**Metabolic profiling of apples from different production systems
before and after controlled atmosphere (CA) storage studied by
¹H high resolution-magic angle spinning (HR-MAS) NMR**

Martina Vermathen^{a}, Mattia Marzorati^{a1}, Gaëlle Diserens^b, Daniel Baumgartner^c, Claudia
Good^{c2}, Franz Gasser^c and Peter Vermathen^b*

^aDepartment of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, CH-3012
Bern, Switzerland.

^bDepartments of Clinical Research and Radiology, University of Bern, CH-3010 Bern,
Switzerland.

^cForschungsanstalt Agroscope Changins-Wädenswil ACW, Schloss 1, Postfach, 8820
Wädenswil, Switzerland.

E-mail addresses: (MM) caverian@gmail.com; (GD) Gaelle.Diserens@insel.ch; (DB)
daniel.baumgartner@acw.admin.ch; (CG) claudia.good@gmx.ch; (FG)
franz.gasser@agroscope.admin.ch; (PV) peter.vermathen@insel.ch

Corresponding Author:

^{*}(M.V.) Phone: +41 031 631 3948. E-mail: martina.vermathen@dcb.unibe.ch

¹ MM: current address: Nestlé S.A., Vevey PTC Konolfingen, Switzerland

² CG: current address: Bundesamt für Bevölkerungsschutz BABS, Zürich, Switzerland

Abstract

Determination of metabolic alterations in apples induced by such processes as different crop protection strategies or storage, are of interest to assess correlations with fruit quality or fruit disorders. Preliminary results proposed the metabolic discrimination of apples from organic (BIO), integrated (IP) and low-input (LI) production. To determine contributions of temporal metabolic developments and to define the type of metabolic changes during storage, ¹H High Resolution-Magic Angle Spinning (HR-MAS) NMR spectroscopy of apple pulp was performed before and after two time points of controlled atmosphere storage. Statistical analysis revealed similar metabolic changes over time for IP-, LI- and BIO-samples, mainly decreasing lipid and sucrose, and increasing fructose, glucose and acetaldehyde levels, which are potential contributors to fruit aroma. Across the production systems, BIO apples had consistently higher levels of fructose and monomeric phenolic compounds but lower levels of condensed polyphenols than LI and IP apples, while the remaining metabolites assimilated.

KEYWORDS

HR-MAS NMR spectroscopy; apples; apple cultivars; controlled atmosphere storage; multivariate analysis; principal component analysis; partial least squares discriminant analysis

1. Introduction

Golden Delicious apples are one of the most important commercial varieties in European apple production (Kellerhals, 2009). Owing to their favourable fruit properties like yellow colour, sweet taste and crisp white fruit flesh, they are not only very popular dessert apples but also often used as breeding stock for other apple varieties (Troggio et al., 2012). The growing interest in environmentally friendly fruit production imposes high demands on conditions of apple cultivation such as organic and integrated production systems (Simon, Brun, Guinaudeau, & Sauphanor, 2011). Storage of apples is an economically indispensable aspect in apple production in order to meet the demand of a year round supply with fresh fruits of high quality. Monitoring the development of apple metabolites during storage is of great interest to ascertain processes and learn potential reasons for storage disorders or fruit quality changes occurring during storage (Bekele, Beshir, Hertog, Nicolai, & Geeraerd, 2015; Jha, Rai, & Shrama, 2012). Specific components can be correlated to taste or nutritional benefits of the fruits (Hecke et al., 2006).

^1H NMR spectroscopy combined with multivariate statistical analyses has become an important and powerful tool for the metabolic profiling of processed and unprocessed foodstuffs. The metabolomic approach has the potential to identify marker compounds, which are indicative of food quality, geographical origin or specific interventions in food production (Monakhova, Kuballa, & Lachenmeier, 2013; Sobolev et al., 2015). In contrast to classical solution state NMR applied to juices or food extracts, HR-MAS NMR allows direct measurement of the original semi-solid food materials such as fruits or vegetables (Santos, Fonseca, Lião, Alcantara, & Barison, 2015).

Previously a preliminary HR-MAS NMR based metabolomic study of Golden Delicious apples has indicated that different cultivation systems could be distinguished due to their specific ^1H NMR metabolic profiles (Vermathen, Marzorati, Baumgartner, Good, &

Vermathen, 2013). However, in this study, apples were just derived from one time point, i.e. taken out of cold storage, and, in addition, apples exhibited a deviation in ripening stage despite same pick dates. Following up on these results, the impact of ripening stage at harvest, and the impact of storage on the cultivation-specific profile needs to be determined. This is particularly important as organically grown fruits can exhibit a different temporal evolution in fruit ripening, compared to growth conditions when applying pesticides for crop protection despite same climate, soil and storage conditions (Peck, Andrews, Reganold, & Fellman, 2006; Róth et al., 2007). In establishing biomarkers for a specific cultivation system, it remains to be clarified if distinguishing parameters emerge during fruit development on the tree, or during storage. Independent from the production system, it is also important to learn more about the temporal evolution of apple metabolites during storage in order to assess their role in fruit quality. Therefore, the aim of the current study was to analyse the metabolic profile of Golden Delicious apples derived from three different previously investigated production systems at three different time points: directly after harvest, and after two different durations of storage in controlled atmosphere (CA). HR-MAS NMR combined with multivariate statistical analysis was applied (i) to probe for storage time dependent metabolite alterations within each production system and (ii) to probe for production type dependent metabolite changes between the different production systems at each time point.

2. Materials and Methods

2.1 Fruit samples

Three groups of Golden Delicious apples, each grown under a different plant protection strategy, were used for the present work: i) Apples grown under organic growth conditions excluding synthetic pesticides and fertilizers (BIO); ii) apples cultivated by the integrated production system (IP) complying with economical production of high quality with priority to

ecologically safe methods; and iii) apples grown using a low-input (LI) plant protection strategy, i.e. combining organic and IP methods with a minimum use of pesticide to prevent apple scab. For IP and LI apple trees, chemically induced thinning was applied at the first bloom stage, while organically grown apple trees were mechanically thinned at the fruitlet stage. The apple trees were grown in the same orchards (N 47° E 8°) of the Agroscope grounds in Wädenswil, Switzerland, assuring the same soil and climate conditions during growth. Buffer zones were placed between the different production methods to avoid cross contaminations. All apples were picked at the optimal harvest maturity window on the same day (28.09.2011). From each of the three cultivation groups, BIO, IP, and LI, 18 apples with a similar ripening index were selected for this study resulting in a number of 54 apples (mean difference in absorbance (DA)-index 1.50 ± 0.05 , DA-definition: see 2.3). One third of the corresponding fruit samples, i.e. 18 apples (6 per group), were directly submitted to HR-MAS NMR analysis (pre-storage, P). The remaining two thirds (36 apples) were put into storage at a constant temperature of 1°C, constant humidity of 92% r.h. and a controlled atmosphere (CA) adjusted to 1% O₂ and 3 % CO₂ content. Eighteen apples were removed from storage after 146 days (S1) and the remaining 18 apples after 209 days (S2) representing commonly applied normal and long term storage times in agricultural farming practice for dessert apple production. Following removal from storage the corresponding fruits were submitted to HR-MAS NMR spectroscopy. All apples were measured in a mixed arbitrary order within a total time of 5 days and were stored at 4°C before use.

2.2 Chemicals

The deuterated solvent D₂O (D 99.9 %) containing 0.75 % TSP (Trimethyl-silyl-3-propionic acid) was obtained from Sigma-Aldrich (Buchs, Switzerland).

2.3 DA-Index

To ensure a uniform maturity of apples used for the present study the DA indices of apples to be picked and selected were determined using a DA meter (Sinteleia, Bologna, Italy, DA = difference in absorbance). The DA meter allows the non-destructive determination of apple maturity by measuring the chlorophyll content in the apple peel. This is achieved by recording the difference in absorption between two wavelengths at 670 nm and 720 nm. In this study, 4 measurements were performed for each apple and the DA indices are given as mean value (Supplementary Table 1).

2.4 Sample preparation

Prior to the HR-MAS NMR measurement each apple was cut along the equatorial plane and from the central cross section 3 pulp samples were taken as indicated in Fig. 1C. HR-MAS samples were prepared as described previously (Vermathen et al., 2013). Briefly, the 3 pulp samples obtained from each apple were prepared simultaneously to reduce the time of air exposure. For this, three HR-MAS rotors were used each to punch a piece of apple pulp with the aid of a rotor coat to ensure similar sample sizes, i.e. cylindrical tissue samples of 4 mm length and 3.0 mm diameter. After the addition of 10 μ L of the lock solvent D₂O (containing 0.75 % TSP) the rotors were sealed using a 50 μ L insert. A total of 162 samples (54 x 3) were submitted to HR MAS NMR.

2.5 ¹H High Resolution Magic Angle Spinning (HR MAS) NMR spectroscopy

The ¹H HR MAS NMR experiments were performed on a Bruker Avance II spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) operating at a resonance frequency of 500.13 MHz for ¹H. The instrument is equipped with a 4 mm HR MAS dual inverse ¹H / ¹³C probe with a Magic Angle gradient. All experiments were carried out at a magic angle (54.7°) spinning rate of 5 kHz and a calibrated temperature of 8°C.

The one-dimensional (1D) ^1H HR MAS NMR spectra were recorded with a 1D nuclear Overhauser enhancement spectroscopy (NOESY) sequence for water suppression (“*noesypr1d*” from the Bruker pulse-program library). Each 1D ^1H NMR spectrum was acquired applying 128 transients, a spectral width of 6002.4 Hz, a data size of 32 K points, an acquisition time of 2.73 s, and a relaxation delay of 3 s. All spectra were processed using the Bruker NMR software Topspin 3.2 patch level 3. The co-added free induction decays (FIDs) were exponentially weighted with a line broadening factor of 1.0 Hz, Fourier-transformed, phase and (polynomial) baseline corrected to obtain the ^1H NMR spectra. All spectra were calibrated to the resonance of the anomeric proton of α -glucose (doublet at 5.22 ppm). Peak assignments were performed according to our previously published results and supported by 2D correlation spectroscopy methods (^1H ^1H -COSY and ^1H ^1H -TOCSY). (Vermathen et al., 2013; Vermathen, Marzorati, Baumgartner, Good, & Vermathen, 2011; Vermathen, Marzorati, & Vermathen, 2012)

2.6 Data Analysis

2.6.1 Multivariate statistical analyses

Spectral bucketing and integration were performed using the Topspin software (version 3.2 patch level 3, Bruker BioSpin AG, Fällanden, Switzerland). Multivariate statistical analyses were performed using MATLAB R2012a (Mathworks[®]) and PLS-Toolbox 7.5.2 (Eigenvector Research, Inc. Wenatchee, Washington, USA). All ^1H HR-MAS NMR spectra were subdivided into individually sized buckets within the range between 0.7 and 9.7 ppm. A total of 100 buckets was selected on an overlay of all averaged spectra according to single resonances or regions of overlapping resonances. The average bucket width was 0.05 ppm (min 0.012 ppm, max 0.217 ppm). Noise regions and the region of the water resonance (4.68 – 5.176 ppm) were excluded. The selected bucket regions are summarized in Supplementary Table 2. Bucket integrals obtained as areas under the curve of the baseline corrected spectra

were imported into the Excel software (Version 14.0, Microsoft, Redmond, USA; see Supplementary Excel file). To account for differences in sample weight probabilistic quotient normalization (PQN) (Dieterle, Ross, Schlotterbeck, & Senn, 2006) was applied to the bucket integrals. The variables (buckets) were mean centred and scaled to unit variance, which results in equal weight of high and low intensity signals. For multivariate statistical analyses all spectra were grouped according to the cultivation method (BIO, LI, IP) of apple samples and according to the time of investigation, i.e. apples before and after 2 different storage time periods (P, S1, S2). This resulted in 9 subgroups with 18 spectra each. Data analysis was then performed on 2 subsets: (i) storage time study evaluating data over three time points (P, S1, S2) for each cultivation method and (ii) production type study comparing data of the three cultivation methods (BIO, LI, IP) for each time point. Principle component analysis (PCA), partial least squares (PLS) regression analysis, and partial least squares discriminant analyses (PLS-DA) were applied to the data to test for clustering and potential separation of the different classes. To visualize significant differences between classes ($p < 0.05$), the 83.4 % confidence intervals (Knol, Pestman & Grobbee, 2011) were calculated and displayed as ellipses in the PCA-, PLS- and PLS-DA score plots. For cross-validation of the PLS and PLS-DA models, the Venetian Blinds procedure with 7 splits and 1 sample per split was applied. Explained and predicted variation parameters (R^2 and Q^2) are summarized in Supplementary Tables 3 and 4. In order to avoid overfitting, permutation tests were performed applying 50 iterations. The resulting probabilities for model significance are also given in Supplementary Tables 3 and 4.

2.6.2 Univariate statistical analyses

For univariate data analysis Box-whisker plots were created for 8 selected metabolites using Excel (Version 14.0, Microsoft, Redmond, USA). The normalized integrals of one typical mostly isolated resonance region for each metabolite were displayed as mean (coloured dots),

second and third quartiles (boxes) and minimum and maximum values (whiskers, lines). In detail, the following resonances were analysed: 4.07-4.15 ppm fructose (Fru), 4.6-4.68 ppm β -glucose (β -Glc), 5.2-5.26 ppm α -glucose (α -Glc), 5.36-5.44 ppm sucrose (Suc), 6.07-6.15 ppm epicatechin (EC), 6.35-6.44 ppm chlorogenic acid (ChA), 6.97-7.0 ppm condensed polyphenols (PP), and 9.63-9.69 ppm acetaldehyde (Acal).

Single factor analysis of variance (ANOVA) was performed on the corresponding integral values to probe for significant differences between the means comparing either BIO, LI, and IP at a given time (P, S1, or S2) or comparing the different times P, S1, and S2 within each cultivar (BIO, LI, IP). Multiple testing correction was performed via false discovery rate (FDR) estimation using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995). Briefly, in this method the p-values are first ranked in ascending order and then each p-value is multiplied by a factor N/R with N being the number of comparisons and R being the assigned rank. Corrected values below 0.05 were considered as statistically significant.

3. Results and Discussion

3.1 *Ripening stage of apples*

Peel chlorophyll content and flesh maturation are correlated in Golden Delicious apples so that the DA-index provides an estimation of the ripening status for the whole fruit (Toivonen, Mostofi, Wiersma, & Hampson, 2011). To ensure a similar ripening stage for all 54 apples used in the present study, their DA indices were determined right before harvest along with the apple weight (Supplementary Table 1). The ripening stage was similar with DA values of 1.45-1.52 for organically grown (BIO), 1.46-1.56 for low-input (LI), and 1.47-1.58 for integrated (IP) apples, respectively. However, BIO fruits had a lower weight ($130.6\text{g} \pm 6.5\text{g}$) as compared to the apples obtained from LI ($232.8\text{g} \pm 38.1\text{g}$) and IP ($212.5\text{g} \pm 11.8\text{g}$) production systems. The impact of the production system on fruit yield, size and weight has

also been reported in previous studies comparing organically and conventionally grown apples where the latter had significantly higher fruit weight despite similar maturation degree and similar fruit quality parameters (Amarante, Steffens, Mafra, & Albuquerque, 2008; Roussos & Gasparatos, 2009). This has been attributed in part to the development of smaller cells in organically grown apple trees (Róth et al., 2007). In addition, it has been reported that the crop load, i.e. the number of fruits per tree, is negatively correlated with fruit weight (Meland, 2009). To achieve optimized crop loads, fruit thinning procedures are commonly applied, which differ depending on the production system. As opposed to chemically induced thinning at the first bloom stage, the mechanical thinning at the fruitlet stage in organically grown apple trees has been found to result in significantly higher crop loads (Meland, 2009) and may in turn also explain the smaller fruit weights in the current study.

3.2 *The ¹H HR-MAS NMR metabolic profile of apple pulp samples*

¹H HR-MAS NMR spectra of pulp samples from Golden Delicious apples have been previously described in detail (Vermathen et al., 2013; Vermathen, Marzorati, Baumgartner, Good, & Vermathen, 2011; Vermathen, Marzorati, & Vermathen, 2012). A representative spectrum is shown in Supplementary Figs. 1 and 2 with resonance assignments summarized in Supplementary Table 2. The apple pulp spectrum is dominated by intense resonances from sugar components between 3 and 6 ppm (Fig. 1A). Relatively low intense resonances in the aromatic region are mainly derived from phenolic compounds. In the aliphatic region, main contributions can be ascribed to organic and amino acids as well as lipid components. Notably, two relatively intense doublets overlapping with the broad lipid methylene resonance at 1.29 ppm (Lip) and at 1.32 ppm also with threonine (Thr) could be recently identified (Fig. 1B). The doublet at 1.32 ppm was assigned to paraldehyde (Par), (Monakhova et al., 2014; Vermathen et al., 2012) which typically seems to accompany acetaldehyde (Acal) as a cyclic trimer. Accordingly, Acal was also present in apple pulp with resonances at 2.23 ppm and

9.65 ppm. More recently, the origin of the doublet at 1.27 ppm could be identified as L-rhamnitol (Rha) (Tomita et al., 2015). This compound was suggested as a potential marker for the geographic origin of specific apple cultivars based on a metabolomic study.

3.3 Principle component analysis (PCA) of apple pulp spectra

For multivariate statistical analysis, all apple pulp HR-MAS NMR spectra were grouped for a production type comparison according to their different cultivation systems, BIO, LI and IP and for a storage time comparison according to the time point of analysis, i.e. pre-storage (P), or after 146 days (S1) and 209 days (S2) of CA storage. Principle component analysis (PCA) was applied to the data covering the whole spectral range for an unsupervised assessment of clustering. The PCA scores plots for a storage time comparison of all BIO, IP and LI apple samples are shown in Fig. 2A. The first two principal components PC-1 and PC-2 accounted together for 44.7 %, 44.7 %, and 53.1 % of the total variance for BIO, IP and LI, respectively. A clustering according to the time point of analysis could be observed for all three cultivation systems. Samples measured pre-storage (P) and after long storage (S2) appeared furthest apart for BIO and LI apples, however, with their confidence levels partly overlapping. For IP apples, the clustering was less pronounced.. Short storage (S1) samples partly overlapped with P and S2 samples – their PC scores were distributed in between them. The PCA score plots applied to BIO, LI and IP samples acquired at each of the three time points before (P) and after storage (S1, S2) are shown in Fig. 2B. The first two principal components PC-1 and PC-2 in sum accounted for 46.4 %, 49.4 %, and 43.3 % of the total variance for BIO, LI, and IP, respectively. At the time point P, the BIO apple samples clearly clustered and partly segregated from LI and IP apples. At both time points, S1 and S2 there was an overlap of all samples deriving from the different cultivation systems, despite a close clustering of BIO apples at S1, pointing towards their similarity. This suggests that the spectral profiles of pulp samples obtained from BIO, LI and IP apples equalize over time during storage.

277

278 **3.4 Storage time dependent metabolite alterations within each production system**

279 **3.4.1 Partial least squares (PLS) regression analysis of apple pulp spectra**

280 Partial least squares regression analysis (PLS) was applied to the data covering the whole
281 spectral range to probe the three cultivation systems for differences between pulp samples
282 depending on the time points. The PLS score plots for a storage time comparison within each
283 of the cultivation systems are shown in Fig. 3 displaying the first two latent variables LV-1
284 and LV-2. Together they accounted for 32.7 %, 40.1 % and 49.5 % of the total variance in
285 BIO, IP and LI apples, respectively. Pre-storage (P) shown in red and S2 post-storage samples
286 shown in blue were separated in PLS for BIO, LI, and IP apples mostly along LV-1. The
287 separation was significant for BIO and LI as indicated by their non-overlapping confidence
288 ellipses (Fig. 3). The three PLS plots looked similar, with P-samples having negative scores
289 and S2-samples positive scores on LV-1, while S1-samples (shown in green) distributed
290 between them with positive and negative scores on LV-1. According to the PLS loadings
291 (Supplementary Fig. 3), the main contribution to the separation between pre-storage (P) and
292 post-storage (S2) samples was derived from the aliphatic and the sugar region. Before storage
293 (P), in particular the lipids and sucrose were higher while after storage (S2) fructose (Fru), α -
294 and β -glucose (α -Glc, β -Glc), and acetaldehyde (Acal) were increased compared to pre-
295 storage. The metabolite contributions for discrimination of different times and the overall
296 pattern of the PLS-loadings for LV-1 were very similar for the three production systems
297 indicating that same physiological processes happen simultaneously during storage
298 independent of the individual production systems. Only in the aromatic region a different
299 trend was observed for IP apples, for which the load values on LV-1 indicate an increase of
300 condensed polyphenolic compounds (PP) after storage (S2), which was inverse for BIO
301 apples and less pronounced for LI apples.

302

3.4.2 *Lipid resonances*

The intense broad lipid resonances around 0.9, 1.3 and 1.6 ppm (Fig. 1B), which decreased during storage according to PLS analysis (Fig. 3 and Supplementary Fig. 3) most likely derive from mobile lipids present in droplets or from free fatty acids. The presence of lipid bodies or droplets in apple pulp has been reported (Wang & Faust, 1992) and the fatty acids, mainly saturated, linoleic- and linolenic acid, have been shown to increase during fruit maturation (Bartley, Stoker, Martin, Hatfield, & Knee, 1985). The latter are important precursors for apple volatile aroma components such as short chain alkyl esters or aldehydes (Dixon & Hewett, 2000). In accordance with our finding, linoleic- and linolenic acid were found to strongly decrease in Golden Delicious apples during ultra-low oxygen (ULO) storage (Harb, Streif, & Bangerth, 1994). Thus, reduced fatty acid synthesis and/or degradation of fatty acids during CA storage of apples have been suggested to be responsible for reduced aroma production (Brackmann, Streif, & Bangerth, 1993; Harb et al., 1994). Similarly, in our study, the storage time dependent changes observed for lipid resonances in BIO, LI and IP apples may also be due to lipid degradation, possibly via β -oxidation, during storage and may possibly play an important role as apple quality parameters.

3.4.3 *Univariate analysis of selected resonance integrals*

For the sugar components and the components deriving from the spectral region between 6 and 10 ppm, the normalized integrals of representative resonances were further analyzed. The corresponding mean values, medians and quartiles are displayed as box-whisker plots in Fig. 4. ANOVA and subsequent multiple testing correction was applied to the bucket integrals to probe for significant differences within each cultivar over time and between cultivars at each time.

During storage, α - and β -glucose and fructose increased in all three production systems, which was significant except for the Fru increase in IP. In all apple samples there was a

simultaneous significant decrease in sucrose levels during storage. A similar trend for sucrose and glucose during CA storage has been reported for Jonagold apples (Róth et al., 2007). The inverse correlation between sucrose on one hand and glucose and fructose on the other hand is a typical result of enzymatic conversion of sucrose to glucose and fructose during post-harvest fruit ripening (Yamaki, 2010). The enzymatic formation of fructose from sorbitol seems to contribute in addition to increasing fructose levels in Golden Delicious, while in Jonagold apples fructose levels remained unchanged during storage and sorbitol was found to be increasing (Róth et al., 2007).

For the phenolic compounds there were no significant time dependent changes before and after storage (S1 and S2) within each cultivar except for epicatechin (EC) in BIO apples (marked with a diamond “♦” in Fig. 4E).

Acetaldehyde (Acal) gave rise to a characteristic isolated quartet resonance at 9.66 ppm and exhibited increased levels over time during storage for each of the cultivation systems, which was significant for BIO and LI (Fig. 4H). The Acal levels were similar between BIO, IP and LI at each time and also similarly, the increase was most pronounced between times S1 and S2. Therefore, the simultaneous formation of Acal seems to be independent of the production system. Acal is a naturally occurring metabolite in apples, which is formed during anaerobic respiration and increases during ripening (Dixon et al., 2000; Pesis, 2005). While it is instable in apple juice due to oxidation or polymerization (Monakhova et al., 2014; Vermathen et al., 2011) it is readily detectable by HR-MAS NMR in apple pulp. Golden Delicious apples were found to have relative high Acal levels as compared to other cultivars (Vermathen et al., 2011). Acal plays an important role as fruit quality parameter not just as a precursor of aroma volatiles but also as a potential polymerizing agent capable to reduce astringency of the fruit (Pesis, 2005). On the other hand, the formation of excessive amounts of Acal and ethanol (Eth) as fermentation products can cause an off-flavor (Pesis, 2005), as it occurs for example in the case of carbon dioxide injury, a storage disorder in apples caused by high CO₂ levels

and related to internal browning (Vandendriessche, Schäfer, Verlinden, Humpfer, Hertog, & Nicolai, 2013). This indicates the importance of well-balanced metabolite concentrations for sensory properties of apples and how this balance is related to optimized CA storage conditions.

3.5 Production type dependent metabolite alterations at each time point

3.5.1 Partial least squares discriminant analysis (PLS-DA) scores plots for a production type comparison

The PLS-DA scores plots for a production type comparison of the 3 different cultivation systems at each time point pre- and post-storage are shown in Fig. 5A displaying the first two latent variables LV-1 and LV-2. They accounted for 43.5 %, 41.2 % and 35.7 % of the total variance at time points P, S1 and S2, respectively. At each time point, BIO samples had negative scores on LV-1 while IP and LI samples predominantly had positive scores on LV-1. The discrimination of BIO apples from the remaining two cultivation systems was best right after harvest (time P) and became less during storage over time. This suggests that the metabolic profile of BIO apples aligns with the profile of IP and LI apples during storage. The discrimination of BIO apples at time P is in agreement with our previous results obtained for BIO, IP and LI apples— after 127 days of cold storage—where BIO apples could likewise be separated from IP and LI apples based on PLS-DA (Vermathen et al., 2013).

To probe for metabolite contributions to the separation in PLS for all times (P, S1, S2), the PLS load values were analyzed. The corresponding loading plots for LV-1 broken down into the aliphatic, sugar and aromatic spectral regions are shown in Fig. 5B. Buckets or integral regions with high positive or negative load values beyond an arbitrary value of > 0.1 or < -0.1 were considered strong contributors.

In the aliphatic region all lipid resonances had strong contributions on positive PLS scores, in particular at P and S1 meaning that they were increased in LI and IP samples as compared to

BIO (because of negative scores on LV-1 for BIO; Fig. 5A). Likewise, high positive load values were found for the amino acids asparagine (Asn), alanine (Ala), valine (Val) as well as for isoleucine (Ile) and leucine (Leu), which partly overlap with the broad lipid resonances. While at S2 the lipid resonances had less influence, the amino acids had still high loadings. Similar to the role of fatty acids some amino acids like Ile and Val are important precursors for aroma production. The accumulation of Ile and Ala in apples under CA conditions has been previously described and has been linked to alterations in the tricarboxylic acid cycle (TCA) under hypoxic conditions (Bekele et al., 2015). Contributions from sugar components are best indicated from the anomeric spectral region (buckets 60 – 70, corresponding to 4.5 – 6 ppm) where there is less signal overlap. Before storage, α - and β -glucose as well as fructose were increased in BIO apples as indicated by their high negative load values. After storage at S1 and S2 only the fructose contribution remained high while sucrose had high positive load values at S2. In the region between 6 and 10 ppm (buckets 71 – 100), condensed polyphenols (PP) have strong contributions to positive scores on LV-1 at all times indicating increased levels in IP and LI samples. In contrast, BIO samples were higher in the low-molecular weight phenolic compounds chlorogenic acid (ChA), epicatechin (EC) and *p*-coumaric acid (pC), in particular before storage (P). After storage (S1, S2) these contributions became less pronounced.

3.5.2 Univariate analysis of selected resonance integrals

While the temporal alterations in sugar components were running largely parallel in BIO, IP and LI apples (Fig. 4A-D), across the production systems only fructose was clearly higher in BIO apples at each time point compared to other samples (Fig. 4A). The contribution of increased fructose in distinguishing BIO from IP and LI apples is in accordance with our previous finding (Vermathen et al., 2013). However, the previously found contributions from higher sucrose and lower α - and β -glucose levels in BIO apples compared to IP and LI could

not be reproduced in the current study. This suggests that these differences were most likely due to different stages in temporal developments (i.e. different ripening stages despite equal pick times) considering the storage time dependent trends of the current study shown in Figure 4A-D. A temporal offset in maturity for IP and LI, i.e. comparing BIO at time P with IP and LI at time S1, would account for the previously found differences in Suc, α - and β -Glc, while high fructose levels seem to be specific throughout for organically grown Golden Delicious apples. Fructose accumulation may be due either to the enzymatic conversion of sorbitol, which is translocated from leaves during fruit development, or to a decrease in fructose consumption, e.g. by reduced fructokinase activity (Yamaki, 2010). These processes may be more expressed in BIO apples as compared to IP and LI fruits leading to higher fructose levels.

When comparing the phenolic compounds among the cultivation systems at the same time (Figure 4E-G), EC and ChA were significantly different before storage (P), while after short (S1) and long storage (S2) there were no significant differences left. Condensed polyphenol (PP) levels remained constantly low in BIO samples at all times, while in IP and LI samples PP levels were higher and particularly in LI exhibited a high variability. At the same time, ChA and EC (pre-storage only) were elevated in BIO as compared to IP and LI apples. This is also visualized in the averaged ^1H HR-MAS NMR spectra that were created from all spectra for each cultivar (Fig. 6). The phenolic compounds EC, ChA and pC give rise to sharp resonances while the condensed polyphenols exhibit broad peaks typical for oligomeric higher molecular weight species. The finding of increased ChA and lower PP levels in BIO compared to IP and LI apples is in agreement with our previous finding (Vermathen et al., 2013) and suggests different conversion rates of monomeric phenols like EC and ChA into oligo- or polymers (PP). It also supports the importance of these compounds as potential markers in the aromatic region for differentiation between the different production systems of Golden Delicious apples right after harvest or short storage. Similar results of enhanced

uncondensed polyphenols in the pulp of organically grown apples as compared to integrated production have been reported (Hecke et al., 2006; Stracke, Rüfer, Weibel, Bub, & Watzl, 2009; Vanzo, Jenko, Vrhovsek, & Stopar, 2013; Veberic, Trobec, Herbinger, Hofer, Grill, & Stampar, 2005) and it was concluded that this up-regulation may be related to exogenous factors of the organic production system, such as a response to stress due to a lack of pesticides or fertilizers (Vanzo et al., 2013; Veberic et al., 2005). On the other hand, Stracke et al (2009) concluded that the yearly climate had a larger impact on the polyphenol content than the production system (Stracke et al., 2009). However, this explanation does not hold for the apples investigated here because they were grown under the same climatic conditions. In contrary to our finding, in one of these studies, condensed proanthocyanidins were also found to be increased in organically grown Golden Delicious apples while in other apple varieties, which were scab resistant, this was not the case and a correlation to scab resistance was postulated (Vanzo et al., 2013). This was in contrast to a previous study reporting higher levels of both, monomer and oligomer phenols in integrated apple pulp (Golden Delicious) as compared to organic (Chinnici, Bendini, Gaiani, & Riponi, 2004). One reason for the discrepancies between these and the present study may be the different analytical approaches. The difficulty in quantifying the highly reactive polymeric phenolic compounds depending on extraction procedures and solubility has been addressed in the literature (Cheynier, 2005; Ignat, Volf, & Popa, 2011). The direct application of HR-MAS NMR to apple pulp has the advantage that it does not require extraction or isolation procedures so that losses due to low solubility or chemical modification like oxidation can be minimized. However, the method is limited with respect to very high molecular weight compounds which become NMR-invisible due to strong signal broadening.

Besides their importance for organoleptic properties (Cheynier, 2005), phenolic compounds in apples are considered important nutritional components with potential health benefits due to their antioxidant activity as radical scavengers (Carbone, Giannini, Picchi, Lo Scalzo, &

Cecchini, 2011; Chinnici et al., 2004). As opposed to the monomeric compounds such as EC and ChA which were found to be higher in BIO, the bioavailability of their condensed polyphenolic counterparts, mainly oligomeric proanthocyanidins, is much lower (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). Nevertheless, beneficial effects are also reported for condensed polyphenols, e.g. on the vascular function (de Pascual-Teresa, Moreno, & Garcia-Viguera, 2010).

In order to clarify the contradictory findings of condensed polyphenolic compounds quantifications reported in the literature, studies directly comparing different analytical methods applied to the same samples would be helpful. In addition, the reproducibility over several harvesting seasons would be required to use the PP amount as reliable biomarker.

4. Conclusion

In the current study, apples derived from organic (BIO), low-input (LI), and integrated (IP) production systems were analyzed before and after two time points of controlled atmosphere (CA) storage by direct ¹H HR-MAS NMR spectroscopy of the apple pulp. While CA storage is aimed at suppressing metabolism, ethylene production and respiration, metabolic processes are still ongoing. Storage time dependent changes of the metabolic profile were found to run parallel in all apple samples and seemed to be mostly independent of the production system. Most pronounced alterations during CA storage were decreasing lipid and sucrose levels and increasing fructose, α - and β -glucose and acetaldehyde levels. The data indicate the extent and direction of the metabolic drift taking place under CA conditions.

Irrespective of these concurrent changes, the metabolic profile of the different production systems converged during CA storage. Accordingly, the discrimination of BIO apples was most pronounced before storage. Those distinguishing features that disappeared during storage were most likely due to different maturation stages that assimilate over time.

However, the consistent higher levels of monomeric phenolic compounds (ChA, EC) and fructose, as well as lower amounts of condensed polyphenols and some amino acids seemed to be rather typical for BIO apples and are most likely linked to the organic cultivation system. The current study demonstrates the importance of the time point of analysis for classification of apples according to production system. In addition, the study shows that the direct application of HR-MAS NMR to apple tissue is a suitable method to gain insights into the metabolic processes taking place during storage without the need of extraction or other laborious sample preparation procedures. The knowledge of metabolite alterations including sugar components and aroma precursors may help optimize processes aimed at avoiding storage disorders and yielding high fruit quality.

Abbreviations Used

Acal, acetaldehyde; α -Glc, α -glucose; Ala, alanine; ANOVA, analysis of variance; Asn, asparagine; β -Glc, β -glucose; BIO, organic production; CA, controlled atmosphere; ChA, chlorogenic acid; DA, difference in absorbance; EC, epicatechin; Eth, ethanol; FDR, false discovery rate; FID, free induction decay; Fru, fructose; HR-MAS, high resolution magic angle spinning; Ile, isoleucine; IP, integrated production; Leu, leucine; LI, low-input production; Lip, lipids; LV, latent variable; NMR, nuclear magnetic resonance; P, pre-storage; Par, paraldehyde; pC, *p*-coumaric acid; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; PP, condensed polyphenolic compounds; Rha, L-rhamnitol; S1, 146 days storage; S2, 209 days storage; Suc, sucrose; TCA, tricarboxylic acid cycle; TSP, trimethyl-silyl-3-propionic acid; ULO, ultra-low oxygen; Val, valine

Conflict of interest

The authors declare no conflict of interest.

510

511 **Acknowledgements**

512

513 Financial support was obtained from the Swiss National Science Foundation (SNF) for the
514 purchase of the HR-MAS probe, grant # 206021-128736 (M.V., P.V.). We thank Julia
515 McFarland for proofreading of the manuscript.

516

517 **Supplementary data**

518 Supplementary data associated with this article can be found in the online version.

519

520

521

References

- Amarante, C. V. T. d., Steffens, C. A., Mafra, Á. L., & Albuquerque, J. A. (2008) Yield and fruit quality of apple from conventional and organic production systems. *Pesquisa Agropecuária Brasileira*, 43, 333-340.
- Bartley, I. M., Stoker, P. G., Martin, A. D. E., Hatfield, S. G. S., & Knee, M. (1985) Synthesis of aroma compounds by apples supplied with alcohols and methyl esters of fatty acids. *Journal of the Science of Food and Agriculture*, 36, 567-574.
- Bekele, E. A., Beshir, W. F., Hertog, M. L. A. T., Nicolai, B. M., & Geeraerd, A. H. (2015) Metabolic profiling reveals ethylene mediated metabolic changes and a coordinated adaptive mechanism of 'Jonagold' apple to low oxygen stress. *Physiologia Plantarum*, 155, 232-247.
- Benjamini, Y. & Hochberg, Y. (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, 57, 289-300.
- Brackmann, A., Streif, J., & Bangerth, F. (1993) Relationship between a reduced aroma production and lipid metabolism of apples after long-term controlled-atmosphere storage. *Journal of the American Society for Horticultural Science*, 118, 243-247.
- Carbone, K., Giannini, B., Picchi, V., Lo Scalzo, R., & Cecchini, F. (2011) Phenolic composition and free radical scavenging activity of different apple varieties in relation to the cultivar, tissue type and storage. *Food Chemistry*, 127, 493-500.
- Cheyrier, V. (2005) Polyphenols in foods are more complex than often thought. *American Journal of Clinical Nutrition*, 81, 223S-229S.
- Chinnici, F., Bendini, A., Gaiani, A., & Riponi, C. (2004) Radical scavenging activities of peels and pulps from cv. Golden Delicious apples as related to their phenolic composition. *Journal of Agricultural and Food Chemistry*, 52, 4684-4689.

548 de Pascual-Teresa, S., Moreno, D. A., & Garcia-Viguera, C. (2010) Flavanols and
 549 anthocyanins in cardiovascular health: A review of current evidence. *International*
 550 *Journal of Molecular Sciences*, *11*, 1679-1703.

551 Dieterle, F., Ross, A., Schlotterbeck, G., & Senn, H. (2006) Probabilistic quotient
 552 normalization as robust method to account for dilution of complex biological
 553 mixtures. Application in ¹H NMR metabonomics. *Analytical Chemistry*, *78*, 4281-
 554 4290.

555 Dixon, J. & Hewett, E. W. (2000) Factors affecting apple aroma/flavour volatile
 556 concentration: A Review. *New Zealand Journal of Crop and Horticultural Science*,
 557 *28*, 155-173.

558 Harb, J., Streif, J., & Bangerth, F. (1994) Synthesis of aroma compounds by controlled
 559 atmosphere (CA) stored apples supplied with aroma precursors: alcohols, acids and
 560 esters. *Acta Horticulturae* 142-149.

561 Hecke, K., Herbinger, K., Veberič, R., Trobec, M., Toplak, H., Štampar, F., Keppel, H., &
 562 Grill, D. (2006) Sugar-, acid- and phenol contents in apple cultivars from organic and
 563 integrated fruit cultivation. *European Journal of Clinical Nutrition*, *60*, 1136-1140.

564 Ignat, I., Volf, I., & Popa, V. I. (2011) A critical review of methods for characterisation of
 565 polyphenolic compounds in fruits and vegetables. *Food Chemistry*, *126*, 1821-1835.

566 Jha, S., Rai, D. R., & Shrama, R. (2012) Physico-chemical quality parameters and overall
 567 quality index of apple during storage. *Journal of Food Science and Technology*, *49*,
 568 594-600.

569 Kellerhals, M. (2009). Introduction to Apple (*Malus × domestica*). In K. M. Foltá & S. E.
 570 Gardiner (Eds.), *Genetics and Genomics of Rosaceae, Plant Genetics and Genomics:*
 571 *Crops and Models* 6 (pp. 73-84). New York: Springer.

572 Knol, M.J., Pestman, W.R., & Grobbee, D.E. (2011) The (mis)use of overlap of confidence
 573 intervals to assess effect modification. *European Journal of Epidemiology*, 26, 253-
 574 254.

575 Manach, C., Williamson, G., Morand, C., Scalbert, A., & Remesy, C. (2005) Bioavailability
 576 and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies.
 577 *American Journal of Clinical Nutrition*, 81, 230S-242S.

578 Meland, M. (2009) Effects of different crop loads and thinning times on yield, fruit quality,
 579 and return bloom in *Malus × domestica* Borkh. 'Elstar'. *The Journal of Horticultural*
 580 *Science and Biotechnology*, 84, 117-121.

581 Monakhova, Y., Kuballa, T., & Lachenmeier, D. W. (2013) Chemometric methods in NMR
 582 spectroscopic analysis of food products. *Journal of Analytical Chemistry*, 68, 755-766.

583 Monakhova, Y., Schütz, B., Schäfer, H., Spraul, M., Kuballa, T., Hahn, H., & Lachenmeier,
 584 D. (2014) Validation studies for multicomponent quantitative NMR analysis: the
 585 example of apple fruit juice. *Accreditation and Quality Assurance*, 19, 17-29.

586 Peck, G. M., Andrews, P. K., Reganold, J. P., & Fellman, J. K. (2006) Apple orchard
 587 productivity and fruit quality under organic, conventional, and integrated
 588 management. *HortScience*, 41, 99-107.

589 Pesis, E. (2005) The role of the anaerobic metabolites, acetaldehyde and ethanol, in fruit
 590 ripening, enhancement of fruit quality and fruit deterioration. *Postharvest Biology and*
 591 *Technology*, 37, 1-19.

592 Róth, E., Berna, A., Beullens, K., Yarramraju, S., Lammertyn, J., Schenk, A., & Nicolaï, B.
 593 (2007) Postharvest quality of integrated and organically produced apple fruit.
 594 *Postharvest Biology and Technology*, 45, 11-19.

595 Roussos, P. A. & Gasparatos, D. (2009) Apple tree growth and overall fruit quality under
 596 organic and conventional orchard management. *Scientia Horticulturae*, 123, 247-252.

597 Santos, A. D. C., Fonseca, F. A., Lião, L. M., Alcantara, G. B., & Barison, A. (2015) High-
 598 resolution magic angle spinning nuclear magnetic resonance in foodstuff analysis.
 599 *Trends in Analytical Chemistry*, 73, 10-18.

600 Simon, S., Brun, L., Guinaudeau, J., & Sauphanor, B. (2011) Pesticide use in current and
 601 innovative apple orchard systems. *Agronomy for Sustainable Development*, 31, 541-
 602 555.

603 Sobolev, A. P., Mannina, L., Proietti, N., Carradori, S., Daglia, M., Giusti, A. M., Antiochia,
 604 R., & Capitani, D. (2015) Untargeted NMR-based methodology in the study of fruit
 605 metabolites. *Molecules*, 20, 4088-4108.

606 Stracke, B. A., Rüfer, C. E., Weibel, F. P., Bub, A., & Watzl, B. (2009) Three-year
 607 comparison of the polyphenol contents and antioxidant capacities in organically and
 608 conventionally produced apples (*Malus domestica* Bork. Cultivar 'Golden Delicious').
 609 *Journal of Agricultural and Food Chemistry*, 57, 4598-4605.

610 Toivonen, P. M. A., Mostofi, Y., Wiersma, P., & Hampson, C. (2011) Evaluation of non-
 611 destructive instruments for assessing apple maturity and quality: 2011 Results.
 612 [http://www.agr.gc.ca/eng/science-and-innovation/results-of-agricultural-](http://www.agr.gc.ca/eng/science-and-innovation/results-of-agricultural-research/other-science-related-publications-2012/evaluation-of-non-destructive-instruments-for-assessing-apple-maturity-and-quality-2011-results/)
 613 [research/other-science-related-publications-2012/evaluation-of-non-destructive-](http://www.agr.gc.ca/eng/science-and-innovation/results-of-agricultural-research/other-science-related-publications-2012/evaluation-of-non-destructive-instruments-for-assessing-apple-maturity-and-quality-2011-results/)
 614 [instruments-for-assessing-apple-maturity-and-quality-2011-results/](http://www.agr.gc.ca/eng/science-and-innovation/results-of-agricultural-research/other-science-related-publications-2012/evaluation-of-non-destructive-instruments-for-assessing-apple-maturity-and-quality-2011-results/) Accessed
 615 24.03.14.

616 Tomita, S., Nemoto, T., Matsuo, Y., Shoji, T., Tanaka, F., Nakagawa, H., Ono, H., Kikuchi,
 617 J., Ohnishi-Kameyama, M., & Sekiyama, Y. (2015) A NMR-based, non-targeted
 618 multistep metabolic profiling revealed L-rhamnitol as a metabolite that characterised
 619 apples from different geographic origins. *Food Chemistry*, 174, 163-172.

620 Troggio, M., Gleave, A., Salvi, S., Chagné, D., Cestaro, A., Kumar, S., Crowhurst, R. N., &
 621 Gardiner, S. E. (2012) Apple, from genome to breeding. *Tree Genetics & Genomes*, 8,
 622 509-529.

623 Vandendriessche, T., Schäfer, H., Verlinden, B. E., Humpfer, E., Hertog, M. L. A. T., &
624 Nicolai, B. M. (2013) High-throughput NMR based metabolic profiling of Braeburn
625 apple in relation to internal browning. *Postharvest Biology and Technology*, 80, 18-24.

626 Vanzo, A., Jenko, M., Vrhovsek, U., & Stopar, M. (2013) Metabolomic profiling and
627 sensorial quality of 'Golden Delicious', 'Liberty', 'Santana', and 'Topaz' apples grown
628 using organic and integrated production systems. *Journal of Agricultural and Food*
629 *Chemistry*, 61, 6580-6587.

630 Veberic, R., Trobec, M., Herbinger, K., Hofer, M., Grill, D., & Stampar, F. (2005) Phenolic
631 compounds in some apple (*Malus domestica* Borkh) cultivars of organic and
632 integrated production. *Journal of the Science of Food and Agriculture*, 85, 1687-1694.

633 Vermathen, M., Marzorati, M., Baumgartner, D., Good, C., & Vermathen, P. (2013). Impact
634 of different cultivation methods on the metabolic profile of apples studied by ¹H HR-
635 MAS NMR spectroscopy. In J.van Duynhoven, H.Van As, P.S.Belton, & G.A.Webb
636 (Eds.), *Magnetic Resonance in Food Science: Food for Thought* (pp. 193-200). RSC
637 Publishing.

638 Vermathen, M., Marzorati, M., Baumgartner, D., Good, C., & Vermathen, P. (2011)
639 Investigation of different apple cultivars by high resolution magic angle spinning
640 NMR. A feasibility study. *Journal of Agricultural and Food Chemistry*, 59, 12784-
641 12793.

642 Vermathen, M., Marzorati, M., & Vermathen, P. (2012) Exploring high-resolution magic
643 angle spinning (HR-MAS) NMR spectroscopy for metabonomic analysis of apples.
644 *Chimia*, 66, 747-751.

645 Wang, S. Y. & Faust, M. (1992) Variation in lipid-composition of apples in relation to
646 watercore. *Journal of the American Society for Horticultural Science*, 117, 829-833.

647 Yamaki, S. (2010) Metabolism and accumulation of sugars translocated to fruit and their
648 regulation. *Journal of the Japanese Society for Horticultural Science*, 79, 1-15.

Figure Captions

Figure 1.

A: Averaged ^1H HR-MAS NMR spectrum of BIO apples (S2); **B:** expansion displaying the aliphatic spectral region between 0.7 and 2.3 ppm; **C:** sketch illustrating the sampling (blue dots) for HR-MAS NMR from the apple cross section, where the five carpels and the surrounding vascular bundles (indicated as grey dots) are shown.

Figure 2.

PCA scores plots **A:** comparing for each cultivation method the 3 different time points, i.e. pre-storage (P) and after storage (S1, S2) and **B:** comparing the cultivation methods BIO, IP, and LI for each time point (P, S1, S2). 83.4% confidence ellipses are shown in dotted lines.

Figure 3.

PLS plots for each of the cultivation methods at 3 different time points, i.e. pre-storage (P) and after storage (S1, S2). 83.4% confidence ellipses are shown in dotted lines. Cross-validation parameters are given in Supplementary Table 3.

Figure 4.

Box-whisker plots for selected integrals from sugars (**A-D**) and from the aromatic region (**E-H**) for the 3 different cultivation methods at each time point pre- (P) and post-storage (S1, S2). Significant differences between integrals according to ANOVA and multiple testing corrections ($p < 0.05$) are marked with ♦ (storage time comparison) and with * (production type comparison).

Figure 5.

A: PLS-DA plots for each time point pre- (P) and post-storage (S1, S2) of the 3 different cultivation methods. 83.4% confidence ellipses are shown in dotted lines. Cross-validation parameters are given in Supplementary Table 4. **B:** PLS-loadings of single buckets for the first PLS component (LV-1) comparing pre- (P) and post-storage (S1, S2) samples of the 3

674 different cultivation methods. Red lines indicate arbitrary limits of LV-1 values <-0.1 and
675 >0.1 .

676 **Figure 6.**

677 Aromatic region of averaged ^1H HR-MAS spectra for BIO, IP, and LI apples after harvest (P).

A PCA - Storage time

B PCA - Production type

PLS - Storage time

revised Figure 6