t tests with a significance threshold of $P < 0.05$. All treatments were performed in triplicate, and data were obtained from three independent experiments.

RESULTS AND DISCUSSION

Influence of B-Ring Hydroxylation on Anthocyanidin (Aglycone) Stability. *Anthocyanidin Degradation*. To assess anthocyanidin stability under simulated physiological conditions, their degradation was determined in both water and buffered matrices (**Figure 2**). In both matrices, an instantaneous loss of >50% of the initial anthocyanidins was observed, with 100% degradation occurring by the first time point measurement (2 h); therefore, rates of degradation were not established. The loss of anthocyanidins over 24 h was associated with the formation of their respective phenolic acids.

Phenolic Acid Formation. In water, the formation of phenolic acids accounted for 30−40% of initial anthocyanidin molar concentrations, while in buffer, the phenolic acid formation accounted for only 8−18% of initial anthocyanidin molar concentrations. In water, pelargonidin (λ_{max} 512 nm; m/z 271), cyanidin (λ_{max} 524 nm; m/z 287), and delphinidin (λ_{max} 528 nm; m/z 303) degraded to yield 53 ± 4.2 μM 4-hydroxybenzoic acid (λ_{max} 254 nm; m/z 138) (accounting for 38 ± 0.1% pelargonidin loss), 58 ± 1.2 μM protocatechuic acid (λ_{max} 260 and 294 nm; m/z 154) (accounting for 39 ± 0.0% of cyanidin loss), and 53 ± 2.0 μM gallic acid [λ_{max} 270 nm; m/z 170] (accounting for 31 ± 0.0% of delphinidin loss), respectively.

In buffered samples, pelargonidin, cyanidin, and delphinidin degraded to yield 25 ± 0.7 μM 4-hydroxybenzoic acid (accounting for 18 ± 0.0% of pelargonidin loss), 32 ± 0.3 μM protocatechuic acid (accounting for 21 ± 0.0% of cyanidin loss), and 14 ± 0.3 μM gallic acid (accounting for 8 ± 0.0% of delphinidin loss), respectively. It should be noted that the formation of gallic acid (associated with delphinidin degradation) increased linearly in water, while in buffer, gallic acid showed first an increase in formation followed by a decrease (loss) in concentration between 6 and 24 h.

Phenolic Aldehyde Formation. All anthocyanin species degraded to their common A-ring constituent, tentatively identified as phloroglucinol aldehyde (λ_{max} 294 nm; m/z 154). After 24 h, phloroglucinol aldehyde formation was 46 ± 1.6 μM in water and 5 ± 1.2 μM in buffered samples (±SD for pelargonidin, cyanidin, and delphinidin) accounting for 30 and 3% of initial anthocyanin concentrations, respectively.

Influence of B-Ring Hydroxylation on Anthocyanin (Glycoside) Stability. *Anthocyanin Degradation*. The influence of B-ring hydroxylation on the rate of anthocyanin degradation and the degree of phenolic acid formation was assessed under simulated physiological conditions (**Figure 3**). The anthocyanins were shown to degrade at a rate of between 3 and 11 μM/h over the first 12 h, representing a total loss of 20, 40, and 100% for pelargonidin-3-glucoside (λ_{max} 502 nm; m/z 433), cyanidin-3-glucoside (λ_{max} 514 nm; m/z 449), and delphinidin-3-glucoside (λ_{max} 524 nm; m/z 465), respectively. Individual rates of degradation were 3.3 μM/h for pelargonidin-3-glucoside, 4.5 μM/h for cyanidin-3-glucoside, and 11.4 μM/h for delphinidin-3-glucoside.

Phenolic Acid Formation. In water, the loss of anthocyanins over 24 h was associated with the formation of their respective phenolic acids, which accounted for 12, 8, and 0% of the initial cyanidin-3-glucoside, delphinidin-3-glucoside, and pelargonidin-3-glucoside concentrations, respectively. Cyanidin-3-glucoside degraded to yield 15 ± 14.4 μM protocatechuic acid (accounting for 65 ± 27.3% of cyanidin-3-glucoside loss), and delphinidin-3-glucoside degraded to yield 12 ± 9.5 μM gallic acid (accounting for 56 ± 41.7% of delphinidin-3-glucoside loss). There was no significant degradation of pelargonidin-3-glucoside or respective formation of phloroglucinol aldehyde over the 24 h incubation.

In buffer, the appearance of phenolic acids accounted for 10, 8, and 2% of initial pelargonidin-3-glucoside, cyanidin-3-glucoside, and delphinidin-3-glucoside concentrations, respectively. Pelargonidin-3-glucoside, cyanidin-3-glucoside, and delphinidin-3-glucoside degraded to yield 16 ± 2.5 μM 4-hydroxybenzoic acid (accounting for 12 ± 1.8% of pelargonidin-3-glucoside loss), 11 ± 1.8 μM protocatechuic acid (accounting for 9 ± 1.3% of cyanidin-3-glucoside loss), and 3 ± 0.4 μM gallic acid (accounting for 2 ± 0.0% of delphinidin-3-glucoside loss), respectively.

Phenolic Aldehyde Formation. The degradation of cyanidin-3-glucoside, delphinidin-3-glucoside, and pelargonidin-3-glucoside was associated with the appearance of a common constituent in buffered matrices; however, its formation was not significant in pelargonidin-3-glucoside samples. The maximum phloroglucinol aldehyde formation was 5 ± 1.1 μM for cyanidin-3-glucoside and 1 ± 0.1 μM for delphinidin-3-glucoside, accounting for 3 and 0.6% of initial anthocyanin concentrations. No significant phloroglucinol aldehyde formation was observed for any of the anthocyanin species in water.

The degradation of the three anthocyanin species as described above was associated with the appearance of a common constituent, which was identified as phloroglucinol aldehyde, based on its retention time, UV−vis, and MS characteristics. It is to be noted that phloroglucinol aldehyde appeared as a split peak, which may be the result of the ionization characteristic of the aldehyde structure under acidic aqueous conditions, where the dynamic equilibrium of the hydrated form and heterodimers is likely to occur (*22*, *23*).

The degradation of all anthocyanin aglycone and glucoside species was also associated with the appearance of three unidentified compounds, eluting at similar retention times. Because these compounds were common to all anthocyanin species in this study, they are likely to be derived from the common anthocyanin A ring. However, mass fragmentation ions could only be obtained for one of the three unidentified compounds (R_t 6.6 min; m/z 198), with the remaining two unidentified compounds showing no ion fragments in both positive and negative ionization modes under the utilized methods.

Anthocyanin Recovery Following Routine Sample Preparation Techniques. To assess the effects of B-ring hydroxylation on the recovery of anthocyanins during routine preanalytical sample preparation, pelargonidin-3-glucoside, cyanidin-3-glucoside, and delphinidin-3-glucoside recovery and degradation were measured following filtration, centrifugation, and SPE in buffer, serum, and cell culture matrices (**Figure 4**). No significant differences in recovery were observed between pelargonidin-3-glucoside and delphinidin-3-glucoside following filtration and centrifugation,

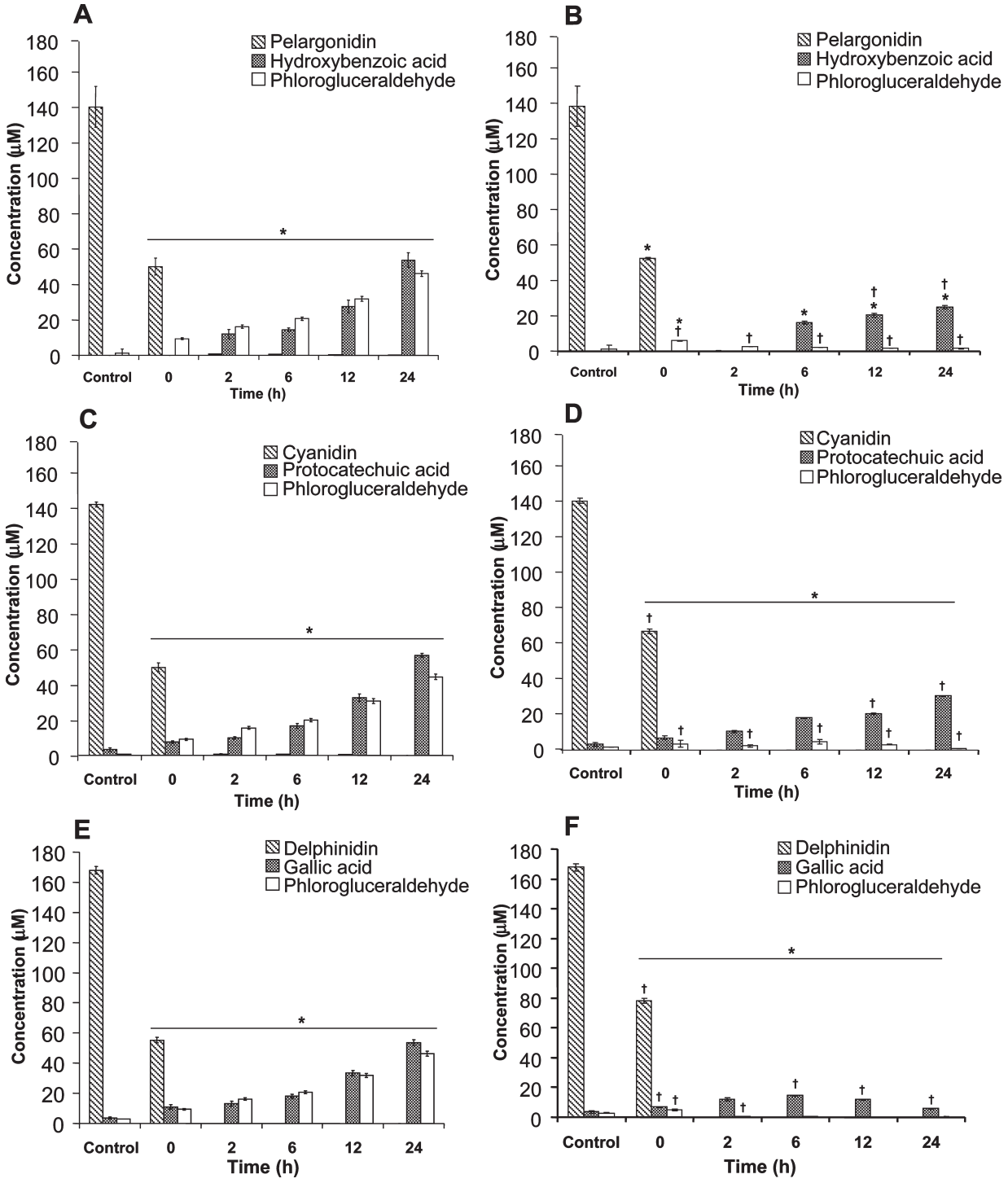

Figure 2. Anthocyanidin degradation over 24 h at 37 °C in water (pH 7 with dilute NaOH) and in physiological buffer (10 mM Na/K phosphate buffer, pH 7.4). (**A**) Pelargonidin degradation in water, (**B**) pelargonidin degradation in buffer, (**C**) cyanindin degradation in water, (**D**) cyanidin degradation in buffer, (**E**) delphinidin degradation in water, and (**F**) delphinidin degradation in buffer. Results expressed as means ± SDs (n = 3). *Significance from controls ($P < 0.05$), which consisted of analytical standards spiked directly into acidified water or buffer matrices at t = 0. †Significant difference ($P < 0.05$) between samples incubated in water and buffer matrices within a given anthocyanin species and incubation time.

although cyanidin-3-glucoside did show significantly lower recovery (relative to pelargonidin-3-glucoside and delphinidin-3-glucoside) in serum and cell media matrices. No significant difference in anthocyanin recovery was observed between filtered and centrifuged samples for a given anthocyanin species. All anthocyanin species showed significant loss following routine SPE, while SPE with an incomplete concentration/evaporation step (i.e., not taken to dryness) significantly improved their stability by 32, 29, and 18% for pelargonidin-3-glucoside (in buffer, serum, and cell media, respectively) and 14, 19, and 0% for delphinidin-3-glucoside (in buffer, serum, and cell media, respectively). Cyanidin-3-glucoside did not show an improvement in recovery. We speculate that this is likely due to the chelating and/or protein binding properties of the catechol group on the anthocyanin B ring (*24*).

The losses of anthocyanins following sample preparation were accounted for by a stoichiometric appearance of their respective phenolic acid constituents, where pelargonidin-3-glucoside and

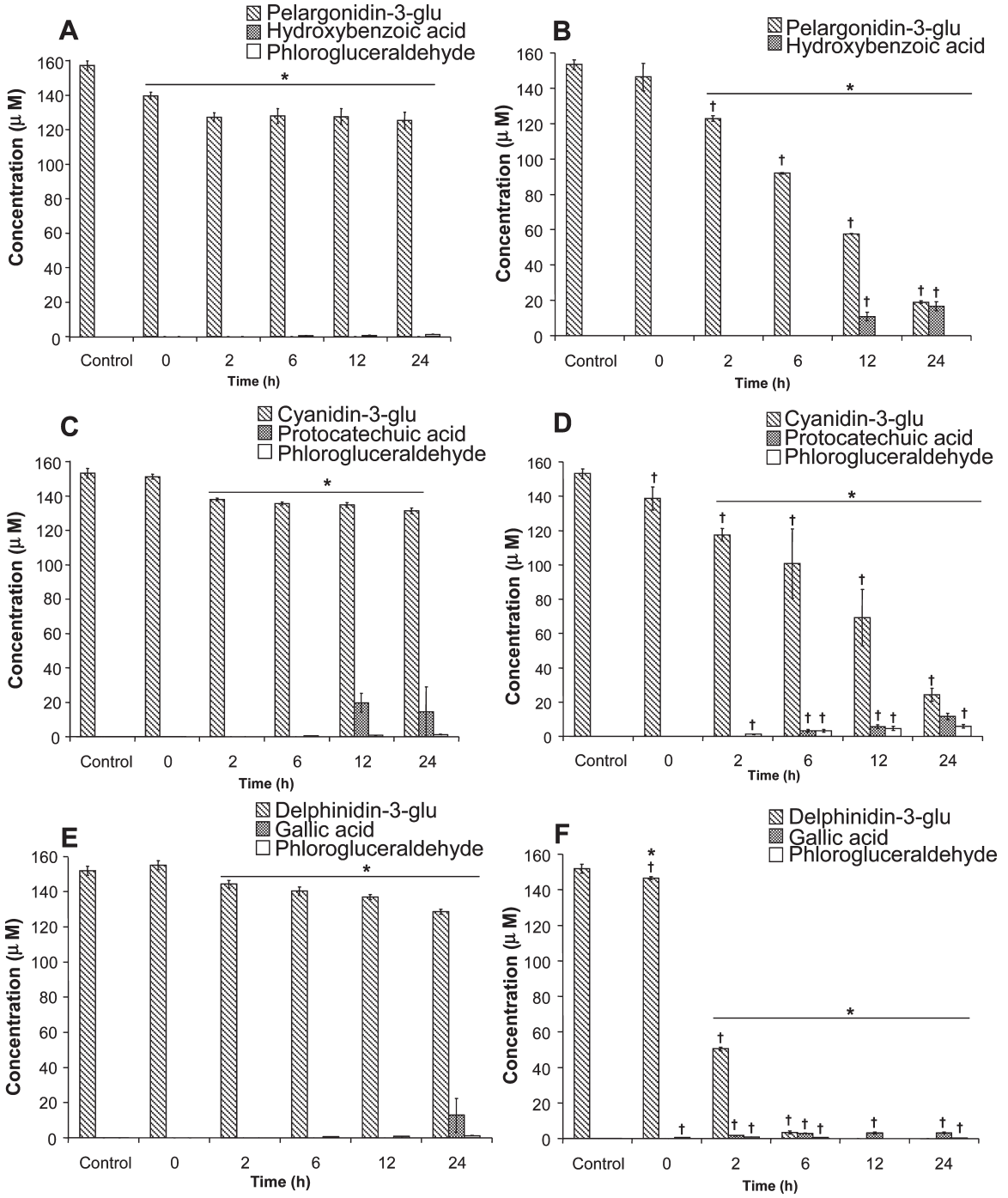

Figure 3. Anthocyanin degradation over 24 h at 37 °C in water (pH 7 with dilute NaOH) and in physiological buffer (10 mM Na/K phosphate buffer, pH 7.4). (**A**) Pelargonidin-3-glucoside degradation in water, (**B**) pelargonidin-3-glucoside degradation in buffer, (**C**) cyanindin-3-glucoside degradation in water, (**D**) cyanidin-3-glucoside degradation in buffer, (**E**) delphinidin-3-glucoside degradation in water, and (**F**) delphinidin-3-glucoside degradation in buffer. Results expressed as means ± SDs ($n = 3$). *Significance from controls ($P < 0.05$), which consisted of analytical standards spiked directly into acidified water or buffer matrices at $t = 0$. †Significant difference ($P < 0.05$) between samples incubated in water and buffer matrices within a given anthocyanin species and incubation time.

cyanidin-3-glucoside were recovered as 4-hydroxybenzoic acid and protocatechuic acid, respectively (**Figure 4**). Delphinidin-3-glucoside degradation to gallic acid was not observed following SPE. To assess the recovery of the phenolic acids following SPE, buffer and serum samples were spiked with 100 μM standards of protocatechuic acid, 4-hydroxybenzoic acid, and phloroglucinol aldehyde, respectively. The recoveries of protocatechuic acid, 4-hydroxybenzoic acid, and phloroglucinol aldehyde standards were established to be 92 ± 2.8, 92 ± 2.5, and 78 ± 2.6% in buffered samples and 89 ± 2.3, 115 ± 2.5, and 68 ± 2.8% in serum samples, respectively. Note that the recovery of 4-hydroxybenzoic acid in serum was >100%, which may have resulted from additional endogenous 4-hydroxybenzoic acid liberated from serum samples following methanol treatment during SPE, as recoveries were compared to spiked buffer controls.

While the loss of anthocyanins could be accounted for by the recovery of their respective B-ring phenolic acid constituents, phloroglucinol aldehyde (the A-ring constituent) was not consistently identified in postextraction samples (i.e., post-SPE). In addition, the disappearance of anthocyanins was associated with

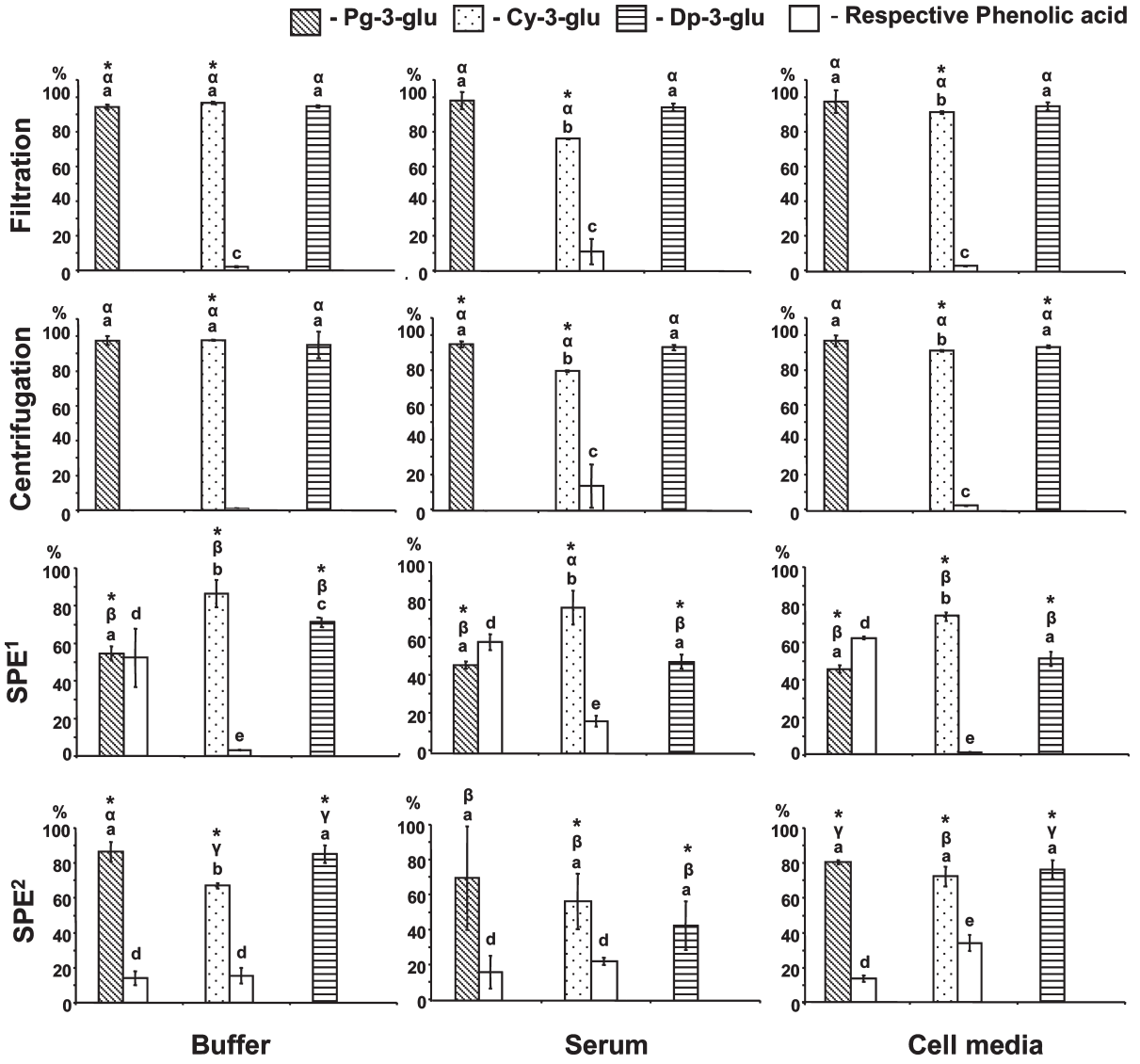

Figure 4. Anthocyanin recovery (%) and the formation of their respective phenolic acid constituents following filtration, centrifugation, and SPE in phosphate buffer (pH 2), fetal calf serum (pH 2), and cell culture media (pH 2). Values are expressed as means ± SDs ($n = 3$). *Significance from controls ($P < 0.05$). Like letters (a−e) represent statistically similar recoveries between anthocyanin species within a given preparation technique and matrix. Like symbols (α, β, and γ) represent statistically similar recoveries between preparation techniques for a given anthocyanin species within a given matrix. [1]SPE method with complete evaporation. [2]SPE method with minimalized evaporation. Controls consisted of anthocyanins spiked into pre-extracted buffer matrices.

the formation of an additional unidentified peak (λ_{max} 272 nm; R_t 21.5 min; m/z 189, 226.9, 248.8), which was similar to the compound formed when phloroglucinol aldehyde was incubated in the presence of acidified methanol (λ_{max} 272 nm; T_R 22.4 min; m/z 187,189).

Anthocyanin and Anthocyanidin Freeze−Thaw Stability. To assess the influence of B-ring hydroxylation on anthocyanin freeze−thaw stability, anthocyanin species, with mono-, di-, and trihyroxylated B rings, were frozen and thawed six times, and their degradation was assessed by HPLC-DAD. It was found that anthocyanin glucosides were stable during storage and freeze−thaw treatment, with no significant losses observed. Of the three aglycone species, both pelargonidin ($P = 0.001$) and cyanidin ($P = 0.026$) showed a significant loss following freeze−thaw cycling, while delphinidin remained stable. Pelargonidin showed a linear rate of degradation, with significant losses demonstrated at four freeze−thaw cycles and a total reduction of 17 μM (11 ± 0.0%) at six freeze−thaw cycles (representing an average loss of 1.8% per cycle). Cyanidin also showed a linear rate of degradation, with a maximum reduction of 10 μM (6 ± 0.0%) at six freeze−thaw cycles (representing an average loss of 1.2% per cycle).

On the whole, anthocyanins have consistently been shown to possess a very low bioavailability (*12*−*14*, *25*). We propose that the poor systemic availability of anthocyanins is due to their instability and physiochemical degradation in vivo or during routine clinical and laboratory sample processing. Therefore, this study aimed to quantitatively investigate the effect of structure on the degradation of anthocyanins under simulated (in vitro) physiological conditions and assess the degradation and recovery of selected anthocyanins following preanalytical sample treatment and storage.

Evidence presented here shows that under simulated (in vitro) physiological conditions (pH and temperature), anthocyanidins degraded at much higher rates than their glucoside counterparts, which was consistent with previously reported data (*7*−*10*). Indeed, the rate of degradation varied according to the B-ring hydroxylation pattern such that an increase in hydroxylation was associated with a decrease in stability. Furthermore, this study demonstrates that anthocyanin degradation under physiological

conditions is associated with the stoichiometric formation of phenolic acid constituents. As the observed rates of anthocyanidin degradation to phenolic acids were rapid, it is likely that these compounds are present at far higher concentrations than anthocyanidins/anthocyanins in processed foods, the gut, and the systemic circulation. Although it is likely that pH and temperature are the chief contributors to anthocyanin degradation under biological conditions, the extent to which other physiological forces, such as protein interactions and enzymatic or microbial transformation, contribute to anthocyanin degradation is yet to be fully elucidated.

Phloroglucinol aldehyde represented the A-ring constituent of anthocyanins in the present investigation; however, it is possible that phloroglucinol aldehyde could also be derived from other sources in the diet, as this A-ring constituent is common to many other flavonoids. Further study is, however, required to fully establish the extent to which phloroglucinol aldehyde formation is derived from the degradation of anthocyanins and other flavonoids within the diet. An apparent lack of appreciation for the rate and extent of anthocyanin degradation under physiological conditions may explain the almost complete absence of data aimed at quantifying anthocyanin breakdown products in human samples (tissue, urine, and plasma) and the current lack of reports concerned with the biological activities of these compounds and their impact, as dietary constituents on human health. We contend that anthocyanins may be regarded as "pro-drugs" for the delivery of bioactive phenolic intermediates, and this should be a focus for future research.

In this report, we provide evidence to show that anthocyanins degrade at a faster rate in buffered solution than in water. However, the rate of end stage degradation product (phenolic acids) formation was relatively slower in buffered solution than in water. These observations imply that the buffered environment confers a greater stability to the pH-dependent anthocyanin isoforms (including chalcone, hemiketal, and α-diketone intermediate structures). However, it is possible that slight differences in sample pH could account for some of the differences in anthocyanin stability as observed in the present investigation. As biological samples (i.e., serum, urine, and cell culture media) possess a degree of buffering agents, the degradation characteristics of anthocyanins in these matrices should be considered in light of the current finding, where anthocyanins may demonstrate differential degradation rates in other matrices such as foods, which will be dependent on their individual buffering characteristics.

The data presented here show that B-ring hydroxylation significantly affected the recovery of anthocyanins following routine SPE. This observation suggests that an underestimation of anthocyanin levels in clinically derived samples is possible when employing SPE. Furthermore, we observed that the disappearance of anthocyanins was accompanied by the stoichiometric formation of their respective phenolic acid breakdown products, demonstrating that degradation rather than poor recovery was the foremost cause of anthocyanin loss following SPE, particularly in the case of cyanidin-3-glucoside. This highlights the importance of establishing method accuracy and precision when estimating anthocyanin levels in clinical studies. Our data suggested that the degradation of anthocyanins observed during SPE may be the result of sample evaporation. We provide direct evidence that evaporation of SPE eluents to dryness contributes significantly to loss of anthocyanins and that recoveries can be substantially improved by partially evaporating SPE eluents. This may be the result of acid concentration and eventual hydrolysis in non-neutralized samples during post-SPE evaporation. Thus, it is clear that both sample preparation technique and anthocyanin structure contribute to their experimental recovery. Consequently, these factors should be considered when analyzing clinical or biological samples (such as cell culture medium, serum/plasma, or urine) to avoid the underestimation of anthocyanin bioavailability.

While some studies have evaluated the storage stability of anthocyanins in various food matrices (*26*−*28*), no studies have formally reported their storage stability under clinically relevant conditions. Thus, this study has shown that anthocyanins are stable when stored at −80 °C and ≤pH 2, while anthocyanidins are relatively less stable. This indicates that the poor recovery of anthocyanins following clinical feeding trials is not likely to be the result of degradation in stored samples but the result of degradation in vivo or during initial sample processing.

In conclusion, this report demonstrates that anthocyanins are rapidly degraded to their phenolic acid and aldehyde constituents under simulated (in vitro) physiological conditions and that increased anthocyanin B-ring hydroxylation is associated with a decrease in stability; these observations have major implications for the design and interpretation of dietary intervention studies utilizing anthocyanins. Furthermore, anthocyanin losses during sample preparation were accounted for by the stoichiometric appearance of their phenolic acid degradation products, particularly during SPE. Hence, we suggest that the degradation of anthocyanins and the representative formation of their phenolic acid constituents be of important consideration when investigating anthocyanins within clinical and laboratory settings and that these degradation products be targeted in future bioavailability and bioactivity studies to establish their true occurrence and influence on human health and disease.

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