IMPACT OF DIFFERENT CULTIVATION METHODS ON THE METABOLIC PROFILE OF APPLES STUDIED BY 1H HR-MAS NMR SPECTROSCOPY

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1 INTRODUCTION

The cultivation of dessert apples has to meet the consumer's demand for high fruit quality and a sustainable mostly residue-free production while ensuring a competitive agricultural productivity.[1,2] It is therefore of great interest to know the impact of different cultivation methods on the fruit quality. Fruit quality attributes like flavor, health properties and texture, in turn largely depend on the chemical composition and the interplay of different specific chemical components. In this study we present the application of ^{1}H High Resolution Magic Angle Spinning (HR-MAS) NMR spectroscopy on apple tissue to analyze the metabolic profiles of apples grown under 3 different cultivation methods, i.e. organic, low-input and conventional integrated methods. Previously, we have demonstrated the feasibility of HR-MAS NMR directly performed on apple tissue as analytical tool for metabonomic studies.[3] HR-MAS NMR requires only a minimum of sample preparation making it possible to study the native, mostly uninfluenced chemical composition of the fruit pulp. Combined with multivariate statistical analysis, this method provides detailed information on the metabolic profile and potential markers for discrimination of the cultivation methods in addition to the conventionally collected fruit quality parameters.

2 METHODS AND RESULTS

2.1 Experimental design

2.1.1 Fruit samples. Golden Delicious apples were grown applying three different plant protection strategies. These included organic growth conditions (BIO) and the commonly used integrated (IP) production system, which allows the application of e.g. pesticides, though integrates in contrast to conventional agriculture natural resources and regulation mechanisms to achieve maximum replacement.[4] Besides, a low-input (LI) plant protection strategy was applied with a minimum use of pesticide focusing on apple scab, a fungal plant disease to which Golden Delicious apples are susceptible (i.e. a production system in between IP and Bio production). All apple trees were situated on orchards of the Agroscope grounds assuring same soil and climate conditions during growth. Apples of each cultivation method were picked within the optimal harvest window for assuring good fruit quality after storage. The corresponding fruits had a ripening index according to Streif[5] between 0.08 and 0.12 based on firmness, starch and soluble solids levels. The

apples were stored at a temperature of 1°C in a normal atmosphere for 127 days. After removal from storage the apples were submitted to HR-MAS NMR spectroscopy in a mixed order.

2.1.2 Fruit quality analysis. Fruit firmness, content of soluble solids, and titratable acidity were determined for 20 apples from each group both, after harvest and after removal from storage. In addition, the starch index was measured after harvest by the starch-iodine staining test.[6] For fruit firmness measurements, the Pimprenelle automatic penetrometer was used. The content of soluble solids (mainly sugar) and acid was determined by Fourier Transform Infrared Spectroscopy (FTIR) of juice samples each pooled from 5 apples (n = 4 for each group).

2.1.3 HR-MAS NMR measurements. A total of 15 apples, i.e. 5 per each of the three cultivation methods BIO, IP, and LI, were investigated by ^{1}H HR-MAS NMR spectroscopy. To account for potential intra-apple heterogeneity, 5 samples were taken along a circular line from the equatorial cross section of each apple as shown in Figure 1. The pulp samples were directly punched with the MAS rotor with a diameter of 4mm. To ensure similar sample sizes a home-made rotor coat was used for achieving a constant punching depth of 4mm (see Figure 1). After gently pushing the fruit pulp to the bottom of the rotor, 10 µL of D_2O containing 0.75% of TSP as lock solvent and internal reference were added. All five samples were immediately taken using 5 rotors simultaneously to reduce the time of air exposure for the cut apple surface. Rotors were closed with a 50µL insert, screw and cap.

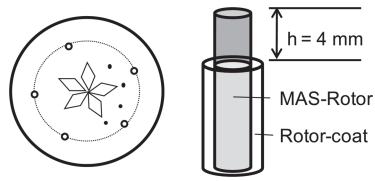

Figure 1 *Sketch of apple cross section showing the distribution of samples (open circles)taken for HR-MAS NMR (left); Sketch of MAS rotor protruding 4mm from the rotor coat for homogenous sampling (right).*

^{1}H HR-MAS NMR measurements were performed on a Bruker AV II 500 MHz system equipped with a 4mm HR-MAS probe. A rotor spinning speed of 5 kHz at the magic angle (54.7°) was applied at a temperature of 8°C. The ^{1}H NMR spectra were recorded with water presaturation using the "*noesypr1d*" pulse sequence of the Bruker pulse program library. For each spectrum, 128 scans were averaged with 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. Spectral postprocessing included exponential multiplication of the FIDs with a line broadening factor of 1.0 Hz, Fourier transformation, phasing, polynomial baseline correction and calibration to the TSP-signal (0 ppm).

2.1.4 Statistical Analysis. A total of 75 ^{1}H HR-MAS NMR spectra were analyzed by means of principle component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) using the Bruker AMIX software. The five spectra of one apple (IP) were clearly identified in the PCA as outliers. These samples turned out to be measured at an incorrect temperature setting leaving a total of 70 ^{1}H NMR spectra. The spectra were bucketed between 0.75 and 9.7 ppm excluding noise regions and the residual water signal (4.7-5.2 ppm). Excluded regions are indicated in Figure 2 as grey boxes. The bucket width

was 0.05 ppm. Two regions containing the malic acid signals each were combined into one single bucket (2.4-3 ppm and 4.25-4.6 ppm) due to pH-dependent shifts. This resulted in a total of 94 buckets which were normalized applying probabilistic quotient normalization (PQN)[7] and scaled to unit variance.

Differences between the mean fruit quality parameters of the three cultivation groups were analysed by using analysis of variance (ANOVA) and pairwise comparisons were performed by the post hoc Duncan's new multiple range test.

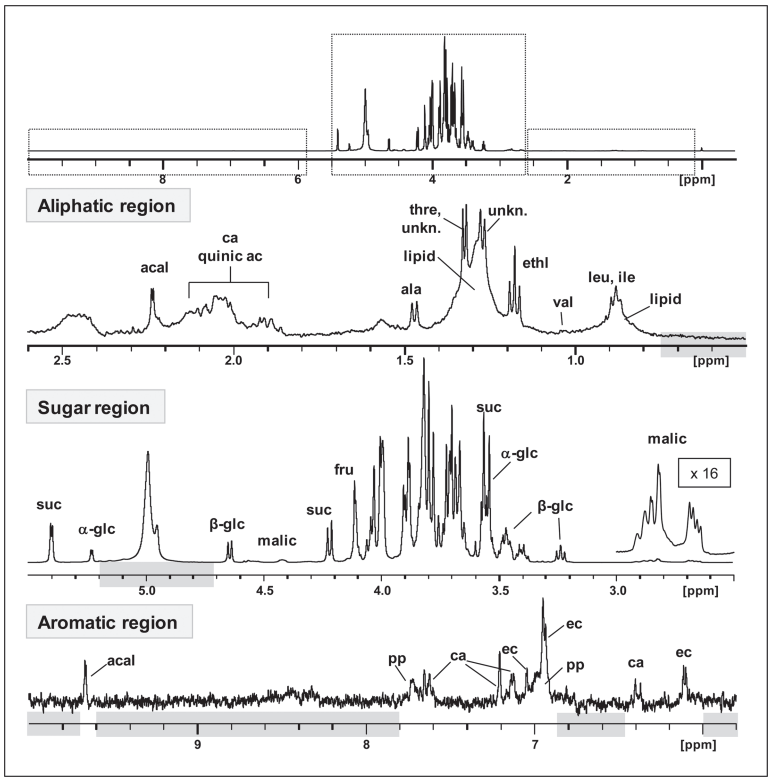

Figure 2 *[1]H HR-MAS NMR spectrum of a BIO apple sample with a spectral overview (top row) and expansions into the aliphatic, sugar and aromatic regions. Assigned signals are: acal: acetaldehyde, ala: alanine, ca: chlorogenic acid, ec: epicatechine, ethl: ethanol, fru: fructose, glc: glucose, ile: isoleucine, leu: leucine, malic: malic acid, pp: polyphenols, quinic ac: quinic acid, suc: sucrose, thre: threonine, unkn: unknown, val: valine.*[3]

2.2 Comparison of the three cultivation methods

2.2.1 Apple metabolites. In Figure 2, a typical [1]H HR-MAS NMR spectrum of an apple pulp sample is shown. The main signals derive from sugar peaks between 3 and 5.5 ppm including the resonances of α- and β-glucose, sucrose, and fructose. Another prominent compound is malic acid with resonances around 3.8 and 4.4 ppm. Signals in the

aliphatic region (0.3-2.6 ppm) can be assigned to the amino acids leucine, isoleucine, valine, alanine and threonine while the broader signals primarily belong to lipids. The relatively intense doublet at 1.33 ppm overlapping with threonine has been previously tentatively assigned to paraldehyde[3] which is related to acetaldehyde (2.23 and 9.65 ppm). In the aromatic region (5.8 – 10 ppm) the signals mainly derive from catechine, epicatechine and polyphenols.

2.2.2 Fruit quality parameters. In Table 1 the data for some fruit quality parameters like ripening index, soluble solids, titratable acidity and fruit firmness at harvest and after removal from storage are shown for the three cultivation methods. The corresponding apples were obtained from the same batch as those apples investigated by HR-MAS NMR. Within the window of optimal maturity for storage, BIO apples were the least ripe and LI apples the ripest fruits at harvest. Firmness and acid were higher in BIO apples while the sugar content was not significantly ($p<0.05$) different for the three production systems at harvest even though BIO apples had not converted the same amount of starch into sugars than IP and LI apples. After 127 days of cold storage at 1°C in a normal atmosphere, the firmness and acidity had decreased for all apples with BIO still exhibiting the highest values. While the total sugar content did not much change for IP and LI apples, it increased for BIO apples during storage.

Table 1 ***Fruit quality parameters (± se, n=20) at harvest and after cold storage (1°C)***

Parameter	*Harvest*			*After storage*		
	BIO	*IP*	*LI*	*BIO*	*IP*	*LI*
Ripening index[a]	0.12 ± 0.01	0.1 ± 0.01	0.08 ± 0.002	–	–	–
Firmness[b] [kg/cm^2]	8.9 ± 0.2	8.3 ± 0.2	7.7 ± 0.1	5.8 ± 0.2	5.1 ± 0.2	5.1 ± 0.1
Sugar[c] [°Brix] (soluble solids)	14.2 ± 0.08	13.6 ± 0.1	13.7 ± 0.11	15.5 ± 0.11	14.0 ± 0.12	13.7 ± 0.04
Acid[c] [g/L] (titratable acidity)	8.22 ± 0.05	6.29 ± 011	6.42 ± 0.13	4.65 ± 0.04	3.74 ± 0.05	4.08 ± 0.05
Starch index	5.23 ± 0.1	6.18 ± 0.24	6.88 ± 0.19	–	–	–

[a]According to Streif[5] (low indices indicate riper apples). [b]By Pimprenelle penetrometer. [c]By FTIR from juice.

2.2.3 Multivariate analysis of HR-MAS NMR data. All HR-MAS ^{1}H NMR spectra were submitted to principle component analysis (PCA). In Figure 3, the PCA scores plot is shown for the first two principle components. While apples from the low-input (LI) and integrated (IP) production systems did not separate, a clear clustering for the organically grown apples (BIO) could be observed. The separation of BIO apples was mostly due to negative scores on PC2 combined with predominantly positive scores on PC1.

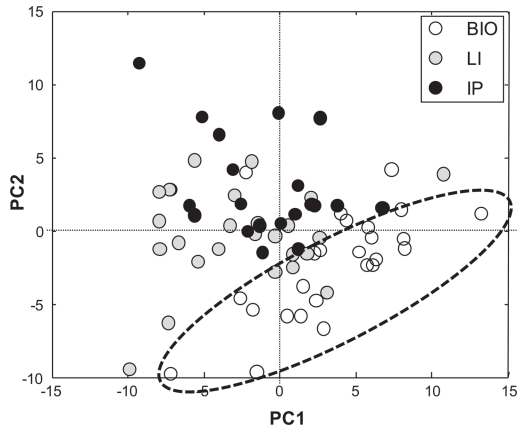

Figure 3 *PCA-scores plot (PC1 vs PC2) based on all ^{1}H HR-MAS NMR spectra for apples derived from BIO-, LI- and IP-cultivation methods.*

To further investigate if apple samples derived from the three cultivation methods could be differentiated based on the HR-MAS NMR data, partial least squares discriminant analysis (PLS-DA) was performed, based on the spectral assignment to one of the three groups, BIO, IP or LI. The corresponding scores plots for the first three PLS components, PLS-1 versus PLS-2 and PLS-3, respectively, are shown in Figure 4 (A). With this model, BIO apples could be well separated from IP and LI apples mainly because of their positive scores on PLS-1. Scores along PLS-2 were predominantly positive for BIO apples and negative for the other samples but were less decisive for separation. The third PLS component discriminated LI and IP apples with LI apples mainly exhibiting negative scores on PLS-3.

To identify the chemical components responsible for the separation of the three cultivation methods the corresponding loading values were analyzed. In Figure 4 (B) the load values of the first three PLS components are shown for those buckets which could be assigned to specific apple components. Resonances from the sugar region have a strong influence on the separation along PLS-1. Sucrose, fructose, and malic acid have high positive load values and are therefore in higher relative amounts present in BIO apples than in IP and LI apples. The latter two (IP and LI) on the other hand have higher levels of α- and β-glucose as well as quinic acid according to their negative load values on PLS-1. The aliphatic region comprising several amino acid resonances (Ile, Leu, Val, Thre, unkn.), which partly overlap with lipid signals, and quinic and chlorogenic acid mainly contributes to the negative PLS-3 loadings of LI-apples. Accordingly, LI apples have higher levels of these compounds in particular as compared to IP apples. The aromatic region also adds to the discrimination of the three cultivation groups. In particular, regions containing the polyphenol resonances are higher in IP and LI apples than in BIO according to PLS-1 load values, while they are in turn higher in IP than in LI according to the positive PLS-3 loadings of IP apples. These results can also be visualized when comparing the averaged spectra for the three apple groups as shown in Figure 5. For example in IP apples the polyphenol signals (pp) are most intense whereas the chlorogenic acid content (ca) seems higher in LI and BIO apples. The increased polyphenol content in IP apples is in agreement with a previous HPLC study comparing integrated and organically grown Golden

Delicious apples[8] whereas another HPLC and spectrophotometric study suggested little effect of the production system on total polyphenols except for procyanidines in Golden Delicious apple pulp.[9]

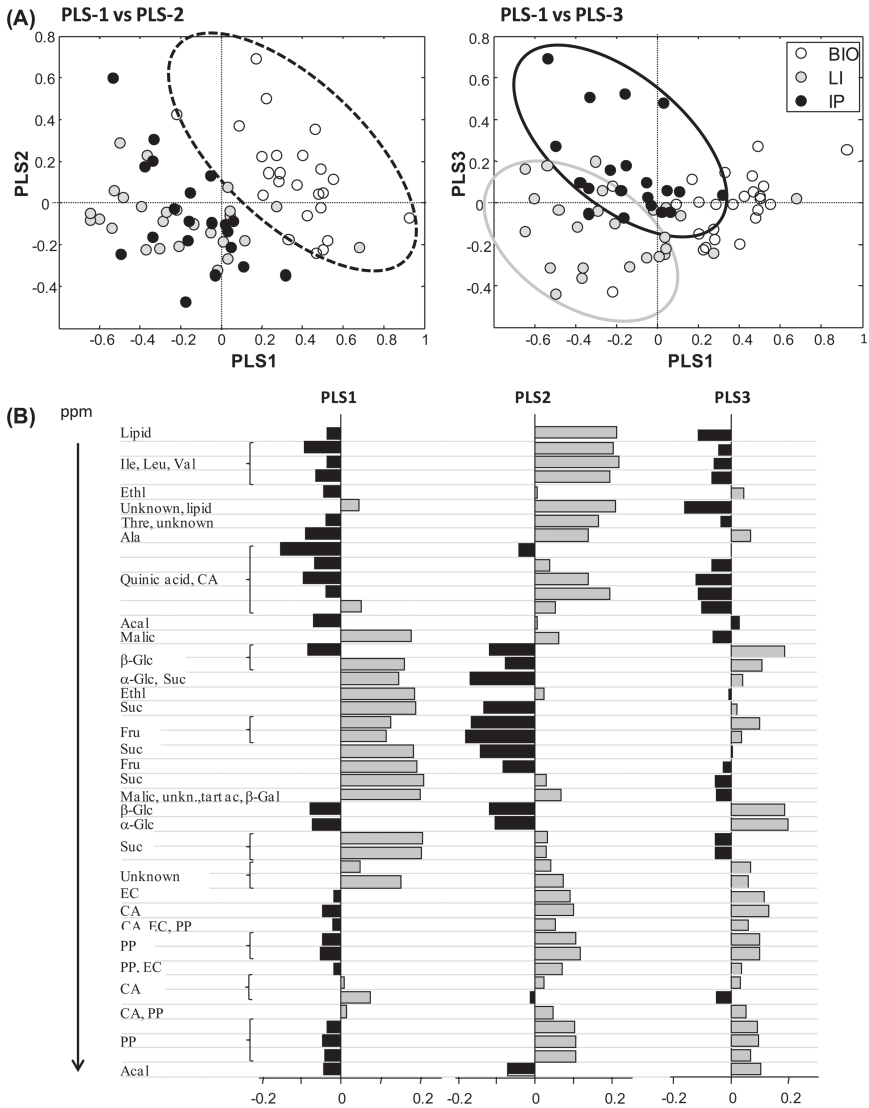

Figure 4 **(A)** *Partial least Squares discriminant analysis (PLS-DA) for all apple spectra .***(B)** *Load values for the first three PLS components and assigned buckets.*

Although unit variance scaling puts same weight onto high and low intense signals, the main contribution for discriminating BIO apples still originates in the sugar region. The higher levels in malic acid are in agreement with the fruit quality parameters assessed by

conventional methods (Table 1). However, while the conventional methods determine the sum of sugars present in the juices from the soluble solid content, the HR-MAS NMR data provide a more detailed picture of the sugar components. Thus, IP and LI apples have significantly higher glc/suc ratios than BIO apples which also becomes evident when comparing the averaged spectra (Figure 5).

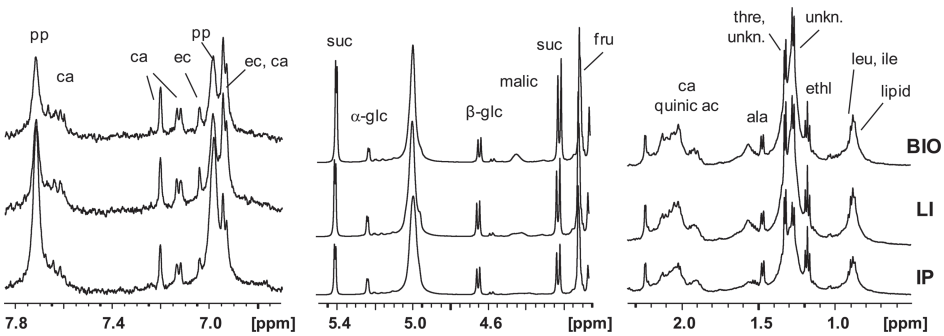

Figure 5 *Expansions from the aromatic, sugar and aliphatic regions of the ^{1}H HR-MAS NMR spectra each averaged for all IP-, LI- and BIO-apple samples, respectively. For signal annotations: see Figure 2.*

The fruit quality parameters shown in Table 1 indicate that the organically grown apples are - within the window of optimal ripening index - less ripe than the other fruits. Also previous studies have suggested that organic cultivation may have an impact on the growth rate and the overall crop load of the apple trees.[10] However, with LI apples having the lowest ripening index, i.e. being ripest, spectral differences seem to be largest between BIO and IP apples, in particular in the aromatic region (Figure 5).

3 CONCLUSION

The present study demonstrates the potential of HR-MAS NMR spectroscopy of fruit tissue as analytical tool for finding markers for specific fruit production conditions like the cultivation method. The results of this study suggest that differences in the chemical composition are induced by the application of different cultivation methods. Thus, the proposed model may now serve as a starting basis for testing further apple samples obtained from subsequent harvest seasons for validation. Moreover, even though apples derived from the three different production systems all had a ripening index within the optimal harvest window, it remains to be tested whether the maturity differences within this range contribute to the HR-MAS based discrimination. For this, we are currently investigating in an ongoing longitudinal HR-MAS NMR study apples from the three different cultivation methods as function of ripening index assessed by conventional analytical methods. Applying HR-MAS NMR spectroscopy allows obtaining a comprehensive metabolic fingerprint of the apple in one shot with a minimum of sample manipulation. Ideally, this will help to estimate how production induced chemical properties correlate with fruit quality, like flavor and taste which are determined by the combination of several fruit constituents. On the other hand the method may serve for distinguishing apple production systems in food control.

Abbreviations

HR-MAS: High Resolution Magic Angle Spinning, BIO: organic production, IP: integrated production, LI: low-input production, PCA: principle component analysis, PLS-DA: partial least squares discriminant analysis, ANOVA: analysis of variance, acal: acetaldehyde, ala: alanine, ca: chlorogenic acid, ec: epicatechine, ethl: ethanol, fru: fructose, glc: glucose, ile: isoleucine, leu: leucine, malic: malic acid, pp: polyphenols, quinic ac: quinic acid, suc: sucrose, thre: threonine, unkn: unknown, val: valine.

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