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Liquid Chromatography/Mass Spectrometry Investigation of the Impact of Thermal Processing and Storage on Peach Procyanidins

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Normal-phase liquid chromatography/mass spectrometry (LC/MS) was used to determine the levels and fate of procyanidins in frozen and canned Ross clingstone peaches as well as in the syrup used in the canning over a 3 month period. Procyanidin oligomers, monomers through undecamers, were identified in Ross clingstone peaches. Optimized methods allowed for the quantitation of oligomers through octamers. The profile of procyanidins in peaches is similar to profiles found in grapes, chocolate, and beverages linked to health benefits such as tea and wine. The monomer content in frozen peeled peaches was found to be 19.59 mg/kg. Dimers (39.59 mg/kg) and trimers (38.81 mg/kg) constituted the largest percent composition of oligomers in the peaches. Tetramers through octamers were present in levels of 17.81, 12.43, 10.62, 3.94 and 1.75 mg/kg, respectively. Thermal processing resulted in an 11% reduction in monomers, a 9% reduction in dimers, a 12% reduction in trimers, a 6% reduction in tetramers, and a 5% reduction in pentamers. Hexamers and heptamers demonstrated an approximate 30% loss, and octamers were no longer detected. Analysis of the syrup after thermal processing indicates that there is a migration of procyanidin monomers through hexamers into the syrup that can account for the losses observed during the canning process. Storage of canned peaches for 3 months demonstrated a time-related loss in higher oligomers and that by 3 months oligomers larger than tetramers are not observed. At 3 months postcanning, levels of monomers had decreased by 10%, dimers by 16%, trimers by 45%, and tetramers by 80%. A similar trend was observed in the canning syrup.

KEYWORDS: Procyanidins; peaches; thermal processing; LC/MS

INTRODUCTION

Peaches contain different types of phenolic compounds, including hydroxycinnamic acids (*1*), flavan-3-ols (*2*), flavonols (*3*), and anthocyanidins (*4*). Phenolic compounds are secondary metabolites naturally present in peaches that are synthesized in response to environmental stress (*5*). They are relevant in terms of quality, as they play in roles in visual appearance and taste, and recently have been linked to the health-promoting properties of fruit (*6*). For example, flavonoids are reported to modulate the cancer process, the immune system, and hemostasis (*6*−*9*).

Flavan-3-ol metabolite and procyanidins are of special interest due to their potent antioxidant activity (*10*), free radical scavenging abilities (*11*), and protective cardiovascular effects (*12*−*14*). Structurally, procyanidins are composed of the polyhydroxyl flavan-3-ol units (+)-catechin or (−)-epicatechin (Figure 1). Fruit and vegetables contain both the flavan-3-ols and more complex oligomeric procyanidins (Figure 2). Singly linked PCs are more common in foods, comprising the major polyphenolics in cocoa, grapes, and apples (*15*). Doubly linked procyanidins have been identified in fruits such as cranberries and blueberries (*16*). Oligomeric procyanidins demonstrate antioxidant activity that increases linearly with the number of reactive catechol and/or pyrogallol groups (*5*, *17*). The content

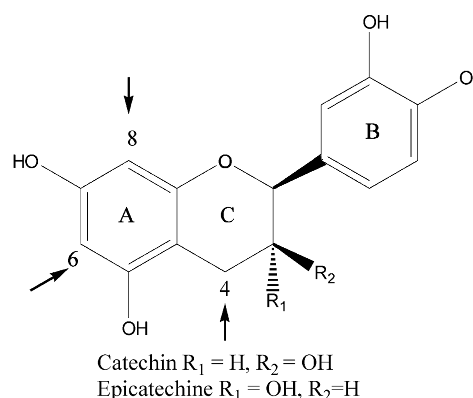

Figure 1. Representative structure of catechin and epicatechin.

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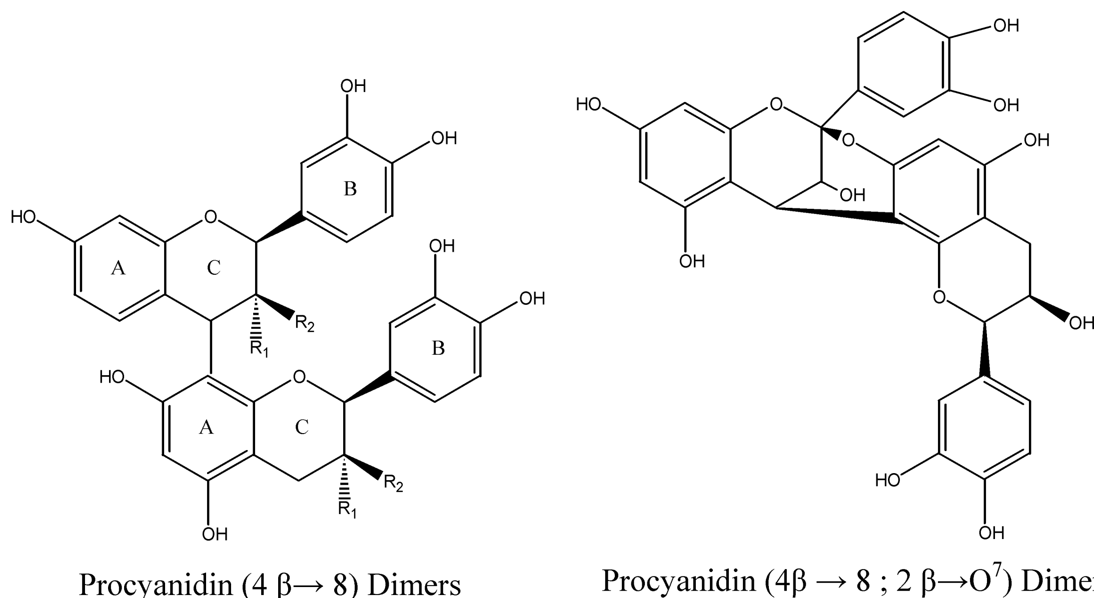

Figure 2. Representative structures of singly linked procyanidin B-type dimers and doubly linked procyanidin A-type dimers.

and composition of catechin or epicatechin and procyanidin oligomers in fruits varies considerably with genetic variation (*18*−*20*) and postharvest processing conditions (*19*−*22*).

Clingstone peaches are commonly preserved by thermal processing methods such as canning. The thermal process applied to clingstone peaches is typically greater than that required for microbiological destruction in order to assist softening the texture to a degree that the fruit is acceptable to the consumer. There have been few studies on the effect of processing on phenolic composition in fruits and vegetables, even though variations in phenolic composition are shown to impact the antioxidant capacity of processed peaches (*23*). Fuleki and Ricardo-da-Silva (*24*) showed that flavan-3-ols in the grape juice were influenced by pressing methods, cultivar, pasteurization, and vintage. Hernández et al. (*21*) investigated variations in the phenolic composition of peach and apple juices with different thermal treatments. These studies demonstrated that different processes gave rise to a series of changes in the phenolic composition and that flavan-3-ol monomers were not detected after the thermal treatments. It is not clear if higher molecular weight oligomers have the same biological activity as lower molecular weight oligomers, and at what specific size their effectiveness as antioxidants declines. However, Lotito et al. (*25*) suggest that the individual procyanidin oligomers have different antioxidant potentials.

Advanced high-performance liquid chromatography (HPLC) detection techniques coupled to LC/MS have allowed for the separation and quantification of procyanidins in cocoa and chocolate as well as several other plant foods (*15*). Recently, Hammerstone et al. (*26*) determined the average levels of procyanidins in red wine (19.5 mg/100 g), chocolate (300 mg/100 g), cranberry juice (18.8 mg/100 g), and apples (147.1 mg/medium-sized apple). Adamson et al. (*27*) quantified procyanidin monomers through decamers in cocoa and chocolates using normal-phase LC/MS and found that levels ranged from 0.7 to 20 mg/g. Nonetheless, a more extensive sampling of foods is still needed.

The profile of procyanidin oligomers (monomers through heptamers) was recently described in clingstone peaches (*23*). This study indicated that both storage and thermal processing conditions affect levels of procyanidins and total polyphenolics in peaches, which are the only constituents that correlated with the total antioxidant capacity (*28*, *29*). Spanos et al. (*30*) demonstrated that the loss of procyanidins is associated more closely with storage than with processing per se. With the increased recognition of the role of plant-based phenolics in human health and nutrition, it is increasingly more important to understand how various postharvest and processing conditions impact phenolic antioxidants such as the procyanidins.

In this study, negative-mode LC/MS methodology was optimized for the analysis of procyanidin oligomers through octamers in clingstone peaches in order to quantitatively evaluate the impact of thermal processing and canned storage (3 months) on the levels of individual procyanidin oligomers.

MATERIALS AND METHODS

Chemicals. Fluorescein was obtained from Sigma (St. Louis, MO). HPLC-grade acetone, methylene chloride, methanol, and acetic acid were obtained from Fisher Scientific (Houston, TX). Reagent-grade, bacteria-free water was generated by a Barnstead E-pure four-module deionization system (Dubuque, IA).

Peaches. Clingstone peaches of the Ross cultivar were hand-picked from the University of California orchard (Winters, CA). Fruits were harvested randomly from both the outer and internal canopy of selected trees in order to obtain a homogeneous sample. Prior to canning, peaches were sliced, pitted, and peeled with 2% lye, rinsed, and packed into number 2.5 cans. Cans were made with enamel-coated bodies and tin-plated lids. Filled cans were weighed and the peach content was adjusted to 538.7 g of fruit/can. Cans were filled with 11.5 oz of 30° Brix syrup prior to pulling a vacuum on the pack and sealing the lids to the cans. Fruits were processed in a Food Manufacturing Corp. Steritort (Madera, CA). Processing conditions were designed to ensure commercial sterility at 220 °F for 10 min. This canning process was started with a 3 min come-up time to evacuate air from the Steritort chamber and to bring the cans up to temperature. Peach samples were analyzed prior to pasteurization (frozen), after canning (day 0), and after storage for either 1, 2, or 3 months at room temperature.

Extraction of Procyanidins. Procyanidins were extracted from peaches with an extraction solvent composed of acetone, water, and acetic acid (70:29.5:0.5 v/v/v). Both peach (5 g sample) and canning syrup (15 mL) were evaluated for procyanidin content. Samples were homogenized for 1 min at maximum speed in a Waring blender in the presence of extracting solvents (100 mL). Individual samples were run in triplicate. Samples were spiked with 200 μL of fluorescein (10 mg/mL) in 80% methanol/water (v/v) as a recovery standard prior to

Table 1. Recoveries of Procyanidin Oligomers in the Cocoa Standard[a]

	procyanidin recovery (%)
monomer	102.39 ± 3.54
dimer	88.34 ± 5.18
trimer	82.92 ± 9.21
tetramer	72.99 ± 8.67
pentamer	64.76 ± 10.08
hexamer	68.58 ± 18.62
heptamer	61.42 ± 13.94

[a] Results presented as mean ± SD for triplicates.

homogenization. The mixture was vortexed and sonicated for 30 min at room temperature to allow for complete solvent extraction. Extracts were centrifuged at 3000 rpm for 15 min at 20 °C. The supernatant was filtered through a Whatman no. 1 filter, after which the filtrate was concentrated in a rotary evaporator under partial vacuum at 40 °C. Samples were concentrated to 30∼35 mL and freeze-dried. Dried peach powders were reconstituted in water (20 mL) and sonicated for 30 min. Samples were loaded onto preconditioned Supercosil Envi-18 6 mL solid-phase extraction cartridges (SPE; Supelco, Inc., Bellefonte, PA) and washed with 15 column volumes (∼90 mL) of Nanopure water. SPE columns were preconditioned by rinsing with 3 column volumes of Nanopure water and 3 column volumes of methanol, followed by 6 column volumes of Nanopure water. The columns were dried under vacuum for 1−2 min. Procyanidins were eluted from the SPE column with 6 mL of acetone, water, and acetic acid (70:29:0.05 v/v/v).

LC/MS Analysis of Procyanidins. Procyanidin oligomers were separated by normal-phase HPLC (Shimadzu Scientific, Columbia, MD) on Phenomenex (Torrance, CA) with a 5 μm Luna silica column (25 cm × 2.0 mm) as previously described (*31*). The binary mobile phase consisted of solvent A, composed of methylene chloride, methanol, water, and acetic acid (82:14:2:2 v/v/v/v), and solvent B, composed of methanol, water, and acetic acid (96:2:2 v/v/v). Separations were performed by linear gradients of B into A at a flow rate of 0.2 mL/min as follows: time 0−30 min, 0−17.6% B in A; time 30−45 min, 17.6−30.7% B in A; and 45−50 min, 30.7−87.8% B in A. In all cases, the columns were reequilibrated between injections with the equivalent of 10 mL of the initial mobile phase. The HPLC system was interfaced through an electrospray interface (ESI) to a ZSPRAY Micromass Quattro LC (Beverely, MA). LC/MS was optimized for peaches with a capillary voltage of 3.2 kV, a cone voltage of 30 V in (+)-mode ESI and −3.2 kV, −30 V, respectively, in (−)-mode ESI at a source temperature of 150 °C and a desolvation gas temperature of 300 °C. The introduction of ammonium acetate (10 mM) as an ionization reagent was attempted in three different flow rates (0.02, 0.03, and 0.04 mL/min) via a tee in the eluant stream. MS data were collected from 100 to 3500 *m*/ *z* and processed with MassLynx v 3.5. The comparison between (+)- and (−)-mode ESI detection was made by filtering total ion chromatogram (TIC) at *m*/*z* corresponding to singly and multiply charged ions produced from each oligomer. Quantification of procyanidins in peaches was achieved by both internal and external calibration. External calibration was achieved by use of a composite standard of procyanidin oligomers through decamers extracted from cocoa.

Determination of Recovery. Extraction recoveries were determined by standard spike and recovery techniques employing fluorescein, which was added to samples prior to extraction. Recoveries of fluorescein were determined from a standard curve based upon HPLC peak areas obtained at 280 nm of a set of known fluorescein standards generated under the same conditions for procyanidins. The recoveries of individual procyanidins were determined from a cocoa standard containing procyanidin oligomers through heptamer for the validation of method. Recoveries are given in Table 1.

Quantification of Individual Procyanidins. Quantification of procyanidins was performed via an external standardization. The standard was obtained from cocoa extracts and contained monomers and procyanidins through hepatmers. Monomers and procyanidin oligomers were identified by UV detection monitoring at 280 nm and by determining the mass-to-charge ratio (*m*/*z*) by negative-mode LC/MS. The areas for the peaks corresponding to molecular ions of each oligomeric class were summed in order to include the contributions from all of the isomers within an oligomeric class. Calibration curves for each oligomer were linear ($0.8 < r^2 < 0.995$) in the range of 0.1−5 mg/mL.

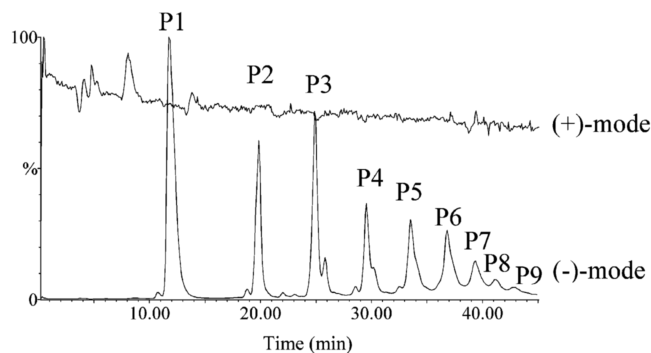

Figure 3. Total ion chromatograms of cocoa procyanidins obtained after postcolumn addition of 10 mM ammonium acetate in both positive and negative electrospray ionization modes. P1–P9 correspond to procyanidin oligomers monomers through nonamers.

Statistical Analysis. HPLC peak areas were obtained in triplicate for each of the three independent measures of the peach or canning syrup. HPLC peak areas were averaged and the standard deviation (SD) was determined from the average of the three measurements.

RESULTS AND DISCUSSION

LC/MS conditions were optimized for the analysis of procyanidins in normal-phase solvents [solvent A, methylene chloride/methanol/water/acetic acid 82:14:2:2 (v/v/v/v); solvent B, methanol/water/acetic acid 96:2:2 (v/v/v)] by use of a composite procyanidin standard extracted from cocoa (*31*). To assist the ionization of procyanidins in normal-phase solvents, a tertiary pump was added in-line for the postcolumn addition of ammonium acetate prior to the ESI interface. Ammonium acetate levels between 0 and 100 mM were evaluated for their ability to enhance ionization monitoring in both positive and negative ESI/MS. The optimal level of ammonium acetate in both modes was determined to be 10 mM (data not shown). Total ion chromatograms (TIC) of the procyanidins extracted from cocoa were obtained in both positive and negative ionization modes with the postcolumn addition of 10 mM ammonium acetate (Figure 3). These chromatograms demonstrate that although the TIC signal is not as intense in negative ESI/MS as compared with positive ESI/MS, it produces ions corresponding to procyanidin oligomers through undecamers without matrix effect. Variation in the flow rate also greatly influenced the separation and ionization of the procyanidin oligomers (Figure 4). Finally, we have determined that the optimal conditions for analyzing procyanidins require a flow rate of 0.03 mL/min along with the postcolumn addition of 10 mM ammonium acetate in negative ESI/MS.

The recovery of individual procyanidin oligomers was determined by use of a previously characterized cocoa standard containing monomers and procyanidin oligomers through heptamers (Table 1). Recoveries of oligomers varied and ranged from 61.42% to 102.39% depending on the degree of polymerization. Greater losses in recoveries were observed with the higher molecular weight oligomers. The procyanidin composition of peaches was determined by monitoring UV at 280 nm and by extracting ions corresponding to each oligomeric group from the TIC and generating reconstructed ion chromatograms (RICs; Figure 5). The RICs were generated by filtering data for *m*/*z* ratios corresponding to singly charged $[M - H]^-$ and

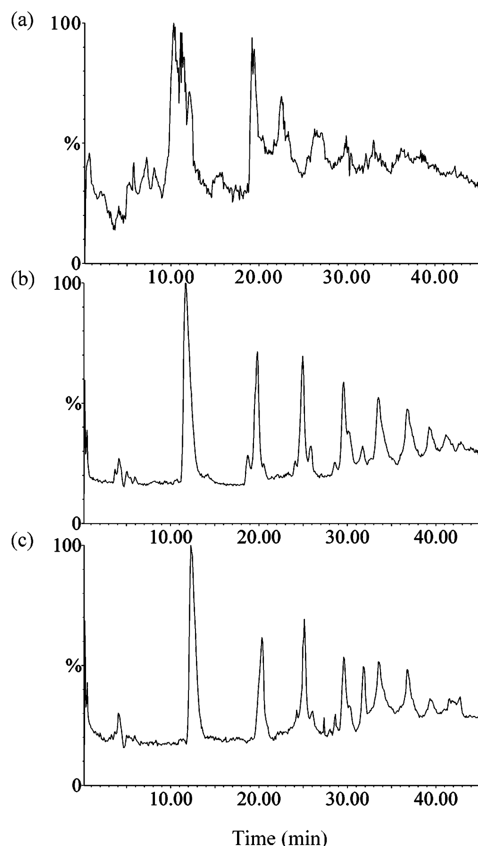

Figure 4. Total ion chromatograms of cocoa procyanidins obtained in negative ESI/MS with postcolumn addition of 10 mM ammonium acetate at flow rates of (a) 0.02 mL/min, (b) 0.03 mL/min, and (c) 0.04 mL/min.

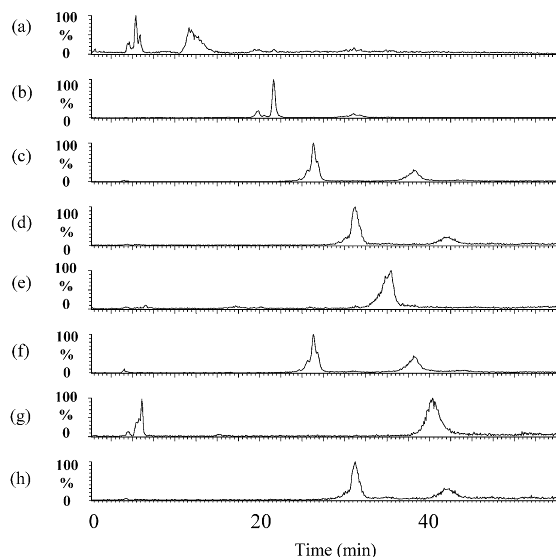

Figure 5. Reconstructed ion chromatograms (RICs) of procyanidin oligomers through octamers in Ross clingstone peaches. RIC were generated by filtering ions corresponding to (a) monomers [*m/z* 289], (b) dimers [*m/z* 577], (c) trimers [*m/z* 865], (d) tetramers [*m/z* 1153], (e) pentamers [*m/z* 1441], (f) hexamers [*m/z* 1729 and double-charged *m/z* 864], (g) heptamers [*m/z* 2017 and double-charged *m/z* 1009], and (h) octamers [*m/z* 2305 and double-charged *m/z* 1152].

multiply charged $[M - nH]^{n-}$ ($n \geq 2$) ions correlating to each procyanidin oligomer. RICs of several oligomers demonstrated peaks that correspond to the multiply charged ions of higher mass oligomers (e.g., Figure 5c,d). RICs demonstrate the presence of procyanidin monomers and oligomers through undecamers in peaches. However, detection limits constrained quantitation to octamers. The detection limit for monomers through pentamers was 0.5 mg/kg wet wt, whereas the detection limit for hexamers through octamers were 1 mg/kg wet wt. Quantification based upon RIC peak areas indicates that the monomer content of frozen peeled peaches is 19.59 mg/kg and that dimers (39.59 mg/kg) and trimers (38.81 mg/kg) constitute the largest percentage composition of oligomers in the peaches. Tetramers through octamers were present in levels of 17.81, 12.43, 10.62, 3.94, and 1.75 mg/kg, respectively. Arts et al. (*32*) reported the total catechin content in peaches as 23.3 mg/kg of fresh edible wt. De Pascual-Teresa et al. (*33*) reported similar levels of monomers (11.8 mg/kg) and higher levels of dimers (45.4 mg/kg) and trimers (28.7 mg/kg) for selected isomers in fresh peeled peaches. In none of these studies were oligomers greater than tetramers quantified. In this study the average amount of total procyanidins in frozen peaches was found to be 91 mg/kg. Tomas-Barberan et al. (*18*) found that the levels of total flavan-3-ols in different varieties of peach ranged from approximately 90 to 700 mg/kg in peeled peaches. These studies indicate that peaches are a significant source of procyanidins as compared with other procyanidin-rich foods such as dark chocolate, 1700 mg/kg; blueberries, 8 mg/kg; cranberries, 17 mg/kg; and apples, 100−400 mg/kg (*16*, *18*, *31*).

Table 2. Content of Procyanidin Oligomers in Frozen and Canned Peaches at 0, 1, 2, and 3 Months of Storage[a]

	frozen peach (mg/kg)[b]	canned peach (mg/kg)[b]			
		0 month	1 month	2 months	3 months
P1	19.59 ± 0.03	17.50 ± 0.02	17.18 ± 0.02	15.77 ± 0.02	15.85 ± 0.01
P2	39.59 ± 0.04	36.50 ± 0.03	34.84 ± 0.02	29.03 ± 0.02	30.55 ± 0.06
P3	38.81 ± 0.01	34.77 ± 0.03	31.33 ± 0.02	19.23 ± 0.02	19.06 ± 0.04
P4	17.81 ± 0.03	16.97 ± 0.01	10.07 ± 0.03	6.26 ± 0.06	3.61 ± 0.06
P5	12.43 ± 0.05	11.85 ± 0.08	7.61 ± 0.08	2.83 ± 0.08	
P6	10.62 ± 0.08	7.87 ± 0.06	3.52 ± 0.06		
P7	3.94 ± 0.09	2.76 ± 0.07			
P8	1.75 ± 0.09				

[a] Results are presented as mean ± SD for triplicates. Maximum relative SDs are 8.2%, 4.1%, 2.8%, 4.4%, and 2.8%, respectively. [b] Based on wet weight.

To date, little is known with the respect to the fate of procyanidins during thermal processing or canned storage of peaches. To address this issue, we monitored levels of procyanidins in canned peaches and the syrup used in the canning over a 3 month period (Table 2). Thermal processing resulted in an 11% reduction in monomers, a 9% reduction in dimers, a 12% reduction in trimers, a 6% reduction in tetramers, and a 5% reduction in pentamers. Hexamers and heptamers demonstrated an approximate 30% loss, and octamers were no longer detected. The impact of canned storage on peach procyanidins revealed that there is a time-related loss in higher oligomers, and that by 3 months oligomers larger than tetramers are not observed. At 3 months postcanning, levels of monomers had decreased by an additional 10%, dimers by 16%, trimers by 45%, and tetramers by 80%. The levels of total phenolics were recently measured in Ross clingstone peaches canned at 220 °F for 10 min, under the same conditions used in this study (*34*). These results demonstrate that thermal processing results in a 21% loss in total phenolics and that a 30−43% loss in total phenolics occurred during the first 3 months of storage at room temperature after canning. It appears that some of these losses can be attributed to a loss in procyanidins. Arts et al. (*32*) showed that a 19.7% loss in catechin (23.3 mg/kg in fresh, 18.7 mg/kg in canned peach) occurred in canned peaches purchased at market. Additional studies on various fruits demonstrate similar trends. For example, Skrede et al. (*35*) found

Table 3. Content of Procyanidin Oligomers in Canning Syrup at 0, 1, 2 and 3 Months of Canned Storage[a]

	syrup (mg/kg)[b]			
	0 month	1 month	2 months	3 months
P1	1.24 ± 0.05	1.24 ± 0.00	1.15 ± 0.00	1.16 ± 0.00
P2	5.65 ± 0.03	5.60 ± 0.01	3.67 ± 0.01	4.10 ± 0.01
P3	7.27 ± 0.01	6.63 ± 0.02	4.11 ± 0.03	3.73 ± 0.08
P4	3.11 ± 0.01	2.55 ± 0.01	1.26 ± 0.04	
P5	2.11 ± 0.02	0.79 ± 0.02		
P6	1.58 ± 0.08			

[a] Results are presented as mean ± SD for triplicates. Maximum relative SDs are 7.7%, 3.8%, 5.2% and 3.4%, respectively. [b] Based on wet weight.

Table 4. Total Procyanidin Content in Frozen and Canned Peaches and in 324.8 g of Canning Syrup at 0 Month of Storage

	total procyanidin content (mg)			
	frozen peach (538.7 g; 19 oz)	canned peach (538.7 g; 19 oz)	canning syrup (324.8 g; 11.5 oz)	canned peach total (863.5 g)
P1	10.55	9.43	0.40	9.83
P2	21.33	19.66	1.84	21.50
P3	20.91	18.73	2.36	21.10
P4	9.59	9.14	1.01	10.15
P5	6.70	6.39	0.68	7.07
P6	5.72	4.24	0.51	4.75
P7	2.12	1.49		1.49
P8	0.94			

that a 20% loss in procyanidins occurred in blueberry after juice processing, and Spanos et al. (*36*) demonstrated that 9 months of storage, after apple juice processing, resulted in a total loss of procyanidins. To determine if the losses we observed could result from a redistribution of procyanidins into the canning syrup, we monitored levels of procyanidins in the canning syrup during the 3 month storage time (Table 3). Analysis of the syrup directly after canning demonstrated that it contained monomers through hexamers, in ratios that were reflective of the composition in canned peaches. In addition, a time-related loss in higher oligomers was found, and indicates that by 3 months oligomers larger than trimers are not observed. Levels of total procyanidins were calculated on a per-can basis (each can contained 538.7 g of peaches and 324.8 g of syrup) and compared to the content calculated for 538.7 g of frozen peaches (Table 4). This comparison indicates that the migration of procyanidins into the canning syrup can account for the losses observed during the thermal processing step. Future work is underway to identify processing methods that will maintain levels of procyanidin oligomers in peaches during longer storage periods.

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