Endogenous Enzymes, Heat, and pH Affect Flavone Profiles in Parsley (*Petroselinum crispum* var. *neapolitanum*) and Celery (*Apium graveolens*) during Juice Processing

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ABSTRACT: Flavones are abundant in parsley and celery and possess unique anti-inflammatory properties in vitro and in animal models. However, their bioavailability and bioactivity depend in part on the conjugation of sugars and other functional groups to the flavone core. The effects of juice extraction, acidification, thermal processing, and endogenous enzymes on flavone glycoside profile and concentration in both parsley and celery were investigated. Parsley yielded 72% juice with 64% of the total flavones extracted, whereas celery yielded 79% juice with 56% of flavones extracted. Fresh parsley juice averaged 281 mg flavones/100 g and fresh celery juice, 28.5 mg/100 g. Flavones in steamed parsley and celery were predominantly malonyl apiosylglucoside conjugates, whereas those in fresh samples were primarily apiosylglucoside conjugates; this was apparently the result of endogenous malonyl esterases. Acidification and thermal processing of celery converted flavone apiosylglucosides to flavone glucosides, which may affect the intestinal absorption and metabolism of these compounds.

KEYWORDS: *flavones, celery, parsley, juice, apigenin, mass balance*

■ INTRODUCTION

Flavones are a class of flavonoids found in a variety of fruits and vegetables and are most abundant in artichoke heads, kumquats, parsley, and celery.[1−3] In their native forms they are conjugated to sugars, simple acids (acetyl and malonyl), and cinnamic acids (Figure 1) rather than existing as aglycones. In vitro studies demonstrate that the flavone apigenin inhibits human lung, colon, breast, prostate, brain, and skin cancer cells;[4−8] tongue cancer;[9] and leukemia.[10] Apigenin and luteolin also reduce monocyte adhesion to LDL in vitro, showing potential to prevent one of the initial stages of atherosclerosis.[11] In addition, animal studies with flavones demonstrate the ability to attenuate the inflammatory response.[12,13]

The glycosylation of flavones in food is important because intestinal hydrolysis of flavonoid glycosides varies according to the sugars and other functional groups attached to the flavone core. Similar to other flavonoids, flavone glucosides can be hydrolyzed by β-glucosidase in the small intestine to their respective aglycones,[14] which can be absorbed in the small intestine and subsequently glucuronidated and sulfated by the small intestine and liver before reaching systemic circulation.[15,16] In contrast, flavonoid glycosides with disaccharide and malonyl moieties are resistant to intestinal β-glucosidase relative to their simple glucoside counterparts, potentially limiting their bioavailability.[14] Therefore, determining the native glycosides is potentially useful when functional foods are developed.

Using a Caco-2 model, apigenin and luteolin aglycones were more readily absorbed into intestinal cells and 10 times more of the aglycone forms were transported across the intestinal epithelium than apigenin 6-*C*-glucoside or luteolin 7-*O*-glucoside.[17] Experiments with isolated rat small intestine showed a similar pattern for isoflavones. Nearly twice as much genistein and daidzein (aglycones) was absorbed and transported to the basolateral side than their 7-*O*-glucoside

counterparts, and the malonyl glucoside conjugates were absorbed in only trace amounts.[18]

A review of the literature and a screening of a variety of fruits and vegetables revealed that parsley and celery were particularly rich in flavones occurring as their apiosylglucosides.[19,20] On the basis of their flavone concentrations and the biological activity of flavones previously cited, parsley and celery can potentially be used in a food-based approach to the prevention of chronic disease. In the current study, the objective is to determine how juice processing, endogenous enzymes, acidification, and thermal processing affect the stability and glycosylation of flavones in parsley and celery. Flavone glycosides were first identified and quantified in fresh and steamed parsley and celery, in pomace, and in juice after extraction. Flavones were then quantified in juices after acidification and thermal processing. These processes were optimized to produce flavone derivatives that may have enhanced bioavailability and efficacy.

■ MATERIALS AND METHODS

Chinese celery (*Apium graveolens* L., Apiaceae) and Italian parsley (*Petroselinum crispum* Mill., Apiaceae) were purchased from local grocery stores in Columbus, OH. Chrysoeriol and luteolin 7-*O*-glucoside were purchased from Extrasynthese (Genay, France); apigenin, luteolin, and citric acid monohydrate from Sigma (St. Louis, MO); and diosmetin and apigenin 7-*O*-glucoside from Chromadex (Irvine, CA). All other reagents were from Fisher Scientific (Fair Lawn, NJ).

Effects of Steaming on Flavone Conjugation in Parsley and Celery. To test for endogenous enzymes and possible effects on flavones, celery leaves or parsley were steamed prior to maceration to inactivate enzymes and compared to unheated control samples. All
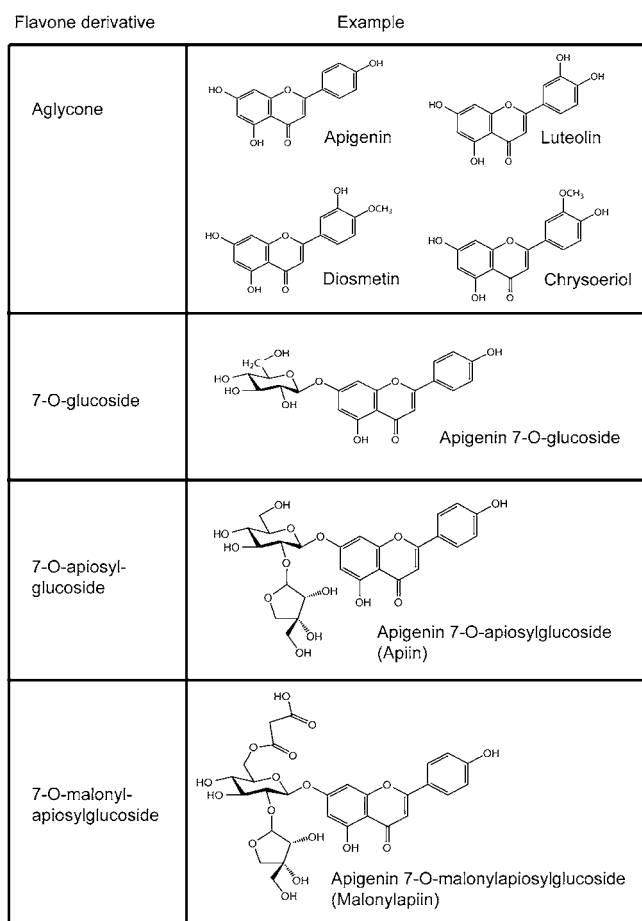
Flavone derivative | Example

Figure 1. Structures of common flavone derivatives.

samples were prepared by first rinsing and spin-drying 5 g of fresh celery leaves or parsley. Steamed samples were held in a sieve over 1 L of boiling water for 10 min to inactivate endogenous enzymes. Steamed or untreated celery leaves and parsley were then ground in liquid nitrogen with a mortar and pestle and incubated at 37 or 20 °C for 3 h to allow any remaining endogenous enzymes to act on flavonoids in the samples. Experiments were done in triplicate and samples extracted as described below.

Juice Extraction and Processing. Juicing experiments were carried out in triplicate, and the steps for processing are outlined in Figure 2. Celery and parsley stalks and leaves were used for juice
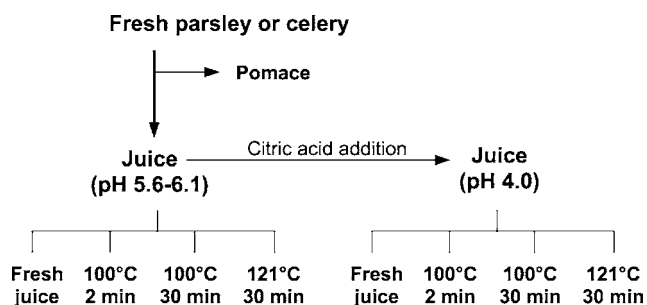

Figure 2. Processes for juicing, acidification, and thermal processing of parsley and celery.

extraction with an Omega 8006 masticating juicer (Harrisburg, PA). To determine the effects of pH and heat on flavones during processing, juice was divided into two lots with one left at its original pH (5.6–6.2) and the other acidified to pH 4.0 with citric acid. Each lot was then processed at 100 °C for 2 or 30 min in a boiling water

bath or at 121 °C for 30 min in a Splendid pressure cooker (Fagor America, Lyndhurst, NJ). To further explore the effects of heat and pH on flavone stability and profiles, celery juices were acidified to pH 3.0, 4.0, or 5.0 and heated at 121 °C for 15, 30, 45, or 60 min (Figure 3).
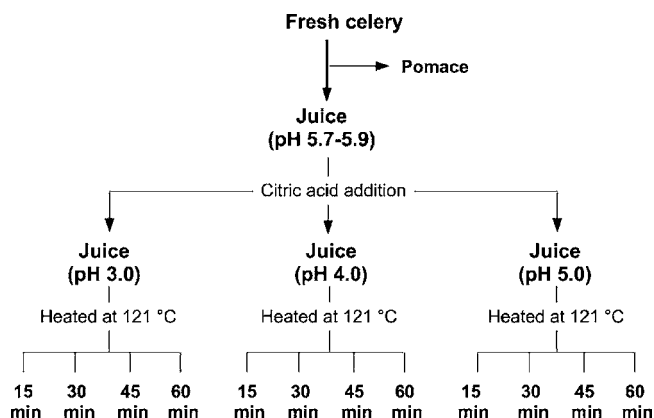

Figure 3. Flow diagram for acidification and thermal processing of celery juice.

Flavone Extraction. Steamed and untreated samples were cooled on ice after incubation, and 0.5 g of each was extracted. To assess the mass balance of flavones in parsley and celery during processing (Figure 2), duplicate samples were lyophilized and 0.1 g of each was extracted. Acidified celery juices were processed at 121 °C (Figure 3), and 1.0 g was extracted after processing and cooling. All samples were extracted three times with 2.5 mL of 70% (v/v) aqueous methanol, and the combined supernatants were analyzed by high-performance liquid chromatography (HPLC).

Identification by HPLC–Mass Spectrometry. Flavone glycosides were tentatively identified by electrospray mass spectrometry (ESI-MS) with a QTOF Premier (Micromass, Manchester, U.K.) using accurate mass and fragmentation patterns in positive and negative ionization modes. Source parameters included the following: capillary voltage, 2.8 kV in negative mode and 3.2 kV in positive mode; cone, 35 V; source block, 110 °C, collision energy, 8 V. Desolvation gas was delivered at 600 L/h and 450 °C, cone gas at 50 L/h, and collision-induced dissociation gas at 2.5×10^{-3} mBar (argon). Sodium formate was used to calibrate and leucine-enkephalin as a lockspray reference. MS^E experiments were applied where alternating low and high collision energies were used to view precursors and fragments in a single run. MS/MS scans were then used to confirm parent–daughter relationships.

HPLC Analysis. Analysis of celery, parsley, and juice samples was conducted by HPLC. Apigenin, luteolin, chrysoeriol, diosmetin, apigenin 7-*O*-glucoside, and apigenin 7-*O*-apiosylglucoside standards were used to identify compounds by UV spectra and retention times. Other flavone glycosides were identified by ESI-MS as described above. For quantification, the HPLC system included a Waters 2695 separation module, a 2996 photodiode array detector, a Symmetry C_{18} column (3.5 μm, 4.6 × 75 mm), and Empower software (Waters Corp., Milford, MA). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), and the column was maintained at 30 °C. For fresh and steamed parsley and celery, the following gradient method was used: 0–1 min, 5–19% B; 1–6.5 min, 19% B; 6.5–7 min, 19–20% B; 7–13 min, 20% B; 13–15 min, 20–55% B; 15–18 min, 55–80% B; 18–20 min, 80–100% B; 20–22 min, 100% B; 22–25 min, 5% B. Juice samples were separated with the following gradient: 0–1 min, 5–18% B; 1–13 min, 18% B; 13–15 min, 18–100% B; 15–17 min, 100% B; 17–20 min, 5% B. Peak areas were measured at 338 nm for apigenin and at 350 nm for luteolin, diosmetin, and chrysoeriol. Because the flavone glycosides in this study share the same chromophore as their aglycone counterparts, concentrations of all flavones were determined with aglycone standard curves.
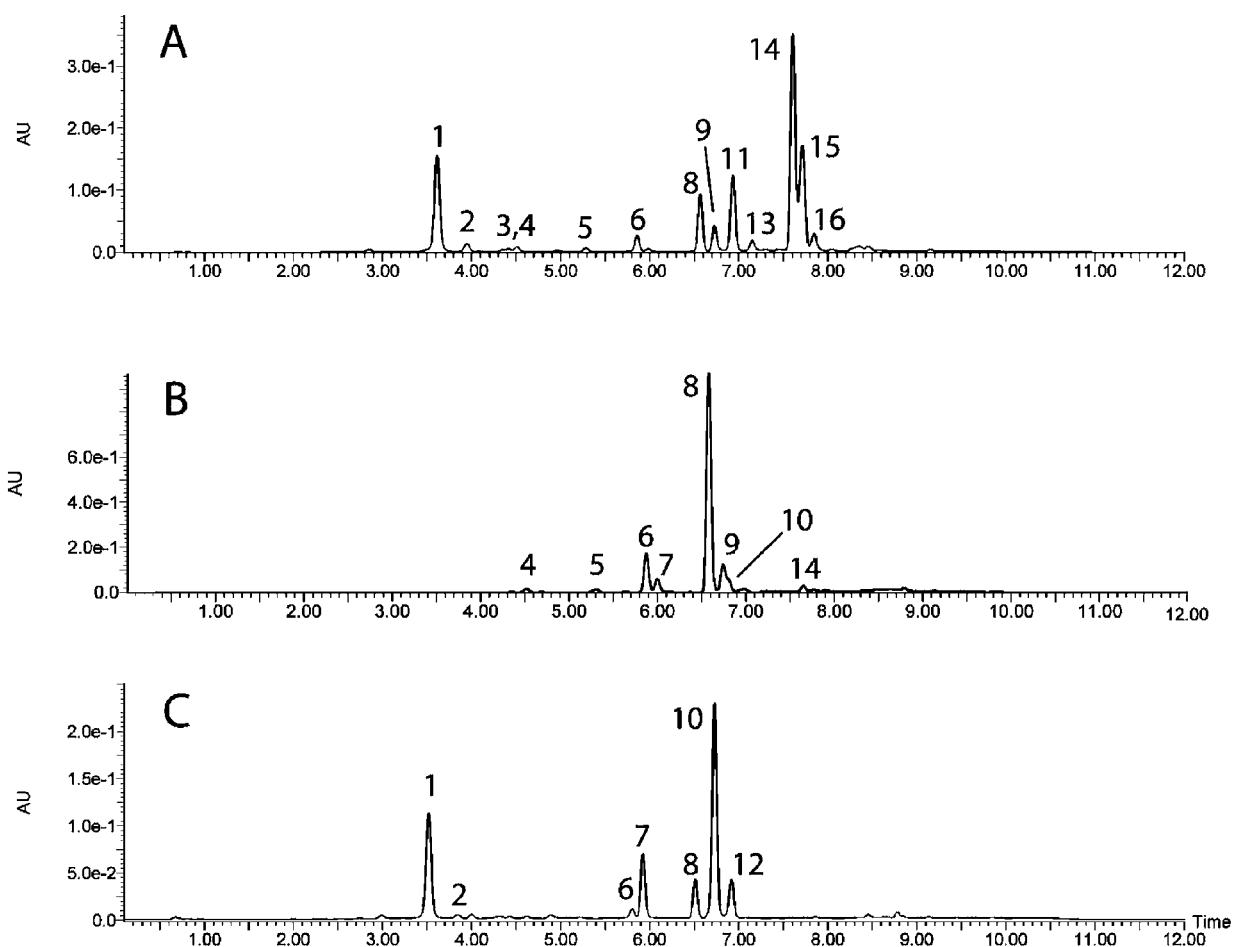
Figure 4. HPLC chromatograms of celery extracts: (A) celery steamed for 10 min, macerated, and incubated for 3 h at 37 °C; (B) celery macerated and incubated for 3 h at 37 °C; (C) celery juice acidified to pH 3.0 and processed for 60 min at 121 °C.

Statistical Analysis. Statistical analysis was performed with R 2.11 for Windows (R Foundation for Statistical Computing, Vienna, Austria) using one- or two-way analysis of variance (ANOVA) blocking for different batches of celery or parsley. Post hoc comparisons were done with Tukey's HSD.

RESULTS

Identification of Flavones by HPLC-MS. Representative HPLC chromatograms for steamed celery, fresh celery, and acidified, thermally processed celery juice are provided in Figure 4. Peaks were identified by retention time (RT), UV–visible absorbance (λ_{max}), mass of parent ions in positive and negative modes ([M + H]$^+$ and [M − H]$^-$), and mass of aglycone ions after fragmentation ([A + H]$^+$ and [A − H]$^-$) (Table 1). Chlorogenic and coumaroylquinic acids were detected only as negative ions, and flavone malonyl glucosides were detected as acetyl glucosides in negative mode due to decarboxylation (Table 1). The compounds identified in fresh and steamed celery and parsley have been previously reported,[19,20] but apigenin 7-O-glucoside in acidified, thermally processed juice samples is newly identified (Figure 4C; Table 1).

Effects of Steaming on Flavone Conjugation in Parsley and Celery. To evaluate the effects of endogenous enzymes on flavone conjugation, celery leaves and parsley were steamed for 10 min prior to incubation and compared with fresh celery and parsley samples. When celery leaves or parsley were steamed before incubation at 37 or 20 °C for 3 h,

significantly greater amounts of flavone glycosides were present as malonyl apiosylglucosides and significantly less were present as apiosylglucosides compared to fresh samples (Tables 2 and 3). Fresh celery leaves and parsley contained predominantly flavone apiosylglucosides, and there were no significant differences associated with incubation temperature. There were no significant differences in total flavone concentrations between any of the treatments (Tables 2 and 3).

Mass Balance of Flavones during Juice Processing. Parsley yielded 72% juice (w/w) with similar amounts of apigenin and diosmetin glycosides. Whereas the majority of apigenin, diosmetin, and total flavones were found in the juice after extraction, nearly one-third of the flavones were retained in the pomace (Table 4). Celery yielded 79% juice, and slightly more apigenin, luteolin, and chrysoeriol were found in the juice than in the pomace (Table 5). In fresh celery and juice, apigenin glycosides were the most abundant, accounting for over half of all flavones.

Effects of pH and Heat Processing on Flavone Conjugation in Parsley and Celery Juices. To determine the effects of pH and thermal processing on parsley and celery juices, untreated juices (pH 5.6–6.2) were compared with acidified juices (pH 4.0) and were heated at 100 or 121 °C for 2–30 min. In parsley juice apigenin concentrations averaged 116–140 mg/100 g fresh weight (FW); diosmetin, 130–158 mg/100 g FW; and total flavones 247–298 mg/100 g FW. There were no significant differences associated with pH and

Table 1. Tentative Peak Assignments for Chromatograms of Celery Extracts Based on HPLC-MS Data

peak	RT[a] (min)	λ_max (nm)	[M + H]⁺/ [M − H]⁻[b] (m/z)	[A + H]⁺/[A − H]⁻[c] (m/z)	identification
1	3.59	326	−/353	−/191	chlorogenic acid
2	3.92	327	−/353	−/191	chlorogenic acid
3	4.41	312	−/353	−/191	chlorogenic acid
4	4.51	312	−/337	−/191	coumaroylquinic acid
5	5.27	306	−/337	−/191	coumaroylquinic acid
6	5.85	255, 349	581/579	287/285	luteolin 7-O-apiosylglucoside
7	6.00	255, 349	449/447	287/285	luteolin 7-O-glucoside
8	6.55	267, 338	565/563	271/269	apigenin 7-O-apiosylglucoside
9	6.70	253, 348	595/593	301/299	chrysoeriol 7-O-apiosylglucoside
10	6.80	267, 338	433/431	271/269	apigenin 7-O-glucoside
11	6.92	254, 348	667/621[d]	287/285	luteolin 7-O-malonyl apiosylglucoside
12	6.99	253, 347	463/461	301/299	chrysoeriol 7-O-glucoside
13	7.14	253, 345	535/489[d]	287/285	luteolin 7-O-malonyl glucoside
14	7.59	267, 337	651/605[d]	271/269	apigenin 7-O-malonyl apiosylglucoside
15	7.70	253, 345	681/635[d]	301/299	chrysoeriol 7-O-malonyl apiosylglucoside A
16	7.83	253, 348	681/635[d]	301/299	chrysoeriol 7-O-malonyl apiosylglucoside B

[a]Retention time. [b][M + H]⁺/[M − H]⁻ positive and negative parent ions. Chlorogenic and coumaroylquinic acids were detected only in negative mode. [c][A + H]⁺/[A − H]⁻ positive and negative aglycone ions. [d]Malonyl glycosides were detected as acetyl glycosides due to decarboxylation in negative mode.

thermal treatments in parsley juice. Celery juice acidified and heated to 121 °C for 30 min had higher apigenin concentrations than those at their native pH, and juice heated to 100 °C for 30 min had higher luteolin concentrations than nonacidified juice heated for only 2 min (data not shown). Flavone apiosylglucosides were most abundant in all juices, followed by malonyl apiosylglucosides. Parsley and celery juices acidified to pH 4.0 and heated at 121 °C for 30 min showed 10−15% conversion of apiosylglucosides to simple glucosides, whereas flavone 7-O-glucosides were not detected in juices left at their native pH and heated at 121 °C (data not shown).

Although parsley juice had higher flavone concentrations than celery juice, celery is more palatable and more economical for juicing. To further explore the effects of processing conditions on the stability of flavone glycosides, celery juice was acidified to pH 3.0, 4.0, or 5.0 and heated at 121 °C for 15−60 min. After variation between lots of celery had been taken into consideration, the pH 3.0 and 4.0 juices had slightly higher total flavone concentrations than the pH 5.0 juices (data not shown). There were no significant differences associated with heating times.

As apigenin glycosides were the most abundant flavones in celery, they were used to represent the change in profiles with acidification and thermal processing. The conversion of apiin to apigenin 7-O-glucoside in celery juice acidified to pH 5.0 or 4.0 increased slightly with time, averaging 15% in pH 5.0 juice and 24% in pH 4.0 juice after 60 min (Figure 5). The most striking conversion of flavone glycosides occurred in juice acidified to pH 3.0. The conversion to apigenin 7-O-glucoside increased rapidly over time, from 38% after 15 min at 121 °C to 83% after 60 min. There was no significant conversion of apigenin glycosides to aglycones and no loss of total apigenin derivatives after acidification and thermal processing (Figure 6).

■ DISCUSSION

In all of the juice samples analyzed, flavone apiosylglucosides were the predominant flavonoids and malonyl apiosylglucosides were minor or undetectable. When celery leaves or parsley was steamed, significantly greater amounts of flavone glycosides were present as malonyl apiosylglucosides and significantly less were present as apiosylglucosides compared to fresh samples (Tables 2 and 3). Given the rapid loss of malonyl groups from the compounds in fresh juices and the preservation of these groups after steaming, this conversion of flavone glycosides is likely due to enzymatic activity. Malonyl esterase activity has previously been reported in parsley,[20] but no reports of similar activity in celery were found after a review of the literature and the BRENDA enzyme database.[21] Demalonylation of flavone glycosides can occur after oven-drying[22] or after the extraction process, as has been noted with other flavonoid glycosides.[23] Heating soy products can lead to demalonlyation (high moisture) or decarboxylation (low moisture) of isoflavone malonyl glucosides,[24] but the parsley and celery in our experiments retained this functional group after steaming.

With a masticating juicer, parsley yielded 72% juice yield (w/w) and celery yielded 79%. The majority of individual and total flavones were found in parsley and celery juices after extraction. However, pomace had higher flavone concentrations than juice, particularly from celery (Tables 4 and 5). This is similar to the retention of flavonoids in the pomace of other fruits and vegetables after juicing, including artichoke hearts[25] and apples.[26]

Acidifying parsley and celery juices to pH 4.0 and heating at 100 or 121 °C for up to 30 min did not significantly affect total flavone concentrations. These results were similar to those of

Table 2. Concentrations of Flavone Derivatives in Steamed and Fresh Parsley after Maceration and Incubation for 3 h[a]

	apiosylglucoside		malonyl apiosylglucoside		
conditions	apigenin	diosmetin	apigenin	diosmetin	total flavones[b]
steamed, incubated at 37 °C, 3 h	78.5 ± 34.7 a	18.7 ± 8.4 a	171 ± 43.5 a	39.2 ± 10.5 a	340 ± 48.9 a
fresh, incubated at 37 °C, 3 h	255 ± 24.8 b	58.5 ± 4.1 b	7.0 ± 1.4 b	0.0 ± 0.0 b	338 ± 29.0 a
fresh, incubated at 20 °C, 3 h	264 ± 22.8 b	60.2 ± 1.7 b	7.7 ± 1.0 b	0.0 ± 0.0 b	348 ± 23.5 a

[a]Values within each column without a common letter differ significantly ($p < 0.05$). Flavone quantities are expressed as aglycone equivalents (mg/100 g fresh weight). Data are the mean ± SD, $n = 3$. ND, not detected. [b]Includes minor flavone glycosides such as acetyl glucosides.

Table 3. Concentrations of Flavone Derivatives in Steamed and Fresh Celery Leaves after Maceration and Incubation for 3 h[a]

conditions	apiosylglucoside			malonyl apiosylglucoside			total flavones[b]
	luteolin	apigenin	chrysoeriol	luteolin	apigenin	chrysoeriol	
steamed, incubated at 37 °C, 3 h	9.0 ± 1.4 a	16.3 ± 1.6 a	11.6 ± 1.0 a	31.0 ± 4.9 a	78.8 ± 9.5 a	45.2 ± 5.7 a	210 ± 24.6 a
fresh, incubated at 37 °C, 3 h	13.3 ± 3.7 a	130 ± 4.7 b	75.2 ± 6.2 b	0.0 ± 0.0 b	3.8 ± 2.1 b	3.4 ± 3.6 b	226 ± 11.7 a
fresh, incubated at 20 °C, 3 h	30.6 ± 2.4 b	128 ± 2.2 b	71.1 ± 4.8 b	0.0 ± 0.0 b	6.4 ± 0.2 b	7.4 ± 0.2 b	244 ± 6.9 a

[a]Values within each column without a common letter differ significantly ($p < 0.05$). Flavone quantities are expressed as aglycone equivalents (mg/100 g fresh weight). Data are the mean ± SD, $n = 3$. ND, not detected. [b]Includes minor flavone glycosides such as acetylglucosides.

Table 4. Yield, Moisture, and Flavones in Fresh Parsley, Pomace, and Juice[a]

step	yield (g)	moisture (%)	total apigenin (mg)	total diosmetin (mg)	total flavones (mg)
fresh parsley	100	88.1 ± 0.8	178 ± 14.5	179 ± 94.7	357 ± 97.0
parsley juice	71.9 ± 2.8	91.8 ± 0.5	92.7 ± 13.0	108 ± 62.7	200 ± 75.6
parsley pomace	25.2 ± 1.0	78.6 ± 1.5	56.8 ± 4.1	55.9 ± 30.2	113 ± 26.3

[a]Flavone quantities are expressed as aglycone equivalents. Data are the mean ± SD, $n = 3$.

Table 5. Yield, Moisture, and Flavones in Fresh Celery (Stalks and Leaves), Juice, and Pomace[a]

step	yield (g)	moisture (%)	total apigenin (mg)	total luteolin (mg)	total chrysoeriol (mg)	total flavones (mg)
fresh celery	100	93.1 ± 0.6	41.1 ± 9.3	16.7 ± 5.5	13.0 ± 4.0	70.8 ± 18.9
celery juice	79.1 ± 0.4	95.4 ± 0.1	13.4 ± 1.4	4.9 ± 0.2	4.3 ± 0.4	22.5 ± 1.8
celery pomace	18.8 ± 0.3	83.7 ± 0.6	11.1 ± 0.3	3.2 ± 0.3	3.6 ± 0.2	18.0 ± 0.8

[a]Flavone quantities are expressed as aglycone equivalents. Data are the mean ± SD, $n = 3$.
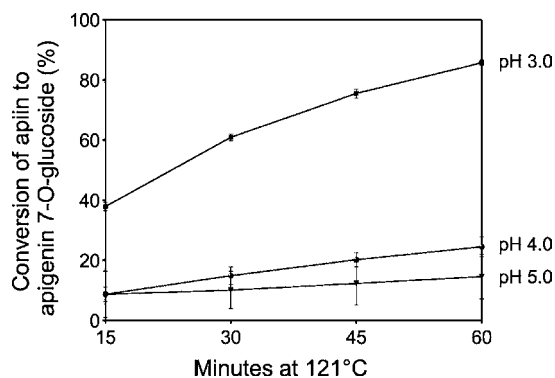

Figure 5. Conversion of apiin to apigenin 7-O-glucoside in celery juice. Juices were processed at pH 3.0, 4.0, or 5.0 at 121 °C for 15−60 min. Data are the mean ± SD, $n = 3$.
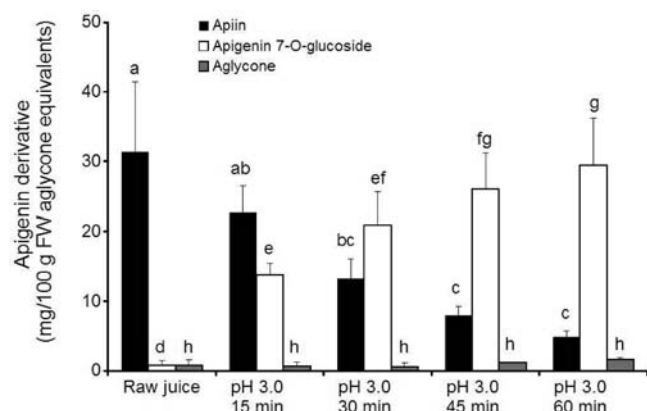

Figure 6. Profile of apigenin derivatives in celery juice. Raw untreated juice was compared with juice processed at pH 3.0 at 121 °C for 15−60 min. Data are the mean ± SD, $n = 3$. Values within each treatment group without a common letter differ ($p < 0.05$). FW, fresh weight.

most other studies on juices. Thermal processing at 95 °C for up to 1 min had little effect on flavonoid concentrations in orange juice,[27,28] and processing at 88 °C for up to 30 min had no effect on total flavonoids in tomato juice.[29] Heating grape juice at 95 °C for 15 min did not degrade anthocyanins.[30] In contrast, up to 49% of isoflavones were lost from soybeans when they were boiled for 20 min during tempeh production.[31]

After acidifying celery juice to pH 3.0, 4.0, or 5.0 and heating at 121 °C for 15−60 min, there was no effect of processing times on total flavone concentrations in this study. Few studies have compared the effects of extensive thermal processing on flavonoids in foods. Isoflavones in soy converted from glycosides to aglycone during extrusion at 110−150 °C, but no loss of total isoflavones was observed.[32] Others have tested the effects of heat and pH treatments on pure flavonoid glycosides with varied results. Quercetin and quercetin rutinoside standards boiled at 100 °C for up to 5 h at pH 5 and 8 showed different stabilities, and both were more stable in the absence of oxygen. Both compounds were more stable at pH 5, and the quercetin glycoside was more stable than the aglycone.[33] Daidzein and genistein (aglycone) standards both degraded when heated at 120 °C for 20 min, but the effect varied with pH. Daidzein was more stable at pH 9, whereas genistein was more stable at pH 7.[34]

Although the concentration of total flavones in celery juice was generally stable after heat processing, the conversion of apiin to apigenin 7-O-glucoside increased rapidly at 121 °C over time at pH 3.0, reaching 83% after 60 min (Figure 6). Previous investigations suggested that this deconjugation to a simple flavonoid glucoside may improve absorption. In human subjects, converting hesperetin 7-O-rhamnosylglucoside to hesperetin 7-O-glucoside in orange juice increased peak hesperetin plasma concentrations 4-fold.[35] The flavonoid apiosylglucosides in parsley and celery are similar to the flavonoid rhamnosylglucosides in orange juice in that they consist of a disaccharide group attached at the same position on the flavonoid core. Experiments with humans fed parsley and

celery containing flavone apiosylglucosides resulted in t_{max} values of 7−8 h,[36,37] compared to <1 h for those fed simple flavone glucoside.[38] These data support the hypothesis that flavone apiosylglucosides are absorbed in the colon, whereas simple glucosides such as apigenin 7-O-glucoside are absorbed in the small intestine.

The bioavailability of flavones, particularly abundant in parsley and celery, depends on both the dose and the conjugation of these compounds to sugars and other functional groups. The current study shows that during juice processing, a substantial portion of the flavones from parsley and celery are retained in the pomace. Perhaps more importantly, total flavone concentrations changed little, but the profile of flavone glycosides in the resulting juices was modified by endogenous enzymes, pH, and thermal processing. Extended thermal processing at low pH resulted in the conversion of apigenin apiosylglucoside to apigenin glucoside, potentially modulating intestinal absorption and metabolism.

Abbreviations Used

ESI-MS, electrospray mass spectrometry; FW, fresh weight; HPLC, high-performance liquid chromatography; RT, retention time; t_{max}, time of maximum plasma concentration.

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