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Determination of amygdalin in apple seeds, fresh apples and the processed apple juices

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Abstract

Cyanogenic glycosides are natural plant toxicants. Action by endogenous plant enzymes can release hydrogen cyanide causing potential toxicity issues for animals including humans. We have quantified amygdalin in seeds from different apple varieties, determined the effects of processing on the amygdalin content of apple juice and quantified amygdalin in commercially-available apple juices. Amygdalin contents of seeds from fifteen varieties of apples ranged from 1 mg g^{-1} to 4 mg g^{-1}. The amygdalin content of commercially-available apple juice was low, ranging from 0.01 to 0.04 mg ml^{-1} for pressed apple juice and 0.001 to 0.007 mg ml^{-1} for long-life apple juice. Processing led to juice with low amygdalin content, ranging from 0.01 mg ml^{-1} to 0.08 mg ml^{-1}. The results presented show that the amygdalin contents of commercially-available apple juices are unlikely to present health problems to consumers.

Keywords: Cyanogenic glycosides, amygdalin, apples, apple juice, pasteurization.

Introduction

Apple (*Malus domestica*) is a member of the *Rosaceae* family that also includes apricots, cherry, peaches, pear and plum and is the most widely consumed fruit in the UK. World apple production in 2011/12 was estimated to be 65.23 million metric tons, out of which 12.2 million metric tons was used for the production of apple juice (Negro & Lojo, 2011). Although apple contains compounds which may confer significant health benefits to humans, apple seeds contain amygdalin (Fig.1), a potentially toxigenic compound.

Commercial apple juice is usually made from a blend of apples to produce an acceptable juice in terms of flavour. Apples are soaked in water to remove soil and other foreign material. The cleaned apples are then inspected, and damaged or decayed fruit should be removed to avoid patulin contamination from the final product. The sorted apples are ground into mash or pulp for extraction, crushing or cutting the apples to appropriate consistency. The mashed apples are pressed by applying pressure to obtain the juice. In some cases, enzymatic mash treatment is used to improve the pressability of the mash and increase juice yield, achieved by adding a pectinase enzyme such as pectinol specifically developed for apple mash pre-treatment. The enzyme acts mainly by breaking down the structure of the cell wall, thus freeing the juice.

Cyanogenic glycosides, including amygdalin, are naturally-occurring plant toxins. They are widely distributed in the plant kingdom, being present in more than 2500 species (Ganjewala, Kumar, Asha, & Ambika, 2010). Cyanogenic glycosides are stored in vacuoles within plant cells. When tissues are disrupted, for example by crushing, the cyanogenic glycosides come into contact with endogeneous enzymes (β-glucosidases and α-

hydroxynitrile lyases) resulting in the release of hydrogen cyanide (Zagrobelny, Bak, Rasmussen, Jørgensen, Naumann, & Møller, 2004). In plants, consequently, cyanogenic glycosides serve as important chemical defence compounds against herbivores (Zagrobelny *et al*., 2004; Ganjewala *et al*., 2010). In humans, consumption of cyanogenic plants can cause sub-acute cyanide poisoning with symptoms including anxiety, headache, dizziness and confusion. Acute poisoning results in decreased consciousness, hypotension, paralysis, coma and even death. Acute cyanide poisoning has been reported from the ingestion of apricot kernels (Sahin, 2011), almonds (Sanchez-Verlaan, 2011) and cassava (Akintonwa & Tunwashe, 1992).

Cyanogenic glycosides are present in economically important food plants such as apple, almond, various beans, cereals, cassava, taro and sorghum (Jones, 1998; Donald, 2009). Processing techniques such as pounding, crushing, grinding, soaking, fermentation, boiling and drying have been used over the years to reduce the cyanide contents of foods. Processing allows contact between cyanogenic glycosides and endogeneous enzymes which results in the hydrolytic breakdown of cyanogenic glycosides to hydrogen cyanide. Because the boiling point of hydrogen cyanide is 26 ^{o}C, it easily volatilizes during food processing (Montagnac, Davis, & Tanumihardjo, 2009). Quantification of cyanogenic glycosides in plants is carried out either indirectly (by determining the amount of hydrogen cyanide released after hydrolysis) or directly (by determining the intact form). The indirect method is the most commonly used analytical method and usually involves enzymatic hydrolysis followed by colorimetric determination of total cyanide (Bradbury, Egan, and Lynch, 1991; Santamour, 1998). Methods for determination of intact cyanogenic glycosides include liquid chromatography with refractive index detection (Sornyotha, Kyu, &

73 Ratanakhanokchai, 2007), gas chromatography/mass spectrometry (Chassagne, Crouzet,
74 Bayonove, & Baumes, 1996), and HPLC with UV detection (Bolarinwa, Orfila & Morgan,
75 2014).

76 Although humans, generally, do not consume apple seeds, apple juice is generally
77 produced from whole apples including the seeds. Apple seeds disintegrate during juice
78 production and contaminate the juice. While there are extensive studies on the antioxidant
79 composition of apple juice (Spanos, Wrolstad, & Heatherbell, 1990; Miller, Diplock, & Rice-
80 Evans, 1995), microbial safety and preservation of apple juice (Evrendilek, Jin, Ruhlman,
81 Qui, Zhang, & Richter, 2000), there has been no study on the amygdalin content of apple
82 juice, and there is limited information on the amygdalin contents of apple seeds
83 (Holzbecher, Moss, & Ellenberger, 1984; Haque & Bradbury, 2002).

84

85 ***2. Materials and Methods***

86 *2.1. Reagents and standards*

87 Amygdalin, ethanol, diethyl ether, and HPLC-grade methanol were all purchased
88 from Sigma-Aldrich (Dorset, UK). Water was prepared using a Millipore Milli-Q purification
89 system. All other reagents were of analytical grade.

90 *2.2. Apples*

91 Fifteen varieties of apples (Braeburn, Bramley, Cox, Elstar, Empire, Egremont
92 Russet, Fuji, Golden Delicious, Granny Smith, Jazz, Pink Lady, Red Delicious, Royal Gala,
93 Rubens and Spartan) were purchased from local supermarkets in Leeds (UK). The apples
94 were stored at 4 ^{o}C immediately after purchase prior to processing.

95 *2.2.1. Extraction of amygdalin from apple seeds*

96 Apples were each cut into four equal parts and apple seeds were separated from
97 other tissues with a knife and extracted immediately. Apple seeds (2 g) were disintegrated
98 with a mortar and pestle, and 1 g was weighed into a round-bottom flask (500 ml). Ethanol
99 (50 ml) was added, and the mixture was boiled under reflux for 100 min. The extract was
100 filtered (Whatman No. 1 filter paper) and transferred into plastic polypropylene tubes (50
101 ml). Ethanol was completely evaporated from the filtrate with a rotary evaporator (low BP,
102 35 ^{o}C, 7 mbar). Diethyl ether (10 ml) was added to the dried sample and the mixture was
103 vortexed (1 min) at room temperature (20^{o} ± 2 ^{o}C) to precipitate amygdalin. The diethyl
104 ether was allowed to evaporate overnight and the extracted amygdalin was dissolved in
105 water (5 ml) and prepared for HPLC analysis (2.5).

106
107 *2.2.2. Apple juice extraction*

108 *2.2.2.1. Apple juice from whole apple*

109 Four samples of apple juice were produced from four apple cultivars (Braeburn,
110 Egremont Russet, Golden Delicious and Royal Gala). Whole apples (10) were each
111 washed, cut into 4 pieces and pressed in a commercial juice extractor (Kenwood JE 600).
112 The extracted juice from each apple variety was divided into portions, each of which was
113 subjected to different processing conditions. Apple juice was also produced from either the
114 flesh with skin or the core of the four apple varieties. The flesh with skin and core of the
115 apples were separated with a knife prior to juice extraction. The juice was stored at −20 ^{o}C
116 until extraction.

117
118 *2.2.2.2. Processing of apple juice and determination of amygdalin content*

119 Apple juice was divided into 20 ml portions and treated as follows, (i) extracted for
120 determination of amygdalin content immediately, (ii) boiled immediately, frozen, thawed and
121 then extracted for determination of amygdalin content, (iii) frozen immediately, thawed then
122 extracted for determination of amygdalin content, (iv) held at room temperature (20 ± 2 ^{o}C)
123 for 10, 30, 60 or 120 min then frozen, thawed and extracted for determination of amygdalin
124 content, (v) pasteurized at 75 ^{o}C for 30 min then held for 10, 30, 60 or 120 min at room
125 temperature (20 ± 2 ^{o}C), then frozen, thawed and extracted for determination of amygdalin
126 content, (vi) held for 10, 30, 60 or 120 min at room temperature (20 ± 2 ^{o}C) then
127 pasteurized at 75 ^{o}C for 30 min then frozen, thawed and extracted for determination of
128 amygdalin content.

129
130 *2.3. Commercially-available apple juice*

131 The amygdalin contents of apple juices from supermarkets in Leeds (UK) were
132 determined. The following juices were purchased locally: Appletiser (100% concentrate),
133 Aspall Apple juice (100% pressed English apple), Copella Apple juice (hand - picked
134 English apples), Del Monte Quality Long Life Apple Juice (100% concentrate), Innocent
135 Juicy Drink (75% pressed apple) and 100% pressed apple Apple Juice, Jucee Long Life
136 Apple Juice (100% concentrate), Juice Tree Long Life Apple Juice (100% concentrate),
137 Morrisons own-brand Cloudy Apple Juice (100% squeezed apple; Jonagold, Elstar, Golden
138 Delicious) and English Pressed Apple juice (100% fruit), Robinsons Long Life Apple Fruit
139 Shoot Juice Drink (8% concentrate), Sainsbury's own-brand Pressed Apple Juice (100%
140 pressed & squeezed fruit) and Long Life Apple Juice (from concentrate), Sun Grown

Cloudy Apple Juice (pure fruit juice) and Long Life Apple Juice (from concentrate), Sun Sip Long Life Apple High Juice (50% fruit juice, 50% concentrate), Tesco own-brand; Fruit Splash Long Life Apple Juice Drink (31% concentrate), Long Life Apple Juice (100% concentrate), Organic Long Life Apple Juice (from concentrate), Long Life Apple Juice (10% concentrate), Pure Apple Juice (100% concentrate; Long Life), Value Apple Juice (100% concentrate; Long Life), Pressed Cloudy Apple Juice, Light Choices Long Life Apple Juice Drink (10% concentrate), Long Life Apple Squash (double strength) and Long Life Apple High Juice (50% fruit juice, 50% concentrate) and Tropicana Pressed Apple Juice (100% pure squeezed apple fruit). All the apple juice was stored at 4 ^{o}C after purchase prior to extraction and analysis.

2.4. *Extraction of amygdalin from apple juice*

The pH of all the apple juices analysed were determined and range from 3.86 to 3.95. Amygdalin solubility at this pH was tested at room temperature and boiling temperature in our preliminary study. The results obtained showed that amygdalin is soluble at the pH range and at both temperature.

Apple juice (10 ml) was weighed into a round-bottom flask (500 ml) and extracted as for the apple seeds as described above (2.2.1). The extract was prepared for HPLC analysis (2.5). In order to achieve complete extraction of amygdalin from the apple juices, extraction was carried out three times using the same sample. Each extracts were analysed separately. Summation of the amount of amygdalin in the three extracts gives the amount of amygdalin in each sample.

2.5. Preparation of extracts for HPLC analysis

Aliquots of the extract was dispensed into eppendorf tubes (1.5 ml), centrifuged (10 min, 22 ^{o}C, 14000 rpm, using a micro centrifuge) and filtered with 0.45 µm PTFE filters (Chromacol, UK).

2.6. *HPLC determination of amygdalin*

Amygdalin contents of desiccated apple seeds, commercial apple juice and experimentally processed apple juice were determined by RP-HPLC, using a Shimadzu HPLC consisting of a 20ADXR pump, SIL-20ACXR autosampler and degasser (Bolarinwa *et al.,* 2014). The column was a Phenomenex C18, Type Nucleosile 3, 120 A (150 mm x 4.60 mm, 3 µm) placed in a column oven set at 40 ^{o}C. The mobile phase was an isocratic elution that consisted of methanol and water (25:75, v:v) and the flow rate was 1 ml/min. The mobile phase was sonicated (20 min, 22^{o} ± 2 ^{o}C) to remove gas bubbles before use. The sample injection volume was 5 µl. Amygdalin was detected using a photodiode array detector at 214 nm. Results were expressed as the amount of amygdalin in mg per gram or mg per milliliter of extracted samples.

2.7. Statistical analysis

The data obtained in this study were assessed statistically by analysis of variance (ANOVA) using LSD at 5% significant level. The IBM SPSS statistics version 20 software was used for the analysis.

3. Results and discussion

3.1. HPLC analysis of amygdalin

Amygdalin detection was achieved by UV detection in an isocratic elution with an excellent linearity (correlation $R^2 = 0.9999$) between the peak area and the concentration of amygdalin (Fig. 2). The amygdalin peak was completely separated from other materials without any pre-treatment. The recovery of amygdalin was greater than 98% (results not shown).

3.2. Amygdalin content of apple seeds

The amygdalin contents of seeds from different varieties of apple are given in Table 1. The amygdalin content of apple seeds ranged from 1 to 3.9 mg g^{-1}. Among the fifteen apple varieties analyzed in this study, Golden Delicious seeds had the highest amygdalin content (3.9 mg g^{-1}) followed by Royal Gala (3 mg g^{-1}), Red Delicious (2.8 mg g^{-1}), Spartan (2.6 mg g^{-1}) and Pink Lady (2.6 mg g^{-1}). The amygdalin contents of Rubens, Elstar, Empire and Jazz apple seeds were 2.4, 2.4, 2.3 and 2.2 mg g^{-1} respectively. Lower amygdalin contents were determined in Fuji (1.9 mg g^{-1}), Cox (1.6 mg g^{-1}), Granny Smith (1.6 mg g^{-1}), Bramley (1.3 mg g^{-1}) and Braeburn (1.2 mg g^{-1}) with Russet having the lowest value at 1 mg g^{-1}. The amygdalin contents of the apple seeds could generate between 0.06 and 0.2 mg cyanide equivalents per gram of apple seeds; these values are relatively high. Acute cyanide toxicity can occur in humans at doses between 0.5-3.5 mg kg^{-1} body weight (Speijers, 1993). In a previous study, Haque & Bradbury (2002) reported the amygdalin contents of Fuji apple seeds to be 5.4 mg g^{-1}. This value is slightly higher than the

208 amygdalin content of Fuji apple seeds (1.89 mg g^{-1}) reported in this study. The variation in
209 the amygdalin content of apple seeds could be due to cultivation practices (e.g. different
210 levels of fertilization, irrigation and use of pesticides) or environmental factors such as
211 drought or infection by pathogens during fruit formation. Application of fertilizer to a field
212 before planting has been reported to decrease cyanogenic glycoside levels in cassava
213 tubers (Omar, Hassan, Yusoff, Abdullah, Wahab, & Sinniah, 2012). An amygdalin content
214 of 4.7 mg g^{-1} was reported for apple seeds from an unknown variety (Holzbecher *et al.,*
215 1984). This value is closer to the value reported for Golden Delicious apple seed (3.9 mg g^{-1})
216 in this study.

217 *3.3. Amygdalin contents of apple juices made from apple flesh with skin, apple core and*
218 *whole apple*

219 Amygdalin was not detected in juice made from apple flesh with skin. This is almost
220 certainly because apple flesh does not contain amygdalin. The flesh of rosaceous fruit is
221 acynogenic (Swain, Li, & Poulton, 1992). Although Voldrich and Kylink (1992) reported that
222 fruits with higher concentration of glycosides in their seeds would contain higher amounts in
223 their pulp, this statement does not appear to be true for apple. The amygdalin content of
224 apple juice made from whole apple fruit was compared with that of juice made from apple
225 core. In all the juices analysed, the results showed that the amygdalin contents of juices
226 made from core were the highest compared with apple juice made from whole apple (Fig.
227 3). Apple juice made from Golden Delicious core had the highest (0.43 mg ml^{-1}) amygdalin
228 content followed by Royal Gala core (0.25 mg ml^{-1}), Braeburn core (0.20 mg ml^{-1}) and
229 Egremont Russet core (0.13 mg ml^{-1}). Amygdalin content of juice made from Golden
230 Delicious core was significantly different ($p<0.05$) from the amygdalin content of juice made

231 from Royal Gala, Braeburn and Egremont Russet core. However, juice made from Royal
232 Gala, Braeburn and Egremont Russet core were not significantly different ($p>0.05$) in terms
233 of their amygdalin contents. The amygdalin content of juice made from whole fruit also
234 followed the same trend, with Golden Delicious apple juice having the highest level (0.09
235 mg ml^{-1}) of amygdalin, followed by Royal Gala juice (0.06 mg ml^{-1}), Braeburn juice (0.06 mg
236 ml^{-1}) and Egremont Russet juice (0.04 mg ml^{-1}). The levels of amygdalin in apple juices
237 made from whole fruits were not significantly different ($p>0.05$) in all the apple varieties
238 tested. Amygdalin contents of apple juice from the core of all the 4 apple varieties was
239 about 75% higher than that of juice from whole apple. Higher amygdalin contents were
240 detected in juice from apple core because apple seeds disintegrated during juice extraction
241 and were diluted with the juice from the limited flesh surrounding the core. Amygdalin
242 content of apple juice from whole apple consists of amygdalin content of apple flesh and
243 disintegrated apple seeds. This study shows that the amygdalin contents of apple juice
244 would depend on the amygdalin content of the seeds (which depends on apple variety), the
245 quantity of seeds that disintegrated during juice extraction and the amount of juice in the
246 fruit (i.e. how juicy is the fruit).

247 *3.4. Effect of processing on amygdalin content of apple juice made from whole apple*

248 We sought to determine effects of processing on the enzymatic degradation of
249 amygdalin in apple juice in order to examine the possibility that certain processing
250 conditions might mitigate against enzymatic breakdown of amygdalin. The amygdalin
251 content of freshly-made apple juice ranged from 0.035 mg ml^{-1} for juice from Egremont
252 Russet apples to 0.088 mg ml^{-1} for juice from Golden Delicious apples (Fig. 4). Freezing of

253 the juice prior to amygdalin analysis did not significantly affect the amygdalin content of any
254 sample, even without prior boiling (data not shown). This indicates that the endogenous β-
255 glycosidase enzyme activity was not affected by freezing. Thus all the processed apple
256 juice samples were frozen immediately after collection.

257 All processing conditions used in this study followed the same trend in that there
258 were no significant differences ($p>0.05$) between the varieties tested (Fig. 4). It can be
259 observed that holding the juice at room temperature for 120 min either before freezing,
260 before pasteurization, or after pasteurizing decreased the amygdalin content by 11-19%
261 compared to the original juice. For example, the amygdalin content of juice from Egremont
262 Russet apple reduced by 2% by holding at room temperature for 10 min and by 13% by
263 holding for 120 min prior to freezing (hold-freeze). In the case of pasteurized-hold and hold-
264 pasteurized, the reductions were 7% in 10 min and 18% and 19% in 120 min respectively.
265 The pasteurized-hold and hold-pasteurized results were close to the hold-freeze at room
266 temperature, thus an indication that endogenous β-glucosidase in Egremont Russet apples
267 has limited activity at both 20 ± 2 ^{o}C and 75 ^{o}C (the pasteurization temperature). Moreover,
268 pasteurization does appear to reduce the activity of β-glucosidse, as the enzyme is not very
269 active after pasteurization. A previous study by Nout, Tunçel and Brimer (1995) reported
270 that endogenous β-glucosidase activity causes a significant degradation of amygdalin in
271 ground apricot seeds soaked at 20 ^{o}C. Although plant enzymes are generally believed to
272 be active at about 20-40 ^{o}C (Tunçel, Nout, Brimer, & Göktan, 1990), there could be
273 variation depending on the optimum conditions required by specific enzymes.

In line with what was observed in Egremont Russet apple juice, there was no significant reduction in the amygdalin contents of juices from the other three varieties (Braeburn, Golden Delicious and Royal Gala) irrespective of the processing conditions and the holding time (Fig. 4). Amygdalin contents of apple juices from Braeburn, Golden Delicious and Royal Gala reduced from 3 to 11%, 3 to 15%, and 2 to 12%, respectively, in 10-120 min hold-freeze at room temperature, 4 to 12%, 4 to 16%, and 2 to 13%, respectively, in 10-120 min pasteurized-hold and hold-pasteurized. This indicates that the endogenous enzyme in these apple juices have limited impact on the degradation of amygdalin, possibly because of low levels of enzyme in the juice. Although β-glucosidase from apple seeds has been reported to have higher thermal stability, with maximum activity temperature at 70 ^{o}C, it is, however, inactivated at temperatures slightly higher than 70 ^{o}C (Yu, Xu, Lu, & Lin, 2007).

3.5. Commercially-available apple juice

3.5.1 Apple juice from pressed/squeezed fruit

The amygdalin contents of commercially-available apple juice from pressed or squeezed fruit are shown in Table 2. Sun-grown cloudy apple juice had the highest amygdalin content (0.039 mg ml^{-1}), followed by Morrison's 100% juice (0.037 mg ml^{-1}), Sainsbury's juice (0.035 mg ml^{-1}), Copella juice (0.032 mg ml^{-1}), Morrison's English apple juice (0.030 mg ml^{-1}) and Tropicana juice (0.030 mg ml^{-1}). The amygdalin contents of Innocent apple juice, Aspall juice, Tesco juice and Innocent juicy drink were 0.027, 0.026, 0.011 and 0.010 mg ml^{-1} respectively. Amygdalin contents of commercially-available apple juice from pressed or squeezed fruit (0.01 - 0.04 mg ml^{-1}) were within the range obtained in our laboratory processed apple juice (0.01 - 0.08 mg ml^{-1}).

297 *3.5.2 Long-life apple juice*

298 Table 3 shows the amygdalin contents of Long-life apple juice. Among the
299 seventeen commercially-available long-life apple juices analyzed, Sun-sip Apple High Juice
300 had the highest amygdalin content (0.007 mg ml^{-1}), followed by Tesco Apple High Juice
301 (0.005 mg ml^{-1}), Appetizer (0.004 mg ml^{-1}), Tesco Apple Squash (0.003 mg ml^{-1}) and Juice
302 Tree Apple Juice (0.003 mg ml^{-1}). Amygdalin contents of Del Monte Juice, Tesco Value
303 Juice, Sainsbury's Juice and Sun-grown Juice were 0.002, 0.002, 0.001 and 0.001 mg ml^{-1}
304 respectively. Amygdalin was not detected in Jucee Apple Juice, Robinson Fruit shoot,
305 Tesco Apple Juice, Tesco Drink, Tesco Everyday Value, Tesco Light Choices, Tesco
306 Organic and Tesco Fruit Splash. Low levels of amygdalin in long-life apple juice when
307 compared with apple juice from pressed or squeezed fruit could be as a result of
308 degradation and loss due to high thermal treatment during processing or very low content
309 of apple (replaced by other fruits or water) in some drinks.

310 A glass of pure apple juice and long-life apple juice would liberate 0.26 – 1.03 and 0.026 –
311 0.18 mg equivalent cyanide per gram, respectively. Since the toxic dose of cyanide
312 depends on body weight, to reach the lethal dose an adult man would have to drink 10 L to
313 40 L of pure apple juice, while a child would have to drink around 8 L at once. Ingestion of
314 such a large volume of apple juice at once is unlikely.

315
316 **4. Conclusion**

317 Serious health problems could occur as a result of intentional or unintentional
318 ingestion of amygdalin from foods, including apple seeds. While amygdalin is toxic at high
319 concentration, no work has been done on its effect on health at low level. The results

presented in this study clearly showed that the amygdalin contents of pure apple juice and long-life apple juice are relatively low, and would be unlikely to pose health problems to consumers. It is recommended that apple seeds should be removed before consumption or processing because of the high content of amygdalin in apple seeds. Although processing conditions employed in this study resulted in slight reduction in amygdalin content of apple juice, the reduction is counter-balanced by a loss of juice quality as a result of increased enzymatic browning.

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Figure Legend

Figure 1: Structure of amygdalin.

Figure 2: Extraction yield of amygdalin from juice made from apple flesh with skin, apple core and whole apple . Juice from apple core was made from apple fruit with most of the flesh removed (i.e core with limited flesh). Juice from whole apple was made from whole apple fruit (i.e all apple flesh including the core). Each vertical rectange represent the average value of triplicate determinations. Error bars indicate the standard deviation.

Figure 3: Effects of processing on whole apple juice produced from different apple varieties. OJ – Original Juice, PH – Pasteurized Hold, HP – Hold Pasteurized, HF – Hold Freeze. Freshly produced apple juices were held for 10 to 120min either before freezing, before pasteurizing or after pasteurizing. Each point represents the average of three determinations. Error bars indicate standard deviation.