Effect of Processing and Storage on the Antioxidant Ellagic Acid Derivatives and Flavonoids of Red Raspberry (*Rubus idaeus*) Jams

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From red raspberries, ellagic acid, its 4-arabinoside, its 4′ (4″-acetyl) arabinoside, and its 4′ (4″-acetyl)xyloside, as well as quercetin and kaempferol 3-glucosides, were identified. In addition, two unidentified ellagic acid derivatives were detected. The free radical scavenging activity of the ellagic acid derivatives was evaluated by using the DPPH method and compared to that of Trolox. All of the isolated compounds showed antioxidant activity. The effect of processing to obtain jams on raspberry phenolics was evaluated. The flavonol content decreased slightly with processing and more markedly during storage of the jams. The ellagic acid derivatives, with the exception of ellagic acid itself, remained quite stable with processing and during 6 months of jam storage. The content of free ellagic acid increased 3-fold during the storage period. The initial content (10 mg/kg of fresh weight of raspberries) increased 2-fold with processing, and it continued increasing up to 35 mg/kg after 1 month of storage of the jam. Then a slight decrease was observed until 6 months of storage had elapsed. The increase observed in ellagic acid could be explained by a release of ellagic acid from ellagitannins with the thermal treatment.

Keywords: *Raspberry; Rubus idaeus; jams; processing; phenolics; antioxidant activity; ellagic acid; flavonols*

INTRODUCTION

Raspberries are a very rich source of phenolic compounds (*1*). The presence of anthocyanins (*2*), flavonols (*3*, *4*), and ellagic acid derivatives (*5*, *6*) has been previously reported. Ellagic acid is present in raspberries in three different forms: as ellagitannins, in which hexahydroxydiphenic acid forms esters with a sugar (the main one in terms of content); as free ellagic acid; and as ellagic acid glycosides (*6*−*8*). The phenolic compositions of different raspberry cultivars have been studied (*7*, *8*) as well as the effect of processing to obtain raspberry juice (*2*, *4*, *6*) and jam (*9*). Processing and storage can have marked effects on the phenolic content of fruits that might affect their health-promoting properties. Thus, industrial processing of pomegranates to obtain juices increases their antioxidant capacity and phenolics content (*10*), whereas strawberry processing to produce jams decreased the total ellagic acid content by 20% (*11*) and the flavonols by 15−20% (*12*). It has also been reported that cold storage of berries increases their anthocyanin content as well as their antioxidant activity (*13*).

In the past few years there has been an increased interest in the study of berry phenolics, due to their antioxidant activity and health-promoting properties that make consumption of these soft fruits and derived processed products a very healthy habit (*13*). The bioactivity of these compounds depends on the release of the compounds from the food matrix during the digestion process and their absorption and metabolism. In the bioavailability and solubility of these compounds, glycosylation has an important effect and, therefore, analysis of the naturally occurring phenolic glycosides in food is essential to evaluate their potential physiological properties (*14*).

We have reported previously the effect of processing to obtain raspberry jam on the color and anthocyanin content of raspberries (*9*).

The aim of the present work is to study the noncolored antioxidant phenolic compounds from raspberry and the effect of processing to obtain jams on these compounds.

MATERIALS AND METHODS

Raspberries and Jam Processing. Fruits of cultivar Heritage produced in Nerpio (Albacete, Spain) were harvested at processing maturity and frozen at −20 °C. Fruits were stored at this temperature until processing (<3 months). Jam was made under the specific conditions of the industry (*9*) to obtain a final product that contains 450 g of fruit kg^{-1}, 2 g of pectin kg^{-1}, 0.4 g of citric acid kg^{-1}, and a final sucrose concentration up to 63 °Brix. Fruit and sugar were mixed with pectin and citric acid, processed for 15 min at 78 °C under vacuum (500 mmHg), heated at 92 °C, and allowed to cool to 88 °C before glass jars (250 g) were filled. Glass jars were closed and cooled gradually with water and kept in the dark at 20 °C for 6 months.

Extraction and HPLC Analysis of Phenolics. Fruit or jam (containing 50% fruit) samples (50 g) were extracted by homogenization with 200 mL of methanol in an Ultra-Turrax T-25 (Jankel & Kunkel, IKA Labortechnik) at 9500 rpm for 1 min. The homogenate was filtered through two layers of cheesecloth. Fifty milliliters of the filtrate was added to 15 mL of distilled water, and the methanol was removed under reduced pressure at 35 °C. The remaining aqueous solution was filtered through a solid phase extraction cartridge (Sep-Pak C18, Waters Associates, Milford, MA) previously activated with methanol. Phenolics were retained in the cartridge, and this was washed with 20 mL of distilled water. Phenolic compounds were then eluted with 10 mL of methanol. The

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Table 1. UV Analysis of the Isolated Ellagic Acid Derivatives (*15*)[a]

	MeOH	+ NaOMe	+ $AlCl_3$	+ $AlCl_3$ + HCl
1	359, 344 sh, 303 sh, 255	370 sh, 353, 283, 257, 243 sh	363, 312, 268, 236	361, 312 sh, 255 sh, 233 sh
2	366, 349 sh, 306 sh, 255	371 sh, 355, 279, 255, 246 sh	364, 316 sh, 272, 236	365, 312 sh, 255 sh, 236
3	360, 248 sh, 305 sh, 269 sh	370 sh, 348, 284, 256, 243 sh	363, 316 sh, 272, 236 sh	361, 312 sh, 255 sh, 233 sh
4	356, 342 sh, 307 sh, 255	370 sh, 350, 282, 258, 245 sh	362, 314 sh, 266, 235	360, 310 sh, 250 sh, 236 sh
5	357, 324 sh, 307 sh, 256	370 sh, 349, 282, 258, 244 sh	363, 313 sh, 266, 236	360, 310 sh, 255 sh, 236 sh
6	355, 337 sh, 306 sh, 256, 248 sh	350, 279, 256, 248 sh	360, 313, 274 sh, 233	360, 313 sh, 275 sh, 236 sh

[a] Values are λ_{max} (nm). sh, shoulder.

methanol extract was then taken to dryness under reduced pressure (35 °C) and redissolved in 1 mL of methanol. This methanol extract was filtered through 0.45 μm (type Millex HV13, Millipore Corp., Bedford, MA) before HPLC analysis. Twenty microliters of every sample was injected for HPLC analysis on equipment using a Merck-Hitachi pump L-6200 (Merck, Darmstadt, Germany) and a diode array detector Shimadzu SPD-M6A (Shimadzu, Kyoto, Japan), using a reversed-phase column Lichrocart 100 RP-18 (Merck) (25 × 0.4 cm, particle size = 5 μm). Elution was performed with water/5% formic acid (solvent A) and methanol (solvent B), with a solvent flow rate of 1 mL min^{-1}, using a gradient that started with 10% B in A to reach 15% B at 5 min, 30% B at 20 min, 50% at 35 min, and 90% at 38 min. The different phenolics were identified by their UV spectra recorded with a diode array detector and by chromatographic comparisons with authentic markers: ellagic acid (Sigma, St. Louis), quercetin 3-glucoside, and kaempferol 3-glucoside (previously isolated and identified in our laboratory). Five unidentified ellagic acid derivatives were isolated and their structures studied (see below). Flavonols were quantified as quercetin 3-glucoside at 360 nm and ellagic acid derivatives as ellagic acid at 255 nm. All of the analyses were replicated (n = 4) and expressed as mean values. The reproducibility of the HPLC analysis was 6%.

Isolation of Ellagic Acid Derivatives. Frozen raspberries (800 g) were homogenized with distilled water (2 L) using an Ultra-Turrax (T25) equipment at 9500 rpm. The homogenate was centrifuged on a Sorvall SS-3 centrifuge at 8000 rpm (11000*g*). The supernatant was filtered through filter paper and the filtrate diluted with distilled water to 3 L. This was mixed with 200 g of nonionic polymeric resin Amberlite XAD-2 (Fluka Chemie; pore size = 9 nm; particle size = 0.3−1.2 mm) and stirred overnight at room temperature to allow phenolic compounds to be adsorbed on the resin particles. The resin was then packed on a column (50 × 4 cm) and washed with distilled water (5 L) to wash sugars and other polar compounds. The phenolic fraction was then eluted with methanol (500 mL). The methanol extract was concentrated to 10 mL and fractionated by column chromatography on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) (40 × 3 cm) using methanol as mobile phase. After the elution of brown compounds, anthocyanins, some UV-absorbing fractions containing the ellagic acid derivatives were visualized under UV light (360 nm) and separated. The nature and composition of the different fractions were controlled by HPLC under the analytical conditions described above. The fractions containing the ellagic acid derivatives were separated by semipreparative HPLC using a reversed-phase column ODS-2 (Teknokroma, Barcelona, Spain) (25 × 0.7 cm; 5 μm particle size) with a solvent flow rate of 2 mL/min^{-1}. Different mixtures of methanol/water were used for the isolation of individual compounds. In all cases purification started with 30% methanol; the methanol percentage was increased gradually until the different compounds eluted. The purity of the different isolated compounds was evaluated by analytical HPLC. The isolated compounds were freeze-dried and stored at −20 °C for further studies.

Isolated Compound Identification. UV spectrophotometric analyses were achieved as reported previously (*15*) in methanol and after the addition of shift reagents NaOMe, $AlCl_3$, and $AlCl_3$ plus HCl on a Shimadzu UV-1603 spectrophotometer. ^{1}H NMR spectra were recorded with a Varian 300 MHz instrument in DMSO-d_6 with TMS as internal standard. FAB-MS was recorded in the negative form using *m*-nitrobenzyl alcohol as matrix in a an Autospec 5000 VG spectrometer. Acid hydrolysis (2 N HCl, 90 °C, 2 h) was performed to identify sugars (glucose, arabinose, and xylose by TLC on silica gel with authentic markers using as mobile phase ethyl acetate/methanol/acetic acid/water, 60:15:15:10), and aglycons were identified by HPLC (same method as reported above).

Evaluation of Free Radical Scavenging Activity of the Isolated Derivatives. The free radical scavenging activity of the isolated ellagic acid derivatives was evaluated after solution of the isolated compounds in methanol by the 2,2-diphenyl-1-picrylhydrazyl) (DPPH) (Sigma, St. Louis, MO) method (*16*). The activities of the different isolated compounds were compared to that of trolox (6-hydroxy-2,5,7,8-tetramethylcarboxylic acid) (Sigma).

RESULTS AND DISCUSSION

HPLC Analysis of Raspberry Phenolic Compounds. The HPLC analysis of raspberry (and raspberry jam) phenolic compounds shows that six compounds (**1**−**6**) with UV spectra of ellagic acid derivatives were detected (Table 1). Compound **2** was identified as ellagic acid by chromatographic comparisons with an authentic marker. The other five ellagic acid derivatives showed similar UV spectra recorded with the diode array detector. The occurrence of different ellagic acid derivatives in raspberries has previously been reported (*4*, *6*), although their structures were not established. These authors suggested that these compounds could be methylated, methoxylated, or glycosylated derivatives of ellagic acid. In addition, quercetin 3-glucoside and kaempferol 3-glucoside have also been detected.

Isolation and Identification of Ellagic Acid Derivatives. The five unknown ellagic acid derivatives were isolated in sufficient amount to study their structures. The UV spectra of the different isolated compounds in methanol were very similar but not identical, suggesting some differences in the substitution of the phenolic hydroxyls of the ellagic acid nucleus (Table 1). After the addition of alkaline and metal reagents (*15*), shifts of the UV maxima are induced, and these shifts could be correlated with some structural characteristics. The UV study indicates that all of the isolated compounds had at least one *o*-dihydroxyl grouping in the molecule, as they allow the formation of complexes with aluminum (bathochromic shift from 255 to 266−272 nm after the addition of aluminum chloride) and complexes that are destroyed after the addition of hydrochloric acid (hipsochromic shift from 266−272 to 255 nm). This sensitivity of the aluminum complexes to acid conditions indicated that there are no carbonyl groups vicinal to free phenolic hydroxyls, as the aluminum complexes formed between carbonyl and hydroxyl groups remain stable after the addition of hydrochloric acid (*15*).

Compound **1** showed a UV spectrum with the characteristic shape of an ellagic acid derivative but differed from that of ellagic acid in which the maximum of the band at larger wavelength was at 359 nm while that of ellagic acid was at 366 nm. This 7 nm difference could

indicate the substitution of one of the hydroxyls of the ellagic acid nucleus, as is well documented in the case of flavonoids (*17*). Compound **1** eluted in HPLC on reversed-phase columns with a retention time slightly shorter than that of ellagic acid, showing that this compound was more polar. After acid hydrolysis, TLC and HPLC demonstrated the occurrence of arabinose and ellagic acid, respectively. The FABMS analysis (negative form) showed a molecular ion at *m/z* 433 (39% relative intensity) consistent with a pentosyl ellagic acid (molecular weight = 434) and a fragment at *m/z* 301 (25%) corresponding to ellagic acid after the loss of 132 mass units (pentose). The ^{1}H NMR analysis showed the presence of singlets at 7.69 and 7.45 ppm, corresponding to the two aromatic protons of the ellagic acid nucleus (H5 and H5′), and a broad singlet at 5.63 ppm, corresponding to the anomeric proton of arabinose. In addition, different signals between 4.10 and 3.27 ppm were observed corresponding to the remaining protons of the arabinose. The ^{1}H NMR analysis of ellagic acid under the same conditions showed one singlet at 7.46 ppm with an integration of two protons corresponding to protons H5 and H5′ of the ellagic acid nucleus. In this case, only one singlet is observed, as this is a symmetric molecule. These results confirm the asymmetric nature of compound **1** as arabinose is linked to one of the phenolic hydroxyls of the ellagic acid nucleus. This linkage would be responsible for the downfield shift of the response of H5 (from 7.45 to 7.69 ppm) and would suggest that the arabinose residue should be linked to the hydroxyl at the 4-position of ellagic acid. This position would be the most favored as only 4-glycosides of ellagic acid have been reported in nature so far. In addition, the coupling constant H1−H2 in the arabinose residue, which is <1 Hz, indicates that this is an α-L-arabinofuranoside (*18*). Thus, compound **1** has been identified as ellagic acid 4-α-L-arabinofuranoside. Compound **2** coincided chromatographically with an ellagic acid standard and was identified as this compound.

Compound **4** showed a UV spectrum similar to those of the other ellagic acid derivatives (Table 1). After acid hydrolysis, xylose and ellagic acid were detected. After acid treatment in mild conditions (4 N HCl, room temperature, 60 h), the original compound (HPLC retention time of 24.7 min) was transformed into two compounds: ellagic acid (t_R = 19.7 min) and another ellagic acid derivative eluting at a shorter t_R (18.8 min). This hydrolysis product showed chromatographic behavior similar to that of compound **1**, suggesting that this could be another ellagic acid pentoside. The FABMS spectrum (negative) showed a molecular ion at *m/z* 475 (M − H) and significant fragments at *m/z* 433 (M − acetyl) and *m/z* 301 (M − acetyl − pentose), coincident with ellagic acid, suggesting that this compound was an acetyl derivative of an ellagic acid xyloside. The absence of a fragment M − 132 (M − pentose) indicates that the acetyl must be linked to one of the hydroxyls of the xylose residue. The ^{1}H NMR analysis clearly shows the asymmetric nature of **4**, as proton H5 is shifted downfield (singlet at 7.64 ppm) for the substitution of the hydroxyl at the 4-position (4-xylosyl), whereas H-5′ is similar to those protons of ellagic acid (singlet at 7.44 ppm). This spectrum shows a doublet at 5.02 ppm (J_{1-2} = 3.7 Hz) corresponding to the anomeric proton of xylose, and a triplet at 4.87 ppm corresponding to the CH of the xylose, which is linked to the hydroxyl where the acetyl residue is located and is shifted downfield by this reason. This triplet indicates that this CH is coupled to the CH_2 in position 5 of xylose, suggesting that the acetylation is located in position 4 of the xylose. The anomeric protons of xyloses in flavonoid glycosides range between 4.8 and 5.1 ppm in the ^{1}H NMR spectra in DMSO-d_6, and acetylation of the hydroxyl in position 2 of xylose induces a downfield shift of 0.4 ppm in the anomeric proton (*18*). As this shift is not observed in compound **4**, the acylation in position 2 is not possible. In addition, the spectrum shows signals for the other protons of xylose between 4.3 and 3.2 ppm. In addition, a singlet integrating three protons at 2.07 ppm corresponds to the methyl group of the acetyl residue is also observed. The chemical shift and the coupling constant of 1−2 of the xylose anomeric proton indicate that this is an α-D-xylopyranose (*18*). Compound **4** is therefore identified as ellagic acid 4-(4″-acetyl)-α-D-xylopyranoside.

Table 2. Antioxidant Activity of Raspberry Ellagic Acid Derivatives

compound (1 mM)	trolox equiv (mM)
ellagic acid (**2**)	2.6
4-arabinosylellagic acid (**1**)	1.1
unknown ellagic acid derivative (**3**)	4.3
4-acetylxylosylellagic acid (**4**)	1.4
4-acetylarabinosylellagic acid (**5**)	1.6
unknown ellagic acid derivative (**6**)	0.5
gallic acid	2.5
caffeic acid	2.2
ferulic acid	1.3
catechin	2.5
epicatechin	2.7
protocatechuic acid	2.1
quercetin	3.3
kaempferol	2.7

Compound **5** showed a UV spectrum similar to those of the other ellagic acid derivatives. After acid hydrolysis, arabinose and ellagic acid were detected. The FABMS spectrum (negative form) showed the same molecular ion and fragments as compound **4**, suggesting that this was a similar compound in which xylose had been replaced by arabinose.

In addition, two other ellagic acid related compounds (**3** and **6**) were detected, although their structures were not fully identified. They had UV spectra similar to that of ellagic acid (Table 1), and their MS and NMR analyses showed that they were polymers of ellagic acid, without sugars in their molecules (data not shown). Their structures are not completely established with the present work, and further studies are needed to complete their structural characterization. Their molecular weights were determined with the FABMS analysis and showed that compound **3** had a molecular weight of 922 *m/z* (corresponding to an ellagic acid trimer plus 16 mass units) and compound **6** a molecular weight of 852 *m/z*.

Free Radical Scavenging Activity of the Isolated Compounds. The ability to neutralize the free radical DPPH by the different ellagic acid derivatives isolated was evaluated and compared to that of trolox, and the results are shown in Table 2. Ellagic acid showed the highest antioxidant activity of the isolated ellagic acid derivatives, as could be expected it being the derivative with two dihydroxyl groupings, whereas the other derivatives (glycosides) had only one dihydroxyl grouping, and their antioxidant activity was divided by two. When these data are compared with those of other food phenolic constituents, the activity of ellagic acid was

Table 3. Effect of Processing on the Content of Raspberry Phenolics[a]

compound	raspberry	raspberry jam
ellagic acid arabinoside (**1**)	22.75 (3.21)	22.40 (1.44)
ellagic acid (**2**)	10.13 (1.10)	25.40 (5.33)
ellagic acid acetylxyloside (**4**)	3.62 (0.71)	2.93 (0.66)
ellagic acid acetylarabinoside (**5**)	2.02 (0.25)	1.85 (0.19)
unknown (**3**)	9.15 (1.32)	10.60 (2.65)
unknown (**6**)	1.10 (0.10)	1.64 (0.36)
quercetin glucoside	70.28 (4.61)	66.13 (4.98)
kaempferol glucoside	10.32 (1.10)	8.28 (0.99)

[a] Values are mg/kg of fruit fresh weight. Values in parentheses are standard deviations ($n = 4$).

similar to those of catechin and kaempferol but was smaller than that of quercetin (Table 2). The ellagic acid glycosides had a smaller antioxidant activity that was similar to that of ferulic acid. These results show that raspberry ellagic acid derivatives have an in vitro antioxidant activity which is similar to that of the well-known antioxidant food phenolics. The polymeric derivative **3** showed an antioxidant capacity that was double that of ellagic acid, as could be expected for a compound with a larger number of phenolic hydroxyls per molecule. On the contrary, compound **6**, which seems to be more lipophilic and to have fewer free hydroxyls, shows a smaller activity.

Effect of Processing and Storage on the Phenolic Content of Jams. The effect of processing to obtain jams on raspberry phenolics shows that ellagic acid glycosides and flavonol glycosides are not much affected by thermal processing, under the specific conditions used by the industries (addition of sugar and pectin and cooking). However, the content of free ellagic acid increases 2.5-fold after processing (Table 3). This is evident in the HPLC chromatogram of the phenolic compounds present in raspberries and raspberry jams (Figure 1), in which the peak corresponding to ellagic acid is clearly increased with respect to the other phenolics in the chromatogram of raspberry jam. When the individual phenolics were evaluated in the produced jams during 6 months of storage at a room temperature of 20 °C and in the dark, ellagic acid continued to increase during the first month of storage up to 45 mg/kg of raspberry and then to decrease slightly over the rest of the storage period, but never reaching values <20−25 mg/kg. A similar trend was observed in the unidentified ellagic acid derivative **3**, which also increased its content although not at the same levels as observed for ellagic acid (Figure 2). The ellagic acid glycosides did not show much variation in content with storage. In the case of flavonoid derivatives, the quercetin glucoside content decreased significantly during the first 3 months of storage to reach levels of ∼40 mg/kg of fresh weight and then became quite stable during the rest of the storage period (Figure 2).

The increase in free ellagic acid observed during jam cooking can be related to a release of hexahydroxydiphenic acid from ellagitannins, which is transformed to ellagic acid, or to an easier extractability of this compound from processed products due to degradation of the cell structures.

Häkkinen et al. (*12*) reported that red raspberries contained 9.5 mg/kg of fresh weight of flavonols (expressed as aglycons). The content found in the present work, expressed as glycosides, is considerably higher (70 mg/kg of fresh weight of quercetin 3-glucoside). In the case of jam processing, these authors found that during

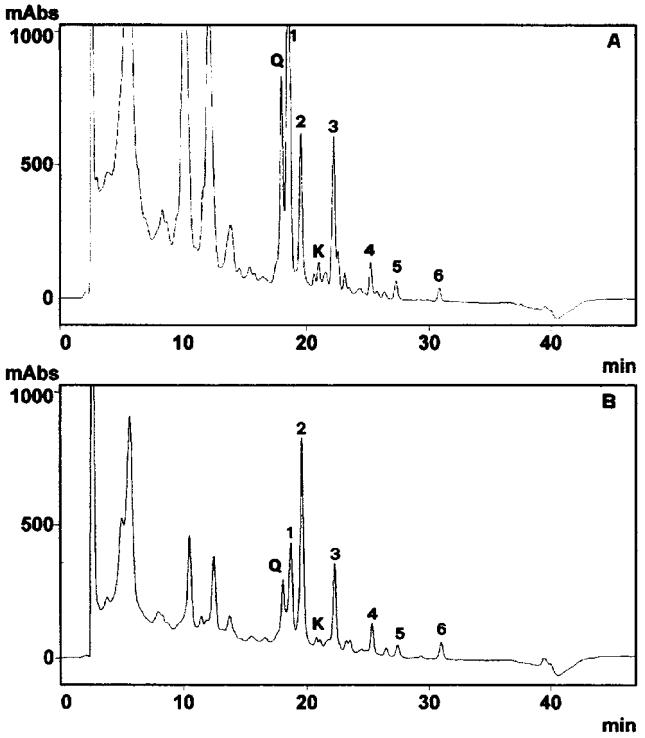

Figure 1. HPLC chromatograms of raspberry phenolics (255 nm): (A) raspberry fruit (50 g); (B) raspberry jam (50 g of jam equivalent to 25 g of fruit). Peaks: (Q) quercetin 3-glucoside; (K) kaempferol 3-glucoside; (**1**) ellagic acid arabinoside; (**2**) ellagic acid; (**3**) unidentified ellagic acid derivative; (**4**) ellagic acid acetylxyloside; (**5**) ellagic acid acetylarabinoside; (**6**) unidentified ellagic acid derivative.

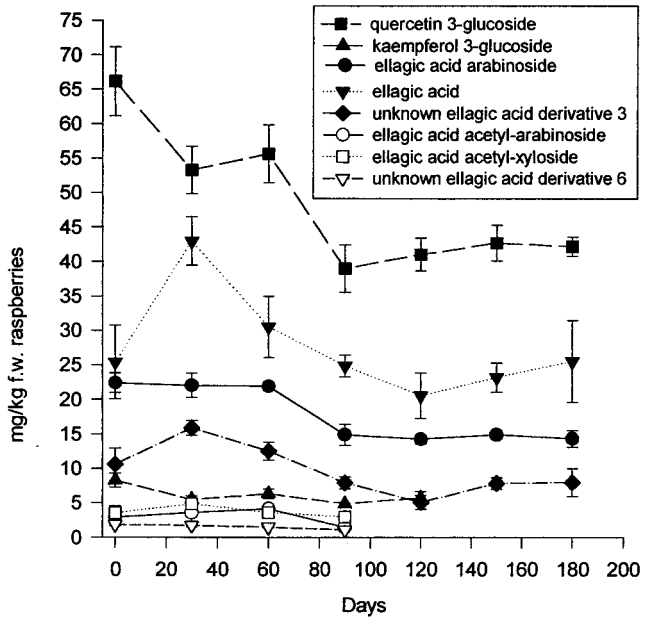

Figure 2. Changes in raspberry jam phenolics during storage over 6 months at 20 °C in the dark.

manufacturing of strawberry jam a 15−20% loss of flavonols is observed (*12*). In the present work raspberry jam processing produced a 6% loss in quercetin 3-glucoside, whereas a 20% loss was observed in the case of kaempferol 3-glucoside. This higher sensitivity of kaempferol to thermal processing coincided with that reported by Häkkinen et al. in the case of strawberry jam flavonols (*12*). During 6 months of storage after jam manufacturing, the loss of quercetin 3-glucoside reached

40%, and in the case of kaempferol 3-glucoside, 50% (Figure 2).

Concerning ellagic acid derivatives we have observed that processing and storage produce an increase in free ellagic acid of raspberries, whereas ellagic acid glycosides are not affected. This increase has already been recently reported in raspberry jams (*19*), although the increase observed in this case was smaller than that observed in the present work. When total ellagic acid derivatives were evaluated after acid hydrolysis, processing led to a significant decrease in ellagic acid, as was reported in strawberry (*11*) and raspberry (*19*). In the case of strawberry jam, values of 238 mg/kg of fresh weight of total ellagic acid (free and bound) were reported (*11*) as well as a 20% decrease in the ellagic acid content of jam compared to that of unprocessed strawberries. The decrease reported in the case of raspberries was 16% (*19*).

These results show that although a decrease in total ellagic acid has been reported with jam processing, an increase in free ellagic acid is also produced, and this could affect the biovailability of these compounds. Ellagic acid monomers are probably better absorbed than high molecular weight ellagitannins, and therefore jam processing could increase ellagic acid bioavailability (*20*). Research on ellagic acid bioavaliability will clarify this point.

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