Targeted metabolomics study of 'Braeburn' apples during long-term storage

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Abstract

'Braeburn' is an apple cultivar susceptible to the occurrence of internal browning (Braeburn Browning Disorder; BBD) during storage. This physiological disorder is characterised by the development of brown spots inside the fruit, eventually resulting in the formation of cavities. The objective of this study was to investigate the effects of the preharvest application of calcium, potassium and triazole fungicides on the postharvest

primary metabolites of 'Braeburn' fruit, and to offer a better understanding of the biochemical processes behind internal browning. The primary metabolites of 'Braeburn' cortex samples at harvest and after 2 weeks, 4 weeks, 4 months and 8 months of storage at browning-inducing conditions were analysed using GC-MS. No significant difference in the primary metabolites was observed between the different levels of the applied preharvest applications. Early during storage, fruit developed browning, with the severity increasing with storage duration. This was correlated to a group of primary metabolites that showed either an increase (e.g., alanine, galactose, mannitol, sorbitol, xylose) or a decrease (e.g., malate, sucrose) in concentration with time. Radial distribution of the metabolites in the fruit tissue was also observed; some metabolites (e.g., galactose, mannitol) were higher in concentration in the inner cortex, while the concentrations of other metabolites (e.g., mannose, sucrose) were higher in the outer cortex.

1. Introduction

'Braeburn' (*Malus* × *domestica* Borkh.) is susceptible to the development of an internal browning disorder during storage, called Braeburn browning disorder (BBD; Elgar et al., 1999). In Belgium, 'Braeburn' apples are stored at a combination of low O_2 (2.5 kPa) and slightly elevated CO_2 (0.7 kPa) at low temperature (1 °C) (Flanders Centre of Postharvest Technology, VCBT). Under these conditions, year-round availability of good-quality fruit can generally be guaranteed. However, some fruit may still develop BBD. The disorder is characterised by brown patches which can lead to cavity formation (Elgar et al., 1998). Off-flavours can also be associated with the disorder (Felicetti et al., 2011). As a result of the brown tissue and the related off flavours, apples are rendered unacceptable in the market, resulting in sometimes unexpected large economic losses.

Internal browning has been associated with the enzyme polyphenol oxidase, which catalyses the oxidation of phenolic compounds, eventually resulting in the formation of brown-coloured pigments (Mathew and Parpia, 1971; Veltman et al., 1999) . In unaffected tissues, the oxidase enzyme and the phenols (enzymatic substrates) are situated in separate subcellular compartments (Toivonen and Brummell, 2008). Local hypoxic regions in the centre of the fruit may be generated as a result of too low O_2 or too high CO_2 partial pressures in the storage atmosphere (Lammertyn et al., 2003; Ho et al., 2006). This may lead to disturbances in the cellular respiration leaving insufficient energy to properly fuel maintenance processes such as of membranes. As a result compartmentalisation may be lost due to which polyphenol oxidases come into contact with their phenolic substrates thus starting the browning reactions (Streif et al., 2003; Franck et al., 2007).

In general, BBD can be controlled by storing the fruit under the recommended controlled atmosphere storage conditions. However, the disorder may still erratically develop in fruit coming from some orchards in certain growing seasons. This fact seems to indicate that several preharvest factors affect the susceptibility of apple to internal browning. Calcium is an plant nutrient that plays an important role in maintaining the postharvest quality of fruit as it affects cell membrane permeability and cell wall stability (Marinos, 1962; Poovaiah et al., 1988; White and Broadley, 2003). When low levels of calcium were used in the soil, an increased incidence of browning in ‘Braeburn’ fruit was observed (Rabus and Streif, 2000). In another study on ‘Braeburn’ apples, calcium application was shown to reduce the browning incidence in fruit (Hatoum et al., 2014). Another factor affecting browning in apple is potassium. Potassium competes with the uptake of calcium from the soil which might therefore lead to higher browning incidence (Nava and Dechen, 2009; Neilsen and Neilsen, 2009). Hatoum et al. (2014), however, observed a decreased browning incidence in ‘Braeburn’ apples when potassium fertilizers were used. The use of triazole-based chemicals

has increased in recent years with the aim of inhibiting fungal growth and controlling plant growth. More browning was observed in fruit when triazole fungicides were used (on 'Cox's Orange Pippin' apples: Johnson 2009; on 'Braeburn' apples: Hatoum et al., 2014).

The above-mentioned factors are also known to affect the metabolic status of plant organs. Calcium, given its role in cell membranes and cell wall stabilization, can alter the fruit metabolic profile (Picchioni et al., 1995). Armengaud et al. (2009) showed that the metabolic profiles of *Arabidopsis thaliana* root and shoot were influenced by the level of potassium fertilizer. Jaleel et al. (2009) observed changes at the metabolic level of *Catharanthus roseus* when treated with plant growth regulators. However, it is still to be elucidated how the above-mentioned preharvest factors can affect the primary metabolites of apple fruit at harvest and beyond.

The objective of this study was two-fold: 1) to understand the metabolic changes associated with the various application levels of calcium and potassium fertilizers, and triazole fungicides; and 2) to offer a better understanding of the biochemical changes that occur in the 'Braeburn' fruit cortex tissue during controlled atmosphere storage in relation to internal browning.

2. Materials and Methods

2.1. Fruit growing, harvesting and CA storage

'Braeburn' apples were grown (grafted on Hillwell rootstock, in their 9^{th} and 10^{th} growth year) and harvested in the orchard of the experimental tree fruit research station (RSF-pcfruit) in Sint-Truiden, Belgium. In this study, 'Braeburn' fruit were grown under 8 different treatment combinations of calcium, potassium and triazole fungicides (Table 1). Details about the composition, timing and dosing of the two treatment levels of calcium, potassium and

triazole (and non-triazole) fungicides can be found in Supplementary Tables 1, 2, and 3. Thirty fruit from each condition (150 fruit from condition 7) were harvested on 28/10/2010. This commercial harvest date for 'Braeburn' fruit was determined by the VCBT (Leuven, Belgium) based on a combination of firmness, starch, sugars and acids measurements. Fruit from condition 7 were, one day after harvest, stored at browning-inducing CA conditions consisting of 2.5 kPa O_2, 3.7 kPa CO_2 and at 4 °C up to 8 months. Fruit were not treated with 1-MCP.

2.2. Browning assessment and fruit selection

To assess the primary metabolites at harvest, 6 'Braeburn' apples were randomly selected from each growing condition at harvest.

For the storage experiment, 'Braeburn' fruit were rated for internal browning immediately at harvest and after 2 weeks, 4 weeks, 4 months and 8 months of CA storage using 30 fruit per time point. Slices (~1 cm thick) were cut from the middle of the fruit perpendicular to the longitudinal axis, and pictures were taken using a digital camera. Corrections for light differences between images were done using a colour chart (Fig. 1). Internal browning was rated using an in-house developed MATLAB program (Matlab R2010, The MathWorks, Inc., Natick, MA, USA) in which the colour of each pixel of the apple cortex (excluding the core, the skin and the reddish coloured outer layer of the cortex directly underneath the skin) was compared to the yellow to brown colours from the colour chart (Fig. 1). A brown index (BI) was then calculated as the average of the squared colour indices of all pixels from the cortical tissue. As the colour scale is based on 10 classes (from yellow = 1 to brown = 10) the calculated brown index ranges from 1 to 100. Four fruit with the highest and four with the lowest BI values were selected for metabolomics analysis (Fig. 2). As browning was first detected after 4 weeks of CA storage, fruit from 4 weeks of storage onwards that had high BI

values will be referred to as brown fruit, whereas those with low BI values will be referred to as sound fruit.

2.3. Fruit sampling

Slices (~1 cm thick) were cut from the middle of the selected fruit perpendicular to the longitudinal axis. Five tissue samples were taken from both the outer and the inner parts of each fruit slice (Fig. 3) using a cork borer (0.4 cm Ø). The samples were immediately frozen by immersion in liquid nitrogen and transferred to 15 mL test tubes. Samples were then stored at -80 °C until further analysis. Homogenization of the tissue samples into fine powder was done using a CryoMill grindomixer MM200 (Retsch, Haan, Germany).

2.4. Sample preparation and GC-MS analysis

The used apple metabolomics protocol is as follows: frozen tissue powder (200 mg) was mixed with 1 mL of ice-cold methanol and 45 µL of 2910 ng/µL phenyl β-D-glucopyranoside (internal standard) and incubated at 70 °C for 15 min while shaken vigorously. After centrifugation at 14,000 rpm for 20 min, 100 µL of the supernatant was transferred to a new microcentrifuge tube and dried under a stream of nitrogen gas. The dried samples were re-dissolved in 120 µL of 20 mg/mL methoxyamine hydrochloride in pyridine and incubated at 30 °C for 90 min while shaking. Finally, derivatization of the mixture was achieved through incubation with 120 µL of BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) at 37 °C for 30 min while shaking. Samples of 1 µL were used for injection on the GC column of an Agilent GC-MS system (GC 7890 with a 5975 single quadrupole MS with electron impact ionization source; Agilent Technologies, Palo Alto, CA, USA). Each sample was analysed twice; a split (1:150) method was used for the abundant compounds and a splitless method was adopted for

147 the less abundant compounds. The GC column used was a HP-5-MS capillary column of 30 m
148 length, 0.25 mm internal diameter and 0.25 µm film thickness (Supelco, Bellefonte, CA,
149 USA). For both methods, the injection and interface temperatures were 220 °C and 280 °C
150 respectively. Helium was used as a carrier gas with an average velocity of 35 cm/s. The GC
151 temperature program started isothermal at 50 °C for 1 min (acids method) or at 120 °C for 1
152 min (sugars method), and was then ramped at a rate of 10 °C/min to 310 °C where it was kept
153 for 13 min (acids method) or to 300 °C where it was kept for 6 min (sugars method). The total
154 run time for the acids method was 40 min and that for the sugars method was 25 min. Mass
155 spectra in the 50 to 600 m/z range were recorded at a scanning speed of 2.66 scan cycles per
156 second. The MS ion source and quadrupole temperatures were 230 °C and 150 °C,
157 respectively.
158

159 *2.5. Data analysis*

160 The automated mass spectral deconvolution and identification system (AMDIS, National
161 Institute of Standards, Gaithersburg, MD, USA) was used to deconvolute the chromatographic
162 peaks. Identification was done by comparing the peak retention indices (RI) and mass spectra
163 to a home-built library of commercial standards. Standards were purchased from Sigma-
164 Aldrich-Fluka (Diegem, Belgium) (pyruvic acid, benzoic acid, phosphoric acid, glyceric acid,
165 glutamic acid, alanine, valine, phenylalanine, asparagine, serine, threonine, sucrose, galactose,
166 glucose, sorbitol, urea), Acros Organics (Geel, Belgium) (lactic acid, succinic acid, fumaric
167 acid, quinic acid, aspartic acid, mannose, fructose, cellobiose, erythritol, ribitol), Merck
168 Chemicals (Overijse, Belgium) (malic acid, xylose) and VWR (BDH Prolabo, Leuven,
169 Belgium) (mannitol). Quantification (peak area determination) of the compounds was done
170 using the MSD ChemStation software (Agilent Technologies, Palo Alto, CA, USA).

171 Raw peak area data were corrected using the actual peak area of the internal standard (phenyl
172 β-D-glucopyranoside) and the sample fresh weight. Partial least squares discriminant analysis
173 (PLS-DA) was performed on the normalized data of the storage experiment using the
174 Unscrambler software (version 10.3, CAMO A/S, Trondheim, Norway) with the metabolites
175 as predictor variables and storage duration (harvest, 2W, 4W, 4M and 8M), internal browning
176 (BI) and tissue position (In / Out) as response variables. All variables were mean centred and
177 weighted by their standard deviation to give them equal variance. An analysis of variance was
178 performed using the GLMSELECT stepwise procedure from SAS (v 9.3, The SAS Institute
179 Inc., Cary, NC, USA), to determine the relation between internal browning, storage duration
180 and position in the apple cortical tissue on one hand, and individual metabolite levels on the
181 other hand. The position in the apple tissue (In versus Out) was used as an independent class
182 variable, while the brown index (BI) and the storage duration were used as independent
183 continuous variables. The relative responses of the metabolites were considered as the
184 dependent response variable. The GLM stepwise selection method ensures that no effect can
185 be added to the model until all non-significant effects currently present in the model are
186 removed. The stepwise process ends when none of the effects outside the model are
187 significant and every effect in the model is significant.
188

189 # 3. Results and Discussion

190 In this study, the effect of three different preharvest factors on the primary metabolites of
191 'Braeburn' fruit cortical tissue was studied using a GC-MS metabolomics approach. In
192 addition, the dynamics of the primary metabolites of fruit stored under browning-inducing
193 controlled atmosphere conditions was analysed. GC-MS enables the identification and relative
194 quantification of primary metabolites resulting in a fairly comprehensive coverage of the
195 central pathways of primary metabolic pathways (Fiehn et al., 2000; Roessner et al., 2001;

196 Halket and Zaikin, 2003). The broad dynamic range of GC-MS makes it suitable to analyse a
197 wide range of compounds, including organic and amino acids, sugars, and sugar alcohols
198 (Sumner et al., 2003). However, and because of the large concentration differences between
199 apple metabolites (sugars and acids), protocol optimization was needed. This was achieved
200 with a metabolomics protocol by Roessner et al. (2000) as a starting point. Due to the large
201 concentration differences between sugars and other metabolites, it was necessary to analyse
202 every sample twice. The split injection was used for the sugars, while the splitless injection
203 was used for the detection of the low abundant compounds. In this study, 29 primary
204 metabolites were unequivocally identified and (relatively) quantified from the polar extracts
205 of the 'Braeburn' apple cortical tissue (Table 2).
206

207 ### *3.1. At harvest metabolic differences due to preharvest factors*

208 The results showed that the primary metabolites of the apple cortex tissue at harvest were not
209 significantly affected by the different preharvest applications of calcium and potassium
210 fertilizers, and triazole fungicides (data not shown). As the studied factors are known to affect
211 the incidence of internal browning in fruit (Rabus and Streif, 2000; Johnson, 2009; Nava and
212 Dechen, 2009; Neilsen and Neilsen, 2009; Hatoum et al., 2014), it seems that the expected
213 changes in the primary metabolites of the fruit only become manifest later during storage.
214 This result is in accordance with Vandendriessche et al. (2013) who reported no significance
215 difference in the metabolic composition of 'Braeburn' apple juice when different levels of
216 fertilizers (calcium, phosphorus and potassium) were applied. From independent fruit mineral
217 analyses (data not shown) confirmation was obtained that the different preharvest treatments
218 did affect the mineral composition of the fruit and thus were effective. In addition Hatoum et
219 al. (2014) has shown the effects of the different applications of calcium, potassium and
220 triazoles on the browning incidence after storage. Treatments with calcium and potassium

fertilizers were shown to reduce the incidence of internal browning in fruit during controlled atmosphere storage, while the triazole fungicides resulted in an increased incidence of internal browning. So while the different preharvest treatments did not alter the primary metabolites of fruit at harvest, they do have effects that become manifest later on, possibly through latent effects on secondary metabolites.

3.2. Postharvest metabolic dynamics under browning inducing conditions

3.2.1. Exploratory data analysis

The primary metabolites of 'Braeburn' apple cortex tissue were shown to change with storage time (from harvest to 8 months of storage), the position in the fruit cortex (inner versus outer part) and the state of the tissue (brown versus sound). This was clearly illustrated by the partial least squares discriminant analysis (PLS-DA) (Fig. 4). In the PLS 18 % and 16 % of the total *X*-variance and 33 % and 17 % of the total *Y*-variance was accounted for by the first two latent variables, whereas the fourth latent variable, that accounted for 5 % of the total *X*-variance and 10 % of the *Y*-variance (Fig. 4A, B), explained the relation with the brown index (BI; Fig. 4C). The further the variables are from the axis origin the more influential the variables are in explaining the differences among the different samples. The PLS analysis revealed divergence of apple flesh metabolome with increasing storage duration and in relation to browning and the position in the apple cortex (Fig. 4A). In the PLS loadings plots (Fig. 4B, C), the associations among the individual metabolites (as X-variables) on one hand and the storage duration, position in the apple cortex, and the browning (BI) development (as Y-variables) on the other hand were revealed. Some metabolites (e.g., alanine, galactose, sorbitol, xylose) were associated with longer storage duration, while the metabolites associated with fruit at harvest included malate and sucrose. With respect to the position in

245 the apple cortex, some metabolites were associated with the inner cortex (e.g., asparagine,
246 galactose, mannitol, phenylalanine, quinate, serine), while others were more abundant in the
247 outer cortex (e.g., mannose, sucrose).
248 In order to obtain information about statistical significance, a univariate analysis of variance
249 (GLM stepwise selection procedure) was performed to determine the relation between internal
250 browning, storage duration and position in the apple cortical tissue on one hand, and
251 individual metabolite levels on the other hand. Only metabolites that showed a significant
252 response to the variables studied (at a $p = 0.05$) were retained and will be discussed in the
253 following sections (Fig. 5A, B).
254

255 ### *3.2.2. Spatial metabolic differences*

256 From the PLS model (Fig. 4A) it could be observed that the primary metabolites differed
257 between samples from the inner and the outer cortex tissue as the samples from the inner
258 cortex tissue (especially those after 4 months and 8 months of storage) are located to the
259 positive sides of the LV1 axis, whereas those from the outer cortex tissue are located more to
260 the negative side of the LV2 axis. The development of browning in 'Braeburn' fruit generally
261 starts from the inner cortical region (near the core) and in more advanced stages spreads into
262 the outer cortex (till just underneath the skin) (Elgar et al., 1998). This is mirrored by the fact
263 that brown index (BI) shares some common direction with the inner cortex tissue (In) along
264 LV1 (Fig. 4C).
265 Nine out of the twenty-one metabolites identified by the univariate statistical approach (Fig.
266 5A, B) were significantly different with respect to position. These results are in agreement
267 with previous work by Franck et al. (2003) and Pedreschi et al. (2009) on 'Conference' pears,
268 and by Biais et al. (2010) on melons who observed a radial distribution of metabolites in the
269 fruit tissue.

The most obvious spatial differences were observed for galactose and mannitol, both increasing during storage, and sucrose, which was decreasing during storage. All three showed a clear separation between the inner and the outer fruit cortex but not between the sound and the brown fruit. The concentration of asparagine in the inner fruit cortex was mostly higher than that in the outer cortex. When the sound and the brown fruit were compared, asparagine was higher in concentration in the inner cortex of the brown fruit than that of the sound fruit. In the outer cortical tissue, however, no clear separation could be observed between the sound and the brown fruit. Mannose showed a higher concentration in the outer than in the inner fruit cortical tissue while for phenylalanine this was the other way around, neither of them showing a clear separation between the sound and the brown fruit In the case of quinate, a higher concentration was observed in the inner than in the outer fruit cortex during the early part of storage. In the case of the amino acids serine and valine, the separation between the inner and the outer fruit cortical tissues at harvest largely disappeared during storage.

The differences between fruit at harvest could, on one hand, explain some of the observed differences in the distribution of the metabolites in the apple cortical tissue. Franck et al. (2003) observed an asymmetrical distribution of ascorbate, with a higher concentration on the side of the pear facing the sun. On the other hand, during CA storage, gas gradients in the fruit are formed as a result of the concentration of O_2 and CO_2 in the atmosphere and the diffusion barrier of the fruit tissue. Ho et al. (2010) observed a decrease of O_2 concentration and an increase of CO_2 concentration towards the centre of the apple fruit as a result of the diffusion barrier of the fruit tissues. On a study on melon fruit, Biais et al. (2010) concluded that the metabolite gradient in the fruit might be a reflection of stress caused by low O_2 concentration. At low O_2 concentration, glycolysis is inhibited and a decrease in the adenylate energy status of the cell is expected while at the same time hypoxia inhibits a range of metabolic processes

295 that consume ATP (Geigenberger et al., 2000). In a study on different apple cultivars, Ho et
296 al. (2013) reported that the low O_2 concentrations inside the fruit, showing a further decrease
297 towards the centre, might result in a switch from respiration to fermentation with insufficient
298 ATP production for the maintenance of cell integrity. In the current study, given the spatial
299 distribution in sucrose only developed over time this might be related to a differential
300 inhibition of the starch and sugar metabolism in response to the O_2 gradients developing
301 inside the fruit. On the outside tissue of the fruit ATP availability might still be high enough
302 to support the release of sugars from starch feeding into the glycolysis while in the centre of
303 the fruit, where ATP availability at the lower O_2 levels might become more restrictive, the
304 fruit starts to utilise its sucrose reserves. In other words, the low O_2 level in the centre of the
305 fruit reduces ATP availability for the cellular repair mechanisms eventually causing cell
306 death.
307
308 *3.2.3 Temporal metabolic differences*

309 The analysis of the apple cortex primary metabolites revealed the difference between the
310 samples at the different sampling time points (harvest and storage duration; Fig. 4A),
311 especially when going from 4 weeks to 4 and 8 months of storage. During the first 4 weeks of
312 storage no clear separation was observed indicating less changes in the primary metabolites.
313 Browning in 'Braeburn' fruit was visually observed after 4 weeks of CA storage, and the
314 severity continuously increased until 8 months. This is in agreement with previous research
315 (Elgar et al., 1998; Lee et al., 2012b). As shown from the correlation loadings (Fig. 4B),
316 browning and storage duration share similar information along LV1 mirroring the fact that
317 browning develops with time in storage.

318 Some of the metabolites retained by the univariate statistical approach (e.g., alanine,
319 galactose, mannitol, sorbitol and xylose) increased in concentration with storage duration,
320 while the concentration of others (e.g., malate and sucrose) decreased (Fig. 5A, B).
321 The concentrations of xylose and mannose increased with storage duration. As xylose and
322 mannose have been identified in xyloglucan which is a primary cell wall hemicellulose
323 (Miller and Fry, 2001), the increase in their concentrations may indicate hemicellulose
324 breakdown, which can be an indication of fruit senescence. A similar increase in xylose and
325 mannose has also been reported by Pedreschi et al. (2009) in brown 'Conference' pear tissue.
326 Galactose also increased in concentration, but unlike xylose, this increase was observed early
327 in storage. Galactose in fruit is mainly bound to the side chains of cell wall polysaccharides
328 (Harholt et al., 2010). During ripening, galactose can be liberated as a result of cell wall
329 breakdown (Knee, 1973). The observed increase in galactose cannot be explained by
330 senescence as this rise in concentration was observed early in storage. Cellobiose is a
331 disaccharide of 2 glucose molecules that is generated through the hydrolysis of cellulose in
332 the plant cell wall (Barras and Stone, 1969). In this study, cellobiose was significantly higher
333 in concentration in the inner cortical tissue in the brown fruit than in the sound fruit, thus
334 indicating cell wall breakdown.
335 The increase in sorbitol concentration is consistent with previous research on 'Jonagold' apple
336 (Roth et al., 2007). Lee et al. (2012a) showed an association between sorbitol accumulation
337 and flesh browning in 'Empire' apple. Fidler and North (1970) suggested that sorbitol
338 accumulation might be an indication of disturbed metabolism. In our study, sorbitol
339 accumulation was associated with flesh browning and storage duration. During CA storage,
340 the concentration of mannitol also increased. Mannitol can protect plants against oxidative
341 damage by hydroxyl radicals (Shen et al., 1997), thus the increase in its concentration might
342 be an indication of stressed cortical tissue. Ribitol also showed an increased concentration

343 with storage time, especially towards the end of the storage (from 4 to 8 months). In a study
344 on 'Empire' apples, Lee et al. (2012a) reported an association between ribitol and flesh
345 browning.
346 Malate decreased in concentration during storage as was also observed by others (Suni et al.,
347 2000; Roth et al., 2007; Vandendriessche et al., 2013). Malate, the major organic acid found
348 in apple fruit, is a major substrate for aerobic respiration that typically decreases as a result of
349 fruit ripening (Ingle et al., 2000; Bai et al., 2005). In a study on 'Conference' pears, Pedreschi
350 et al. (2007) reported an up-regulated expression of malic enzyme (catalyses the oxidative
351 decarboxylation of malate to pyruvate, CO_2 and NADPH) as well as a down-regulation of the
352 expression of fumarase (catalyses the hydration of fumarate to malate). In both cases, a
353 decrease in malate concentration would result together with an increase in pyruvate (malic
354 enzyme up-regulation) or fumarate (fumarase down-regulation). In the current work, however,
355 the absence of a significant difference in the concentration of pyruvate or fumarate between
356 brown and sound 'Braeburn' apple tissues indicates that the observed decrease in malate
357 concentration might not be associated with internal browning in fruit, but is the result of fruit
358 ripening during storage. This is in accordance with Lee et al. (2012a,b) who stated that the
359 levels of organic acids, including the decrease in malate, are not associated or are not directly
360 involved in the development of browning disorders. The concentration of threonine decreased
361 until 4 months of storage. In a study on 'Jonagold' apples, Sugimoto et al. (2011) observed a
362 decrease in threonine concentration which was related to fruit ripening. As threonine serves as
363 a substrate for isoleucine biosynthesis, its decreased concentration might indicate an increased
364 isoleucine synthesis. Pedreschi et al. (2009) observed an increased isoleucine concentration in
365 'Conference' pear brown tissue. In our study, however, isoleucine could not be measured in
366 the apple cortical tissue samples. The concentration of sucrose also decreased in storage.
367 Decreased sucrose concentration was also observed in ripening tomato fruit (Oms-Oliu et al.,

368 2011), in ‘Jonagold’ apple (Roth et al., 2007), and in ‘Empire’ apple (Lee et al., 2012a). The
369 concentration of glucose increased slightly during storage. An increase in glucose
370 concentration with storage duration was also observed in ‘Empire’ apple (Lee et al., 2012a).
371 The decrease in sucrose and the increase in glucose concentrations are due to the hydrolysis of
372 sucrose into fructose and glucose during the storage of apple fruit (Rouchaud et al., 1985;
373 Suni et al., 2000).
374 During storage, an increase in the concentration of the amino acid alanine was observed. On
375 an NMR based metabolomics study on ‘Braeburn’ apple juice, Vandendriessche et al. (2013)
376 reported similar results. The increased alanine concentration might, therefore, indicate the
377 activation of fermentation pathways in the centre of the fruit due to O_2 limitation as alanine
378 can be formed from pyruvate by reductive amination (Biais et al., 2009). However, given the
379 fact that in our work pyruvate was not significantly different between sound and brown fruit
380 tissues or as a result of storage, the increase in alanine concentration with storage time cannot
381 be explained by reductive amination of pyruvate. The observed accumulation of alanine might
382 therefore be the result of proteolysis provoked by cell death (Muntz, 2007). During storage,
383 and especially in brown fruit, glycerate showed an increase in concentration. This is similar to
384 results on ‘Empire’ apple reported by Lee et al. (2012a). Succinate concentrations typically
385 increase in fruit with CO_2 injury (Hulme, 1956; Fernández-Trujillo et al., 2001). Hulme
386 (1956) suggested that the observed cell death in CO_2-damaged apples is the result of succinate
387 toxicity. Other studies, however, reported lower levels of succinate in brown as compared to
388 healthy pear tissue (Pedreschi et al., 2007). In our study, an increased succinate concentration
389 in brown fruit was observed. Thus, succinate accumulation might be the result of the
390 increased CO_2 concentration used in the CA storage of the apple fruit in this study, which
391 leads to the inhibition of the enzyme succinate dehydrogenase (Gonzàlez-Meler et al., 1996).
392 Besides, succinate accumulation can also be explained via activation of the GABA shunt

393 pathway under stress conditions (Fait et al., 2008). The concentration of glutamate increased
394 early in storage until after 4 weeks. After that, a steep decrease in glutamate concentration
395 was observed. Under stress conditions, glutamate can be converted to GABA (γ-
396 aminobutyrate) as a result of the stimulated glutamate decarboxylase enzyme (Ferreira de
397 Sousa and Sodek, 2002). Elevated levels of GABA have been linked to internal browning in
398 'Conference' pear (Pedreschi et al., 2009). However, in our study GABA was not detected in
399 the apple cortical tissue. Finally, a decrease in the concentration of aspartate was observed
400 after 4 weeks of storage. Aspartate is synthesised from oxaloacetate which is an intermediate
401 of the citric acid cycle. The decrease in aspartate concentration might therefore indicate that
402 oxaloacetate is being used for the production of other compounds. For instance, it has been
403 suggested that high CO_2 concentration during hypoxia may facilitate the conversion of
404 oxaloacetate from phosphoenolpyruvate, which via the reversal of Krebs cycle might lead to
405 fumarate accumulation (Pedreschi et al., 2009). In our study, however, fumarate did not
406 significantly accumulate in brown apple tissue or as a result of storage. This contradicts with
407 the hypothesis of the reversal of Krebs cycle induced by high CO_2 levels. Therefore, we
408 suggest that the decrease in aspartate concentration is likely the result of a partial blocking of
409 the Krebs cycle due to O_2 limitation at the reaction catalysed by succinate dehydrogenase.
410 This is, indeed, in line with the observed accumulation of succinate in the brown tissue (see
411 above).
412

413 # 4. Conclusions

414 In this study, a GC-MS based metabolomics approach was used to study the metabolic
415 response of 'Braeburn' apple cortex tissue to preharvest treatments with calcium, potassium
416 fertilizers, and triazole fungicides, and up to 8 months storage at browning-inducing
417 controlled atmosphere conditions. Browning was detected starting from 4 weeks of storage.

418 The metabolomics analysis resulted in the identification of 29 compounds from the apple
419 cortex tissue.
420 No significant effects of the different preharvest treatments on the primary metabolites at
421 harvest could be detected. The analysis of fruit after different storage times resulted in
422 differences at the metabolite level coinciding with the internal browning of the fruit. The
423 primary metabolites of the samples from the inner and the outer apple cortex were also
424 divergent indicating a spatial differentiation of the primary metabolites. This work indicated
425 how certain processes are associated with storage duration and browning development in
426 apple fruit. The increase in cell wall constituents (cellobiose, galactose, mannose and xylose)
427 indicated collapsed cell wall architecture; the increase in sugar alcohols (mannitol, ribitol and
428 sorbitol) indicated a stressed state of the tissue; the decrease in malate, sucrose and threonine
429 and the increase in glucose were associated with ripening of the fruit; alanine accumulation
430 indicated cell death, while the increase in succinate and the decrease in aspartate suggested a
431 disturbed citric acid cycle. Taken together, our data provide an overview of the biochemical
432 mechanisms taking place in the 'Braeburn' apple fruit cortical tissue during storage and in
433 relation to internal browning.
434 In the current study, the storage time and the browning incidence are covariates which makes
435 it difficult to unambiguously unravel their relationship to the various metabolic changes.
436 Further studies will be directed towards the analysis of 'Braeburn' fruit subjected to different
437 combinations of pre- and postharvest treatments inducing different levels of browning with
438 time in order to be able to discriminate between general fruit ripening related changes and
439 specific browning related changes.
440

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447

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Tables

Table 1. Overview of the different combinations of the applications of calcium and potassium fertilizers and triazole fungicides. Two treatment levels (none versus maximum) of each factor were applied.

Condition	1	2	3	4	5	6	7	8	Explanation label (0 / 1)
Calcium	1	1	0	1	0	1	0	0	None / Maximum
Potassium	0	1	1	1	0	0	1	0	None / Maximum
Triazoles	1	0	1	1	1	0	0	0	None / Maximum

Table 2. Primary metabolites identified by GC-MS as components of a methanol extract from apple cortex tissue.

Organic acids	Amino acids	Sugars	Sugar alcohols	Others
Pyruvate	Aspartate	Sucrose	Erythritol	Urea
Lactate	Glutamate	Mannose	Ribitol	
Malate	Alanine	Galactose	Mannitol	
Benzoate	Valine	Fructose	Sorbitol	
Phosphate	Phenylalanine	Glucose		
Succinate	Asparagine	Xylose		
Glycerate	Serine	Cellobiose		
Fumarate	Threonine			
Quinate				

Figure legends

Fig. 1. Cross-section of 'Braeburn' apples with visible internal browning (left picture) and without visible internal browning (right picture). Included in the pictures is the calibration colour card which was used for brown index calculations (class 1 (yellow, BI =1) to class 10 (brown, BI = 100)).

Fig. 2. Selection of 'Braeburn' fruit from the storage experiment for metabolomics analysis. From the 30 fruit used for brown index (BI) analysis, four apples with the highest BI values (indicated by the letter H) as well as those four with the lowest values (indicated by the letter L) were selected for analysis from fruit after 2 weeks (2W), 4 weeks (4W), 4 months (16W) and 8 months (32W) of controlled atmosphere storage at 2.5 kPa O_2/3.7 kPa CO_2 at 4 °C.

Fig. 3. Cross-section of a 'Braeburn' apple fruit. Samples (1-5) were taken from the outer cortex tissue while samples (6-10) were taken from the inner cortex tissue of the fruit slice.

Fig. 4. (A) PLS scores plot illustrating changes in the metabolomes of 'Braeburn' apple cortex tissue at harvest and after 2 weeks, 4 weeks, 4 months and 8 months of controlled atmosphere storage at 2.5 kPa O_2/3.7 kPa CO_2 at 4 °C. (B, C) PLS overlaid metabolites and Y-variables (Browning (BI), Storage Time, Position (In / Out)) loading plots illustrating the identified metabolites in the apple cortex tissue of the fruit stored at 2.5 kPa O_2/3.7 kPa CO_2 at 4 °C. In (A) and (B) the first 2 latent variables (LV1 and LV2) are shown, whereas in (C) the first and the fourth latent variables (LV1 and LV4) are shown because these were the most relevant for predicting the Y-variable (BI).

Fig. 5. Relative response ratios for the metabolites selected by the GLMSELECT procedure at *p*-0.05 significance level. The compounds are divided between Fig. 5A and Fig. 5B so that the graphs are made easier to read. The relative response ratio is obtained by dividing the metabolite peak area by the peak area of phenyl β-D-glucopyranoside, the internal standard, and by the sample fresh weight. Each data point represents an average of 4 samples with the error bars representing the standard errors of the means. Samples were taken from apples at harvest, and after 2 weeks (2W), 4 weeks (4W), 4 months (4M), and 8 months (8M) of CA storage at 2.5 kPa O_2/3.7 kPa CO_2 at 4 °C. Squares represent samples from the inner cortex and triangles represent samples from the outer cortex; at every time point, filled symbols

denote samples from apples with highest brown index, and open symbols denote samples from apples with lowest brown index.