Effects of enzymatic and thermal processing on flavones, the effects of flavones on inflammatory mediators in vitro, and the absorption of flavones in vivo

DISSERTATION

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By

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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. Two studies were conducted to determine the effects of processing on stability and profiles of flavones in celery and parsley, and a third explored the effects of deglycosylation on the anti-inflammatory activity of flavones in vitro and their absorption in vivo.

In the first processing study, celery leaves were combined with β-glucosidase-rich food ingredients (almond, flax seed, or chickpea flour) to determine test for enzymatic hydrolysis of flavone apiosylglucosides. Although all of the enzyme-rich ingredients could convert apigenin glucoside to aglycone, none had an effect on apigenin apiosylglucoside. Thermal stability of flavones from celery was also tested by isolating them and heating at 100 °C for up to 5 hours in pH 3, 5, or 7 buffer. Apigenin glucoside was most stable of the flavones tested, with minimal degradation regardless of pH or heating time. Apigenin, luteolin, and chrysoeriol were stable at pH 3, but degraded steadily at pH 5 or 7. Apigenin apiosylglucoside was least stable at pH 3, but its primary degradation product was apigenin glucoside. Further experiments with thermal processing and enzyme treatments showed that apigenin apiosylglucoside could be
converted stepwise to apigenin glucoside and then to aglycone, resulting in over 95% conversion of total apigenin derivatives.

The second processing study explored the effects of juice extraction, acidification, thermal processing, and endogenous enzymes on flavone profiles and concentrations in parsley and celery. Parsley yielded 72% juice with 64% of the total flavones extracted, while 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, while 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, resulting in a juice that could improve the bioavailability and efficacy of these potentially beneficial compounds.

In the third study the anti-inflammatory activities of celery extracts, some rich in flavone aglycones and others rich in flavone glycosides, were tested on the inflammatory mediators TNF-α and NF-κB in LPS-stimulated macrophages. Pure flavone aglycones and aglycone-rich extracts effectively reduced TNF-α production and inhibited transcriptional activity of NF-κB, while glycoside-rich extracts showed no significant effects. Deglycosylation of flavones increased cellular uptake and cytoplasmic localization as shown by HPLC and by staining with DPBA. Celery diets with different glycoside or aglycone contents were formulated, and absorption was evaluated in mice fed with 5% or 10% celery diets. Relative absorption was significantly higher in mice fed
aglycone-rich diets as determined by HPLC-MS/MS. The results demonstrated that deglycosylation increases absorption of dietary flavones in vivo and modulates inflammation by attenuating TNF-α and NF-κB, suggesting the potential use of flavone-rich functional foods for the treatment or prevention of inflammatory diseases.
Acknowledgments

I would like to first acknowledge my advisor, Steven Schwartz, for his patience, support, and guidance throughout my time here. I would also like to thank Andrea Doseff for her work on my committee and her ongoing support in lab work and writing. Luis Rodriguez-Saona and Erich Grotewold also served on my committee and provided useful feedback on results. Sheryl Barringer helped navigate the many details of graduate school and stepped up to the plate to serve on my exam committee. Ken Riedl has been immensely helpful over the years, providing constant advice on lab techniques, experimental design, and writing. Other members of the Schwartz lab that deserve special recognition are Rachel Kopec and Jennifer Ahn-Jarvis, whose technical and moral support have helped me every step of the way. Current and past members of the Doseff lab that were instrumental in this work include Courtney Nicholas, Horacio Cardenas, Mayra Dios-Tora, and Daniel Arango. Monica Giusti was an excellent mentor during the years that I helped with the Introduction to Food Processing class.
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Publications


Fields of Study

Major Field: Food Science and Technology
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INTRODUCTION

Flavonoids are widespread throughout the plant kingdom, and several reviews have examined their food sources and the bioavailability, metabolism, and biological activity of these compounds in humans (1-4). Although extensive, these reviews omitted or only briefly mentioned flavones, likely because of the limited information available in comparison to other flavonoids. Flavones have been the subject of numerous promising in vitro studies, and many recent publications have further expanded our knowledge of their pharmacokinetics and activity in humans and in animal models.

This review explores the most common flavones in the diet, their biological role in plants, and the most abundant food sources containing them. It will also delve into the bioavailability of flavones from foods and their metabolism in humans. Finally, the biological activity of flavones in animals and humans will be surveyed with an attempt determine the dose required for beneficial effects.

1 Written as a manuscript for The American Journal of Clinical Nutrition, in preparation.
FLAVONE FUNCTIONS AND VARIABILITY WITH GROWING CONDITIONS

Flavones differ from other flavonoids in that they have a double bond between C2 and C3 in the flavonoid skeleton, there is no substitution at the C3 position (5), and they are oxidized at the C4 position (Fig. 1.1). These compounds play a variety of roles in plants. Along with flavonols, they are the primary pigments in white and cream colored flowers (6) and act as co-pigments with anthocyanins in blue flowers (7). Flavones and other flavonoids can also act as UV-B protectants in plants, since they absorb in the 280-315 nm range (7). This is likely the primary role of flavones in parsley, as they are synthesized and stored in the epidermal cells (8) and flavonoid synthesis is upregulated by UV light in parsley cells (9). This might explain why apigenin and luteolin concentrations in celery leaves are more than 20 times higher than those in stalks (10) and why field grown parsley and chamomile leaves have much higher flavone concentrations than greenhouse grown plants (11,12). However, lettuce grown in polyethylene high tunnels had similar luteolin concentrations to those grown in open fields (13), indicating that UV light may not stimulate flavone synthesis in all plants.

A few studies suggest that flavones can act as natural pesticides in plants. Luteolin aglycone and apigenin C-glycoside deter aphids (6), and cucumber plants resistant to powdery mildew contain higher quantities of apigenin and luteolin C-glycosides (14). Post-harvest trials have also shown that apigenin and luteolin O-glucosides inhibit mycelia growth of some common storage fungi (15). Plants grown under organic conditions (with presumably more pest pressure), however, have in most cases shown no higher concentrations of flavones than their conventional counterparts. While biodynamic chicory had greater apigenin glucuronide concentrations than
conventionally grown plants for part of the season, there were no differences in luteolin glucuronide concentrations between the growing methods (16). Trials of organic and conventionally grown lettuce, pac choi, and collard greens did not show significantly different concentrations of apigenin or luteolin between the two methods (17). A comparison of flavonoid concentrations in conventional and organically grown bell peppers over three years found no difference in luteolin (18), and another study found no differences in luteolin concentrations in lettuce grown organically or conventionally (13).

Other studies on the effects of growing conditions on flavone production are limited. The application of zinc to the soil did not affect apigenin concentrations in chamomile flowers (19). Although the mechanisms are not fully understood, treating citrus fruits with the hormones 6-benzylaminopurine (6-BA) or 2,4-dichlorphenoxyacetic acid (2,4-D) at fruit set significantly increased diosmetin 7-O-rutinoside concentrations in the fruit (20).

Flavones also act as signaling molecules for plants. Apigenin and luteolin are secreted by the roots of several legume species to signal nitrogen-fixing bacterial to colonize their roots (21). Similarly, flavones such as chrysin have been shown to promote root colonization of mycorrhizal fungi (22).

**FOOD SOURCES OF FLAVONES**

Flavones from plants are typically conjugated as 7-O-glycosides (Fig. 1.1) (5) and may also have acetyl or malonyl moieties. Flavone C-glycosides are most commonly detected as 6-C- and 8-C-glucosides. While flavone O-glycosides can be hydrolyzed with enzymes or acid prior to analysis, flavone C-glycosides are resistant to both processes.
and must be analyzed in their native forms (23). Both glycosides and aglycones are commonly used for quantitation in the literature.

Concentrations of flavones in dry tea and herbs are listed in Table 1.1. Chamomile and parsley have the highest flavone concentrations, with as much as 4190 mg apigenin/100 g dried chamomile flowers or 1350 mg/100 g dried parsley leaf (24,25). The apigenin conjugates in chamomile have been identified as acetyl, malonyl, and caffeoyl derivatives of apigenin 7-O-glucoside (26), while those in parsley are predominantly apigenin 7-O-malonyl apiosylglucoside (malonylapiiin) and apigenin 7-O-apiosylglucoside (apiin) (24). Luteolin 8-C-glucoside (orientin) and luteolin 6-C-gluconsode (isoorientin) are most abundant in rooibos tea (27). Green, black, and oolong teas contain orientin and isoorientin, as well as a variety of apigenin mono- and di-C-glycosides (23). Flavone O-glycosides are also reported in many plants from the mint family (Lamiaceae), but their reported concentrations vary widely between sources. For example, luteolin glycosides in peppermint ranged from 42-3070 mg luteolin glycosides per 100 g dry leaf, varying with the source and the analytical methods used (28,29).

Table 1.2 shows a survey of flavones reported in juice and wine. All of the juices with appreciable flavone concentrations are in the citrus family and contain methylated flavones, e.g. acacetin, diosmetin, and chrysoeriol glycosides. Bergamot juice contains the highest concentrations of total flavone glycosides, including both flavone O- and C-glycosides (30,31). Apigenin and diosmetin 6,8-di-C-glucosides were found in bergamot, mandarin orange, orange, and citron juices (30). Although kumquats (Fortunella crassifolia) contain over 21 mg apigenin per 100 g fresh weight (32), kumquat juice (F. japonica) has less than 1 mg/100 g (33). This may be due to differences in species or to
the amount of peel included in the sample. In grapefruit, peels contained more than 100
times the concentration of naringin than the juice (34).

The concentrations of flavones in fruits, vegetables, and honey are listed in Table
1.3. Most of the studies reported values for aglycones after acid hydrolysis, although
some gave concentrations of flavone O-glycosides. Fresh parsley had the highest flavone
concentrations among the fresh foods, with up to 1484 mg apigenin per 100 g (35).
Chicory had up to 333 mg luteolin per 100 g in one study (16), but was not detected in
another (36). Wide variations in flavone concentrations between studies were also
observed for many other fresh foods. Vegetables with the highest concentrations were in
the sunflower family (Asteraceae) and carrot family (Apiaceae).
Figure 1.1. Structures of flavone aglycones and common flavone derivatives.
<table>
<thead>
<tr>
<th>Botanical name (Family)</th>
<th>Common name</th>
<th>Flavone</th>
<th>mg/100 g dry wt</th>
<th>mg/serving</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Matricaria chamomilla, Anthemis nobilis</em> (Asteraceae)</td>
<td>Chamomile flowers</td>
<td>apigenin O-glycosides luteolin O-glycosides</td>
<td>2926-4190</td>
<td>59-190</td>
<td>(25,37)</td>
</tr>
<tr>
<td><em>Tanacetum vulgare</em> (Asteraceae)</td>
<td>Tansy leaf</td>
<td>apigenin luteolin</td>
<td>165</td>
<td>3</td>
<td>(38)</td>
</tr>
<tr>
<td><em>Aspalathus linearis</em> (Fabaceae)</td>
<td>Rooibos/red bush tea</td>
<td>apigenin C-glycosides luteolin C-glycosides</td>
<td>46-116</td>
<td>1-2</td>
<td>(27,39,40)</td>
</tr>
<tr>
<td><em>Fenugreek seed</em> (Fabaceae)</td>
<td>Fenugreek seed</td>
<td>apigenin luteolin</td>
<td>731</td>
<td>15</td>
<td>(38)</td>
</tr>
<tr>
<td><em>Mentha x piperita</em> (Lamiaceae)</td>
<td>Peppermint</td>
<td>apigenin O-glycosides luteolin O-glycosides</td>
<td>42-3070</td>
<td>1-61</td>
<td>(28,29)</td>
</tr>
<tr>
<td><em>Salvia officinalis</em> (Lamiaceae)</td>
<td>Sage</td>
<td>luteolin/luteolin O-glycosides</td>
<td>49.6-1110</td>
<td>1-22</td>
<td>(29,38)</td>
</tr>
<tr>
<td><em>Rosmarinus officinalis</em> (Lamiaceae)</td>
<td>Rosemary</td>
<td>apigenin luteolin/luteolin O-glycosides diosmetin O-glycosides</td>
<td>ND -43.8 10-661 ND -250</td>
<td>&lt;1-1 0-13 &lt;1-5</td>
<td>(38,41)</td>
</tr>
<tr>
<td><em>Perilla frutescens</em> (Lamiaceae)</td>
<td>Shiso</td>
<td>apigenin O-glycosides luteolin O-glycosides</td>
<td>280-920</td>
<td>6-18</td>
<td>(42)</td>
</tr>
<tr>
<td><em>Camellia sinensis</em> (Theaceae)</td>
<td>Green tea</td>
<td>apigenin C-glycosides luteolin C-glycosides</td>
<td>47.8-246.8 2.5-21.9</td>
<td>1-5 &lt;1</td>
<td>(23)</td>
</tr>
<tr>
<td><em>Camellia sinensis</em> (Theaceae)</td>
<td>Black tea</td>
<td>apigenin C-glycosides luteolin C-glycosides</td>
<td>41.4-175.7 5.9-14.0</td>
<td>1-4 &lt;1</td>
<td>(23)</td>
</tr>
<tr>
<td><em>Camellia sinensis</em> (Theaceae)</td>
<td>Oolong tea</td>
<td>apigenin C-glycosides luteolin C-glycosides</td>
<td>198.6 14.8</td>
<td>4 &lt;1</td>
<td>(23)</td>
</tr>
<tr>
<td><em>Petroselinum crispum</em> (Apiaceae)</td>
<td>Parsley</td>
<td>apigenin O-glycosides</td>
<td>1200-1350</td>
<td>24-27</td>
<td>(24)</td>
</tr>
</tbody>
</table>

ND not detected.
### Table 1.2. Concentrations of flavones in juices and wines.

<table>
<thead>
<tr>
<th>Botanical name (Family)</th>
<th>Common name (serving size)</th>
<th>Flavone</th>
<th>mg/100 g fresh wt</th>
<th>mg/serving</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Citrus bergamia</em> (Rutaceae)</td>
<td>Bergamot juice (250 ml)</td>
<td>apigenin C-glycosides</td>
<td>3.8-7.2</td>
<td>10-18</td>
<td>(30,31)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>apigenin O-glycosides</td>
<td>6.1-7.7</td>
<td>15-19</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>luteolin C-glycosides</td>
<td>0.6-0.8</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>chrysoeriol C-glycosides</td>
<td>1.3-1.5</td>
<td>3-4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>chrysoeriol O-glycosides</td>
<td>5.4-6.9</td>
<td>14-17</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>diosmetin C-glycosides</td>
<td>2.9-5.9</td>
<td>7-15</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>diosmetin O-glycosides</td>
<td>1.9-2.7</td>
<td>5-7</td>
<td></td>
</tr>
<tr>
<td><em>Citrus deliciosa</em> (Rutaceae)</td>
<td>Mandarin orange juice (250 ml)</td>
<td>apigenin C-glycosides</td>
<td>2.3-2.7</td>
<td>6-7</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>diosmetin C-glycosides</td>
<td>0.6-0.8</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>Citrus sinensis</em> (Rutaceae)</td>
<td>Orange juice (250 ml)</td>
<td>apigenin C-glycosides</td>
<td>1.0-8.0</td>
<td>3-20</td>
<td>(30,43-45)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>apigenin O-glycosides</td>
<td>0.2-0.7</td>
<td>1-2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>luteolin C-glycosides</td>
<td>0.2-0.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>chrysoeriol C-glycosides</td>
<td>0.2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>diosmetin C-glycosides</td>
<td>0.2-0.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><em>Citrus medica</em> (Rutaceae)</td>
<td>Citron juice (250 ml)</td>
<td>apigenin C-glycosides</td>
<td>0.6-0.8</td>
<td>2</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>diosmetin C-glycosides</td>
<td>6.1-6.8</td>
<td>15-17</td>
<td></td>
</tr>
<tr>
<td><em>Fortunella japonica</em> (Rutaceae)</td>
<td>Kumquat juice (250 ml)</td>
<td>acacetin O-glycosides</td>
<td>0.1</td>
<td>&lt;1</td>
<td>(33)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>acacetin C-glycosides</td>
<td>0.1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>apigenin C-glycosides</td>
<td>0.03</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td><em>Vitis vinifera</em> (Vitaceae)</td>
<td>Red and white wine (100 ml)</td>
<td>apigenin</td>
<td>ND</td>
<td>ND</td>
<td>(46-49)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>luteolin</td>
<td>0.03-0.39</td>
<td>ND&lt;1</td>
<td></td>
</tr>
</tbody>
</table>

ND not detected.
Table 1.3. Concentrations of flavones in foods.

<table>
<thead>
<tr>
<th>Botanical name (Family)</th>
<th>Common name (serving size)</th>
<th>Flavone</th>
<th>mg/100 g fresh wt</th>
<th>mg/serving</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactuca sativa</em> (Asteraceae)</td>
<td>Lettuce (100 g)</td>
<td>luteolin</td>
<td>ND-8.8</td>
<td>&lt;1-8.8</td>
<td>(36,50)</td>
</tr>
<tr>
<td><em>Cynara scolymus</em> (Asteraceae)</td>
<td>Artichoke heads (200 g)</td>
<td>apigenin glycosides</td>
<td>6.0</td>
<td>12.0</td>
<td>(51)</td>
</tr>
<tr>
<td><em>Chicorium intybus</em> (Asteraceae)</td>
<td>Chicory (100 g)</td>
<td>apigenin/apyrin/apyrin O-glycosides luteolin/luteolin O-glucosides</td>
<td>1.8</td>
<td>1.8-68</td>
<td>1.8-68</td>
</tr>
<tr>
<td><em>Petroselinum crispum</em> (Apiaceae)</td>
<td>Parsley (5 g)</td>
<td>apigenin</td>
<td>184-1484</td>
<td>9-74</td>
<td>(10,35,52)</td>
</tr>
<tr>
<td><em>Apium graveolens dulce</em> (Apiaceae)</td>
<td>Celery stalks (200 g)</td>
<td>apigenin</td>
<td>1.3-10.8</td>
<td>2.6-21.6</td>
<td>(10,53,54)</td>
</tr>
<tr>
<td><em>Brassica oleracea</em> var. botrytis (Brassicaceae)</td>
<td>Broccoli (200 g)</td>
<td>luteolin/luteolin glycosides</td>
<td>0.3-7.45</td>
<td>0.6-14.9</td>
<td>(32,55,56)</td>
</tr>
<tr>
<td><em>Pisum sativum</em> (Fabaceae)</td>
<td>Peas (100 g)</td>
<td>apigenin</td>
<td>ND-17.6</td>
<td>&lt;1-17.6</td>
<td>(55,56)</td>
</tr>
<tr>
<td><em>Capsicum annuum</em> (Solanaceae)</td>
<td>Bell peppers (100 g)</td>
<td>luteolin</td>
<td>ND-0.4</td>
<td>&lt;1</td>
<td>(10,32,36)</td>
</tr>
<tr>
<td><em>Citrus lanatus</em> (Cucurbitaceae)</td>
<td>Watermelon (200 g)</td>
<td>luteolin</td>
<td>ND-1.84</td>
<td>&lt;1-3.7</td>
<td>(54,57)</td>
</tr>
<tr>
<td><em>Cucumis melo</em> (Cucurbitaceae)</td>
<td>Muskemelon (200 g)</td>
<td>luteolin</td>
<td>ND-2.58</td>
<td>&lt;1-5.2</td>
<td>(54,57)</td>
</tr>
<tr>
<td><em>Cucurbita sp.</em> (Cucurbitaceae)</td>
<td>Pumpkin (200 g)</td>
<td>luteolin</td>
<td>ND-1.63</td>
<td>&lt;1-3.3</td>
<td>(55,57)</td>
</tr>
<tr>
<td><em>Citrus paradisi</em> (Rutaceae)</td>
<td>Grapefruit (200 g)</td>
<td>apigenin/aprin/aprin glycosides</td>
<td>ND-4.86</td>
<td>&lt;1-9.7</td>
<td>(32,54,56)</td>
</tr>
<tr>
<td><em>Citrus sinensis</em> (Rutaceae)</td>
<td>Orange (200 g)</td>
<td>luteolin</td>
<td>ND-1.5</td>
<td>&lt;1-3</td>
<td>(56-58)</td>
</tr>
<tr>
<td><em>Fortunella crassifolia</em> (Rutaceae)</td>
<td>Kumquat (100 g)</td>
<td>apigenin glycosides</td>
<td>21.89</td>
<td>21.9</td>
<td>(32)</td>
</tr>
<tr>
<td><em>Vitis sp.</em> (Vitaceae)</td>
<td>Grapes (200 g)</td>
<td>luteolin</td>
<td>ND-2.6</td>
<td>&lt;1-5.2</td>
<td>(10,56,59)</td>
</tr>
<tr>
<td><em>Vaccinium sp.</em> (Ericaceae)</td>
<td>Blueberry (100 g)</td>
<td>luteolin</td>
<td>ND-1.8</td>
<td>&lt;1-1.8</td>
<td>(10,54,56)</td>
</tr>
<tr>
<td><em>Olea europaea</em> (Oleaceae)</td>
<td>Black olives (50 g)</td>
<td>apigenin</td>
<td>6.5</td>
<td>3.3</td>
<td>(60)</td>
</tr>
<tr>
<td><em>Actinidia deliciosa</em> (Actinidiaceae)</td>
<td>Kiwifruit (100 g)</td>
<td>luteolin</td>
<td>ND-2.2</td>
<td>&lt;1-2.2</td>
<td>(32,54,57)</td>
</tr>
<tr>
<td><em>Honey</em> (20 g)</td>
<td>luteolin</td>
<td>ND-29.3</td>
<td>&lt;1-1.59</td>
<td>(61-63)</td>
<td></td>
</tr>
<tr>
<td><em>Honey</em> (20 g)</td>
<td>luteolin</td>
<td>ND-3.19</td>
<td>&lt;1-0.6</td>
<td>(61-63)</td>
<td></td>
</tr>
</tbody>
</table>

ND not detected.
EFFECTS OF PROCESSING ON FLAVONE GLYCOSIDES

Like other flavonoids, flavones are typically present in plants as glycosides. Flavone O-glycosides are composed of the aglycone moiety with one or more sugars attached with a β linkage (Fig. 1.1). These compounds may be modified slightly by endogenous enzymes such as malonyl esterases (e.g. conversion from malonylapiin to apiin) (64), but remain as glycosides after processing such as shredding (65), juicing (66), and heating (44). Flavone C-glycosides are reportedly stable to fermentation (39) as well as juicing, concentration, spray drying, and pasteurization (44,67). Significant degradation of flavone C-glycosides, however was found after processing for 15 minutes at 121 °C or 4 minutes at 135 °C (67).

ABSORPTION AND METABOLISM OF FLAVONE O-GLYCOSIDES

Given that dietary flavones are ingested predominantly as glycosides, their fate is determined by how and where they are absorbed, metabolized, transported, and excreted. The absorption of orally delivered flavone O-glycosides and aglycones has been the subject of many animal studies, particularly in rats (Table 1.4). Most studies show that apigenin, luteolin, and their simple glucosides are absorbed quickly. The time of maximum plasma concentration (T_{max}) was generally one hour or less, with maximum concentrations (C_{max}) of 1-100 µM (68-71), depending on the dose and matrix. The shortest T_{max} were seen with luteolin dissolved in DMSO/polyethyleneglycol, propyleneglycol, or ethanol (68-70). Longer Tmax was seen with luteolin and apigenin from Verbena officinalis extract and although the flavone form was not specified (72), the native forms are flavone mono- and diglucuronides (73). The longest T_{max} and greatest
were seen in rats fed with radiolabelled apigenin, where the values included all metabolites of apigenin detected as radioactivity (74). Mice showed very low plasma concentrations (<1 µM after a 300 mg/kg dose) (75), but comparisons with rats are difficult because the small weight of mice only allows one blood sample rather than a pharmacokinetic study.

In contrast to pharmacokinetic studies of flavones in rats, humans show plasma concentrations of less than 1 µM following feeding (Table 5). Many used celery leaves or parsley as their flavone source (76-79), which contain primarily flavone apiosylglucosides (24,80). While an older study using ultraviolet detection did not find flavones in serum (77), two newer reports using electrochemical detection found concentrations averaging less than 0.2 µM with $T_{\text{max}}$ values of more than 7 hours (76,78). The latter suggests that the flavone apiosylglucosides are absorbed in the colon, which may contribute to their low bioavailability. Subjects fed comparable amounts of flavones as simple glucosides from artichoke leaf extract had $T_{\text{max}}$ values of less than one hour and maximal plasma concentrations of 0.2-0.5 µM (81). While less than 1% of flavones from parsley were found in urine (78,79), after feeding alycone-rich hydrolyzed Chrysanthemum morifolium extract, urinary excretion was 2% of luteolin and 6% of apigenin intakes (82). Greater absorption of apigenin relative to luteolin was also observed as higher plasma concentrations after feeding flavone-rich plant extracts to rats (72,83).

While numerous studies have shown that flavones are absorbed systemically (Tables 1.4 & 1.5), they are typically found in plasma or urine as glucuronide or sulfate metabolites rather than the original flavone O-glycosides (81,84,85). Like many other
flavonoid glycosides in foods, flavones must first be hydrolyzed to aglycones for absorption (86), and are then metabolized to glucuronidated or sulfated forms before reaching systemic circulation (87). A brief summary of the sites of metabolism and enzymes involved is presented in Table 1.6.

Although ex-vivo models show that flavonoid glycosides can be absorbed through the human stomach and can be cleaved by stomach-specific β-glucosidase (88), few compounds are absorbed there because of its relatively small surface area (89). And while the stomach pH is very low in the fasted state and could theoretically hydrolyze some flavonoid glycosides to aglycones, in vitro models with pH 1.2-2 buffer solution and rat gastric contents found that quercetin and apigenin glycosides were very stable under gastric conditions (71,90). Furthermore, the gastric contents are typically pH 3-7 after a meal (89), not much different from the pH of most foods.
Table 1.4. Absorption of flavone aglycones and O-glycosides in animal models.

<table>
<thead>
<tr>
<th>No. of animals per treatment group</th>
<th>Dose</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; plasma</th>
<th>Plasma conc.</th>
<th>AUC</th>
<th>Urinary excretion</th>
<th>Recovery from feces</th>
<th>Elimination half-life</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>h</td>
<td>µmol/L</td>
<td></td>
<td>% of intake</td>
<td>% of intake</td>
<td>h</td>
<td></td>
</tr>
<tr>
<td>4-10 C57BL/6J mice</td>
<td>0.2% apig (1.1 mmol/kg, ~300 mg/kg) for 7 days</td>
<td>NR</td>
<td>0.09 (steady state)</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>(75)</td>
</tr>
<tr>
<td>6 male Wistar rats</td>
<td>100 mg apig or apiin</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>for apig: 0.38 (24 h) 1.18 (3 d) for apiin: 1.5 (24 h) 2.28 (3 d)</td>
<td>n/a</td>
<td>n/a</td>
<td>(91)</td>
</tr>
<tr>
<td>11-14 Sprague-Dawley rats</td>
<td>66 µmol apig 7-O-glucoside /kg (28.5 mg/kg)</td>
<td>0.5-1</td>
<td>1.5</td>
<td>NR</td>
<td>0.2-0.3 (24 h)</td>
<td>3-4 (48 h)</td>
<td>NR</td>
<td>(71)</td>
</tr>
<tr>
<td>10 Wistar rats</td>
<td>10 mg apig/kg coupled with 10 µCi/kg bw (radio-labeled)</td>
<td>24</td>
<td>1591 (whole blood)</td>
<td>NR</td>
<td>16-18 (24 h) 51 (10 d)</td>
<td>6-7 (24 h) 12-13 (10 d)</td>
<td>91.8</td>
<td>(74)</td>
</tr>
<tr>
<td>3-5 SD rats</td>
<td>50 µmol lut/kg (14.3 mg/kg)</td>
<td>0.5</td>
<td>15.5</td>
<td>NR</td>
<td>4 (24 h)</td>
<td>n/a</td>
<td>NR</td>
<td>(68)</td>
</tr>
</tbody>
</table>

Continues
Table 1.4. Continued

<table>
<thead>
<tr>
<th>No. of animals per treatment group</th>
<th>Dose</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; plasma</th>
<th>Plasma conc.</th>
<th>AUC</th>
<th>Recovery from feces</th>
<th>Elimination half-life</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>h</td>
<td>µmol/L</td>
<td></td>
<td>% of intake</td>
<td>% of intake</td>
<td>h</td>
</tr>
<tr>
<td>6 Sprague-Dawley rats</td>
<td>3.32 mg apig/kg and 2.06 mg lut/kg (as Verbena officinalis extract)</td>
<td>4.33</td>
<td>24.8</td>
<td>28.77</td>
<td>NR</td>
<td>NR</td>
<td>3.30 (72)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.97</td>
<td>5.7</td>
<td>µg/mL h (0-∞)</td>
<td>10.67</td>
<td>µg/mL h (0-∞)</td>
<td>4.02</td>
</tr>
<tr>
<td>8-11 Sprague-Dawley rats</td>
<td>50 mg lut/kg</td>
<td>0.08-0.25</td>
<td>39.3</td>
<td>16.64</td>
<td>4.8 (24 h)</td>
<td>n/a</td>
<td>6.57 (69)</td>
</tr>
<tr>
<td>5 Sprague-Dawley rats</td>
<td>14.3 mg lut/kg (as peanut hull extract or purified)</td>
<td>0.52 / 1.02</td>
<td>29.13 / 6.88</td>
<td>20.3 / 10.7</td>
<td>n/a</td>
<td>n/a</td>
<td>2.77 / 4.94 (70)</td>
</tr>
<tr>
<td>5 male SD rats</td>
<td>15.2 mg lut 7-O-glucoside and 10.38 mg apig 7-O-glucoside/kg (as 200 mg Chrysanth. morifolium extract/kg)</td>
<td>1.1 lut 3.9 apig</td>
<td>13.9 lut 62.9 apig</td>
<td>23.03 lut 237.6 apig</td>
<td>6.6 lut 16.6 apig</td>
<td>31.3 lut 28.6 apig</td>
<td>2.7 lut 3.4 apig (83)</td>
</tr>
<tr>
<td>3 Beagle dogs</td>
<td>5.29 mg lut/kg (as 102 mg Chrysanth. morifolium extract/kg)</td>
<td>1.54</td>
<td>1.62</td>
<td>3095</td>
<td>n/a</td>
<td>n/a</td>
<td>1.79 / 6.97 (92)</td>
</tr>
</tbody>
</table>

AUC area under the curve, NR not reported, apig apigenin, lut luteolin.
Table 1.5. Bioavailability of flavone O-glycosides in humans.

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of subjects</th>
<th>Dose</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; plasma h</th>
<th>Plasma conc. µmol/L</th>
<th>AUC µmol/L</th>
<th>Urinary excretion % intake</th>
<th>Elimination half-life h</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parsley</td>
<td>5 women and 6 men</td>
<td>2 g parsley/kg (0.24 mg apiq/kg)</td>
<td>7.2</td>
<td>0.127</td>
<td>61.98 min *</td>
<td>0.22</td>
<td>n/a</td>
<td>(78)</td>
</tr>
<tr>
<td>Parsley</td>
<td>7 men and 7 women</td>
<td>20 g parsley/day (4.5 mg apiq/day) for 7 days</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>0.58</td>
<td>n/a</td>
<td>(79)</td>
</tr>
<tr>
<td>Parsley</td>
<td>9 men and 9 women</td>
<td>4.9 g dried parsley/day (84 mg apiq/day) for 7 days</td>
<td>n/a</td>
<td>&lt;1.1</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>(77)</td>
</tr>
<tr>
<td>Celery leaf</td>
<td>10 men and 10 women</td>
<td>2 g celery leaf/kg (bolus dose)</td>
<td>7.70</td>
<td>0.190</td>
<td>89.02 min *</td>
<td>n/a</td>
<td>n/a</td>
<td>(76)</td>
</tr>
<tr>
<td>100 ml chamomile extract (ethanol/water)</td>
<td>1 woman</td>
<td>225.5 mg apiq 7-glucoside, 10.6 mg lut 7-glucoside, 22.5 mg apiq, 6.0 mg lut</td>
<td>n/a</td>
<td>&lt;0.74</td>
<td>n/a</td>
<td>ND</td>
<td>n/a</td>
<td>(93)</td>
</tr>
</tbody>
</table>

Continues
Table 1.5. Continued

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of subjects</th>
<th>Dose</th>
<th>$T_{\text{max}}$</th>
<th>Plasma conc.</th>
<th>AUC</th>
<th>Urinary excretion</th>
<th>Elimination half-life</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$h$</td>
<td>$\mu\text{mol/L}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chrysanthemum morifolium</em> tablets</td>
<td>4 men and 4 women</td>
<td>85.56 mg lut 65.04 mg apig</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>2.30 lut 6.09 apig (12 hr)</td>
<td>n/a</td>
<td>(82)</td>
</tr>
<tr>
<td>Cooked artichoke heads (61.7 g)</td>
<td>2 men and 3 women</td>
<td>4.9 mg lut glycoside 6.0 apig glycoside</td>
<td>n/a</td>
<td>ND</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>(51)</td>
</tr>
<tr>
<td><em>Artichoke leaf extract</em></td>
<td>7 men and 7 women</td>
<td>14.4 or 35.2 mg lut eq.</td>
<td>0.36 / 0.46</td>
<td>0.206 / 0.546</td>
<td>168.6 / 499.6 ng/ml * h</td>
<td>1.72 / 1.99</td>
<td>2.50 / 2.45</td>
<td>(81)</td>
</tr>
<tr>
<td><em>Diosmin</em> (diosmetin 7-O-rutinoside)</td>
<td>1 man</td>
<td>1000 mg (493 dios eq.)</td>
<td>3.00</td>
<td>0.170</td>
<td>142.84 ng/ml * h</td>
<td>0.003</td>
<td>1.10</td>
<td>(94)</td>
</tr>
<tr>
<td>Diosmin</td>
<td>2 men and 3 women</td>
<td>10 mg/kg diosmin 4.93 mg/kg dios eq.</td>
<td>1</td>
<td>1.388</td>
<td>5617 ng/ml * h</td>
<td>ND</td>
<td>31.5</td>
<td>(84)</td>
</tr>
</tbody>
</table>

AUC area under the curve, ND not detected, apig apigenin, lut luteolin, dios diosmetin.
Table 1.6. Most common metabolic conversions of flavone O-glycosides.

<table>
<thead>
<tr>
<th>Site of metabolism</th>
<th>Enzyme</th>
<th>Conversion</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small intestine</td>
<td>β-glucosidases (LPH, CBG)</td>
<td>Flavone 7-O-glucoside to aglycone</td>
<td>(95)</td>
</tr>
<tr>
<td>Small intestine, liver</td>
<td>UDP-glucuronyltransferases (UGT1A1, UGT1A8, UGT1A9)</td>
<td>Aglycone to flavone glucuronide</td>
<td>(96)</td>
</tr>
<tr>
<td>Liver</td>
<td>Sulfotransferases (SULT1A1, SULT1E1)</td>
<td>Aglycone to flavone sulfate</td>
<td>(75,97,98)</td>
</tr>
<tr>
<td>Large intestine</td>
<td>Intestinal microbiota</td>
<td>Flavone 7-O-glucoside to aglycone, 3-(4-hydroxyphenyl) propionic acid (4-HPPA)</td>
<td>(71)</td>
</tr>
</tbody>
</table>

The small intestine, which is longer and contains villi and microvilli, is much more effective at absorbing compounds (89). There is also evidence that the small intestine is a major site of hydrolysis and metabolism of flavone O-glycosides. Cleavage of flavonoid glycosides by β-glucosidase in the small intestine was tested in vitro with a Caco-2 model and cultured small intestinal cells from 10 people. The human small intestinal cells showed up to 87-fold interindividual variation in β-glucosidase activity, with variation also depending on the flavonoid conjugate being hydrolyzed (86). Two β-glucosidases in small intestinal cells can cleave flavonoid glycosides (86). Lactase-phlorizin hydrolase (LPH) is a membrane-bound enzyme that can act on flavonoid glycosides in the lumen, including apigenin and luteolin O-glycosides, but the number and position of glucose moieties can substantially slow the reaction rate. Cytosolic β-glucosidase (CBG) resides inside cells and would require active transport into the cytoplasm. LPH did not hydrolyze rhamnose, xylose, arabinose, and galactose from the flavonoids tested (86). CBG is found in human liver, intestine, spleen, and kidney. It
preferentially hydrolyzes β-D-fucose, β-D-glucose, α-L-arabinose, and β-D-galactose, but can also hydrolyze β-L-arabinose and β-D-xylose linkages. Of the flavone glycosides tested, it had a greater specificity for luteolin 4-glucoside over apigenin 7-glucoside and luteolin 7-glucoside (95). In vitro and in vivo studies with rats indicated that quercetin glucoside was more readily hydrolyzed and absorbed than quercetin galactoside (90), but it is not clear which enzyme is predominantly responsible for hydrolysis. LPH also hydrolyzes lactose, and is therefore not produced in lactose-intolerant individuals; this is a possible source of interindividual variation (86).

After flavone O-glycosides are cleaved to aglycones, they are absorbed into the small intestinal epithelial cells. It has been proposed that flavonoids such as quercetin are transported into cells by passive diffusion (99-101), which would favor the transport of less polar (e.g. aglycone) molecules through the lipid bilayer membrane. While the sodium-dependent glucose transporter (SGLT1) is involved in the uptake of some flavonoid glucosides (102), it does not appear to transport flavones and in fact may be inhibited by luteolin (103).

Flavones are extensively metabolized by intestinal cells to glucuronidated and sulfated forms and then effluxed both back to the intestinal lumen and to the bloodstream. Efflux of apigenin sulfate is mediated by both multidrug resistance-related proteins (MRPs) and organic anion transporters (OATs), while efflux of apigenin glucuronide is mediated by MRPs. This efflux of flavone metabolites is likely a rate-limiting step in transport across the intestinal membrane (87). In vitro studies with human liver and intestinal microsomes found that luteolin was glucuronidated primarily at the 7 position in liver cells and at the 3’ and 4’ positions in intestinal cells. This differed markedly from
rats, where liver microsomes glucuronidate luteolin primarily at the 3’ position. Intestinal microsomes conjugated nearly three times as much luteolin as liver microsomes. When individual enzymes were tested, some glucuronidated luteolin much more efficiently than others, with UGT1A1, UGT1A8, and UGT1A9 being especially effective (96).

After flavones and their metabolites are transported to the basolateral side of the small intestine, they are carried to the liver via the hepatic portal vein (89). When apigenin was incubated with human liver microsomes and cytosol, monoglucuronides were produced by microsomes and monosulfates were produced in the cytosol. Apigenin was more rapidly glucuronidated than sulfonated (75). In contrast, an in vitro study using HEPG2 liver cells found that luteolin absorbed into the cells was predominantly methylated, with some conjugated with sulfate or glucuronide groups and some left as aglycone. Most of the apigenin absorbed into the cells was left unmetabolized, with a small amount conjugated to sulfate or glucuronide. Luteolin, apigenin, chrysin, and their metabolites were all taken into nuclei (97).

Flavonoids that reach the colon can be further hydrolyzed and metabolized by bacteria. Rats were either kept germ-free or associated with human intestinal microbiota. After giving the rats apigenin as an intragastric apigenin 7-glucoside dose, different levels of apigenin, apigenin conjugates, and other metabolites were excreted in the urine and feces (71). Rats with human intestinal microbiota excreted primarily conjugated apigenin and free 4-HPPA in urine and free apigenin in feces, compared with conjugated apigenin in urine and free and conjugated apigenin in feces of germ-free rats. Rats with human intestinal microbiota excreted more of the following metabolites: free and conjugated naringenin, free phloretin, conjugated luteolin, free 3,4-
dihydroxyphenylpropionic acid (3,4-DHPPA), free and conjugated 3-(4-hydroxyphenyl)propionic acid (4-HPPA) and 4-hydroxycinnamic acid (HCA), and free 3-hydroxyphenylpropionic acid (3-HPPA) in their urine. These are likely intermediates in the conversion to 4-HPPA (71).

In vitro experiments with *Eubacterium ramulus*, an obligate anaerobe found in the human colon, showed that eriodictyol and 3-(3,4-dehydroxyphenyl)propionic acid were metabolites of luteolin (104). Studies with *Clostridium orbiscindens*, found in similar numbers to *E. ramulus* in the intestinal tract, also showed that eriodictyol was a product of luteolin metabolism and naringenin a product of apigenin. Phloretin, 3-(4-dehydroxyphenyl)propionic acid and phloroglucinol were end products of both apigenin and luteolin degradation (105).

**ABSORPTION AND METABOLISM OF FLAVONE C-GLYCOSIDES**

As with flavone O-glycosides, rats are the most common animal model used for flavone C-glycoside absorption (Table 1.7). The $T_{\text{max}}$ for vitexin (apigenin 8-C-glycoside) derivatives was less than one hour, and the $C_{\text{max}}$ was 1-29 µM, varying with the dose (106-108). Urinary excretion of less than 1% confirms that flavone C-glycosides are poorly absorbed (107), and 10-88% recovery from feces indicates that they may be resistant to degradation by gut bacteria in rats (107,109). Like flavone O-glycosides, the C-glycosides are less bioavailable in humans than in rats (Tables 1.7 & 1.8). Although metabolites of apigenin or luteolin C-glycosides were found in plasma after human subjects drank rooibos tea, the concentrations were less than 1 nM (40). Gastrointestinal and feces extracts showed three primary metabolites by intestinal bacteria:
phloroglucinol, hydrocaffeic acid, and phloretic acid (109). When luteolin 6-C-glucoside (isoorientin) was incubated with human intestinal bacteria, only a small amount of luteolin aglycone was produced. The most abundant metabolites were 3,4-DHPPA and eriodictyol, with small amounts of 6-C-glucosyleriodictyol and phloroglucinol (110).
**Table 1.7.** Absorption of flavone C-glycosides in animal models.

<table>
<thead>
<tr>
<th>No. of animals per treatment group</th>
<th>Dose</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; plasma</th>
<th>Plasma conc.</th>
<th>AUC</th>
<th>Urinary excretion</th>
<th>Recovery from feces</th>
<th>Elim. half-life</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>h</td>
<td>µmol/L</td>
<td></td>
<td>% of intake</td>
<td>% of intake</td>
<td>h</td>
<td></td>
</tr>
<tr>
<td>5 Wistar rats</td>
<td>120 mg vitexin O-rhamnoside/kg</td>
<td>0.5</td>
<td>1.39</td>
<td>82,010 Μµg * min/ ml</td>
<td>n/a</td>
<td>n/a</td>
<td>0.3</td>
<td>(108)</td>
</tr>
<tr>
<td>3 Sprague-Dawley rats</td>
<td>50 mg vitexin rhamnoside/kg</td>
<td>0.92</td>
<td>~1.7</td>
<td>1745 Μµg * h/L (0-∞)</td>
<td>n/a</td>
<td>n/a</td>
<td>1.15</td>
<td>(106)</td>
</tr>
<tr>
<td>4 Sprague-Dawley rats</td>
<td>1 g/kg bamboo leaf extract containing vitexin, isovitexin, orientin, isoorientin</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>24.0 orientin 24.0 isoorientin 9.7 vitexin 20.9 isovitexin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-5 SPR Wistar rats</td>
<td>83.3 mg/kg vitexin glucoside, 342.5 mg/kg vitexin rhamnoside (as <em>Crataegus pinnatifida</em> leaf extract)</td>
<td>0.75</td>
<td>6.9/28.5</td>
<td>11.7/47.9 Μµg/m 1*h</td>
<td>0.70/0.72</td>
<td>64.2/88.3</td>
<td>2.53/2.3</td>
<td>(107)</td>
</tr>
<tr>
<td>Beagle dogs</td>
<td>576 mg vitexin rhamnoside (as Yixintong tablets)</td>
<td>1.0-4.0</td>
<td>NR</td>
<td>5880-6792 ng*h / ml</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>(111)</td>
</tr>
</tbody>
</table>

AUC area under the curve, NR not reported.
Table 1.8. Bioavailability of flavone C-glycosides in humans.

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of subjects</th>
<th>Dose</th>
<th>$T_{\text{max}}$</th>
<th>Plasma conc.</th>
<th>AUC</th>
<th>Urinary excretion</th>
<th>Elimination half-life</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rooibos teas (fermented and un-fermented leaves)</td>
<td>5 women and 5 men</td>
<td>0.1-0.2 mg lut, 12.2-12.6 mg lut C-glycosides, 2.0-2.2 mg apig C-glycosides</td>
<td>n/a</td>
<td>ND</td>
<td>n/a</td>
<td>ND</td>
<td>n/a</td>
<td>(112)</td>
</tr>
<tr>
<td></td>
<td>10 men</td>
<td>43 mg lut C-glycosides, 5.6 mg apig C-glycosides, 0.9 mg lut O-glycosides</td>
<td>1.5-3</td>
<td>&lt;0.001</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>(40)</td>
</tr>
</tbody>
</table>

*ND, not detected.*
Several animal studies have explored the effects of orally administered flavones in vivo (Table 1.9). Most of these studies were conducted in mice and used purified apigenin or luteolin. A comparison of apigenin to genistein and equol found that in contrast to the isoflavones, apigenin reduced the concentration of the estrogen receptor ERα in the uteri of mice, indicating an anti-estrogenic effect (113). However, a longer term study in rats found that apigenin improved many biomarkers associated with osteoporosis, a disease associated with declining estrogen levels and increasing pro-inflammatory cytokines (114). The protective effect against osteoporosis may be due to the higher absorption of flavones in rats (Table 1.4) or to the anti-inflammatory effects of flavones, which are documented in other animal studies. Mice fed apigenin had lower serum IL-6 and TNF-α concentrations following LPS stimulation (115), and luteolin reduced TNF-α production and ear edema following immune stimulation (116,117).

Oral doses of apigenin also showed the ability to counter stress and depression in rats and mice (118). In rats exposed to chronic mild stress, those fed luteolin had normal brain levels of corticosterone, serotonin, and dopamine compared to control animals. Mice given forced swim tests had the same mobility after treatment either apigenin or fluoxetine, a common antidepressant drug (118). Dietary apigenin and flavone C-glycosides can also reduce serum triglyceride and cholesterol levels in mice, showing potential to prevent atherosclerosis (119-121).
Table 1.9. Biological activity of flavones and flavone-containing foods in animal models.

<table>
<thead>
<tr>
<th>Flavones administered</th>
<th>Flavone dose/day</th>
<th>Days</th>
<th>No. animals per group</th>
<th>Biomarkers affected</th>
<th>Biomarkers not affected</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apigenin in DMSO/ethanol administered orally</td>
<td>10 mg/kg 3 times per week</td>
<td>105</td>
<td>6 female Sprague-Dawley rats</td>
<td>Following ovariectomy: increased femur and tibia weights; increased mineral content and bone density; reduced osteocalcin, alkaline phosphatase, and fragments of type I collagen C-terminus</td>
<td>None</td>
<td>(114)</td>
</tr>
<tr>
<td>Apigenin in water, orally administered</td>
<td>7 or 14 mg/kg</td>
<td>28</td>
<td>8 male Wistar rats</td>
<td>Following mild chronic stress: reversed decrease in sucrose intake, prevented increase in serum corticosterone, prevented reduction in adenyl cyclase activity, and attenuated changes in serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), and dopamine in various regions of the brain</td>
<td>Noradrenaline in the brain</td>
<td>(118)</td>
</tr>
<tr>
<td>Apigenin by water gavage</td>
<td>10 or 20 mg/kg</td>
<td>7 or 14</td>
<td>10 male ICR mice</td>
<td>Anti-depressant like effects after 14 days in forced swim test</td>
<td>No difference in swim test with 10 mg/kg for 7 days</td>
<td>(118)</td>
</tr>
<tr>
<td>AIN-93G diet + 0.6% luteolin</td>
<td>19.9 mg</td>
<td>28</td>
<td>12-14 adult and aged male Balb/c mice</td>
<td>Reduced IL-1β RNA in hippocampus of aged mice; improved spatial working memory in aged mice</td>
<td>IL-6, TNF-α, and MHC class II in hippocampus</td>
<td>(122)</td>
</tr>
<tr>
<td>Apigenin by DMSO gavage</td>
<td>100 mg/kg</td>
<td>4</td>
<td>5 C57B6 female mice</td>
<td>Reduced cytosolic and overall ERα in uterus</td>
<td>Estrogenic potential (uterine wt.)</td>
<td>(113)</td>
</tr>
<tr>
<td>Apigenin by 50 µL DMSO gavage</td>
<td>50 mg/kg</td>
<td>1</td>
<td>12 B6C3F1 female mice</td>
<td>Decrease in LPS-induced serum IL-6 and TNF-α production</td>
<td>None</td>
<td>(115)</td>
</tr>
</tbody>
</table>

Continues
Table 1.9. Continued

<table>
<thead>
<tr>
<th>Flavones administered</th>
<th>Flavone dose/day</th>
<th>Days</th>
<th>No. animals number per group</th>
<th>Biomarkers affected</th>
<th>Biomarkers not affected</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF diet + 0.025% apigenin</td>
<td>14</td>
<td>5-6</td>
<td>C57BL/6 N male mice</td>
<td>Reduced serum IgE levels</td>
<td>Body and organ weights; serum IgG, IgM, IgA, 32 serum cytokines</td>
<td>(123)</td>
</tr>
<tr>
<td>AIN-93M diet + 0.025% apigenin</td>
<td>21</td>
<td>5-6</td>
<td>BALB/c mice</td>
<td>Following ovalbumin immunization: reduced serum IgE, CTACK, eotaxin, G-CSF, IL-4, leptin, MCP-1, TPO, and VEGF levels</td>
<td>Body and organ weights; serum IgG, IgM, IgA, 24 serum cytokines</td>
<td>(124)</td>
</tr>
<tr>
<td>Luteolin administered orally</td>
<td>0.1-1 mg</td>
<td>1</td>
<td>3 male ICR mice</td>
<td>Inhibited serum and systemic TNF-α production and ear edema</td>
<td>None</td>
<td>(116)</td>
</tr>
<tr>
<td>Luteolin, apigenin administered orally</td>
<td>1 mg</td>
<td>1</td>
<td>3 male ICR mice</td>
<td>Luteolin reduced TPA-induced ear edema and romurtide/OK-432-induced TNF-α activity</td>
<td>Apigenin did not significantly affect ear edema or TNF-α activity</td>
<td>(117)</td>
</tr>
<tr>
<td>Luteolin, administered orally</td>
<td>1.2 mg/kg per week</td>
<td>21</td>
<td>6 male Balb/c mice</td>
<td>In AOM-induced colon cancer model: increased body weight and reduced liver weight, reduced aberrant crypt foci in colon, reduced plasma peroxyl radicals and lipid peroxidation, reduced MDA-DNA adducts, increased colon enzymatic and non-enzymatic antioxidants, increased plasma non-enzymatic antioxidants</td>
<td>None</td>
<td>(125)</td>
</tr>
<tr>
<td>High fat diet + 0.05% apigenin</td>
<td>1.5-1.8 mg</td>
<td>30</td>
<td>6-9 C57BL/6 Cr Slc male mice</td>
<td>Reduced food intake, body weight, visceral fat, and triglyceride levels</td>
<td>Plasma glucose, total and HDL cholesterol, gonadal fat, liver weight</td>
<td>(119)</td>
</tr>
</tbody>
</table>

Continues
Table 1.9. Continued

<table>
<thead>
<tr>
<th>Flavones administered</th>
<th>Flavone dose/day</th>
<th>Days</th>
<th>No. animals number per group</th>
<th>Biomarkers affected</th>
<th>Biomarkers not affected</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buckwheat sprout extract in propylene glycol containing flavone C-glycosides</td>
<td>100 mg/kg solution (30% rutin, 12% orientin, 17% isoorientin, 16% vitexin, 25% isovitexin)</td>
<td>3</td>
<td>10-11 female ddY mice</td>
<td>Reduced plasma corticosterone, glucose, total cholesterol concentration and reduced plasma and liver lipid peroxidation following restraint stress</td>
<td>Plasma insulin, triglyceride, and HDL cholesterol concentrations, hepatic lipid concentration, plasma glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase activities</td>
<td>(120)</td>
</tr>
<tr>
<td>AIN-93G + 5% or 10% lyophilized buckwheat sprouts containing flavone C-glycosides</td>
<td>4850 mg/100 g dry sprouts (30% rutin, 12% orientin, 17% isoorientin, 16% vitexin, 25% isovitexin)</td>
<td>21</td>
<td>9-10 male diabetic and non-diabetic C57BL mice</td>
<td>In diabetic mice: reduced weight gain and liver weight; reduced blood HbA1c, total cholesterol, and HDL cholesterol; increased bile acid concentration in feces; increased hepatic lipids and reduced total cholesterol and triglycerides in liver tissue; increased mRNA of HMG-CoAR and CYP7A1; reduced plasma and liver lipid peroxidation</td>
<td>Epididymal adipose tissue weight, plasma glucose, mRNA of other enzymes of lipid metabolism</td>
<td>(121)</td>
</tr>
</tbody>
</table>
BIOLOGICAL ACTIVITY OF FLAVONES IN HUMANS

Several intervention studies have been carried out with flavones and flavone-containing foods in humans (Table 1.10). A trial using fresh and cooked parsley as a flavone source found that the treatment group had higher blood antioxidant enzyme activities (79). Other interventions with flavone-rich foods showed that they differed in their effects on biomarkers for heart disease and stroke. In a crossover study, subjects showed no differences in platelet aggregation after consuming a dried parsley supplement for 7 days, even though apigenin appeared effective at doses as low as 2.5 µM in vitro (77). An 84-day study using artichoke leaf extract as an apigenin source found that the treatment group had lower total cholesterol than those taking placebo, but there were no significant differences in HDL cholesterol, LDL cholesterol, or triglycerides (126). Supplementation with vitexin-rich hawthorn extract lowered total and LDL cholesterol after 6 months, but also had no effect on HDL cholesterol or triglycerides (127). Diosmetin rutinoside supplements have been successfully used in Europe to reduce bleeding and improve wound healing (128-130). The proposed mechanisms of action include increasing venous tone and countering inflammatory mediators (130), congruent with the anti-inflammatory activity of flavones already demonstrated in animal studies (Table 1.9).

The efficacy of flavones in human clinical trials cannot always be predicted by in vitro, animal, or bioavailability studies. One reason is that although flavones are found in circulation as glucuronide or sulfate conjugates, most in vitro experiments are done with aglycones or glucoside conjugates. Human neutrophils can deconjugate luteolin glucuronide to aglycone in circulation, releasing the biologically active aglycone (131),
but the extent of this process in vivo is unknown. Flavones may also be active in the intestinal lumen rather than in circulation (132), providing an explanation for the biological activity of the poorly available flavone C-glycosides.

CONCLUSION

Flavones occur in a wide variety of fruits, vegetables, and beverages. In their native forms, they are found as both O- and C-glycosides, with O-glycosides being more common and usually better absorbed. Relative to other polyphenols, flavones are not well absorbed, with plasma concentrations typically less than 1 µM in humans. However, flavones have demonstrated many potentially beneficial activities in animal studies and human trials, and these should be further explored. Future clinical trials should not only take into consideration the dose but also the flavone form, as some native glycosides are poorly available and could be improved through processing.
<table>
<thead>
<tr>
<th>Substance given</th>
<th>Flavone(s)</th>
<th>Dose/day</th>
<th>Days</th>
<th>No. of subjects per group</th>
<th>Biomarkers affected</th>
<th>Biomarkers not affected</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parsley</td>
<td>Apigenin</td>
<td>20 g parsley (4.5 mg apigenin)</td>
<td>7</td>
<td>7</td>
<td>Increase in erythrocyte glutathione reductase and erythrocyte superoxide dismutase activities</td>
<td>2-adipic semialdehyde, erythrocyte glutathione peroxidase, erythrocyte catalase</td>
<td>(79)</td>
</tr>
<tr>
<td>Parsley</td>
<td>Apigenin</td>
<td>4.9 g dried parsley (84 mg apigenin)</td>
<td>7</td>
<td>18</td>
<td>None</td>
<td>Factor VII activity, fibrinogen, plasminogen activity, PAI-1 activity, thromboxane B&lt;sub&gt;2&lt;/sub&gt; production, collagen-induced aggregation, ADP-induced aggregation, platelet number</td>
<td>(77)</td>
</tr>
<tr>
<td>Artichoke leaf extract</td>
<td>Luteolin 7-glucuronide</td>
<td>1280 mg extract (5.1 mg luteolin glucuronide)</td>
<td>84</td>
<td>37-38</td>
<td>Reduced total plasma cholesterol</td>
<td>LDL and HDL cholesterol, triglycerides</td>
<td>(126)</td>
</tr>
<tr>
<td>Artichoke leaf extract</td>
<td>Luteolin 7-glucoside</td>
<td>320 or 640 mg extract (~2.4 or 4.8 mg luteolin glucoside)</td>
<td>60</td>
<td>231-223</td>
<td>Reduced mild dyspepsia: digestion, abdominal pains, nausea</td>
<td>None</td>
<td>(133)</td>
</tr>
</tbody>
</table>

Continues
<table>
<thead>
<tr>
<th>Substance given</th>
<th>Flavone(s)</th>
<th>Dose/day</th>
<th>Days</th>
<th>No. of subjects per group</th>
<th>Biomarkers affected</th>
<th>Biomarkers not affected</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venex tablet</td>
<td>Diosmetin rutinoside</td>
<td>900 mg</td>
<td>7</td>
<td>74-76</td>
<td>Reduced rhinorrhoea, sneezing, nasal obstruction, and eosinophils in nasal smear</td>
<td>None</td>
<td>(134)</td>
</tr>
<tr>
<td>Micronized purified flavonoid fraction (MPFF)</td>
<td>Diosmetin rutinoside (90%), hesperetin rutinoside</td>
<td>1800 mg for 10 days, then 900 mg for 20 days</td>
<td>30</td>
<td>25</td>
<td>Following hemorrhoidectomy: reduced bleeding, pain, tenesmus, and pruritis</td>
<td>None</td>
<td>(129)</td>
</tr>
<tr>
<td>Micronized purified flavonoid fraction (MPFF)</td>
<td>Diosmetin rutinoside (90%), hesperetin rutinoside</td>
<td>1st phase: 2700 mg for 4 days &amp; 1800 mg for 3 days; 2nd phase: 900 mg for 83 days</td>
<td>90</td>
<td>50</td>
<td>Cessation of bleeding from internal hemorrhoids</td>
<td>Blood pressure; serum creatinine, aspartate aminotransferase, alanine aminotransferase, and bilirubin</td>
<td>(130)</td>
</tr>
<tr>
<td>Micronized purified flavonoid fraction (MPFF)</td>
<td>Diosmetin rutinoside (90%), hesperetin rutinoside</td>
<td>1000 mg</td>
<td>60</td>
<td>52-53</td>
<td>Reduced healing time of leg ulcers</td>
<td>None</td>
<td>(135)</td>
</tr>
</tbody>
</table>

Continues
<table>
<thead>
<tr>
<th>Substance given</th>
<th>Flavone(s)</th>
<th>Dose/day</th>
<th>Days</th>
<th>No. of subjects per group</th>
<th>Biomarkers affected</th>
<th>Biomarkers not affected</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venex tablet</td>
<td>Diosmin</td>
<td>500 mg</td>
<td>9</td>
<td>12</td>
<td>Pretreatment with diosmin increased metronidazole in circulation, reduced acid and hydroxyl metabolites</td>
<td>None</td>
<td>(136)</td>
</tr>
<tr>
<td><em>Vigna radiata</em> seed extract (Vitexina)</td>
<td>Vitexin and isovitexin</td>
<td>360 mg vitexin, 40 mg isovitexin</td>
<td>42</td>
<td>36</td>
<td>Following radiation therapy; increased erythrocyte and platelet counts</td>
<td>Hemoglobin and leukocytes</td>
<td>(137)</td>
</tr>
<tr>
<td>Hawthorn extract</td>
<td>Vitexin 2'-O-rhamnoside (≥1.8%)</td>
<td>500 mg</td>
<td>70</td>
<td>9</td>
<td>None</td>
<td>Blood pressure (resting, after stress, after exercise), anxiety</td>
<td>(138)</td>
</tr>
<tr>
<td><em>Crataegus laevigata</em> extract (hawthorn)</td>
<td>Vitexin 2'-O-rhamnoside (1.4%)</td>
<td>1200 mg extract (17 mg vitexin rhamnoside)</td>
<td>180</td>
<td>21-24</td>
<td>Reduced total, LDL, and non-HDL cholesterol after 6 mos; reduced neutrophil elastase after 6 mos</td>
<td>Blood glucose, leukocyte and neutrophil counts, HDL cholesterol, triglycerides, malondialdehyde, C-reactive protein</td>
<td>(127)</td>
</tr>
</tbody>
</table>
Chapter 2

Effects of food formulation and thermal processing on flavones in celery

Gregory Hostetler, Ken Riedl, and Steven Schwartz

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 \((10,32,51)\). The flavone apigenin exhibits toxicity to cancer cells in vitro \((139-143)\) and animal studies with flavones demonstrate the ability to attenuate the inflammatory response \((117,144)\). However, human trials with flavone rich foods such as parsley and celery show limited bioavailability of these compounds, with maximum plasma concentrations of less than one micromolar \((76,78)\). The time of maximum plasma concentration after eating these foods was over 7 hours \((76,78)\), indicating that the flavones are likely absorbed in the colon rather than the small intestine. Future use of flavone-rich foods for health benefits requires a better understanding of the forms that are most readily absorbed and their stability during food processing.

Both parsley and celery are rich in flavone glycosides, particularly apigenin 7-O-apiosylglucoside \((apiin) (24,80)\). Because intestinal absorption of flavonoid glycosides varies according to the aglycone core and the sugars and others functional groups

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2 Written as a manuscript for the Journal of Agricultural and Food Chemistry, in preparation.
attached, the glycosylation of flavones may determine their site and efficiency of absorption. Similar to other flavonoids, flavone glucosides can be hydrolyzed by intestinal $\beta$-glucosidase (86) and absorbed in the small intestine. In contrast, flavonoid glycosides with disaccharide and malonyl moieties are more resistant to intestinal $\beta$-glucosidase than their simple glucoside counterparts, potentially limiting their bioavailability (86). Caco-2 models show that flavone aglycones were more readily absorbed into intestinal cells than flavone glucosides, with 10 times more apigenin or luteolin transported across the intestinal epithelium than apigenin 6-C-glucoside or luteolin 7-O-glucoside (145).

Flavone glycosides in foods may be modified slightly by endogenous enzymes such as malonyl esterases (e.g. conversion from malonyl apiin to apiin) (64), but remain as glycosides after processing such as shredding lettuce (65), juicing artichoke heads (66), and heating orange juice (44). Rhamnosidase has been used to convert hesperetin rhamnosyl glucoside to hesperetin glucoside, thereby improving bioavailability 4-fold (146). The beta linkage of flavonoid glycosides can also be cleaved by $\beta$-glucosidase to release flavonoid aglycones (147), and $\beta$-glucosidase-rich ingredients such as almond (148), flax seeds (149), and chickpeas (150) can potentially be used for this purpose in food processing. In an effort to develop a flavone-rich functional food, we tested several processing parameters for their effects on flavone glycosylation in order to improve the bioavailability of flavones from celery. In addition, we evaluated the thermal stability of purified flavone derivatives from celery (Fig. 2.1) to better understand processing effects on flavone-rich foods.
Figure 2.1. Flavone aglycones and glycosides derived from celery.
MATERIALS AND METHODS

Chinese celery (*Apium graveolens* L., Apiaceae), raw almonds (*Prunus dulcis* Mill., Rosaceae), flax seeds (*Linum usitatissimum* L., Linaceae), and chickpea flour (*Cicer arietinum* L., Fabaceae) were purchased from local grocery stores in Columbus, Ohio. Flavo-Natin tablets with chamomile extract were obtained from Koehler-Pharma GmbH (Alsback-Naehnlein, Germany). Formic acid, apigenin, and luteolin were from Sigma (St. Louis, MO), and apigenin 7-O-glucoside from Chromadex (Irvine, CA). Ammonium acetate was from J. T. Baker (Phillipsburg, NJ). Phosphoric acid was obtained from Corco Chemical Corp. (Fairless Hills, PA). All other reagents were from Fisher Scientific (Fair Lawn, NJ).

Effects of Food Ingredients on Flavone Deglycosylation. Celery leaves were separated from stalks, macerated, and lyophilized. Almonds and flax seeds were ground in a coffee grinder. Crushed chamomile tablet (250 mg) or lyophilized celery leaves (125 mg) were combined with an equal weight of ground almond, ground flax seed, or chickpea flour in 2.5 mL sodium acetate buffer (1.4 M, pH 5.0). The mixtures were incubated at 37 °C for 20 hours to allow enzymes in the ingredients to deconjugate flavone glycosides in the chamomile tablets or celery leaves. After incubation, the samples were extracted once with 2.5 mL methanol and twice with 2.5 mL 70% (v/v) aqueous methanol. The supernatants were combined and analyzed by HPLC.

Preparative HPLC of Flavones. Celery leaves were crushed, lyophilized, and extracted with 2.5 mL 70% (v/v) aqueous methanol. To isolate flavone apiosylglucosides, the original extract was used. An aliquot of the original 70% methanol extract was hydrolyzed with HCl at 90 °C to hydrolyze flavone apiosylglucosides to flavone
glucosides and aglycones. The hydrolyzed extract was dried under N$_2$ and reconstituted in 50% aqueous methanol. Extracts were filtered through 0.45 µm nylon and separated by preparative high performance liquid chromatography (HPLC) using an HP 1050 separations module (Agilent Technologies, Santa Clara, CA), SunFire C$_{18}$ column (5 µm, 19 x 150 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 following gradient method was used: 0-1 min, 5% B; 1-16 min, 21% B; 16-35 min, 30% B; 35-40 min, 100% B; 40-45 min, 5% B. Analytical HPLC confirmed that each compound was >97% pure.

**Thermal Stability of Purified Flavones.** Ammonium acetate buffer (0.1 M) was adjusted to pH 3, 5, or 7 with ammonium hydroxide or formic acid. Apigenin 7-O-apiosylglucoside (apiin), apigenin 7-O-glucoside, apigenin, luteolin, and chrysoeriol isolated from celery from dissolved in pH 3, 5 or 7 buffers to make 25 µM solutions of 4 ml each. Solutions were heated for 5 h in a boiling water bath (100 °C), taking 400 µL samples at 0, 60, 120, 180, 240, and 300 min. Aliquots were diluted 1:1 with methanol, sonicated, and analyzed directly by HPLC.

**Thermal Stability of Flavones in Celery.** To determine the effects of heat and acidity on flavones in celery, fresh celery leaves (500 mg) were macerated, acidified with 1.5 N H$_3$PO$_4$ (1 mL), and left at room temperature (25 °C) for 90 min or heated in a boiling water bath (100 °C) for 90 min. After processing the samples were extracted three times with 2.5 mL 70% (v/v) aqueous methanol, and the supernatants were combined and analyzed by HPLC.
Effects of Food Ingredients on Flavone Glycosides in Thermally Processed Celery. Fresh celery leaves were macerated, acidified to pH 2.7 with 1.5 N \( \text{H}_3\text{PO}_4 \), and heated in a boiling water bath (100 °C) for 90 min. Samples were then neutralized to pH 5 with 100 µL 10% NaOH and combined with almond powder, ground flax seed, or chickpea flour. Blended samples were incubated at 50 °C for 2 h and cooled on ice. After processing the samples were extracted three times with 2.5 mL 70% (v/v) aqueous methanol, and the supernatants were combined and analyzed by HPLC.

**HPLC Analysis.** Analysis of celery, chamomile tablets, and purified flavones was conducted by HPLC. Commercial apigenin, luteolin, chrysoeriol, apigenin 7-O-glucoside, and apigenin 7-O-apiosylglucoside standards were used to identify compounds by UV spectra and retention times. For quantification, the HPLC system included a Waters 2695 separations module, a 2996 photodiode array detector, a Symmetry C\(_{18}\) column (3.5 µm, 4.6 x 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 celery, chamomile tablets, apiin, apigenin 7-O-glucoside, apigenin, and chrysoeriol, 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. Luteolin isolated from celery was quantified with the following gradient: 0-10 min, 5-30% B; 10-13 min, 30-80% B; 13-15 min, 80-100% B; 15-17 min, 100% B; 17-20 min, 5% B. Peak areas were measured at 338 nm for apigenin and 350 nm for luteolin and chrysoeriol. Because the flavone glycosides in this study shared the same wavelengths of maximum absorption and extinction coefficients as their aglycone
counterparts, concentrations of all flavones were determined with aglycone standard curves.

**Statistical Analysis and Lines of Best Fit.** Statistical analysis was performed with R 2.11 for Windows (R Foundation for Statistical Computing, Vienna, Austria) using one-way analysis of variance (ANOVA). Post hoc comparisons were done with Tukey’s HSD. For flavone thermal stability studies, lines of best fit were calculated with Microsoft Excel (linear and polynomial equations) or OpenOffice Calc (exponential equations).

RESULTS

**Effects of Food Ingredients on Flavone Deglycosylation.** The chamomile extract contained predominantly apigenin 7-O-glucoside with smaller concentrations of apigenin 7-O-acetyl glucoside and apigenin (Fig. 2.2). After incubation with almond, flax seed, and chickpea flour for 20 h at 37 °C, concentrations of apigenin 7-O-glucoside decreased from over 400 mg/100 g to less than 50 mg/100 g. In the same samples, apigenin concentrations increased from less than 100 mg/100 g to over 500 mg/100 g. The concentration of apigenin acetyl glucoside was not significantly different from the control with any of the treatments (Fig. 2.2).
Lyophilized celery leaves contained primarily apiin, with smaller concentrations of apigenin (Fig. 2.3). While apigenin glycosides were the most abundant in the celery samples (75% of total flavone aglycone equivalents by weight), luteolin glycosides (14%) and chrysoeriol glycosides (11%) were also found. Almond and flax seed treatments led to significantly higher apigenin aglycone concentrations after incubation (Fig. 2.3), but the concentrations only increased from 2.7% of all apigenin derivatives to 3.6% and 5.0% for almond and flax seed respectively.

**Figure 2.2.** Conversion of apigenin glycosides in chamomile extract to aglycone after incubation for 20 h at 37 °C alone and combined 1:1 (w/w) with other food ingredients. Data are mean ± SD, n=3. *P<0.001 versus control.
Figure 2.3. Conversion of apigenin glycosides in celery to aglycone after incubation for 20 h at 37 °C alone and combined 1:1 (w/w) with other food ingredients. Data are mean ± SD, n=3. *P<0.05 versus control.

**Thermal Stability of Purified Flavones.** After observing the stability of apigenin 7-O-apiosylglucoside (apiin) to incubation with enzyme-rich food ingredients, we tested several flavone derivatives for their thermal stability. Apiin, apigenin 7-O-glucoside, apigenin, luteolin, and chrysoeriol were heated at 100 °C for up to 300 min at pH 3, 5, or 7. Apiin was relatively stable to thermal processing at pH 5 and 7, but its concentration fell rapidly after heating 1 h at pH 3 (Fig. 2.4A). The primary degradation product of apiin at pH 3 was apigenin 7-O-glucoside (data not shown).
Figure 2.4. Stability of flavones at 100 °C for up to 300 min at pH 3, 5, or 7. (A) apiin, (B) apigenin 7-O-glucoside, (C) apigenin, (D) luteolin, (E) chrysoeriol. Data are mean, n=3.

Of all flavones tested, apigenin 7-O-glucoside was the most stable to thermal processing at every pH tested, retaining 90-95% of the original flavone concentrations.
after 5 h of heat treatment (Fig. 2.4B). The flavone aglycones were all relatively stable to at pH 3 and degraded linearly (Fig. 2.4C,D,E; Table 2.1). Both apigenin and chrysoeriol showed similar thermal stability and pH 5 and 7 and degraded asymptotically (Fig. 2.4C,E). Luteolin degraded exponentially at pH 5 and 7, and only 2% remained after heating 2 h (Fig. 2.4E). Lines of best fit for degradation curves are listed in Table 2.1.

**Table 2.1.** Lines of best fit describing thermal stability of flavones in Figure 2.4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH</th>
<th>Best fit line</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>apiin</td>
<td>3</td>
<td>$y = 150.80 \cdot 0.7^{-0.35x}$</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>$y = -1.82x + 102.49$</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>$y = -3.82x + 104.02$</td>
<td>0.99</td>
</tr>
<tr>
<td>apigenin 7-O-glucoside</td>
<td>3</td>
<td>$y = -1.00x + 101.51$</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>$y = -1.27x + 103.07$</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>$y = -1.87x + 102.21$</td>
<td>0.95</td>
</tr>
<tr>
<td>apigenin</td>
<td>3</td>
<td>$y = -2.09x + 102.14$</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>$y = 1.28x^3 - 13.67x^2 + 22.31x + 90.40$</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>$y = 1.21x^3 - 14.13x^2 + 29.79x + 83.17$</td>
<td>0.99</td>
</tr>
<tr>
<td>luteolin</td>
<td>3</td>
<td>$y = -2.53x + 104.17$</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>$y = 291.63 \cdot 0.48^x$</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>$y = 556.15 \cdot 0.17^x$</td>
<td>0.99</td>
</tr>
<tr>
<td>chrysoeriol</td>
<td>3</td>
<td>$y = -2.64x + 103.33$</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>$y = 1.31x^3 - 14.56x^2 + 27.24x + 85.79$</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>$y = 1.04x^3 - 11.50x^2 + 17.55x + 93.02$</td>
<td>0.99</td>
</tr>
</tbody>
</table>

$x=(\text{minutes}/60)+1$

**Thermal Stability of Flavones in Celery.** Because apigenin 7-O-glucoside was the residual product of thermal degradation of apiin at pH 3 and apigenin 7-O-glucoside was very resistant to thermal degradation (Fig. 2.4B), we decided to thermally treat celery to maximize this conversion. When celery was acidified to pH 2.7 and left at room
temperature (25 °C), the predominant apigenin derivative was apiin (Fig. 2.5), as was demonstrated previously with untreated celery. After acidification to pH 2.7 and thermal processing for 90 min at 100 °C, apigenin 7-O-glucoside became the most abundant derivative, with a small amount of aglycone also present (Fig. 2.5). The total concentration of derivatives was similar with both treatments, showing no loss of total flavones. This demonstrates that both heat and low pH are necessary for the hydrolysis of apiin to apigenin 7-O-glucoside in celery.

**Figure 2.5.** Conversion of apiin in celery to apigenin 7-O-glucoside and aglycone after acidification to pH 1.2 and thermal processing for 1 h at 100 °C Data are mean ± SD, n=3.
Effects of Food Ingredients on Flavone Glycosides in Thermally Processed Celery. After apiin in celery was converted to apigenin 7-O-glucoside, we neutralized it to pH 5 and combined it with the same enzyme-rich food ingredients used previously in order to convert apigenin 7-O-glucoside to apigenin (aglycone). After blending lyophilized celery 1:1 (by weight) with almond, flax seed, or chickpea flour and incubating at 50 °C for 2 h, almond and flax seed treatments contained predominantly aglycone as an apigenin derivative (Fig. 2.6). Although the chickpea flour treatment also contained apigenin aglycone, it retained a greater amount of apigenin 7-O-glucoside (Fig. 2.6).

![Bar graph showing conversion of apigenin 7-O-glucoside in celery to aglycone after incubation for 2 h at 50 °C alone and combined with other food ingredients 1:1 (w/w). Data are mean ± SD, n=3.](image)

**Figure 2.6.** Conversion of apigenin 7-O-glucoside in celery to aglycone after incubation for 2 h at 50 °C alone and combined with other food ingredients 1:1 (w/w). Data are mean ± SD, n=3.
To further test the ability of enzyme-rich ingredients to convert apigenin 7-O-glucoside to aglycone, celery was thermally processed and neutralized as before. The lyophilized apigenin 7-O-glucoside-rich celery was then blended 5:1 (w:w) with almond or flax seed and incubated at 50 °C for 2 h. The predominant apigenin derivative in the heated celery control (pH 2.7, 90 min at 100 °C) contained primarily apigenin 7-O-glucoside (Fig. 2.7). Both almond and flax seed resulted in nearly complete conversion of apigenin 7-O-glucoside to aglycone, with less than 5% of apigenin 7-O-glucoside remaining (Fig. 2.7). The concentration of total apigenin derivatives was 30-35% lower with the almond or flax seed treatments, indicating a substantial loss of total flavones.

![Figure 2.7](image)

**Figure 2.7.** Conversion of apigenin 7-O-glucoside in acid-treated celery (pH 2.7) to aglycone after incubation for 2 h at 50 °C alone and combined with other food ingredients 5:1 (w/w). Data are mean ± SD, n=3.
DISCUSSION

To test the ability of β-glucosidase-rich ingredients on flavone glycosides, we combined almond powder, ground flax seeds, or chickpea flour with chamomile extract or lyophilized celery. Chamomile extract contained primarily apigenin 7-O-glucoside, which was readily hydrolyzed by all three added enzyme sources (Fig. 2.2). In contrast, the same added ingredients had no hydrolytic effects of apiin in celery after incubation at 37 °C for 20 h (Fig. 2.3). Although the β-glucosidases from chickpeas can cleave isoflavone 7-O-apiosylglucosides (150), we did not see this activity on apiin in our experiments. Similarly, β-glucosidase from almonds can cleave isoflavone glucosides (151) but had no effect on celery flavone apiosylglucosides. We are not aware of other studies using flax seeds to hydrolyze flavonoid glycosides. Here we report that it is effective on apigenin 7-O-glucoside but not apigenin 7-O-apiosylglucoside.

After testing the stability of celery flavone glycosides to β-glucosidases, we isolated individual flavone glycosides and aglycones to test their thermal stability. Apiin, apigenin 7-O-glucoside, apigenin, luteolin, and chrysoeriol were heated at 100 °C for up to 5 h at pH 3, 5, or 7. We found that while apiin was stable at pH 5 or 7, in degraded steadily at pH 3 (Fig. 2.4A). Apigenin 7-O-glucoside was very stable at 100 °C regardless of pH (Fig. 2.4B). Apigenin glycosides are also more stable at pH 2 rather than pH 7 at 25 °C (26). Our results differed from those reported for other flavonoid glycosides. Quercetin rutinoside, another flavonoid diglycoside, had greater thermal stability at pH 5 than at pH 8 (152). Cyanidin 3-O-glycosides in carrots and elderberries had half lives of only 1.2-2.6 h when heated at 95 °C and pH 3.5 (153).
The flavone aglycones apigenin, luteolin, and chrysoeriol were all stable at pH 3, but degraded steadily at pH 5 or pH 7 (Fig. 2.4C,D,E). The most rapid degradation occurred with luteolin, which contains a catechol group on the B ring, at pH 7 (Fig. 2.4D). Quercetin was more stable at pH 5 than at pH 8 (152), and anthocyanins are known to be more stable at lower pH values (154).

The main conversion product of apiin at pH 3 was apigenin 7-O-glucoside, and these results combined with the ability of enzyme-rich foods to hydrolyze apigenin 7-O-glucoside (Fig. 2.2) suggested that aglycone-rich celery could be produced by combining the two processes. By acidifying the celery to pH 2.7, heating 90 min at 100 °C was necessary for nearly complete conversion of apiin to apigenin 7-O-glucoside (Fig. 2.5). After neutralization to pH 5, apigenin glucoside-rich celery was then incubated with almond powder, ground flax seeds, or chickpea flour to convert apigenin glucoside to aglycone. At a ratio of one part fresh celery to one part β-glucoside-rich ingredient, both almond and flax seed successfully converted apigenin glycosides to aglycone, although with some loss of total apigenin derivatives (Fig. 2.6). Further experiments showed that fresh celery can be mixed with as little as 5:1 almond or flax seeds to achieve over 95% conversion of apigenin glycosides to aglycone (Fig. 2.7). However, this again resulted in up to 30-35% loss of total apigenin derivatives, indicating degradation of apigenin during processing.

In conclusion, we show that although apiin from celery is resistant to β-glucosidase activity, it can be effectively hydrolyzed to apigenin 7-O-glucoside with a combination of acid and heat. Apigenin 7-O-glucoside can then be enzymatically converted to apigenin, resulting in an aglycone-rich food. We also show that apigenin
glycosides are very resistant to degradation during thermal processing, while aglycones are labile above pH 3. Together these results provide insights into making functional foods with maximum flavone concentrations and bioavailability.
Chapter 3

Endogenous enzymes, heat, and pH affect flavone profiles in parsley (*Petroselinum crispum var. neapolitanum*) and celery (*Apium graveolens*) during juice processing

Gregory Hostetler, Ken Riedl, and Steven Schwartz

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 (10, 32, 51). In their native forms they are conjugated to sugars, simple acids (acetyl and malonyl) and cinnamic acids (Fig. 3.1) rather than existing as aglycones. *In vitro* studies demonstrate that the flavone apigenin inhibits human lung, colon, breast, prostate, brain, and skin cancer cells (139-143); tongue cancer (155); and leukemia (156). Apigenin and luteolin also reduce monocyte adhesion to LDL *in vitro*, showing potential to prevent one of the initial stages of atherosclerosis (157). In addition, animal studies with flavones demonstrate the ability to attenuate the inflammatory response (117, 144).

The glycosylation of flavones in food is important because intestinal absorption of flavonoid glycosides varies according to the aglycone core and the sugars and others functional groups attached. Similar to other flavonoids, flavone glucosides can be hydrolyzed by β-glucosidase in the small intestine (86), absorbed in the small intestine,

3 Written as a manuscript for the Journal of Agricultural and Food Chemistry, in preparation.
and subsequently glucuronidated and sulfated by the small intestine and liver before reaching systemic circulation \((96,158)\). In contrast, flavonoid glycosides with disaccharide and malonyl moieties are more resistant to intestinal \(\beta\)-glucosidase than their simple glucoside counterparts, potentially limiting their bioavailability \((86)\). Therefore, determining the native glycosides is potentially useful when developing functional foods.

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 \((145)\). Experiments with isolated rat small intestine showed a similar pattern for isoflavones. Nearly twice as much genistein and daidzein (aglycones) were absorbed and transported to the basolateral side than their 7-O-glucoside counterparts, and the malonyl glucoside conjugates were absorbed in only trace amounts \((159)\).
Both parsley and celery are rich in flavone apiosylglucosides and can potentially be used in a food-based approach to prevention of chronic disease. In the current study, our objective was to determine how juice processing, endogenous enzymes, acidification, and thermal processing affect the stability and profiles of flavones in parsley and celery.
We first identify and quantify flavone glycosides in fresh and steamed parsley and celery, in pomace, and in juice after extraction. We then quantify flavones in juices after acidification and thermal processing. These processes can be optimized to produce flavone derivatives with 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, Ohio. 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 in order to inactivate enzymes and compared to unheated control samples. All samples were prepared by first rinsing and spin drying 5 g fresh celery leaves or parsley. Steamed samples were held in a sieve over 1 L 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 °C 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 were extracted 3 times with 2.5 mL 70% (v/v) aqueous methanol, and the combined supernatants analyzed by HPLC.
**Juice Extraction and Processing.** The steps for processing are outlined in Figure 3.2. Celery and parsley stalks and leaves were used for juice extraction with an Omega 8006 masticating juicer (Harrisburg, PA). To determine 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 min or 30 min in a boiling water bath or at 121 °C for 30 min in a Splendid pressure cooker (Fagor America, Lyndhurst, NJ). Juicing experiments were conducted in triplicate and each juice sample analyzed in duplicate. Samples were lyophilized, extracted 3 times with 2.5 mL 70% (v/v) aqueous methanol, and the combined supernatants analyzed by high performance liquid chromatography (HPLC). 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 (Fig. 3.3). Juicing experiments were carried out in triplicate and samples were extracted 3 times with 2.5 mL 70% (v/v) aqueous methanol, and the combined supernatants analyzed by HPLC.
Figure 3.2. Processes for juicing, acidification, and thermal processing of parsley and celery.

![Flow diagram for juicing, acidification, and thermal processing of parsley and celery.](image)

Figure 3.3. Flow diagram for acidification and thermal processing of celery juice.

Identification by HPLC-Mass Spectrometry. Flavone glycosides were tentatively identified by ESI-MS with a QTOF Premier (Micromass, Manchester, UK) using accurate mass and fragmentation patterns in positive and negative ionization modes. Source parameters included: 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, collision-induced dissociation gas at 2.5 x 10⁻³ mBar (Argon). Sodium formate was used to calibrate and
leucine-enkephalin as a lockspray reference. MS\textsuperscript{E} experiments were applied where alternating low and high collision energy was 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 electrospray mass spectrometry (ESI-MS) as described above. For quantification, the HPLC system included a Waters 2695 separations module, a 2996 photodiode array detector, a Symmetry C\textsubscript{18} column (3.5 µm, 4.6 x 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 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.

**Statistical Analysis.** Statistical analysis was performed with R 2.11 for Windows (R Foundation for Statistical Computing, Vienna, Austria) using one-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 3.4. 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 only detected as negative ions, and flavone malonyl glucosides were detected as acetyl glucosides in negative mode due to decarboxylation (Table 3.1). The compounds identified in fresh and steamed celery and parsley have been previously reported (80), but apigenin 7-O-glucoside in acidified, thermally processed juice samples was newly identified (Fig. 3.4C).
Table 3.1. Peak assignments for chromatograms of celery extracts based on HPLC-MS data.

<table>
<thead>
<tr>
<th>Peak</th>
<th>RT (min)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>[M+H]&lt;sup&gt;+&lt;/sup&gt;/[M-H] (m/z)</th>
<th>[A+H]&lt;sup&gt;+&lt;/sup&gt;/[A-H] (m/z)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.59</td>
<td>326</td>
<td>-/353</td>
<td>-/191</td>
<td>chlorogenic acid</td>
</tr>
<tr>
<td>2</td>
<td>3.92</td>
<td>327</td>
<td>-/353</td>
<td>-/191</td>
<td>chlorogenic acid</td>
</tr>
<tr>
<td>3</td>
<td>4.41</td>
<td>312</td>
<td>-/353</td>
<td>-/191</td>
<td>chlorogenic acid</td>
</tr>
<tr>
<td>4</td>
<td>4.51</td>
<td>312</td>
<td>-/337</td>
<td>-/191</td>
<td>coumaroylquinic acid</td>
</tr>
<tr>
<td>5</td>
<td>5.27</td>
<td>306</td>
<td>-/337</td>
<td>-/191</td>
<td>coumaroylquinic acid</td>
</tr>
<tr>
<td>6</td>
<td>5.85</td>
<td>255, 349</td>
<td>581/579</td>
<td>287/285</td>
<td>luteolin 7-O-apisylglucoside</td>
</tr>
<tr>
<td>7</td>
<td>6.00</td>
<td>255, 349</td>
<td>449/447</td>
<td>287/285</td>
<td>luteolin 7-O-glucoside</td>
</tr>
<tr>
<td>8</td>
<td>6.55</td>
<td>267, 338</td>
<td>565/563</td>
<td>271/269</td>
<td>apigenin 7-O-apisylglucoside</td>
</tr>
<tr>
<td>9</td>
<td>6.70</td>
<td>253, 348</td>
<td>595/593</td>
<td>301/299</td>
<td>chrysoeriol 7-O-apisylglucoside</td>
</tr>
<tr>
<td>10</td>
<td>6.80</td>
<td>267, 338</td>
<td>433/431</td>
<td>271/269</td>
<td>apigenin 7-O-glucoside</td>
</tr>
<tr>
<td>11</td>
<td>6.92</td>
<td>254, 348</td>
<td>667/621&lt;sup&gt;a&lt;/sup&gt;</td>
<td>287/285</td>
<td>luteolin 7-O-malonyl apiosylglucoside</td>
</tr>
<tr>
<td>12</td>
<td>6.99</td>
<td>253, 347</td>
<td>463/461</td>
<td>301/299</td>
<td>chrysoeriol 7-O-glucoside</td>
</tr>
<tr>
<td>13</td>
<td>7.14</td>
<td>253, 345</td>
<td>535/489&lt;sup&gt;a&lt;/sup&gt;</td>
<td>287/285</td>
<td>luteolin 7-O-malonyl glucoside</td>
</tr>
<tr>
<td>14</td>
<td>7.59</td>
<td>267, 337</td>
<td>651/605&lt;sup&gt;a&lt;/sup&gt;</td>
<td>271/269</td>
<td>apigenin 7-O-malonyl apiosylglucoside</td>
</tr>
<tr>
<td>15</td>
<td>7.70</td>
<td>253, 345</td>
<td>681/635&lt;sup&gt;a&lt;/sup&gt;</td>
<td>301/299</td>
<td>chrysoeriol 7-O-malonyl apiosylglucoside A</td>
</tr>
<tr>
<td>16</td>
<td>7.83</td>
<td>253, 348</td>
<td>681/635&lt;sup&gt;a&lt;/sup&gt;</td>
<td>301/299</td>
<td>chrysoeriol 7-O-malonyl apiosylglucoside B</td>
</tr>
</tbody>
</table>

RT retention time.

[M+H]<sup>+</sup>/[M-H] positive and negative parent ions. Chlorogenic and coumaroylquinic acids were only detected in negative mode.

[A+H]<sup>+</sup>/[A-H] positive and negative aglycone ions.

<sup>a</sup>Malonyl glycosides were detected as acetyl glycosides due to decarboxylation in negative mode.
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 °C 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 3.2 & 3.3). 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 3.2 & 3.3).

**Table 3.2.** Concentrations of flavone derivatives in steamed and fresh parsley after maceration and incubation for 3 h. Flavone quantities are expressed as aglycone equivalents (mg/100 g fresh weight).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Apigenin</th>
<th>Diosmetin</th>
<th>Apigenin</th>
<th>Diosmetin</th>
<th>Total flavones&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steamed, incubated at 37 °C, 3 h</td>
<td>78.5 ± 34.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.7 ± 8.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>171 ± 43.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.2 ± 10.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>340 ± 48.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fresh, incubated at 37 °C, 3 h</td>
<td>255 ± 24.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58.5 ± 4.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.0 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>338 ± 29.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fresh, incubated at 20 °C, 3 h</td>
<td>264 ± 22.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.2 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.7 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>348 ± 23.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are mean ± SEM, n=3. Values within each column without a common letter differ significantly (p<0.05). ND not detected.<br><sup>a</sup>Includes minor flavone glycosides such as acetyl glucosides.
Table 3.3. Concentrations of flavone derivatives in steamed and fresh celery leaves after maceration and incubation for 3 h. Flavone quantities are expressed as aglycone equivalents (mg/100 g fresh weight).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Lut</th>
<th>Apig</th>
<th>Chrys</th>
<th>Lut</th>
<th>Apig</th>
<th>Chrys</th>
<th>Total flavones&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steamed, incubated at 37 °C, 3 h</td>
<td>9.0 ± 1.4 a</td>
<td>16.3 ± 1.6 a</td>
<td>11.6 ± 1.0 a</td>
<td>31.0 ± 4.9 a</td>
<td>78.8 ± 9.5 a</td>
<td>45.2 ± 5.7 a</td>
<td>210 ± 24.6 a</td>
</tr>
<tr>
<td>Fresh, incubated at 37 °C, 3 h</td>
<td>13.3 ± 3.7 a</td>
<td>130 ± 4.7 b</td>
<td>75.2 ± 6.2 b</td>
<td>0.0 ± 0.0 b</td>
<td>3.8 ± 2.1 b</td>
<td>3.4 ± 3.6 b</td>
<td>226 ± 11.7 a</td>
</tr>
<tr>
<td>Fresh, incubated at 20 °C, 3 h</td>
<td>30.6 ± 2.4 b</td>
<td>128 ± 2.2 b</td>
<td>71.1 ± 4.8 b</td>
<td>0.0 ± 0.0 b</td>
<td>6.4 ± 0.2 b</td>
<td>7.4 ± 0.2 b</td>
<td>244 ± 6.9 a</td>
</tr>
</tbody>
</table>

Data are mean ± SEM, n=3. Values within each column without a common letter differ significantly (p<0.05). ND not detected, Lut luteolin, Apig apigenin, Chrys chrysoeriol.

<sup>a</sup>Includes minor flavone glycosides such as acetylglucosides.

**Mass Balance of Flavones during Juice Processing.** Parsley yielded 72% juice (w/w) with similar amounts of apigenin and diosmetin glycosides. While 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 3.4). Celery yielded 79% juice, and slightly more apigenin, luteolin, and chrysoeriol were found in the juice than in the pomace (Table 3.5). In fresh celery and juice, apigenin glycosides were the most abundant, accounting for over half of all flavones.
Table 3.4. Yield, moisture, and flavones in fresh parsley, pomace, and juice. Flavone quantities are expressed as aglycone equivalents.

<table>
<thead>
<tr>
<th>Step</th>
<th>Yield (g)</th>
<th>Moisture (%)</th>
<th>Total apigenin (mg)</th>
<th>Total diosmetin (mg)</th>
<th>Total flavones (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh parsley</td>
<td>100</td>
<td>88.1 ± 0.8</td>
<td>178 ± 14.5</td>
<td>179 ± 94.7</td>
<td>357 ± 97.0</td>
</tr>
<tr>
<td>Parsley juice</td>
<td>71.9 ± 2.8</td>
<td>91.8 ± 0.5</td>
<td>92.7 ± 13.0</td>
<td>108 ± 62.7</td>
<td>200 ± 75.6</td>
</tr>
<tr>
<td>Parsley pomace</td>
<td>25.2 ± 1.0</td>
<td>78.6 ± 1.5</td>
<td>56.8 ± 4.1</td>
<td>55.9 ± 30.2</td>
<td>113 ± 26.3</td>
</tr>
</tbody>
</table>

Data are mean ± SD, n=3.

Table 3.5. Yield, moisture, and flavones in fresh celery (stalks and leaves), juice, and pomace. Flavone quantities are expressed as aglycone equivalents.

<table>
<thead>
<tr>
<th>Step</th>
<th>Yield (g)</th>
<th>Moisture (%)</th>
<th>Total apigenin (mg)</th>
<th>Total luteolin (mg)</th>
<th>Total chrysoeriol (mg)</th>
<th>Total flavones (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh celery</td>
<td>100</td>
<td>93.1 ± 0.6</td>
<td>41.1 ± 9.3</td>
<td>16.7 ± 5.5</td>
<td>13.0 ± 4.0</td>
<td>70.8 ± 18.9</td>
</tr>
<tr>
<td>Celery juice</td>
<td>79.1 ± 0.4</td>
<td>95.4 ± 0.1</td>
<td>13.4 ± 1.4</td>
<td>4.9 ± 0.2</td>
<td>4.3 ± 0.4</td>
<td>22.5 ± 1.8</td>
</tr>
<tr>
<td>Celery pomace</td>
<td>18.8 ± 0.3</td>
<td>83.7 ± 0.6</td>
<td>11.1 ± 0.3</td>
<td>3.2 ± 0.3</td>
<td>3.6 ± 0.2</td>
<td>18.0 ± 0.8</td>
</tr>
</tbody>
</table>

Data are mean ± SD, n=3.

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 °C 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 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 non-acidified 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, while flavone 7-O-glicosides 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 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 accounting for variation between lots of celery, 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 pH 4.0 increased slightly with time, averaging 15% in pH 5.0 juice and 24% in pH 4.0 juice after 60 min (Fig. 3.5). 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 minutes at 121 °C to 83% after 60 minutes. There was no significant conversion of apigenin glycosides to aglycones and no loss of total apigenin derivatives after acidification and thermal processing (Fig. 3.6).
Figure 3.5. 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 minutes. Data are mean ± SD, n=3.
Figure 3.6. 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 minutes. Data are mean ± SD, n=3. Values within each treatment group without a common letter differ (p<0.05). FW fresh weight.

DISCUSSION

In all of our juice samples, flavone apiosylglucosides were the predominant flavonoids and malonyl apiosylglucosides were minor or undetectable. When celery leaves or parsley were steamed, significantly greater amounts of flavone glycosides were present as malonyl apiosylglucosides and significantly less were present as apiosylglucosides compared to fresh samples (Tables 3.2 & 3.3). 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 (64), but we are not
aware of any reports of similar activity in celery after reviewing the literature and the BRENDA enzyme database (160). Demalonylation of flavone glycosides can occur after oven drying (24) or after the extraction process, as has been noted with other flavonoid glycosides (65). Heating soy products can lead to demalonlyation or decarboxylation of isoflavone malonyl glucosides (161), but the parsley and celery in our experiments retained this functional group after steaming.

With a masticating juicer, we obtained 72% juice yield (w/w) from parsley and 79% yield from celery. 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 3.4 & 3.5). This is similar to the retention of flavonoids in the pomace of other fruits and vegetables after juicing, including artichoke hearts (66) and apples (162).

We found that acidifying parsley and celery juices to pH 4.0 and heating at 100 ºC or 121 ºC for up to 30 min did not significantly affect total flavone concentrations. Our results were similar to those of most other studies on juices. Thermal processing at 95 ºC for up to one minute had little effect on flavonoid concentrations in orange juice (44,163), and processing at 88 ºC for up to 30 min had no effect on total flavonoids in tomato juice (164). Heating grape juice at 95 ºC for 15 min did not degrade anthocyanins (165). In contrast, up to 49% of isoflavones were lost from soybeans when boiled for 20 minutes during tempeh production (166).

After acidifying celery juice to pH 3.0, 4.0, or 5.0 and heating at 121 ºC for 15-60 min, we found no effect of processing times on total flavone concentrations. 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 ºC, but no loss of total isoflavones was observed (167). Others have tested the effects of heat and pH treatments on pure flavonoid glycosides and their results varied. Quercetin and quercetin rutinoside standards boiled at 100 ºC 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 (152). Daidzein and genistein (aglycone) standards both degraded when heated at 120 ºC for 20 min, but the effect varied with pH. Daidzein was more stable at pH 9, while genistein was more stable at pH 7 (168).

Although the concentration of total flavones in celery juice was generally stable after heat processing, we found that conversion of apiin to apigenin 7-O-glucoside increased rapidly at 121 ºC over time at pH 3.0, reaching 83% after 60 min (Fig. 3.6). 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 (146). 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. Humans fed parsley and celery containing flavone apiosylglucosides resulted in \( T_{\text{max}} \) values of 7-8 hours (76,78), compared to less than one hour for those fed simple flavone glucoside (81). These data support the hypothesis that flavone apiosylglucosides are absorbed in the colon, while 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. Here we show 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 while 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 improving its intestinal absorption and efficacy.
Chapter 4

Deglycosylated plant-derived flavones reduce inflammatory mediators in vitro and are better absorbed in vivo

Gregory Hostetler, Ken Riedl, Horacio Cardenas, Mayra Diosa-Toro, Daniel Arango, Steven Schwartz, and Andrea I. Doseff

INTRODUCTION

The innate immune system provides an important first line of defense to infection by promoting an inflammatory response that mediates pathogen clearance (169). Thus, inflammation is a normal central mechanism of host immune surveillance in response to infections (170). Dysregulated inflammation is central in sepsis and other acute inflammatory pulmonary inflammatory diseases (171,172). In addition, systemic activation of the host immune response has been associated with many chronic diseases, including type 2 diabetes (173), rheumatoid arthritis (174), cancer (175), and atherosclerosis (176). Several anti-inflammatory oral drug therapies are currently available, but their adverse effects include gastrointestinal side effects, high costs, and the potential for cardiovascular damage (177). Thus, there is growing interest in using naturally occurring dietary compounds to modulate inflammation.

\[\textsuperscript{a}\] Written as a manuscript for Molecular Nutrition and Food Research, in preparation.
Flavonoids, common in a variety of fruits and vegetables (2), have anti-
inflammatory activities and can potentially play a key role in nutraceuticals and
functional foods (178). Like other flavonoids, flavones are typically found in plants as
glycosides (2). Flavone O-glycosides are composed of the aglycone moiety with one or
more sugars attached with a β linkage (Fig. 4.1). These compounds may be modified
slightly by endogenous enzymes such as malonylsterases (conversion from malonylapiin
to apiin) (64), but remain as glycosides after processing such as shredding (65), juicing
(66), and heating (44). For many flavonoid glucosides, brush border beta-glucosidase
activity in the small intestine is sufficient to hydrolyze the aryl glycosidic bond and allow
absorption of the aglycone (146,179). Intestinal β-glucosidase, however, cannot
hydrolyze oligosaccharide moieties (146) and although colonic bacteria can cleave these
bonds (71), absorption is reduced. Given that dietary flavones are ingested predominantly
as glycosides, their fate and biological effect are determined by how and where they are
hydrolyzed, absorbed, metabolized, transported, and excreted. Flavone glycosides in
celery occur as apiosylglucosides (Fig. 4.1) and might also be resistant to brush border β-
-glucosidase action. We therefore hypothesized that converting flavone glycosides to
aglycones would result in increased absorption.

Although the metabolic fate of flavones such as luteolin and apigenin has not
been studied as extensively as that of other flavonoids (3,4), flavones have been shown to
have anti-proliferative and anti-inflammatory properties (144,155,156). Treatment of
murine macrophages with LPS, followed by the flavones apigenin, luteolin, or diosmetin,
reduced tumor necrosis factor-α (TNF-α) or IL-6 release (115,180,181). In addition, we
showed that apigenin inhibits LPS-induced TNF-α production in primary human
monocytes and macrophages by reducing the activity of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), a key transcription factor in inflammation (144). We also found that when injected, apigenin reduced TNF-α in vivo in a model of acute inflammation (144). Moreover, mice given purified apigenin orally or in the diet showed lower serum concentrations of the pro-inflammatory cytokines IL-6 and TNF-α (115). While it is clear that purified flavone aglycones have anti-inflammatory activities, less apparent is the role of flavone glycosides or flavone-rich foods in modulating inflammation.

Here we examine the effects of flavone aglycones, glycosides and flavone-rich food extracts on the immune response. Our results demonstrate that flavone aglycones and celery extracts processed to contain flavone aglycones, unlike flavone glycosides and celery extracts containing native flavone glycosides, inhibit LPS-induced production of TNF-α and reduce the transcriptional activity of NF-κB. We also found that targeted formulation of celery-supplemented diets with higher aglycone concentrations resulted in higher relative absorption of flavones in vivo. Collectively these studies provide evidence that diets crafted to augment aglycone content increase absorption and might selectively reduce inflammation, uncovering novel aspects for the potential use of naturally occurring dietary compounds to modulate inflammation.
<table>
<thead>
<tr>
<th>Flavone derivative</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aglycone</strong></td>
<td><img src="image1" alt="Flavone" /> <img src="image2" alt="Apigenin" /></td>
</tr>
<tr>
<td></td>
<td><img src="image3" alt="Luteolin" /> <img src="image4" alt="Chrysoeriol" /></td>
</tr>
<tr>
<td><strong>7-O-glucoside</strong></td>
<td><img src="image5" alt="Apigenin 7-O-glucoside" /></td>
</tr>
<tr>
<td></td>
<td><img src="image6" alt="Luteolin 7-O-glucoside" /></td>
</tr>
<tr>
<td><strong>7-O-apiosyl-glucoside</strong></td>
<td>![Apigenin 7-O-apiosylglucoside (Apiin)]</td>
</tr>
<tr>
<td><strong>7-O-malonyl-apiosylglucoside</strong></td>
<td>![Apigenin 7-O-malonylapiosylglucoside (Malonylapiin)]</td>
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</tbody>
</table>

**Figure 4.1.** Structures of aglycone flavones and common derivatives.
MATERIALS AND METHODS

**Plant Material, Standards, and Reagents.** Chinese celery (*Apium glaveolens*) and raw almonds (*Prunus dulcis*) were purchased from local grocery stores. Chrysoeriol and luteolin 7-O-glucoside (Extrasynthese, Genay, France), apigenin and luteolin (Sigma, St. Louis, MO), and diosmetin and apigenin 7-O-glucoside (Chromadex, Irvine, CA) were dissolved in DMSO for in vitro experiments. Beta-glucuronidase/sulfatase from *Helix pomatia* (lyophilized powder) was from Sigma. Phosphoric acid was obtained from Corco Chemical Corp (Fairless Hills, PA). All other reagents were from Fisher Scientific (Fair Lawn, NJ).

**Preparation of Celery Extracts and Diets.** Lyophilized fresh celery leaves, naturally abundant in apigenin 7-O-apiosylglucoside (apiin), were used for extracts and diets. For fresh celery extract (FCE), 200 mg lyophilized sample was extracted with 6 mL 70% ethanol, centrifuged at 450 x g for 10 min at room temperature, and the supernatant collected. For acid hydrolyzed celery extract (ACE), 1 mL of FCE was combined with 0.5 mL 37% HCl and heated for 1 h at 90 °C. For enzyme treated celery extract (ECE), lyophilized fresh celery leaves were first heated in 1.5 N H₃PO₄ for 90 min at 100 °C to convert apiin to apigenin 7-O-glucoside. After neutralization with 10% KOH, the celery leaves were incubated with ground almond (20% dry wt) for 2 h at 50 °C to convert apigenin 7-O-glucoside to apigenin aglycone by β-glucosidase present in the almonds. The resulting aglycone-rich mixture was then lyophilized to make enzymatically hydrolyzed celery powder and extracted in 70% ethanol as described above. All extracts were dried under nitrogen gas (N₂) and reconstituted in 300 µL DMSO (Fig. 4.4A). After
determining flavone concentration by HPLC analysis, samples were diluted in DMSO to 5 mM apigenin equivalents and stored at -20 °C.

Preparation of celery supplemented diets is outlined in Figure 4.4C. Powdered AIN-93G diet was purchased from Dyets, Inc. (Bethlehem, PA). Lyophilized fresh celery and enzyme-treated celery were combined with AIN-93G powder diet to make 5% or 10% (w/w) celery-supplemented diets. After dry ingredients were mixed, water was added to make a paste and pellets were formed with a Simple Tablet Maker (Wholistic Research Co., Royston, Herts, UK). The same procedure was used to make pellets of the AIN-93G control diet. Pellets were dried at 40 °C to a constant weight and stored at -20 °C for 1-4 weeks until used for feeding. The following diets were made: AIN-93G pellets supplemented with 5% fresh celery (AIN-5FCP), AIN-93G with 10% fresh celery pellets (AIN-10FCP), AIN-93G with 5% enzyme celery pellets (AIN-5ECP), and AIN-93G with 10% enzyme celery pellets (AIN-10ECP). The flavone contents of the diets were confirmed by HPLC analysis prior to feeding.

**Cell Culture.** RAW 264.7 murine macrophages from ATCC were cultured as previously described (144). Briefly, cells were seeded at a density of 5 x 10^5 cells/mL, incubated for 16 h, and then treated with either 100 ng/mL LPS (E. coli O127:B8, Difco, Detroit, MI) or DMSO as diluent control in the presence or absence of different concentrations of flavones or food extracts.

**Immunodetection of Inflammatory Cytokines.** TNF-α in cell media was determined by enzyme-linked immunosorbent assay (ELISA) using the DuoSet kit (R&D Systems, Minneapolis, MN) and TNF-α standards provided by the manufacturer as we previously described (144). Absorbance was measured on an ELX-808 microplate reader.
with KC Junior software (excitation wavelength 450 nm, emission wavelength 540 nm) and concentrations were determined with a 4-parameter standard curve.

**NF-κB Activity.** Macrophages (6 x 10^5/ml) were transfected with 0.6 μg NF-κB-SEAP or vector control-SEAP (BD Clontech) and pCMV-β-galactosidase vector using Lipofectamine 2000 in OPTI-MEN (Invitrogen) according to manufacturer’s specifications. Cells were incubated 24 h and treated with LPS, different flavones, celery extracts, or DMSO for 16 h. NF-κB activity in supernatants was determined using a SEAP fluorimetric reporter gene assay (AnaSpec, Fremont, CA) following the manufacturer’s instructions and read on a VICTOR X3 multilabel plate reader (PerkinElmer). Beta-galactosidase activity in cell lysates was measured with the β-Gal assay kit (Invitrogen) as previously reported (144). Relative units (R.U.) of NF-κB activity are expressed as relative units of SEAP per ml/β-galactosidase activity per mg protein. All experiments were done in triplicate.

**Subcellular Fractionation of Macrophages.** Subcellular fractionations of RAW 264.7 cells were obtained as previously described (156). Briefly, media was harvested and 5 x 10^6 cells were lysed in KPM buffer by 5 freeze/thaw cycles and centrifuged at 100,000 x g for 1 h at 4 °C to obtain cytoplasmic and membrane fractions. To assess the flavone content in each fraction, the media, cytoplasmic and membrane fractions were extracted twice an equal volume acetonitrile, centrifuged at 2500 x g, and the pellet extracted with 70% acetonitrile. The supernatants were dried under N₂ and reconstituted in methanol prior to HPLC analysis.

**DPBA Staining and Fluorescence Microscopy.** Cells were treated with flavonoids or DMSO for 3 h at 37 °C in supplemented RPMI-1640 (without phenol red).
Nuclear staining was done with 1 µg/ml DAPI (4’,6-diamidino-2-phenylindole, Sigma) for 30 min at 37 °C. Cells were rinsed 3 times with PBS and stained with 0.1% (w/v) diphenylboric acid 2-aminoethyl ester (DPBA; Sigma, USA; excitation 490 nm, emission 530 nm) for 1 min as previously described (156). Fluorescence was detected by microscopy (Olympus IX-81 microscope, Olympus, Melville, NY) and digital images were generated with a QCAM Retiga Exi FAST 1394 camera and Slidebook software (Intelligent Imaging Innovations, Denver, CO).

**Mouse Feeding Experiments.** Male C57BL/6J mice, 6-8 weeks old, were purchased from Harlan (Madison, WI), cared for according to the Ohio State University Institutional Animal Care and Use Committee (IACUC) regulations, and used after 10 days acclimation. For dietary intervention, mice housed into individual cages were fed ad libitum for 7 days with either AIN-93G control, AIN-5FCP, AIN-10FCP, AIN-5ECP, or AIN-10ECP. Individual food weight and consumption were recorded daily. Urine was collected at day 7, immediately prior to euthanasia. A blood sample was obtained by cardiac puncture and serum isolated as previously described (144). All samples were stored at -80 °C until analysis.

**Flavone Extraction and HPLC-MS/MS Analysis.** HPLC-DAD analysis was done with a Waters 2695 separations module with a 2996 detector, Symmetry C18 column (3.5 µm, 4.6 x 75 mm), and Empower software (Waters Corp., Milford, MA). The mobile phase consisted of 0.1% formic acid in water and 0.1% formic acid in ACN, using a gradient method with increasing acetonitrile. Concentrations were measured at 338 nm for apigenin and 350 nm for luteolin and chrysoeriol through external calibration with aglycone standards. Apigenin 7-O-glucoside was identified using a commercial
standard, and HPLC-DAD-MS (QToF Premier; Micromass, UK) was used to tentatively identify other flavone glycosides by accurate mass and fragmentation patterns.

Urine and serum samples were analyzed by HPLC-MS/MS (Acquity UPLC, Quattro Ultima; Waters, Milford, MA). Each urine sample (25-100 µL) was combined with twice its volume of sodium acetate buffer (1.4 M, pH 5.5), incubated for 2 h at 37 °C with β-glucuronidase/sulfatase to deconjugate flavones as described previously (82). The resultant flavone aglycones were extracted three times with diethyl ether, supernatants dried under N₂, and reconstituted in methanol prior to HPLC-MS/MS analysis. Serum was prepared as previously described (182) with some modifications. Each sample (30-100 µL) was extracted with twice its volume of -20 °C acetonitrile, centrifuged, and the supernatant collected. The pellet was extracted in 70% ACN, sonicated, and centrifuged. Supernatants from the two extractions were combined, dried under N₂, and reconstituted in sodium acetate buffer (1.4 M, pH 5.5). Samples were digested with β-glucuronidase/sulfatase, extracted four times with diethyl ether, and reconstituted in methanol.

HPLC-MS/MS analysis was done at 0.65 mL/min of 0.1% formic acid versus 0.1% formic acid in ACN with a gradient method, using an Acquity BEH C18 column (2.1 x 50 mm, 1.7 µm) at 40 °C. Eluent was interfaced with the MS via an electrospray probe operated in both negative and positive modes after a 1:10 flow splitter. Specific flavones were detected by multiple reaction monitoring (MRM) with an 84 ms dwell time. Source parameters included: capillary voltage 2.8 kV in negative mode and 3.2 kV in positive mode; cone 35 V; source block 110 °C, desolvation 500 °C; desolvation gas 760 L/hr, cone gas 105 L/hr; collision energies 20-25 V. Desolvation gas was delivered
at 600 L/hr and 450 °C, cone gas at 100 L/hr, collision-induced dissociation at 3x10⁻³ mBar (Argon).

**Statistical Analysis and Graphical Presentation.** Statistical analysis was done with R 2.11 for Windows (R Foundation for Statistical Computing, Vienna, Austria) using one-way analysis of variance (ANOVA) for parametric data sets and the Kruskal-Wallis test for non-parametric data. Post hoc comparisons were done with the corresponding parametric or non-parametric Tukey’s HSD. When necessary, data were log or square root transformed to achieve a normal distribution and equal variances for the ANOVA tests. P values <0.05 were indicative of significant differences. Chemical structures were produced with ChemDraw Pro 12.0 (CambridgeSoft, Cambridge, MA).

RESULTS

**Deglycosylation of Flavones Determines Their Ability to Inhibit Cytokines and NF-κB Activity.** To examine the effect of structural modifications of flavones in their anti-inflammatory activities, macrophages were stimulated with 100 ng/ml LPS in the presence of different doses of the aglycones apigenin, luteolin, and chrysoeriol or diluent control for 8 h. Stimulation with LPS induced the release of TNF-α, undetectable in the presence of diluent controls (Fig. 4.2A). Stimulation with LPS in the presence of any of the aglycones resulted in a significant reduction of TNF-α at doses as low as 10 μM (Fig. 4.2A).

Next, we evaluated the effect of glycosylated flavones on their ability to modulate inflammatory cytokines. Macrophages stimulated with 100 ng/ml LPS in the presence of 10 to 25 μM of apigenin 7-O-glucoside or luteolin 7-O-glucoside showed no effect on
TNF-α (data not shown). To further determine their immunomodulatory activity doses were increased, but even doses as high as 100 μM had no effect on reducing LPS-induced TNF-α (Fig. 4.2B).

**Deglycosylation of Flavones Increases their Ability to Inhibit LPS-induced NF-κB Transcriptional Activity.** LPS induction of TNF-α involves the transcription factor NF-κB, so we next studied NF-κB activity with a reporter gene assay. Macrophages transiently expressing NF-κB SEAP reporter vector were stimulated with 100 ng/ml LPS in the presence of flavone aglycones or diluent control for 16 h. LPS treatment induced NF-κB activity, while cells treated with diluent or aglycones alone had no effect on NF-κB (Fig. 4.3A). We found that treatment of LPS-stimulated cells with different concentrations of the aglycones apigenin, luteolin or chrysoeriol significantly reduced LPS-stimulated NF-κB activity at doses of 25 μM (Fig. 4.3A).
Figure 4.2. Effects of glycosylated and deglycosylated flavones on TNF-α. Macrophages were treated for 8 h with 100 ng/ml LPS in the presence of flavones or diluent control DMSO. Treatments marked with (-) denote addition of diluent. (A) Deglycosylated flavones used at indicated concentrations were apigenin, luteolin and chrysoeriol. (B) Glycosylated flavones used at indicated concentrations include apigenin 7-O-glucoside, luteolin 7-O-glucoside. Data are mean ± SEM, n = 3, *P<0.001 versus control +LPS.

We next examined the effect of flavone glycosides on LPS stimulation of NF-κB. Macrophages treated with apigenin 7-O-glucoside or luteolin 7-O-glucoside in the presence of diluent control had no effect on NF-κB activity (Fig. 4.3B). Moreover, macrophages treated with apigenin 7-O-glucoside or luteolin 7-O-glucoside at doses as high as 100 μM had no effect on LPS-induced NF-κB activity (Fig. 4.3B). Although there was a decrease of NF-κB activity with 50 μM flavone glycosides, the differences were
not significant from the control \((P=0.11, 0.21\) for apigenin 7-O-glucoside and luteolin 7-O-glucoside respectively). These results indicate that deglycosylation of flavones increases their ability to modulate NF-\(\kappa\)B activity.

**Figure 4.3.** Effects of flavones on NF-\(\kappa\)B activity after 16 h. (A) Macrophages were treated with LPS and apigenin, luteolin, chrysoeriol, or DMSO or with control (DMSO + PBS: \(-/-\)). (B) Macrophages were treated with LPS and apigenin 7-O-glucoside, luteolin 7-O-glucoside, or DMSO or with control (DMSO + PBS: \(-/-\)). Data are mean \(\pm\) SEM, \(n = 3\), \(*P<0.05\) versus control +LPS.

**Formulation of Aglycone-rich Food Extracts.** The different ability of pure flavone aglycones and glycosides to modulate inflammation suggests that flavone-rich food extracts might also effectively reduce inflammation if their native flavone...
glycosides are converted to aglycone form. To test this hypothesis, we formulated food extracts enriched in aglycone content. Several foods are abundant in flavones, including artichokes, parsley, celery, and kumquat \((10,32,51)\). We decided to pursue celery as a flavone source because it is relatively palatable and its leaves have very high flavone concentrations \((10)\). To make fresh celery extracts, celery leaves were first macerated, lyophilized, and extracted in 70\% ethanol (Fig. 4.4A). This extract was then dried under \(\text{N}_2\) and reconstituted in DMSO and referred to hereafter as fresh celery extract or FCE. Extracts with enriched aglycone content were formulated by two independent strategies: acid or enzyme treatment. Acid treated celery extracts (named ACE) were obtained by treating ethanol extracts with HCl at 90 °C to convert flavone glycosides to aglycones before drying under \(\text{N}_2\) and reconstitution in DMSO. Enzyme treated celery extracts (ECE) were made by first heating the lyophilized celery with \(\text{H}_3\text{PO}_4\) at 100 °C followed by incubation with almond (rich in beta-glucosidase) at 50 °C to convert flavone glycosides to aglycones. The resultant celery was extracted in 70\% ethanol and reconstituted in DMSO (Fig. 4.4A). HPLC analysis showed that the majority peaks in FCE corresponded to luteolin apiosylglucoside, apigenin 7-O-apiosylglucoside (apiin), and chrysoeriol apiosylglucoside (Fig. 4.4B, peaks A, B, and C). In contrast, ACE and ECE had almost exclusively aglycone forms luteolin, apigenin, and chrysoeriol (Fig. 4.4B, peaks D, E, and F respectively). The celery leaves used in this study had 5-fold greater concentrations of apigenin derivatives relative to luteolin or chrysoeriol (Table 4.1, FCP), so purified apigenin was used as a control for in vitro studies.

**Effect of Food Extracts in Inflammatory Mediators.** To investigate the ability of flavone-rich celery extracts to reduce inflammation, macrophages were treated with
100 ng/ml LPS or PBS diluent for 8 h in the presence of FCE, ACE, ECE, or DMSO. LPS-treated macrophages released TNF-α (Fig. 4.5A, back bar LPS+DMSO), while cells treated with food extracts and diluent PBS did not induce TNF-α release, indicating that the extracts are not pro-inflammatory (Fig. 4.5A, white bar). FCE containing predominantly apiin inhibited LPS-induced TNF-α release by only 30%, while ACE and ECE led to approximately 90% reductions (Fig. 4.5A).

Next, we evaluated the effect of flavone-rich celery extracts on NF-κB transcriptional activity. Macrophages transiently expressing NF-κB SEAP reporter vector were stimulated with 100 ng/ml LPS or PBS control in the presence of FCE, ACE, ECE or DMSO diluent control for 16 h. Macrophages treated with DMSO and LPS induced NF-κB activity compared to PBS-treated cells (Fig. 4.5B, DMSO black and white bars respectively), while cells treated with either food extracts and PBS had no effect on NF-κB (Fig. 4.5B, FCE, ACE and ECE white bars). FCE had no effect on LPS-induced NF-κB activity when compared with LPS and DMSO treated cells (Fig 4.5B). In contrast, both ACE and ECE significantly inhibited NF-κB activity (Fig. 4.5B, ACE, ECE, black bars). Collectively, these results indicate that conversion of flavones in food extracts from glycoside to aglycone greatly enhances their ability to modulate inflammatory mediators.
Figure 4.4. Formulation of flavone-rich food extracts and diets. (A) Flowchart for processing of FCE, ACE, and ECE. (B) HPLC chromatograms of celery food extracts and mouse diets. Peaks represent: A luteolin apiosylglucoside, B apiin, C chrysoeriol apiosylglucoside, D luteolin, E apigenin, F chrysoeriol. (C) Process for making AIN-93G, AIN-5FCP, AIN-10FCP, AIN-5ECP, and AIN-10ECP mouse diets.
Figure 4.5. Effects of flavone-rich celery extracts on inflammation. Macrophages were treated with PBS control or LPS in the presence of DMSO, FCE, ACE, or ECE at 25 µM apigenin equivalents. (A) TNF-α was measured in the media after 8 h. (B) NF-κB activity was measured in the media after 16 h. Data are mean ± SEM, n = 3, *P<0.05, **P<0.001 versus DMSO+LPS.

**Cellular Uptake of Flavones.** To investigate whether the difference in anti-inflammatory activity of glycoside and aglycone flavones was due to cellular uptake, macrophages were cultured for 3 h in the presence of 25 µM apigenin, apigenin 7-O-glucoside, flavone-rich extracts or DMSO diluent control and stained with DPBA as previously described (183). Apigenin-treated cells showed DPBA staining as previously reported (183), while cells treated with DMSO lacked DPBA staining but showed DAPI nuclear staining (Fig. 4.6d-f and 4.6a-c respectively). In contrast, apigenin 7-O-
glucoside-treated cells had negligible DPBA staining (Fig. 4.6g-i). In addition, we found that FCE enters cells as suggested by the DPBA staining, but to a lesser extent than ACE and ECE which showed higher staining (Fig. 4.6j,m,p). Nuclear staining with DAPI showed that there were no changes in nuclear morphology associated with any of the treatments (Fig. 4.6e,h,k,n,q).

**Figure 4.6.** Cellular localization of dietary flavones in macrophages. Macrophages treated with DMSO, apigenin, apigenin 7-O-glucoside, FCE, ACE, or ECE at 25 µM for 3 h were stained with DPBA and DAPI. Representative of 3 independent experiments.
To further study cellular uptake of flavones, subcellular fractionation of cytoplasm and membrane factions were used to determine the content of flavones by HPLC-DAD in macrophages treated for 3 h with flavone-rich diets at a 25 µM apigenin equivalent. Consistent with our previous findings in leukemia cells (156), we found that pure apigenin was absorbed by macrophages at concentrations of approximately 10 µM, with 90% of the absorbed compound present in the membrane fraction. Although apigenin aglycone was detected in the apigenin 7-O-glucoside treated cells at concentrations of 1 µM (Fig. 4.7), this may have been disproportionately absorbed from the purified compound, which contained 0.14% aglycone. In macrophages cultured with flavone-rich extracts, flavones were found mainly in the membrane fractions reaching 20 to 40 times higher levels than in cytoplasm. Apigenin in cells treated with FCE was detected as apiin and concentrations were lower, reaching 3-4 µM (Fig. 4.7). In contrast, ACE and ECE-treated macrophages had up to 10-fold higher accumulation of apigenin, reaching 20 µM in ECE-treated cells. Luteolin and chrysoeriol were found in the membrane fractions of ACE- and ECE-treated cells proportional to their concentration in the extracts (Fig. 4.4B, Fig. 4.7). Together with the DPBA results, the HPLC analysis suggests that flavone aglycones and flavones from aglycone-rich extracts are taken into cells more efficiently than flavone glycosides, potentially impacting their biological effects.

**Effects of Celery Supplemented Diets on Flavone Absorption in Mice.** Based on the ability of flavones to counter inflammation, we prepared foods with high flavone concentrations. Mouse diets were made as described in the materials and methods (Fig.
4.4C) and described in Table 4.1. Mice were fed the diets for 7 days, and steady-state levels of flavones in serum were determined by measuring concentrations in urine and serum at sacrifice. For all diets, apigenin, luteolin, and chrysoeriol were detected in urine proportional to their concentration in celery. Mice fed AIN-5ECP had higher urine flavone concentrations than those fed AIN-5FCP; however, no difference was observed between mice fed AIN-10ECP and AIN-10FCP diets (data not shown). Serum luteolin, apigenin and chrysoeriol concentrations followed a dose-response pattern with each diet formulation at 5% and 10%, and were highest in mice fed AIN-10ECP diet (Fig. 4.8). After accounting for the lower flavone concentrations in the enzyme-treated diets, relative apigenin and luteolin absorption was significantly higher in enzyme-treated diets relative to their fresh celery counterparts. Relative absorption of chrysoeriol was also higher in AIN-5ECP diets than AIN-5FCP diets. These results indicate that conversion of dietary flavones from glycoside to aglycone forms improves absorption into systemic circulation.
Figure 4.7. Subcellular distribution of dietary flavones. HPLC analysis of flavone standards and cytoplasmic and membrane fractions of macrophages treated with DMSO, apigenin, apigenin 7-O-glucoside, FCE, ACE, or ECE at 25 µM for 3 h. Representative of 3 independent experiments.
Figure 4.8. Concentration of flavones in serum of mice fed 5% FCP, 5% ECP, 10% FCP, or 10% ECP diets ad libitum for 7 days. Data are mean ± SEM, n = 4-8. Flavone concentrations were determined by HPLC-MS/MS.
Table 4.1. Concentrations of total flavones and aglycones in five experimental diets. Flavones were quantified as aglycone equivalents (mg/100 g dry weight).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Luteolin Total (mg)</th>
<th>Luteolin Aglycone (mg)</th>
<th>Apigenin Total (mg)</th>
<th>Apigenin Aglycone (mg)</th>
<th>Chrysoeriol Total (mg)</th>
<th>Chrysoeriol Aglycone (mg)</th>
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<tbody>
<tr>
<td>AIN-93G</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FCP</td>
<td>288 ± 20.3</td>
<td>ND</td>
<td>1491 ± 74.6</td>
<td>ND</td>
<td>216 ± 14.8</td>
<td>ND</td>
</tr>
<tr>
<td>AIN-5FCP</td>
<td>13.6 ± 0.1</td>
<td>ND</td>
<td>62.3 ± 0.3</td>
<td>ND</td>
<td>9.0 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>AIN-10FCP</td>
<td>29.2 ± 3.5</td>
<td>ND</td>
<td>138 ± 4.4</td>
<td>0.2 ± 0.2</td>
<td>24.9 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>ECP</td>
<td>143 ± 14.8</td>
<td>115 ± 17.6</td>
<td>631 ± 9.4</td>
<td>551 ± 5.2</td>
<td>95.2 ± 0.6</td>
<td>95.2 ± 0.6</td>
</tr>
<tr>
<td>AIN-5ECP</td>
<td>5.3 ± 0.4</td>
<td>3.4 ± 0.4</td>
<td>22.0 ± 0.2</td>
<td>18.7 ± 0.1</td>
<td>3.7 ± 0.0</td>
<td>3.7 ± 0.0</td>
</tr>
<tr>
<td>AIN-10ECP</td>
<td>9.2 ± 0.8</td>
<td>6.7 ± 0.6</td>
<td>46.9 ± 0.2</td>
<td>39.6 ± 0.2</td>
<td>7.5 ± 0.1</td>
<td>7.5 ± 0.1</td>
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</table>

Data are mean ± SEM, n=3. ND not detected. AIN-5FCP 5% fresh celery pellets, AIN-10FCP 10% fresh celery pellets, AIN-5ECP 5% enzyme-treated celery pellets, AIN-10ECP 10% enzyme-treated celery pellets.

DISCUSSION

Flavones, a class of natural flavonoids with a large number of derivatives in herbs, fruits, and vegetables, are common components of our diet (32,38). Potential nutraceutical anti-inflammatory activity for flavones is emerging but the mechanisms responsible for their action remain unclear (184). Here we demonstrate using a number of flavones and flavone-rich food extracts that the anti-inflammatory activity is dependent on deglycosylation of the compounds. We further show that flavone glycosylation reduces intestinal absorption into systemic circulation.

Flavones including apigenin, luteolin, and chrysoeriol have been shown to reduce
TNF-α secretion in vitro at 10-50 µM concentrations (180,181,185). In agreement with these studies, we found that as little as 10 µM apigenin, luteolin, or chrysoeriol reduced TNF-α secretion compared to the control (Fig. 4.2A). We previously reported that apigenin inhibits TNF-α production by suppressing p65 phosphorylation and subsequent NF-κB activity (144), and our current study shows that flavone aglycones at 25 µM concentrations significantly reduce NF-κB activity (Fig. 4.3A). In contrast, the flavone glycosides apigenin and luteolin 7-O-glucoside had no effect on TNF-α release or NF-κB activity even at concentrations up to 100 µM (Fig. 4.2B and 4.3B). The higher efficacy of aglycones rather than glycosides to reduce TNF-α and NF-κB activity is consistent with other studies showing that neither diosmetin 7-O-rutinoside nor apiin reduced nitric oxide (NO) and TNF-α in response to LPS, whereas apigenin was effectively decreased these inflammatory mediators (181,186). Apiin and fresh celery extracts, however, reduced nitrite (NO₂) and inducible nitric oxide synthase (iNOS) in macrophages, though under different experimental conditions and at much higher concentrations (187).

Adverse effects associated with current anti-inflammatory compounds has prompted the interest in implementation of diets with nutraceutical value for treatment and prevention of inflammatory diseases (188). To determine whether flavone-rich foods can effectively attenuate inflammation, we developed food formulations with different levels of aglycone and glycoside flavones. We found that acid and enzyme-treated celery extracts (rich in aglycones) reduced TNF-α release and NF-κB activity while fresh celery extracts (rich in apiin) did not (Fig. 4.5).

To investigate the mechanisms responsible for the differential anti-inflammatory effect of aglycone and glycoside flavones, we studied their cellular uptake and
distribution. Localization using fluorescence microscopy showed a higher cellular uptake of apigenin compared to apigenin 7-O-glucoside glycoside (Fig. 4.6). Consistent with these results, DPBA staining showed higher levels of fluorescence in macrophages treated with ACE and ECE rich in aglycones than in FCE-treated macrophages. Previous studies using fluorescent microscopy showed that flavonol aglycones rather than glycosides are taken into hepatocytes accumulating in the nucleus (189). In contrast, we found flavones mainly excluded from the nucleus. HPLC-DAD analysis of membrane and cytoplasmic fractions showed that flavones are in particular associated with membrane fractions, reaching concentrations 10 times higher than in soluble cytoplasmic fractions (Fig. 4.7). Consistent with results obtained using fluorescent microscopy, HPLC analysis showed higher uptake of apigenin than the glucoside counterpart. Moreover, concentrations of apigenin in macrophage membranes treated with aglycone-rich celery extracts, ECE and ACE, are higher than in FCE treated cells (Fig. 4.7). These results indicate that most apigenin is not stored in the cytoplasm or nucleus but remains associated with organelle membranes. Although signaling through membrane components cannot be excluded, it has been shown that flavones counter LPS-induced inflammation through several pathways in the cytosol. In addition to inactivating NF-κB through suppression of NF-κB-p65 subunit phosphorylation (144), flavones can reduce TNF-α by interfering with mitogen-activated protein kinases (MAPK). In particular, reductions in extracellular signal-regulated kinase (ERK), p38, and casein kinase 2 (CK2) activation were associated with reductions in TNF-α release from macrophages (190). Our results indicate that aglycones either pure or as part of a diet can be readily be taken up by cells of the immune system and can effectively regulate immune responses. In this
context, the mechanisms for cellular influx of flavonoids are not well understood, and they may vary with the type of flavonoid and membrane transporters found in specific cell types. It has also been proposed that flavonoids are transported into cells by passive diffusion (99,100), which would favor the transport of less polar (e.g. aglycone) molecules through the lipid bilayer membrane. Further study is needed to understand the mechanisms of cellular uptake of flavonoids.

Dietary intervention studies using flavone-rich diets showed increased serum flavone concentrations in mice fed AIN-5ECP and AIN-10ECP diets, averaging nearly twice those of their fresh celery counterparts (Fig. 4.8) even though they contained approximately one third the concentration of flavones measured as apigenin equivalents (Table 4.1). After adjusting for these differences in flavone intake, AIN-5ECP and AIN-10ECP diets resulted in significantly greater relative absorption of luteolin and apigenin than their fresh celery counterparts. These data clearly indicate that dietary flavone aglycones are more efficiently absorbed into serum than their corresponding glycosides, which is consistent with observations that flavonoids glycosides must first be deconjugated to aglycones prior to intestinal absorption (86). Apigenin and luteolin are absorbed primarily in the small intestine (191), and apigenin is more than five times more permeable than apigenin 7-O-glucoside in Caco-2 models (101).

We found that the flavone-rich diets here resulted in much higher concentrations of apigenin in serum than those previously reported in mice fed pure apigenin. The mice fed AIN-10ECP in our study consumed 0.05% apigenin for 7 days and averaged 773 nM apigenin in serum, compared with 90 nM reported in mouse plasma following a diet containing 0.2% pure apigenin for 7 days (75). Studies with flavones and flavone-rich
diets found much greater absorption in rats, although these reported maximum rather than steady state concentrations in blood. The AIN-10ECP mice in our study consumed ~68 mg apigenin/kg body weight daily, while rats fed only 14 mg luteolin/kg body weight by gavage had a peak plasma concentration of 6.9 µM after 1 hour and those fed luteolin in the form of peanut hull extract had concentrations four times as high (70). Rats consuming 10 mg apigenin equivalents/kg body weight in the form of chrysanthemum had 63 µM apigenin in plasma after 4 hours (83). These studies show that flavone concentrations in circulation are much higher within a few hours after consumption but fall to lower steady state levels. There may also be differences in intestinal absorption between species, making comparisons difficult.

In summary, we have demonstrated that deconjugation of flavone glycosides to aglycones determines their anti-inflammatory activity in vitro and their systemic absorption in vivo. Our study also indicates that administration of dietary flavone aglycones rather than glycosides results in more efficient uptake of flavones into serum, which will be important in designing food-based approaches to reducing chronic inflammation.
SUMMARY

In the literature review (Chapter 1), we noted that flavones are commonly found in a wide array of fresh foods, herbs, and juices and naturally occur as glycosides rather than aglycones. Although they are absorbed into system circulation at levels of up to 63 µM in rat plasma, they have not been measured at more than 3 µM in human plasma. Flavone 7-O-glucosides are cleaved by β-glucosidase to aglycones prior to absorption in the small intestine, and are then conjugated to form flavone glucuronide or flavone sulfate in the small intestine and liver. Flavone glycosides that pass intact to the large intestine can be hydrolyzed to flavone aglycones or further broken down to simple phenolics such as (4-hydroxyphenyl) propionic acid. A number of studies with orally administered flavones in animals have demonstrated their biological activities, including anti-depressant effects, anti-inflammatory effects, and lower cholesterol. In human clinical trials, flavones have shown the ability to lower cholesterol, reduce bleeding, and reduce healing times.

As our goal was to develop a flavone-rich functional food with anti-inflammatory activity in human clinical trials, we first tested the effects of processing on flavone stability in two naturally rich sources of flavones, parsley and celery. In the first
processing study (Chapter 2), celery leaves were combined with β-glucosidase-rich food ingredients (almond, flax seed, or chickpea flour) to determine their ability to enzymatically hydrolyze flavone apiosylglucosides. Although all of the enzyme-rich ingredients could convert apigenin glucoside to aglycone, none had an effect on apigenin apiosylglucoside. Thermal stability of flavones from celery was also tested by isolating them and heating at 100 °C for up to 5 hours in pH 3, 5, or 7 buffer. Apigenin glucoside was most stable of the flavones tested, with minimal degradation regardless of pH or heating time. Apigenin, luteolin, and chrysoeriol were stable at pH 3, but degraded steadily at pH 5 or 7. Apigenin apiosylglucoside was least stable at pH 3, but its primary conversion product was apigenin glucoside. Further experiments with thermal processing and enzyme-rich foods showed that apigenin apiosylglucoside could be converted to apigenin glucoside by heating 90 min at 100 °C at pH 2.7, and then to aglycone by glucosidase-rich ingredients. Almond and flax seed were the most effective, resulting in 97% conversion when mixed one part to five parts dry celery and incubated at 50 °C for 2 h. With both almond and flax seed treatments, there was 30-35% degradation of total apigenin derivatives. Taken together, these results provide useful data on the stability of different flavones derivatives and demonstrate that both thermal processing at low pH and incubation with other ingredients can convert flavone apiosylglucosides to simple flavone glucosides and aglycones.

The second processing study (Chapter 3) explored the effects of juice extraction, acidification, thermal processing, and endogenous enzymes on flavone profiles and concentrations in parsley and celery. Parsley yielded 72% juice with 64% of the total flavones extracted, while celery yielded 79% juice with 56% of flavones extracted.
Flavones in steamed parsley and celery were predominantly malonyl apiosylglucoside conjugates, while 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, resulting in a juice that could improve the bioavailability and efficacy of naturally occurring flavones.

In the third study (Chapter 4), the anti-inflammatory activities of celery extracts, some rich in flavone aglycones and others rich in flavone glycosides, were tested on the inflammatory mediators TNF-α and NF-κB in LPS-stimulated macrophages. Pure flavone aglycones and aglycone-rich extracts effectively reduced TNF-α production and inhibited transcriptional activity of NF-κB, while glycoside-rich extracts showed no significant effects. Deglycosylation of flavones increased cellular uptake and cytoplasmic localization as shown by HPLC and by staining with DPBA. Celery diets with different glycoside or aglycone contents were formulated, and absorption was evaluated in mice fed with 5% or 10% celery diets. Relative absorption was significantly higher in mice fed aglycone-rich diets as determined by HPLC-MS/MS. These results demonstrate that deglycosylation increases absorption of dietary flavones in vivo and modulates inflammation by attenuating TNF-α and NF-κB, suggesting the potential use of flavone-rich functional foods for the treatment or prevention of inflammatory diseases.

FUTURE RESEARCH

When formulating flavone-rich functional foods from parsley or celery, the native flavone apiosylglucosides appear to be absorbed less efficiently than aglycones. Our
processing studies showed a variety of strategies to convert native flavone glycosides to aglycones, including using endogenous enzymes for demalonylation, thermal processing at low pH for conversion of flavone apiosylglucosides to flavone glucosides, and incubating with β-glucosidase rich ingredients (e.g. almond, flax seed) to convert flavone glucosides to aglycones. The thermal conversion of flavone apiosylglucosides to flavone glucosides were more efficient at higher temperatures, lower pH, and longer cooking times. To lower pH, acetic, citric, and phorsphoric acids were initially tested and all had the same effect at the same pH. Although phosphoric acid was ultimately used for the celery used in the mouse study because it was easy to neutralize, formic acid could potentially be used. Although formic acid is not considered a GRAS food ingredient, it could be removed from the sample by freeze-drying prior to incubation with almond or flax seed. This would eliminate the need to add potassium hydroxide to neutralize the phosphoric acid and the resultant dilution with potassium phosphate salts. Regardless of the acid used, a smaller amount of acid could be added if the food is cooked at a higher temperature (e.g. 121 °C in a pressure cooker or retort) or for a longer time.

Another consideration in formulating the diet is the addition of almond or flax seed to convert flavone glucosides to aglycones. Both appear to be effective at a ratio of 5:1 (celery:enzyme source) by dry weight at 50 °C for 2 h. Further tests could determine if different pH or temperatures would allow a smaller addition of enzyme-rich ingredient. As lipids can aid in the absorption of flavonoids, the enzyme source should also be added to the other diets to control for this variable. Although we only compared celery diets rich in flavone apiosylglucosides and flavone aglycones, a diet rich in flavone glucosides would also be worth evaluating. It may be possible to improve absorption by simply
using the flavone glucoside-rich diet, and this would avoid dilution with almond or flax seed in the final product.

After further determining the best food formulation for maximum absorption of dietary flavones, animal studies should be done to see if a flavone-rich diet can reduce acute or chronic inflammation. This could be accomplished by monitoring background levels of inflammatory cytokines with or without a flavone-rich diet. Alternatively, an inflammatory response could be induced by injecting the animals with LPS, feeding LPS, or injecting them with bacteria. Levels of cytokines would then be measured in blood after feeding the control diet or the flavone-rich diet.

If the animal models for anti-inflammatory activity show the diet to be effective, human clinical trials should be considered. More processing studies would be necessary to develop a functional food that has a high concentration of readily available flavones and has consumer appeal. Lyophilized celery leaves might be blended in a tomato juice, for example. In a human study, an inflammatory response might be induced by subjecting participants to stressful situations or by feeding them a pro-inflammatory diet. Further literature review is necessary to determine which foods are most pro-inflammatory. Once this is determined, subjects can be fed a pro-inflammatory diet with or without the flavone-rich functional food and blood cytokines can be measured to determine if the flavone-rich food has an anti-inflammatory effect.
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