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Effects of domestic cooking on flavonoids in broccoli and calculation of retention factors

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Abstract

The flavonoid contents in vegetables are strongly influenced by domestic cooking. The objective of this study was to evaluate the effects of domestic cooking on the structurally complex flavonoids in broccoli. Raw broccoli was cooked by boiling, steaming and microwaving. Seven kaempferol (Km) glycosides and one quercetin (Qn) glycoside were identified and quantified in raw and cooked broccoli by HPLC-MS. Boiling resulted in significant loss of all flavonoids, while steaming and microwaving led to minor loses or even increases of the flavonoids. Apparent retention factors (AR) and true retention factors (TR) were calculated for individual flavonoids. AR ranged from 35.6% to 147.5% and TR ranged from 30.4% to 174.1%, respectively, depending on the cooking method and chemical structures of flavonoids. Two different ways to calculate total retention factors, "Retention Factor by Glycoside" and "Retention Factor by Aglycone", were also calculated. In conclusion, domestic cooking significantly altered the flavonoid contents in broccoli, with cooking method and chemical nature being key influential factors. Acylated Km tri- or tetra-glycosides appeared to be more resistant to domestic cooking.

Keywords: Food science, Food analysis, Chemistry

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1. Introduction

Flavonoids are a large group of polyphenolic compounds found in fruits and vegetables. They have been shown to be strong antioxidant and anti-inflammatory agents in human diet (Agati et al., 2012; Pan et al., 2010), and consumption of foods high in flavonoids has been associated with reduced risk of several chronic diseases (Liu, 2013). Estimating flavonoid contents in foods is a first critical step toward understanding their health benefits. Since 1999, USDA has been producing Special Interest Databases (SID) on major flavonoid compounds in foods (Bhagwat et al., 2015). The USDA flavonoid databases have since been widely used as a research tool to estimate the contents of flavonoids in foods and their intake (Bai et al., 2014; Chun et al., 2007; Kim et al., 2016). In addition, the USDA flavonoid databases have provided fundamental information in assessing the association between the flavonoid intakes and the incidences of different chronic diseases, such as cardiovascular disease (Goetz et al., 2016; Jacques et al., 2015 #183; Peterson et al., 2012), in different epidemiologic and cohort studies. However, most of the data in the current USDA flavonoid databases were obtained from the raw foods, whereas many flavonoid containing vegetables are commonly consumed after domestic cooking. Therefore, the information in flavonoid databases should account for the variability in content that occurs as a result of different cooking techniques (Cassidy and Minihane, 2017).

Thermal processing including domestic cooking have long been known to influence the flavonoid contents of the vegetables. The effects of cooking are affected by many factors, including food matrix, cooking method/condition and the chemical nature of the flavonoids (Palermo et al., 2014). As a highly consumed vegetable in the US, broccoli is commonly prepared by different domestic cooking methods. The effects of cooking on various nutrients (Bongoni et al., 2014; dos Reis et al., 2015b) and certain groups of bioactive phytochemicals (e.g. glucosinolates and polyphenols) (Moreno et al., 2007; Yuan et al., 2009; Zhang and Hamauzu, 2004) in broccoli have been investigated. However, the data of the cooking effects on individual flavonoids remain scarce. To our knowledge, only one early study compared individual flavonoids in broccoli before and after cooking. In this study, merely kaempferol (Km) and quercetin (Qn) mono- or diglucosides in broccoli florets were analyzed after undefined cooking procedure (Price et al., 1999). In other recent studies, either total polyphenols by non-specific colorimetric assays and/or Km and Qn aglycone after acid hydrolysis were measured (dos Reis et al., 2015a; dos Reis et al., 2015b; Faller and Fialho, 2009; Gliszczynska-Swiglo et al., 2006; Pellegrini et al., 2010; Severini et al., 2016; Turkmen et al., 2005; Wachtel-Galor et al., 2008; Zhang and Hamauzu, 2004). The bioavailability, metabolism, and biological activity of flavonoids depend largely on their chemical structures (Panche et al., 2016). And research have also shown that the chemical structure is an important factor

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determining the fate of flavonoids during domestic cooking (Palermo et al., 2014). As such, it is important to understand the fate of individual flavonoids as their original glycoside forms during domestic cooking.

The first objective of this study was to determine how common cooking methods alter individual flavonoids in broccoli, and to compare the effects of different cooking methods. Secondly, the results were used to calculate and to compare the retention factors that can be used to estimate flavonoids contents in cooked broccoli.

2. Material and methods

2.1. Chemicals and reagents

Formic acid for mass spectrometry, ~98% pure, was purchased from Sigma-Aldrich (St. Louis, MO). Optima® LC/MS grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Fair Lawn, NJ). HPLC-grade water was prepared from distilled water using a Milli-Q system (Millipore Laboratories, Bedford, MA). Rutin was obtained from Indofine Chemical Co. (Somerville, NJ).

2.2. Plant material

Broccoli were purchased at local Giant Food in Beltsville, MD. After cleaning, the leaves and hard stems were removed and the broccoli heads were cut into small florets (heads ~5 cm and stems ~3 cm). The florets were mixed thoroughly, and a small portion of the raw broccoli florets were lyophilized immediately as raw samples. The samples were weighed before (fresh/wet weight, FW) and after lyophilization (dry weight, DW) to calculate moisture loss of drying. Dried sample was kept at -20 °C until analysis.

2.3. Cooking procedures

The thoroughly mixed broccoli florets were cooked immediately by three cooking methods commonly used by American consumers − boiling, steaming and microwaving. Cooking parameters were selected and modified based on combined information from the literature, online cooking recipes in the US (Christensen, 2016; Ramsey, 2017; Roskelley, 2018) and preliminary testing.

2.3.1. Boiling

Broccoli florets (150 g) were added to boiling tap water in a stainless steel pot (1:5, w/w, broccoli/water), then covered. After boiling for 85 seconds, the broccoli were drained off for 45 s and gently wrapped by paper towel.

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2.3.2. Steaming

Broccoli florets (150 g) were steamed in a single layer in a steaming basket. The basket was suspended in a stainless steel pot of boiling water (one inch), making sure that the water did not touch the sample, and steamed for 5 minutes. After steaming, the broccoli were drained off for 45 s and gently wrapped by paper towel.

2.3.3. Microwaving

Broccoli florets (150 g) were put in a microwave safe bowl with a 1 table spoon of water and covered with a plastic microwave cover. Microwave treatment was carried out in a Panasonic NN-SN651 B Genius 1.2 cubic feet 1200-W microwave (Panasonic, Japan) at full power for 1 min. After microwaving, the broccoli were gently wrapped by paper towel.

After cooking, the cooked samples were lyophilized immediately and the dried samples were kept at -20 °C until analysis. The cooked samples were weighed before and after drying to calculate moisture loss. Each cooking treatment was repeated three times.

2.4. Flavonoid analysis

Freeze-dried broccoli sample (0.2 g) was extracted with 10 mL methanol/water (60:40, v/v). The supernatant was filtered through a 0.45 μm PVDF syringe filter (VWR Scientific, Seattle, WA), and was transferred into a 2 mL HPLC vial for flavonoid analysis. The UHPLC−PDA−HRMS system consisted of an LTQ Orbitrap XL mass spectrometer with an Accela 1250 binary pump, a PALHTC-Accela1-TMO autosampler, an Accela PDA detector (Thermo Fisher Scientific, San Jose, CA), and a G1316A column compartment (Agilent, Santa Clara, CA)). Separation was carried out on a Synergi Hydro-RP column (250 mm × 2.00 mm, 4 μm. Phenomenex, Torrance, CA) at a flow rate of 0.3 mL/min. The mobile phase consisted of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v).

The quantification was carried out using molar response factors with rutin as reference standard as described in previous report (Lin et al., 2012). The concentrations of individual flavonoids based on DW were obtained from the analyses of the freeze-dried samples, and the values were further converted to the concentrations as FW by calculating the moisture losses from FW to DW.

2.5. Calculation of retention factors

Apparent retention (AR) and true retention (TR), based on the concentrations of individual flavonoids analyzed as DW, were calculated with modification for each individual flavonoids (Murphy et al., 1975).

https://doi.org/10.1016/j.heliyon.2019.e01310

$$\text{AR}\ (\%)\ =\ \frac{\text{flavonoid glycoside content per g of cooked broccoli (DW)}}{\text{flavnoid glycoside content per g of raw broccoli (DW)}}\ \times\ 100$$

$$\text{TR}\ (\%) = \frac{\text{flavonoid glycoside content per g of cooked broccoli (FW)x grams of cooked broccoli}}{\text{flavonoid glycoside content per g of raw broccoli (FW) x grams of raw broccoli}}\ \times\ 100$$

Two total true retention factors "Retention Factor by Glycoside" and "Retention Factor by Aglycone" were calculated for total Km flavonoids, total Qn flavonoids and total flavonoids using the formula above for TR.

2.6. Statistical analysis

The effect of different cooking methods (Boiling, Steaming and Microwaving) on eight different flavonoids present in broccoli were statistically compared. A one-way analysis of variance with 'cook method' as a factor with 4 different levels (Raw, Boiling, Steaming, and Microwaving) and nutrient value as the continuous variable was conducted. The data was checked for homogeneity of variances using Fligner-Killeen test with an alpha level of 0.05. It was further checked for Normality using the quantile-quantile plot. If the data met the above two criteria successfully a one-way analysis of variance with repeated measures was performed on it since the samples can be considered as paired. Whenever the tests yielded a significant result it was followed by a paired pairwise t-test analysis with holm correction. The statistical analysis was conducted using R version 3.5.1. (Team, 2018).

3. Results and discussion

Broccoli was found to contain complex acylated tri- or tetra-glycosides of Km and Qn (Vallejo et al., 2004). In the broccoli material used in this study, seven Km tri- or tetra-glycosides and one Qn tri-glycoside, mostly acylated forms, were identified and quantified as major flavonoids. The chemical structures were elucidated based on the HRMS data, retention time, and by comparing the data to the published reports (Cartea et al., 2010; Vallejo et al., 2004). Most of these flavonoids are acylated by three hydroxycinnamic acids: caffeic acid, ferulic acid and sinapic acid (Table 1).

With the same cooking method, retentions of flavonoids are greatly affected by the actual cooking conditions, such as temperature and time (Tiwari and Cummins, 2013). The purpose of this study was not to compare different conditions of each cooking methods (e.g. different time, temperature, etc.), but to focus on retentions of the individual flavonoids under common cooking conditions in the US. For boiling, cooking time was found to range from 30 seconds to 8 minutes in the

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Table 1. Mass spectrometry data and the Identification of flavonoids in broccoli.

t_R (min)	HRMS m/z [M-H]	Calcd Formula [M-H]-	Identification
6.44	771.1968	$C_{33}H_{39}O_{21}$	Km 3-O-diglucoside-7-O-glucoside
9.42	933.2278	$C_{42}H_{45}O_{24}$	Km 3-O-caffeoyldiglucoside-7-O-glucoside
10.03	993.2490	$C_{44}H_{49}O_{26}$	Qn 3-O-sinapoyldiglucoside-7-O-glucoside
11.38	977.2542	$C_{44}H_{49}O_{25}$	km 3-O-sinapoylsophoroside-7-O-glucoside
11.86	947.2436	$C_{43}H_{47}O_{24}$	Km 3-O-feruloylsophoroside-7-O-glucoside
18.12	1477.4040	$C_{66}H_{77}O_{38}$	Km 3-O-sinapolylferuloyltriglucoside-7-O-diglucoside
18.34	1345.3651	$C_{61}H_{69}O_{34}$	Km 3-O-disinapolyltriglucoside-7-O-glucoside
18.79	1315.3550	$C_{60}H_{67}O_{33}$	Km 3-O-sinapolylferuloyltriglucoside-7-O-glucoside

Km: kaempferol; Qn: quercetin.

literature (Pellegrini et al., 2010; Severini et al., 2016). Nonetheless, both the US online cooking recipes and our tests suggested that boiling for about two minutes would help bring out the bright color and the tasty flavor of the vegetable. So we chose to use 85 seconds. For steaming, the cooking time ranged from 30 seconds to 20 minutes in the literature (dos Reis et al., 2015b; Severini et al., 2016). However, US online cooking recipes generally suggested 4−5 minutes. Our preliminary test showed that steaming for longer than 10 minutes significantly changed the color, texture and flavor of the broccoli, all of which made it less favorable to consumers. Therefore, a 5-minute steaming time was used in this study. For microwaving, the cooking time in the literature was reported from 30 seconds to 30 minutes (Pellegrini et al., 2010; Zhang and Hamauzu, 2004). While almost all US online recipes suggest 2−4 minutes. The cooking time of microwaving is also affected by the power of the microwave oven. Considering the literature data, the online recipe and our preliminary test, as well as the specification of the microwave oven used in this study, we adopted a 1-minute cooking time.

All raw and cooked broccoli samples were lyophilized for better flavonoid preservation and sample handling. The losses of moisture contents from the raw/wet samples to the freeze-dried samples were 89.2 ± 0.1% for raw broccoli, 90.8 ± 0.2% for boiled broccoli, 90.6 ± 0.4% for steamed broccoli and 87.3 ± 0.1% for microwaved broccoli. Compared to the raw broccoli, boiled and steamed broccoli slightly gained moisture whereas microwaved broccoli lost moisture. The moisture values were used to convert the flavonoid contents from DW basis to FW basis.

The quantification of individual flavonoids in raw and cooked broccoli based on FW is presented in Table 2. Overall, boiling led to significant losses in all eight flavonoids, whereas steaming and microwaving resulted in much less losses and even

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Table 2. Concentrations of individual flavonoids in raw and cooked broccoli.

Flavonoids	Raw broccoli	Boiled broccoli	Steamed broccoli	Microwaved broccoli
	mg/100g FW	mg/100g FW	mg/100g FW	mg/100g FW
Km 3-O-diglucoside-7-O-glucoside	0.84 ± 0.02[a]	0.25 ± 0.03[b]	0.58 ± 0.01[c]	0.88 ± 0.06[a]
Km 3-O-caffeoyldiglucoside-7-O-glucoside	0.23 ± 0.02[ab]	0.14 ± 0.01[a]	0.30 ± 0.01[bc]	0.39 ± 0.04[c]
Qn 3-O-sinapoyldiglucoside-7-O-glucoside	0.19 ± 0.01[a]	0.10 ± 0.01[b]	0.17 ± 0.01[ab]	0.20 ± 0.003[a]
km 3-O-sinapoylsophoroside-7-O-glucoside	0.23 ± 0.02[a]	0.12 ± 0.004[b]	0.21 ± 0.01[a]	0.34 ± 0.02[c]
Km 3-O-feruloylsophoroside-7-O-glucoside	0.48 ± 0.02[a]	0.24 ± 0.01[b]	0.46 ± 0.01[a]	0.69 ± 0.06[c]
Km 3-O-sinapolylferuloyltriglucoside-7-O-diglucoside	0.27 ± 0.01[a]	0.13 ± 0.01[b]	0.28 ± 0.004[a]	0.39 ± 0.005[c]
Km 3-O-disinapolyltriglucoside-7-O-glucoside	0.44 ± 0.03[a]	0.24 ± 0.01[b]	0.39 ± 0.003[a]	0.59 ± 0.03[c]
Km 3-O-sinapolylferuloyltriglucoside-7-O-glucoside	0.12 ± 0.02[ab]	0.09 ± 0.01[a]	0.12 ± 0.01[ab]	0.17 ± 0.01[b]

Data was expressed as mean ± SD (n = 3).
Km: kaempferol; Qn: quercetin.
[a-c] Different letters in the same row indicated significant statistical differences ($P < 0.05$).

apparent gains for most complex flavonoids. The significant loss of flavonoids after boiling may be due to the combined effects of thermal degradation and leaching of flavonoid compounds into the cooking water (Tiwari and Cummins, 2013). Steaming and microwaving have been suggested as the preferred cooking methods over boiling for green vegetables (Rennie and Wise, 2010), due to generally rated higher consumer acceptability, lower nutrient losses (dos Reis et al., 2015b), and better availability of phenolic compounds (Turkmen et al., 2005). Under the cooking conditions used in this study, microwaving appeared to be a better way to preserve flavonoids than steaming.

Literature data on the effects of these three cooking methods on the phenolic compounds in broccoli are not consistent. To facilitate discussion, the data of cooking effects on total phenolics and/or flavonoids of broccoli in the literature were summarized to identify the major influential factors (Table 3). Among all available publications, only the studies that provided complete information regarding the food material, cooking methods (equipment, cooking time, food: water ratio) and numerical results were included. As being shown in Table 3, total phenolics and/or flavonoid contents could decrease, increase or remain unchanged in broccoli after domestic cooking, largely depending on the factors discussed below:

a) Cooking methods. Among the 9 studies included, the cooking method varied significantly. In general, there are two major opposite phenomena associated with cooking: thermal degradation that cause reduction of phytochemicals, or matrix softening effects that increases the extractability of phytochemicals (Palermo et al., 2014). The net effects of the cooking methods are determined by several important factors. The first one is the cooking time, which ranged

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Table 3. Summary of the literature data of the cooking effects on phenolic compounds in broccoli.

Broccoli sample	Cooking method	Cooking condition and time	Analytical method	Changes in contents	Refs
Florets	Boiling	Sample: water 1:10, 30, 60, 90, 120, 180 s	Total phenolics (TP) by Folin-Ciocalteu method (F-C)	TP ↔	(Severini et al., 2016)
	Steaming	30, 60, 90, 120, 180 s		TP ↔	
	Microwaving	900W, 40, 50, 60, 70 s		TP ↔	
Inflorescences	Boiling	Sample: water 1:5, 5 min	Qn and Km by HPLC after acid hydrolysis	Qn ↓, Km ↓	(dos Reis et al., 2015a)
	Steaming	20 min		Qn ↓, Km ↓	
	Microwaving	200g sample + 16 mL water, 800W, 4 min		Qn ↓, Km ↓	
Inflorescences	Boiling	Sample: water 1:5, 5 min	Qn and Km by HPLC after acid hydrolysis, TP by F-C	TP ↓, Qn ↑, Km ↑	dos Reis et al., 2015b)
	Steaming	20 min		TP ↔, Qn ↔, Km ↔	
	Microwaving	200 g sample + 16 mL water, 800W, 4 min		TP ↔, Qn ↔, Km ↔	
Florets and stems	Boiling	Sample: water 1:5, 8 min	Qn and Km by HPLC after acid hydrolysis	Qn ↓, Km ↓	(Pellegrini et al., 2010)
	Steaming	13 min		Qn ↓, Km ↓	
	Microwaving	300W, 30 min		Qn ↓, Km ↓	
Stalks, leaves and inflorescences	Boiling	100 g Sample + 150 mL water, 6 min	TP by F-C	TP(OG) ↓, TP (CV) ↓	(Faller and Fialho, 2009)
	Steaming	8 min		TP(OG) ↓, TP (CV) ↑	
	Microwaving	2450W, 4 min		TP(OG) ↓, TP (CV) ↑	
Edible parts	Boiling	5 g Sample + 100 mL water, 5 min	TP by F-C	TP ↓	(Wachtel-Galor et al., 2008)
	Steaming	5 min		TP ↔	
	Microwaving	5 g Sample + 100 mL water, 750W, 5 min		TP ↓	
Florets	Boiling	300 g Sample + 1000 mL water, 5 min	FL by HPLC after acid hydrolysis, TP by F-C	TP ↓, FL ↓	(Gliszczynska-Swiglo et al., 2006)
	Steaming	10 min		TP ↑, FL ↑	
Edible parts	Boiling	100 g sample + 150 mL water, 5 min	TP by F-C	TP ↔	(Turkmen et al., 2005)
	Steaming	7.5 min		TP ↑	
	Microwaving	100 g Sample + 6 mL water, 1000W, 1.5 min		TP ↑	
Florets	Boiling	10 g sample + 400 mL water, 30, 60, 90, 120, 300 s	TP by F-C	TP ↓	(Zhang and Hamauzu, 2004)
	Microwaving	10 g sample + 200 mL water, 600W, 30, 60, 90, 120, 300 s		TP ↓	

↓, decrease; ↑, increase; ↔, no change; Km: kaempferol; Qn: quercetin.

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from 30 seconds to 8 min for boiling, 30 seconds to 20 min for steaming and 30 seconds to 30 min for microwaving in previous studies (Table 3). Shorter cooking time generally resulted in unchanged or increase of total phenolics (Severini et al., 2016; Turkmen et al., 2005). Longer cooking times, on the other hand, not only dramatically changed the appearance and flavor, also reduced the total phenolics and flavonoids contents (dos Reis et al., 2015a; Pellegrini et al., 2010) (Table 3). The second factor is the sample to water ratio, especially for microwaving. Broccoli can be microwaved with or without water. The sample to water ratio was found from 100:6 (Turkmen et al., 2005) up to 5:100 (Gliszczynska-Swiglo et al., 2006) in previous studies. Microwaving without water or with small amount of water tended to retain or increase total phenolics and/or flavonoids. When large amount of water was added during microwaving, to some extent resembling boiling, total phenolics and/or flavonoids decreased (Table 3). The third factor is the specification of the cookware and equipment. For example, microwave ovens with different power (300−2450W) were used in above mentioned studies. More powerful microwaves cook faster, tend to improve the retentions of total phenolics and/or flavonoids. Nevertheless, the exact effects of the power are hard to elucidate since they confounded with other factors such as cooking time.

b) Quantitative analysis of flavonoids. In six of the nine studies listed in Table 3, classical Folin-Ciocalteu (F-C) assay was used to quantify total phenolics. Being a colorimetric method, F-C assay is not specific. Chemically, it does not just measure phenols, but reacts with other reducing agents. Therefore, it can be interfered by various reducing substances (such as ascorbic acid) presenting in the plant extracts (Sanchez-Rangel et al., 2013) thus generates false results. This characteristic might contribute to the inconsistency of the data in the literature.

c) Food samples. Broccoli is usually consumed after removing inedible parts. In previous studies, different parts of the vegetable, such as florets, florets and stem, and even stalks and leaves, were used in the investigation (Table 3). This would undoubtfully add variability to the data inconsistency. In addition, the dramatic differences of cooking effects on total polyphenols between conventional and organic broccoli were reported (Faller and Fialho, 2009). Polyphenol content (measured by F-C method) in organically grown broccoli suffered much greater loss comparing to that in conventionally grown broccoli under the same cooking conditions. This interesting observation may suggest that for the same vegetables, different agriculture practices may somehow affect the way of flavonoids existence and their interactions with other compounds, which in turn alter their sensitivity to heat treatments.

According to an earlier paper (Murphy et al., 1975), the retentions of nutrients in cooked foods can be calculated as apparent retention (AR) based on dry form, or true retention (TR) based on fresh/wet form. TR represents the actual consumption

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forms and also takes the weight change after cooking into consideration. It was thus suggested that to make the data more meaningful, TR, rather than AR, should be reported whenever possible. In this study, both AR and TR were calculated for the individual flavonoids (Table 4). Since TR represents the actual consumption forms, it was used to compare the effects of different cooking methods on individual flavonoids in broccoli. Depending on the moisture loss from raw/cooked broccoli to their dried forms, TRs are lower than ARs for the boiled and steamed broccoli, but higher than AR for the microwaved broccoli. TRs were found to be significantly different between three cooking methods for all eight flavonoids as the following order: TR of microwaving > TR of steaming > TR of boiling. Among all flavonoids and all cooking methods, TR ranged dramatically from 30.4 ± 2.8% for Km 3-*O*-diglucoside-7-*O*-glucoside after boiling to 174.1 ± 2.8% for Km 3-*O*-caffeoyldiglucoside-7-*O*-glucoside after microwaving. The possible explanation for TR over 100% is that the thermal processing may increase the extractability and/or the release from binding to other compounds as a result of matrix softening.

The chemical nature of the flavonoids is another important factor that determines their fates during cooking. Of the eight flavonoids identified in broccoli, only one is non-acylated tri-glycosides and the rest are acylated tri- or tetra-glycosides. Notably, non-acylated Km 3-*O*-diglucoside-7-*O*-glucoside underwent greater loss after boiling than all acylated flavonoids, and minor loss after steaming and microwaving (Table 4). It is possible that acylation may lead to increased resistance to thermal processing. Since only one non-acylated flavonoid was identified, whether

Table 4. Calculation of apparent retention factors and true retention factors for individual flavonoids in cooked broccoli.

Flavonoids	Boiled broccoli		Steamed broccoli		Microwaved broccoli	
	AR (%)[†]	TR (%)[‡]	AR (%)	TR (%)	AR (%)	TR (%)
Km 3-O-diglucoside-7-O-glucoside	35.6 ± 3.4	30.4 ± 2.8[a]	80.1 ± 7.2	69.6 ± 3.2[b]	89.1 ± 8.3	105.2 ± 10.2[c]
Km 3-O-caffeoyldiglucoside-7-O-glucoside	72.8 ± 9.3	62.2 ± 9.1[a]	154.2 ± 18.9	134.3 ± 16.2[b]	147.5 ± 4.2	174.1 ± 2.9[c]
Qn 3-O-sinapoyldiglucoside-7-O-glucoside	62.3 ± 2.6	53.1 ± 1.7[a]	104.0 ± 10.6	90.5 ± 6.6[b]	92.0 ± 3.1	108.7 ± 4.9[c]
km 3-O-sinapoylsophoroside-7-O-glucoside	64.5 ± 5.3	55.0 ± 3.9[a]	109.2 ± 8.1	95.0 ± 4.7[b]	126.8 ± 5.9	149.7 ± 6.5[c]
Km 3-O-feruloylsophoroside-7-O-glucoside	57.8 ± 2.0	49.3 ± 2.8[a]	111.2 ± 5.7	96.7 ± 2.0[b]	122.1 ± 13.0	144.0 ± 14.2[c]
Km 3-O-sinapolylferuloyltriglucoside-7-O-diglucoside	55.7 ± 4.2	47.4 ± 2.7[a]	116.1 ± 2.0	101.1 ± 4.2[b]	120.2 ± 2.9	141.9 ± 4.7[c]
Km 3-O-disinapolyltriglucoside-7-O-glucoside	65.1 ± 6.1	55.6 ± 6.2[a]	102.1 ± 2.2	89.0 ± 5.7[b]	113.1 ± 1.7	133.5 ± 2.7[c]
Km 3-O-sinapolylferuloyltriglucoside-7-O-glucoside	84.4 ± 8.2	72.0 ± 7.5[a]	112.8 ± 10.3	98.0 ± 4.2[b]	121.8 ± 21.8	143.6 ± 24.3[c]

Data was expressed as mean ± SD (n = 3).
Km: kaempferol; Qn: quercetin.
[a-c] Different letters in the same row indicated significant statistical differences for TR (%) ($P < 0.05$).
[†] AR: apparent retention factor, calculated by dry weight.
[‡] TR: true retention factor, calculated by fresh/wet weight.

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and how much this phenomenon applies to other non-acylated flavonoids needs further investigation. The effects of the sugar moiety have not been studied in broccoli because in nearly all previous publications, flavonoids were quantified by HPLC as the aglycone forms after acid hydrolysis to remove the sugar moieties (Justesen et al., 1998) (Table 3). Our data suggested that sugar number between 3 and 4 did not appear to make a difference, while the acylated groups that attached to the sugar might be. Particularly, concentrations of Km 3-O-caffeoyldiglucoside-7-O-glucoside, the only caffeoyl glycoside, significantly increased in steamed and microwaved broccoli comparing to the raw broccoli (Table 2), and its TRs are much higher than that of all other Km glycosides (Table 4). Furthermore, the types of aglycone may also play an important role. Broccoli contains Km and Qn as major flavonoid aglycones. In one previous study, Km and Qn were found to behave differently under the same boiling condition (dos Reis et al., 2015b). In this study, only one Qn acylated tri-glycoside were identified. Interestingly, compared to all other Km acylated tri- and tetra-glycoside, Qn 3-*O*-sinapoyldiglucoside-7-*O*-glucoside is the only one that showed loss after microwaving for AR. Again, whether this applies to all Qn flavonoids or is specific to this compound is yet to be determined.

Two methods were used to calculate total retention factors of total Km flavonoids, total Qn flavonoids and total flavonoids based on their concentrations on FW (Table 5). "Retention Factor by Glycoside" was calculated directly using the concentrations of total glycosides. For "Retention Factor by Aglycone", the glycosides were firstly converted into the aglycone by their molecular weights, and then the concentrations of total aglycone were used in the calculation. "Retention Factor by Glycoside" represents the alterations of the actual flavonoids. While "Retention Factor by

Table 5. Calculation of retention factors for total kaempferol glycoside, total quercetin glycosides and total flavonoids.

Flavonoids	Retention Factor by Glycoside	Retention Factor by Aglycone
Boiling		
Km glycosides	46.6%	44.6%
Qn glycosides	53.2%	53.2%
Total flavonoids	47.0%	45.1%
Steaming		
Km glycosides	90.1%	88.2%
Qn glycosides	90.3%	90.3%
Total flavonoids	90.2%	88.3%
Microwaving		
Km glycosides	132.4%	129.8%
Qn glycosides	108.5%	108.5%
Total flavonoids	130.8%	128.4%

Km: kaempferol; Qn: quercetin.

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Aglycone" reflects the true changes of flavonoid core structures and complies with current data expression format in USDA Special Interest Database on Flavonoids (Haytowitz et al., 2018). Hence, the "Retention Factor by Aglycone" was proposed to be an appropriate method and is recommended to be reported in the future studies.

4. Conclusion

Retention of individual complex flavonoids in cooked broccoli were evaluated in this study. Several key influential factors, including cooking methods, conditions and chemical nature were found to significantly affect the flavonoid concentrations in cooked broccoli. Boiling led to considerable loss of flavonoids, whereas only minor loss or even apparent gain were observed in steamed and microwaved broccoli. Of the three cooking methods, microwaving appeared to be the best method to retain complex flavonoids in broccoli, followed by steaming. Characteristics of the chemical structure, including acylation, aglycone, and the sugar moiety, play important roles in determining the fate of flavonoids in cooked broccoli. AR and TR were calculated for individual flavonoids. TR reflects the consumption forms thus was the preferred way to report the retention factors. Of the two methods to calculate total retention factors, "Retention Factor by Aglycone" reflects the true changes of flavonoid core structures and complies with current data expression format in USDA Special Interest Database on Flavonoids. Finally, retention factors for flavonoids in other vegetables are likely to be different due to the different food matrix, compound profile and specific cooking methods and conditions. Therefore, results from this study cannot be simply extrapolated to other vegetables that contain similar or even same compounds. The cooking effects on phytochemical/flavonoid of different type of vegetables ought to be investigated and evaluated on a case-by-case basis. The understanding of cooking effects on flavonoids in vegetable is crucial to accurately estimate their daily intake and further investigate their health benefits.

Declarations

Author contribution statement

Xianli Wu: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yang Zhao: Performed the experiments; Analyzed and interpreted the data.

David B. Haytowitz: Conceived and designed the experiments.

Pei Chen: Contributed reagents, materials, analysis tools or data.

Pamela R. Pehrsson: Conceived and designed the experiments; Wrote the paper.

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Competing interest statement

The authors declared no conflict of interest.

Additional information

No additional information is available for this paper.

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