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Investigation of Different Apple Cultivars by High Resolution Magic Angle Spinning NMR. A Feasibility Study

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ABSTRACT: ^{1}H HR-MAS NMR spectroscopy was applied to apple tissue samples deriving from 3 different cultivars. The NMR data were statistically evaluated by analysis of variance (ANOVA), principal component analysis (PCA), and partial least-squares−discriminant analysis (PLS−DA). The intra-apple variability of the compounds was found to be significantly lower than the inter-apple variability within one cultivar. A clear separation of the three different apple cultivars could be obtained by multivariate analysis. Direct comparison of the NMR spectra obtained from apple tissue (with HR-MAS) and juice (with liquid-state HR NMR) showed distinct differences in some metabolites, which are probably due to changes induced by juice preparation. This preliminary study demonstrates the feasibility of ^{1}H HR-MAS NMR in combination with multivariate analysis as a tool for future chemometric studies applied to intact fruit tissues, e.g. for investigating compositional changes due to physiological disorders, specific growth or storage conditions.

KEYWORDS: HR-MAS NMR spectroscopy, apples, apple cultivars, multivariate analysis, PCA, PLS

1. INTRODUCTION

While high resolution NMR of liquid foods like beverages or extracts has become an established method, high resolution (HR) magic angle spinning (MAS) NMR spectroscopy of semisolid materials is gaining increasing interest in the field of food science for the analysis of semisolid food samples.[1−4] The main advantage of this technique is that it provides direct access to the mostly unaffected chemical composition of the food product without the necessity of time-consuming and selective sample preparation steps like extraction or chromatographic separation, which in addition potentially modify substance quantities and composition. Moreover, technical improvements of the HR-MAS methods over the past years facilitate the application of most of the NMR techniques commonly applied in liquid-state NMR, including advanced 2D methods. Thus, HR-MAS NMR may become a promising alternative to the conventional methods in the qualitative and quantitative analysis of food samples.[3]

Solution-state ^{1}H NMR spectroscopy combined with statistical multivariate analysis has been successfully applied to liquid food samples like fruit juices,[5−8] beer,[9−12] or wine.[13−15] However, to date, only a limited number of studies exist combining ^{1}H HR-MAS NMR on food samples with multivariate statistical data analysis such as the discrimination studies on cheese,[16,17] wheat flour,[18] or beef[19,20] according to their geographical origins. Furthermore, only very few unprocessed fruits, e.g. tomatoes,[21] mangoes,[22] and strawberries,[23] have been investigated by HR-MAS so far. In the current paper we report on a preliminary study of HR-MAS NMR combined with multivariate statistical analysis on three different apple cultivars.

Several chemometric studies performed on apple juices have shown that it is possible to discriminate apple juices originating from different apple cultivars based on liquid-state NMR spectroscopy data.[5,6,8] While these studies provide important information on apple juices, they may not necessarily represent the metabolic state in the apples, since the process of juice production may cause metabolic changes in the apple. It is therefore also of great interest to obtain a direct metabolic image of the apple tissue with a minimum of intervention. This may particularly become useful for the study of dessert apples which are intended for direct consumption.

Therefore, the goal of the present study was to probe the feasibility of HR-MAS NMR applied to intact apple samples for chemometric studies. This is in particular of interest in view of anticipated follow-up studies investigating the impact of different growth and storage conditions or of potential physiological disorders like internal browning on the metabolic profile of apples. The preliminary work presented in this paper is aimed at evaluating the variability of apples as inherently heterogeneous samples with respect to their ^{1}H NMR spectral appearance. Specifically, the intra- and inter-apple variabilities within the same cultivar have been probed by applying both analysis of variance (ANOVA) and principal component analysis (PCA). The importance of such a combined approach of statistical significance testing and PCA of NMR data has been recently pointed out.[24] The final aim was to test whether different apple cultivars can be discriminated based on PCA and partial least-squares−discriminant analysis (PLS−DA) of HR-MAS NMR data and to determine the metabolic origin for a potential separation. The determination of variabilities between individual apples and between different cultivars is a prerequisite of future studies investigating the origin of potential changes in the metabolic profile.

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2. MATERIALS AND METHODS

Materials. Three groups of 6 apples, each representing a different apple cultivar, "Golden Delicious", "Braeburn", and "Rubens", were purchased at a local food retail market. All apples were grown in Switzerland according to declaration. However no further information, e.g. on storage duration and condition, was available. All apples were measured in arbitrary order within a total time of 15 days and were stored at 4 °C before use. The deuterated solvents (used in liquid-state NMR spectroscopy) D_2O (D 99.9%) and MeOD-d_4 were obtained from Cambridge Isotopes Laboratories, Inc. Trimethyl-silyl-3-propionic acid sodium salt D4 (d_4-TSP, D 98%), obtained from Euriso-Top, was used as internal ^{1}H NMR reference. D_2O (D 99.9%) containing 0.75% TSP was obtained from Sigma-Aldrich (used in HR-MAS NMR spectroscopy). The pure amino acids isoleucine, leucine, valine, and threonine used as reference compounds were purchased from Alfa Aesar.

Sample Preparation. For each apple, 5 samples from one central cross section of the apple were taken and each was submitted to HR-MAS NMR measurement resulting in a total of 90 single measurements (3 groups of 6 apples). For this purpose, the apple was cut along the equatorial plane. To account for potential inhomogeneities across the slice, the five samples were distributed evenly along a circle as indicated in Figure 1. Transfer of apple pulp samples into a 50 μL MAS rotor was achieved by directly punching the sample with the rotor. For standardizing this procedure, a homemade "rotor coat" was used which was designed such that the rotor always punched out a cylindrical tissue sample of 4 mm length. Thus apple punches were obtained with an average weight of 20 mg. For each sample, the exact apple weight in the rotor was determined. The 4 mm pulp punch was gently pushed to the bottom of the rotor and was then covered with 10 μL of D_2O (containing 0.75% TSP) serving as lock solvent. To minimize the time during which the freshly cut apple slice was exposed to air, 5 MAS rotors were filled and sealed simultaneously as fast as possible before the samples were submitted to the NMR acquisitions.

Juice Preparation. For a direct comparison of juice and pulp originating from the same apple, a part of the apple (pulp only) used for MAS-sample preparation was manually squeezed through a garlic press to collect about 2 mL of fresh apple juice. An aliquot of the juice (540 μL spiked with 60 μL of D_2O containing 0.1% TSP) was immediately transferred to a 5 mm NMR tube and submitted to liquid-state (static) NMR spectroscopy.

^{1}H High Resolution Magic Angle Spinning (HR-MAS) NMR Spectroscopy. The ^{1}H HR-MAS NMR experiments were performed on a Bruker Avance II spectrometer operating at a resonance frequency of 500.13 MHz for ^{1}H. The instrument is equipped with a 4 mm HR-MAS dual inverse ^{1}H/^{13}C probe with a magic angle gradient. All experiments were carried out at a magic angle spinning rate of 5 kHz and a temperature of 281 K. In a preceding experiment the temperature control was calibrated under the same MAS conditions using a sample of 4% MeOH in MeOD-d_4. Bruker TOPSPIN software (version 2.1, patch level 5) was used to acquire and process the NMR data.

The 1D ^{1}H HR-MAS NMR spectra were recorded using a 1D NOESY two-step presaturation sequence for water suppression ("*noesypr1d*" from the Bruker pulse-program library). Each 1D ^{1}H 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. The coadded 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 ^{1}H NMR spectra. For signal assignment, gradient-enhanced 2D ^{1}H−^{1}H-COSY in magnitude mode and phase-sensitive 2D ^{1}H−^{1}H-TOCSY both with presaturation during relaxation delay were applied.

^{1}H High Resolution Solution (Static) NMR Spectroscopy. The ^{1}H NMR experiments of apple juices were performed on a Bruker Avance II spectrometer operating at a resonance frequency of 400.13 MHz for ^{1}H and equipped with a 5 mm ATM BBI probe with *z*-gradient. All experiments were carried out at ambient temperature (298 K) in order to reduce the time between juice preparation and data acquisition. This resulted in an upfield shift of the water and polyphenol signals as compared to the HR-MAS spectra acquired at 281 K. Otherwise, the 1D ^{1}H NMR spectra were recorded using the same pulse sequence and similar parameters as applied for the apple pulp samples under MAS (i.e., 1D NOESY with presaturation, 128 transients, spectral width of 4795.4 Hz, data size of 32 K points, acquisition time of 3.42 s, and a relaxation delay of 3 s). For signal assignment, gradient-enhanced 2D ^{1}H−^{1}H-COSY and 2D ^{1}H−^{1}H-TOCSY, both phase-sensitive, as well as 2D ^{1}H−^{13}C-HSQC (gradient-enhanced) with carbon multiplicity editing were applied. To prove the assignment for some of the amino acids, ^{1}H NMR spectra of apple juice were also run after addition of the pure reference amino acids isoleucine, leucine, valine, and threonine. ^{1}H chemical shift prediction was performed using the ACD (Advanced Chemistry Development, Inc.) software, release 11.00 version 11.02 (2008).

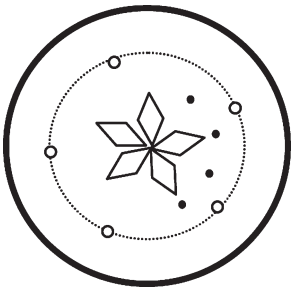

Figure 1. Scheme of sampling from an apple slice (cross section). The open circles indicate the regions from which apple tissue samples were punched for HR-MAS NMR spectroscopy.

Data Analysis. Statistical analyses were performed using the statistical software package SPSS v. 18.0.0 (Chicago, IL) and Excel 2002 (Microsoft, Redmond, WA). Multivariate analysis (PCA and PLS−DA) of the spectra was performed using the Bruker software package AMIX (version 3.8.6). *P*-values of less than 0.05 were considered statistically significant. Where appropriate, the *p*-values were adjusted for multiple comparisons employing the Dubey/Armitage−Parmar procedure, which takes into account correlations among the end points.[25]

Analyses of Variance. ***Analysis of Variance within and between Apples for Each Cultivar.*** To compare the variations of NMR data derived from the 5 different samples of one apple with those derived from the 6 different apples within each cultivar, the mean values and standard errors were calculated for the integrals (normalized to the total of the selected integrals) of some characteristic and exemplary metabolite signals and spectral regions of the apple spectrum (Figure 2). The following 13 integral regions, which are indicated in Figure 2, have been taken into account: 9.634−9.689 ppm (acetaldehyde, AcCHO, peak 15b), 7.172−7.242 ppm (chlorogenic acid, CA, peak 14e), 6.077−6.133 ppm - (epicatechin, EC, peaks 28a,b), 5.358−5.442 ppm (sucrose-1, Suc 1, peak 19e), 5.201−5.250 ppm (α-glucose, α-Glc, peak 18b), 4.600−4.685 ppm - (β-glucose-1, β-Glc 1, peak 17c), 4.250−4.600 ppm (containing malic acid-1, tartaric acid, β-galactose, and two unknown components, Malic 1, peaks 16c, 21−24), 4.184−4.243 ppm (sucrose-2, Suc 2, peak 19d), 4.072−4.130 ppm (fructose, Fru, peak 20b), 3.197−3.270 ppm (β-glucose-2, β-Glc 2, peak 17a), 2.600−3.000 ppm (malic acid-2, Malic 2, peaks 16a,b), 1.430−1.500 ppm (alanine, Ala, peak 12), 0.950−1.050 ppm (leucine, isoleucine, valine, Leu-Ile-Val, peaks 2−4).

After testing for normal distribution with a Kolmogorov−Smirnov test applied to all 30 measurements (5 punches × 6 apples) for each

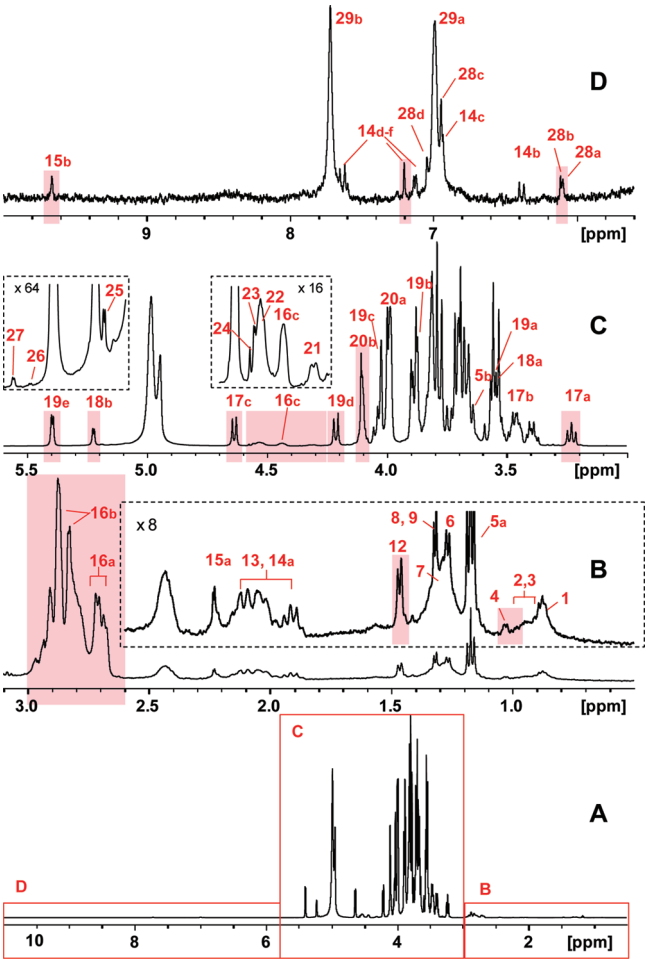

Figure 2. ^{1}H HR-MAS NMR spectra of apple tissue from a Braeburn cultivar. (A) full spectral region (0−10.5 ppm), (B) amino acid region (0.5−3.1 ppm), (C) sugar region (3−5.6 ppm), and (D) aromatic region (5.6−10 ppm), regions for manual integration are indicated (shadowed in red).

cultivar, a one-way analysis of variance (ANOVA) was conducted to compare the within-apple variability with the between-apple variability. For this, *F*-values were calculated for each of the above integral regions and each cultivar. For the given number of apples and measurements per apple *F*-values larger than 2.8 ($p < 0.05$) indicated differences being significantly larger between apples than within single apples. In addition to the ANOVA, PCA (see "Multivariate Statistical Analysis") was performed to test if clustering of samples derived from the same apple could be detected. Linear regression analysis was used to test for pairwise correlations between the selected peak integrals.

Analysis of variance within and between cultivars. To compare the variations of NMR data within and between different cultivars a one-way ANOVA was applied to the normalized integral values of the 13 peak regions (Figure 2) comparing the 3 different cultivars (3 × 30 spectra). In addition, Tukey's HSD tests were applied to compare pairwise the means of the three cultivars.

Multivariate Statistical Analysis. All 90 ^{1}H HR-MAS NMR spectra were subdivided in the range between 10 and 0.5 ppm into buckets of 0.05 ppm each (total of 190 buckets). Because of their pH-dependent shifts, signals deriving from malic acid, i.e. −CH (4.25−4.6 ppm) and $-CH_2$ (2.4−3 ppm), were each combined into a single bucket with the 4.25−4.6 ppm bucket also containing tartaric acid, β-galactose, and two unknown compounds. The spectral region (and corresponding buckets) of 4.7−5.2 ppm containing the water signal was excluded from the statistical analysis. These two procedures reduced the number of buckets to 163. To account for differences in sample weight all signal integrals were normalized to the total integral of the investigated spectral range. The variables (buckets) were mean centered and scaled to unit variance, which results in equal weight of high and low intensity signals.

In order to test first whether or not the samples originating from the same apples exhibit less variation than samples originating from different apples within the same cultivar, a PCA was separately applied to the bucket tables obtained from six apples and five spectra per apple for each of the cultivars resulting in three data matrices each of which representing 30 spectra derived from one apple cultivar.

Second, in order to determine if the different cultivars can be separated, PCA as unsupervised method was initially applied to the bucket tables obtained from all apple samples representing a total of 90 spectra (3 cultivars, 6 apples per cultivar, 5 samples per apple). PLS−DA was then performed using the 90 spectra as *X*-variables and the three cultivar groups as *Y*-variables to test for the discriminant power of the NMR data.

Finally, in order to reduce variability and to account for the fact that the values for the repeated measurements on the identical apple may not be independent from each other (thus not representing independent data points), all five spectra derived from different regions of one apple were averaged. This reduced the number of spectra submitted to PCA and PLS−DA from 90 to a total of 18, with each spectrum derived from one apple out of the 3 groups of different apple cultivars.

The loading values obtained for the PLS−DA model were analyzed in order to find those buckets which mainly contributed to the separation of the classes.

3. RESULTS AND DISCUSSION

^{1}H HR-MAS NMR Spectra of Apple Pulp. A typical 1D ^{1}H HR-MAS NMR spectrum of fresh apple tissue obtained from a Braeburn cultivar is shown in Figure 2. The peak assignments were performed according to the literature[6,21,26−28] and corroborated by our own additional 2D correlation spectroscopy methods (^{1}H^{1}H-COSY, ^{1}H^{1}H-TOCSY, and ^{1}H^{13}C-HSQC). The assignments are summarized in Table 1. The ^{1}H HR-MAS NMR spectrum of the apple pulp is dominated by signals between 3 and 5.6 ppm (Figure 2A,C) mainly deriving from sugars. In this region, the most intense signals derive from β- and α-glucose (peaks 17a−c and 18a,b), sucrose (peaks 19a−e), and fructose (peaks 20a,b). In addition to a much lower content though, β- and α-galactose (peaks 24 and 25) as well as two unknown compounds (peaks 26 and 27) could be detected. The latter indicate most likely also sugar compounds due to the chemical shift and coupling values of their proton signals (see Table 1).

The spectral region between 0 and 3 ppm (Figure 2B) contains signals mainly from amino acids and other organic acids, of which malic acid is the most prominent one (peaks 16a,b). The assignment of isoleucine, leucine, valine, and threonine (peaks 2−4 and 8) in this spectral region could be confirmed by standard addition of the corresponding reference compounds to an apple juice sample measured with solution-state ^{1}H NMR. The presence of threonine in the apple pulp (peak 8) could be proved by its corresponding COSY cross peak at 4.26 ppm/1.32 ppm. However, the signal of the threonine methyl group overlaps in the apple pulp with one yet unknown compound at 1.32 ppm (peak 9), which shows a cross peak at 5.24 ppm in the COSY spectrum. In this region, besides peak 9 another doublet peak (peak 6) at 1.27 ppm could not be assigned yet. The unknown peaks 6 and 9 derive from methyl groups according to

Table 1. Signal Assignment of Protons from Apple Tissue/Juice of Braeburn Cultivar

peak	chem shift [ppm]	multipl *J* [Hz]	compound	group
		Amino Acid Region (0−3 ppm)		
1	0.88 broad		lipid	ω-CH_3
2	0.93, 1.00 unres		isoleucine (Ile)	δ-, γ-CH_3
3	0.94, 0.96 unres		leucine (Leu)	δ-, δ'-, γ-CH_3
4	1.03	d	valine (Val)	γ-CH_3
5a	1.17	t	ethanol	CH_3
6	1.27	d [6.4]	unknown[a]	CH_3
7	1.29 broad		lipid	$(-CH_2)_n$
8	1.32	d [6.5]	threonine[b] (Thre)	γ-CH_3
9	1.32	d [5.2]	unknown[c]	CH_3
10	1.39	d [6.8]	lactic acid[d]	β-CH_3
11	1.43	s	citramalic acid[d]	CH_3
12	1.47	d [7.2]	alanine[e] (Ala)	β-CH_3
13	1.89−2.10		quinic acid	CH_2, −CH
14a	1.89−2.10		chlorogenic acid (CA)	CH_2, −CH
15a	2.23	d [2.9]	acetaldehyde (AcCHO)	CH_3
16a	2.69	dd	malic acid (malic)	β-CH_2
16b	2.84	dd	malic acid (malic)	β-CH_2
		Sugar Region (3−5.6 ppm)		
17a	3.23	dd	β-glucose (β-Glc)	H−C(2)
17b	3.47		β-glucose (β-Glc)	H−C(3)
18a	3.53		α-glucose (α-Glc)	H−C(2)
19a	3.54		sucrose (Suc)	H−C(2)
5b	3.64		ethanol	CH_2
19b	3.87		sucrose (Suc)	H−C′(5)
20a	4.0		fructose (Fru)	H−C(4)
19c	4.03		sucrose (Suc)	H−C′(4)
20b	4.1	d	fructose (Fru)	H−C(3)
19d	4.2	d [8.8]	sucrose (Suc)	H−C′(3)
21	4.31	d [8.6]	unknown	
16c	4.44		malic acid (Malic)	CH
22	broad		unknown	
23	4.55	s	tartaric acid	CH
24	4.57	d [8.1]	β-galactose	H−C(1)
17c	4.64	d [7.9]	β-glucose (β-Glc)	H−C(1)
25	5.19	d [3.4]	α-galactose (α-Gal)	H−C(1)
18b	5.22	d [3.7]	α-glucose (α-Glc)	H−C(1)
19e	5.4	d [3.7]	sucrose (Suc)	H−C(1)
26	5.5	d [3.8]	unknown	CH
27	5.56	d [3.4]	unknown	CH
		Aromatic Region (5.6−10 ppm)		
28a	6.10	s	epicatechin (EC)	CH, arom
28b	6.11	s	epicatechin (EC)	CH, arom
14b	6.39	d [16]	chlorogenic acid (CA)	CH
14c	6.94		chlorogenic acid (CA)	CH, arom
28c	6.95	s	epicatechin (EC)	CH, arom
29a	6.99		cond. polyphenols (PP)	
28d	7.05	s	epicatechin (EC)	CH, arom
14d	7.13	d [8.3]	chlorogenic acid (CA)	CH, arom
14e	7.20	s	chlorogenic acid (CA)	CH, arom
14f	7.64	d	chlorogenic acid (CA)	CH
29b	7.72		cond. polyphenols (PP)	
30	8.33	s	formic acid[d]	$CHCO_2$
15b	9.66	q	acetaldehyde (AcCHO)	CHO

[a] COSY (3.85), TOCSY (3.57). [b] Apple tissue: overlap with peak 9, COSY (4.26). [c] COSY (5.24) in apple tissue only. [d] In juice only. [e] COSY (3.77).

COSY, TOCSY and $^1H^{13}C$-HSQC. The corresponding connectivities to methine protons suggest amino acid like moieties such as CH_3−CHX and CH_3−CHX−CHX with X being either oxygen or nitrogen, respectively. Accordingly, peak 9 may be assigned to paraldehyde, a condensed cyclic trimer of acetaldehyde, which could also be detected in the apple tissue (peaks 15a,b). The predicted ^1H NMR spectrum of paraldehyde (1.3 ppm d [5 Hz], 5.14 ppm) is in good agreement with the experimental data (1.32 ppm d [5.2 Hz], 5.24 ppm) shown in Table 1.

In the aromatic region between 5.6 and 10 ppm (Figure 2D) the two main broad peaks (peaks 29a,b) derive from condensed polyphenolic compounds while the sharper peaks of lower intensities can be assigned to epicatechin (peaks 28a−d), chlorogenic acid (peaks 14b−f) and acetaldehyde (peak 15b) at 9.66 ppm.

Variability of Data within and between Apples of the Same Cultivar. The spatial distribution of compounds within the apple is reported to be inhomogeneous.[29] Compositional gradients across the apple are likely to occur as a result of locally different exposure to sunlight during growth. Moreover, differences in metabolic composition may likewise occur along the radial line from the apple core toward the outer skin.[29] Therefore, care was taken that the pulp samples were always retrieved from a similar region along the radial line of the apple cross section, as is illustrated in Figure 1 and described in Materials and Methods. The normalized integrals derived from the NMR spectra of the 5 samples obtained from each of six apples of all three cultivars were compared for the within-apple variability and the between-apple variability by ANOVA. On average, the inter-apple variability was found to be significantly larger than the intra-apple variability for the selected integral regions and the three different cultivars. The *F*-values were greater than 2.8 ($p < 0.05$) for 5, 10, and 3 of the analyzed 13 integrals in Golden Delicious, Braeburn, and Rubens, respectively (after correction for multiple comparisons corresponding to a multiplication of the *p*-values by approximately 12.6 at the significance level of $\alpha = 0.05$). The mean *F*-values averaged over the 13 integral regions were 8.2 for Golden Delicious, 11.9 for Braeburn, and 3.5 for Rubens.

To cover the whole spectral range, PCA was carried out on the corresponding NMR data acquired for each cultivar. In Figure 3 the first two PC scores are shown (PC1 vs PC2) for each of the three cultivars. Samples taken from the same apple are shown in same color. The dispersion of data points demonstrates for most of the apples a clustering for those samples derived from the same apple. This could be confirmed by ANOVA on the corresponding PC scores (PC1 and PC2). In all cases but one (Braeburn, PC2), the *F*-values were clearly greater than 2.6 ($p < 0.05$), showing a significantly larger inter- than intra-apple variability. Thus, both, analysis of variance of selected spectral regions indicating statistical

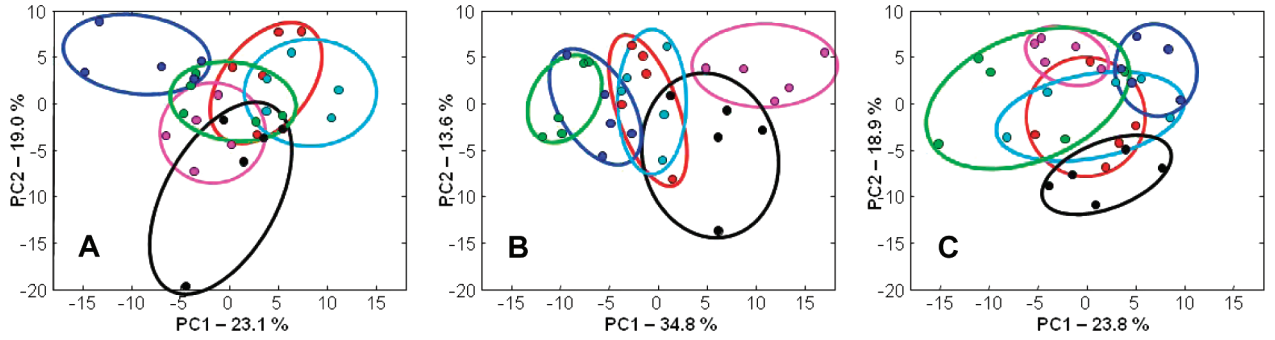

Figure 3. PCA scores plots for the three different apple cultivars. (A) Golden Delicious, (B) Braeburn, and (C) Rubens. Each plot is based on 30 spectra obtained from 6 different apples (of one cultivar) and 5 pulp samples per apple (samples derived from the same apple have the same color).

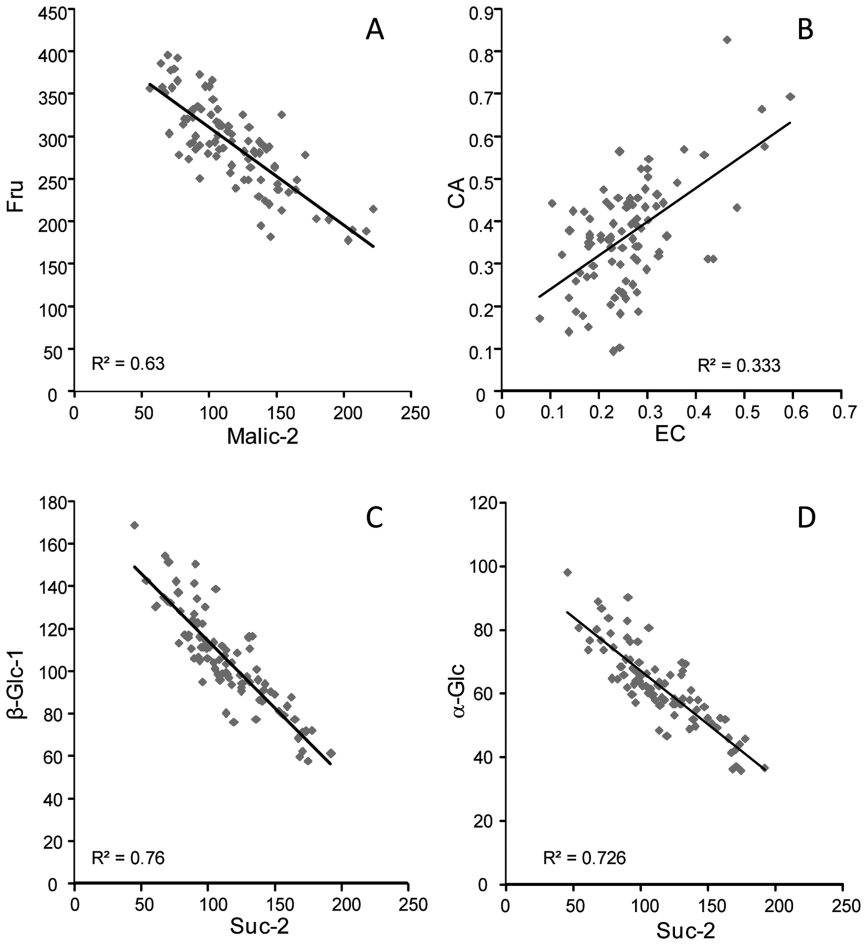

Figure 4. Interdependence of metabolites as determined from manual integration of ^{1}H HR-MAS NMR spectra obtained from each single apple pulp sample (total of 90 samples, i.e. 90 data points). Significant correlations ($p < 0.001$) between (A) Fru and Malic-2, (B) EC and CA, (C) β-Glc-1 and Suc-2, and (D) α-Glc and Suc-2.

significance as well as PCA of the full spectral range indicate that, despite the above-mentioned expected heterogeneity within an apple, the statistical dispersion of compound concentrations is smaller within apples than between different apples of the same cultivar. This seems to be an important prerequisite for applying HR-MAS NMR based chemometrics aimed at finding discriminants between different apple cultivars or between apples of different growth or storage conditions.

Analysis of Correlations between Different Metabolites within All Apple Samples. The 13 selected peak integrals from all samples were probed for correlations between specific metabolites. Since spurious correlations may evolve, due to the applied normalization procedure, all correlations were also tested (a) without any normalization, and (b) after probabilistic quotient normalization.[30] Although as expected most correlation coefficients were lower than with total integral normalization, the correlations, which are described in the following, remained highly significant. Selected correlations between some metabolites are shown in Figure 4. Apple samples with high levels of

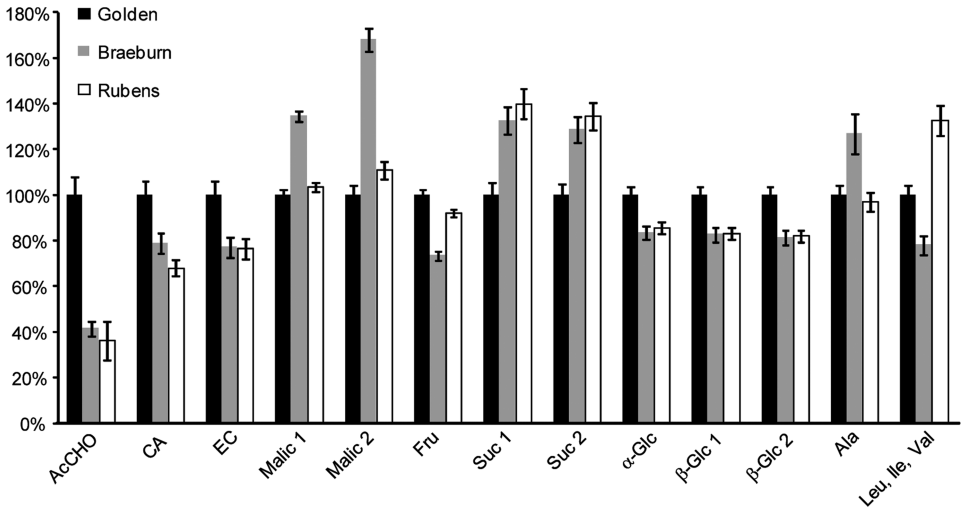

Figure 5. Relative mean values and standard error for integrals of selected resolved peaks or peak regions of the corresponding ^{1}H HR-MAS NMR spectra of apples for each cultivar (black bars, Golden Delicious; gray bars, Braeburn; white bars, Rubens). Integrals for Golden Delicious were set to 100%.

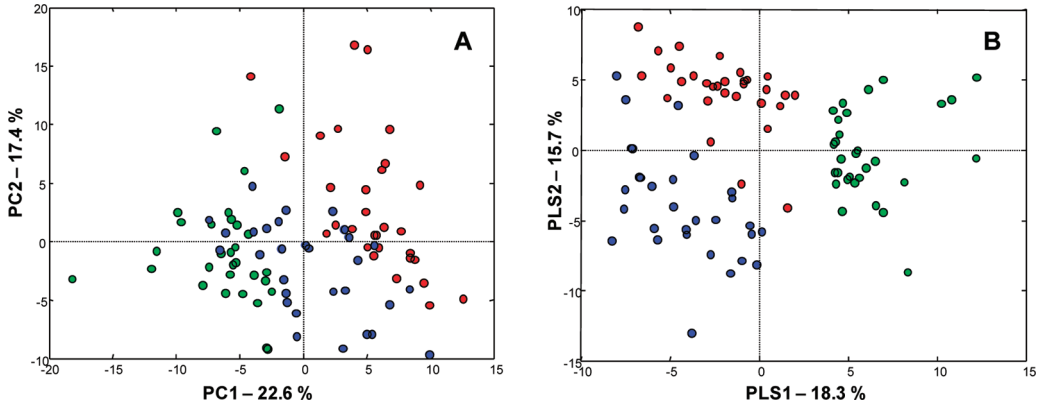

Figure 6. (A) PCA scores plot (PC1 vs PC2) and (B) PLS−DA scores plot (PLS1 vs PLS2) based on apple HR-MAS NMR spectra as X-variables and the three cultivars as *Y*-variables for all apple samples (90 entries). Green, Golden Delicious; blue, Rubens; and red, Braeburn.

fructose were found to have low levels of malic acid (Figure 4A). An inverse relationship between malate accumulation and sugar formation in apples has been reported in the literature.[31−33] This has been attributed to the enzymatic transformation of malate into sugars via gluconeogenesis during fruit development.[33] Among the sugars, fructose is most accumulated in the growing apple, while glucose accumulation is reduced due to enhanced glycolysis.[32] The two phenolic compounds chlorogenic acid (CA) and epicatechin (EC) were positively correlated (Figure 4B). This correlation may be explained by the shared initial biosynthetic pathway steps of CA and EC both having coumaric acid as common precursor. Moreover, it has been shown that, other than in fruit skin, the reduction of chlorogenic acid and flavanols (including EC) runs parallel in apple flesh during fruit ripening.[34] Very strong inverse correlations were also found for sucrose on one hand and β- and α-glucose on the other hand (Figures 4C and 4D, respectively). The converse levels of sucrose and glucose may be due to their interconversion while fructose is mainly formed from sorbitol and is much more highly abundant in apples than glucose.[32]

Variability of Data within One Cultivar and between Different Cultivars. In order to determine the variation of compounds within apple samples from the same cultivar as compared to the variation of compounds between different cultivars, in a first step the 13 selected peak integrals were again evaluated. For each cultivar, the mean integral values and standard errors were calculated for each compound. Figure 5 shows the mean integrals referenced to the integrals of Golden Delicious, which were set to 100%. ANOVA revealed significant group differences for all 13 selected compounds (F-values > 3.1; $p < 0.01$ after correction for multiple comparisons) at least between two of the three cultivars. AcCHO, CA, EC, Suc, and Glc were rather similar in Braeburn and Rubens, but different in Golden Delicious. On the other hand, Malic and Ala were similar in Golden Delicious and Rubens but different in Braeburn. The results prove that the inter-cultivar variability is larger than the intracultivar variability, making it possible to distinguish different cultivars according to their chemical composition.

PCA and PLS−DA of Samples from Three Different Cultivars. *PCA and PLS−DA Applied to All 90 Spectra.* In addition to the analysis of selected compounds, the complete HR-MAS NMR spectra were investigated by basic PCA and subsequent PLS−DA modeling to probe if apples from the three different cultivars can be discriminated and to determine the spectral regions and corresponding compounds mainly responsible for the separation. The PCA scores plot of the first two principal

components explaining 40% of the total variance is shown in Figure 6A for the three different cultivars. Even though not completely separated, a clear clustering could be observed for the three different cultivars. In particular, Golden Delicious (green) and Braeburn apples (red) already separate well with Golden Delicious samples exclusively contributing negative scores on PC1 while all but two Braeburn samples have positive scores on PC1. The Rubens apple samples (blue) are grouped in between the two other cultivars with both positive and negative contributions on PC1. Application of PLS−DA resulted in a better separation of the three cultivar groups. The corresponding PLS−DA scores plot for the first two components is shown in Figure 6B. Golden Delicious samples (green) are clearly separated from Rubens (blue) and Braeburn (red) apple samples along the first PLS component while the separation of the two latter occurs when including the second PLS component.

PCA and PLS−DA Applied to All 18 Averaged Spectra. Since a clustering for all samples deriving from the same apple could be observed (Figure 3) and the intra-apple variability was found to be significantly lower than the inter-apple variability, the five spectra obtained from the same apple were averaged. PCA and PLS−DA were applied to the resulting 18 averaged spectra deriving from the three different cultivars. Even though this procedure introduces a strong reduction in the number of observations, it takes into account that spectra deriving from the same apple may not be treated as independent data points. In PCA, inclusion of the first three principal components (PC1−PC3 explaining 73% of the total variance) leads to a complete separation of the three apple cultivars based on the averaged spectra. While Golden Delicious and Braeburn apples could already be satisfactorily separated according to their PC1 scores, Rubens apples are mainly distinguished because of their positive PC3 scores combined with mainly negative PC2 scores. The corresponding PLS−DA scores plot for the first two components is shown in Figure 7A. The result obtained for the 18 averaged spectra is very similar to that obtained for the 90 single spectra shown in Figure 6B. Again, a clear separation between Golden Delicious on one side and Rubens and Braeburn apples on the other side is observed along the first PLS component while Rubens and Braeburn apples mainly discriminate due to their negative and positive *X*-scores of the second component, respectively.

In summary, this multivariate analysis clearly demonstrates the potential of the application of HR-MAS NMR and subsequent chemometric analysis for investigating intact apple tissue. This is in particular supported by the PCA, which as fully unsupervised approach without application of any selection criteria already yielded clear clustering of apple samples according to their cultivar assignment.

Both the analysis of variance applied to selected spectral regions and the PCA applied to the whole spectral range indicate that differences in the metabolic profile of apples seem to be larger between different cultivars than within one cultivar and can help to distinguish cultivars based on their NMR data.

PLS-Loading Plot. In order to determine the variables, i.e. apple components assigned to the corresponding buckets that are mainly responsible for the separation of the three different cultivars, the load values or weights of the PLS−DA given in Figure 7A were analyzed. The loading plot for all buckets containing peaks which have been assigned (Table 1) is shown in Figure 7B for the first two PLS components.

Signals mainly contributing to positive loadings on PLS1 are those deriving from acetaldehyde (peaks 15a,b) and fructose (peak 20a).

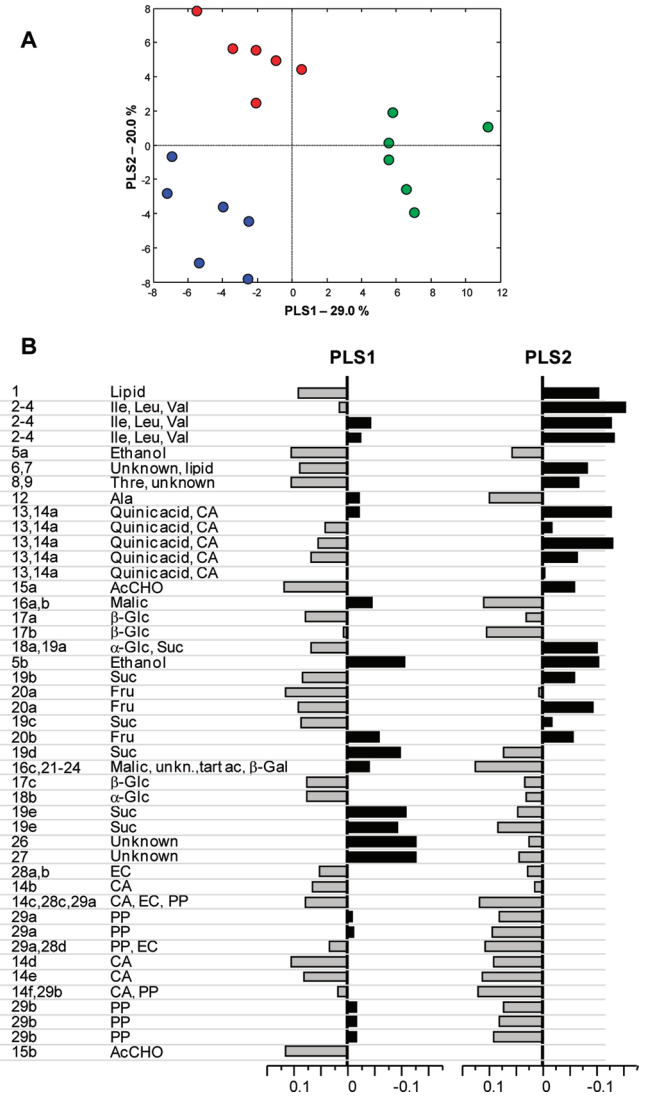

Figure 7. (A) PLS−DA scores plot (PLS1 vs PLS2) based on 18 entries with each point representing the averaged spectrum obtained from the 5 single spectra of one apple (*X*-variables) for the three cultivars (*Y*-variables). Green, Golden Delicious; blue, Rubens; and red, Braeburn. (B) Loading plots of PLS1 and PLS2 for all buckets containing assigned peaks (Table 1). Gray, positive scores; black, negative scores. The metabolite order and peak numbers on the vertical axis correspond to the ppm scale of the spectrum.

Besides, but less pronounced, chlorogenic acid (peaks 14d,e), ethanol (peak 5a), lipid (peak 1), and the two unknown compounds (peaks 6 and 9), one overlapping with lipid (peak 7) and the other—tentatively assigned as paraldehyde—with threonine (peak 8), are high on the positive PLS1 component. In the crowded sugar region (3−5.6 ppm), most bucket loadings contain contributions from several peaks. This may explain that for some compounds with multiple resonances the loading values are not consistent, e.g. for ethanol (peaks 5a,b) or sucrose (peaks 19a−e). However, in this sugar region there is much less overlap for those buckets containing the signals of the anomeric sugar protons. The corresponding signals of α- and β-glucose (peaks 17c, 18b) have positive load values while those of sucrose (peak 19e) and the two yet unidentified compounds resonating around 5.5 ppm (peaks 26 and 27) have strong negative loadings on

PLS1. All these components are responsible for the separation of Golden Delicious from Rubens and Braeburn apples, since they are well separated by their PLS1 scores. The results correlate well with the ANOVA performed on selected spectral regions shown in Figure 5. In particular, large differences between Golden Delicious on one hand and Rubens and Braeburn on the other hand were found for acetaldehyde, which is much higher in Golden Delicious and sucrose, which is higher in Rubens and Braeburn (Figure 5) with both compounds also having strong influence on positive and negative PLS1 loadings, respectively.

The separation of Rubens and Braeburn apples was mainly due to their scores on PLS2. The amino acids leucine, isoleucine and valine (Leu-Ile-Val, peaks 2−4) are the main contributors to negative PLS2 indicating high levels in Rubens. This region (Leu-Ile-Val) was also found to be significantly higher for Rubens compared to Braeburn in the analysis of variance (Figure 5). On the other hand, alanine (Ala, peak 12), malic acid (Malic, peaks 16a−c) and the phenolic compounds (CA, EC and PP, peaks 14, 28, 29) mainly contribute to positive PLS2 indicating high levels in Braeburn. According to ANOVA, alanine and malic acid were also found to be highest in Braeburn apples (Figure 5).

Some of the discriminating compounds are also visualized in Figure 8 comparing details of the ^{1}H NMR spectra from three apples each representing one cultivar. The methyl groups of the amino acids leucine, isoleucine, and valine, which appear around 1 ppm (peaks 2−4), are clearly more intense in Rubens apples than in the other two cultivars, even though this region partly overlaps with some broader components deriving from lipid-methyl groups (peak 1). Likewise, it can be seen that the signals at 2.2 and 9.7 ppm assigned to acetaldehyde (peaks 15a,b) are most intense in Golden Delicious apples. This cultivar also seems to have highest levels of the two yet unidentified compounds with doublets resonating at 1.27 and 1.32 ppm (peaks 6 and 9) also containing threonine (both positive loadings on PLS1). The simultaneous occurrence of compounds 15 (AcCHO) and 9 (unknown) supports the proposed assignment of peak 9 to paraldehyde, which as a condensation product is closely related to acetaldehyde. However, this conclusion is uncertain since the region strongly overlaps with broad signals deriving from lipid methylene groups (peak 7).

In summary, the PCA and PLS−DA of the current data suggest that different apple cultivars can be distinguished based on their different chemical compositions as obtained from ^{1}H HR-MAS NMR. However, other factors than solely the type of cultivar may have contributed to the separation of the three cultivar groups in the current study. Observed changes may also be due to different ripening stages or growth and storage conditions which are not exactly known for the used samples. Accordingly, it has been shown that metabolite concentrations of sugars, organic and amino acids, as well as phenolic compounds show characteristic changes during fruit development.[29] However, it has also been postulated that the cultivar is a more important source of compositional variations than many other factors like growth and storage.[8]

Several liquid-state HR NMR studies of apple juices combined with statistical analyses have yielded cultivar discrimination which was attributed to characteristic differences in chemical composition.[5,6,8] Between different cultivars the variation in acidity of juices, which is mainly determined by malic acid,[26] was reported to be considerably higher than within cultivars.[6] Accordingly, malic acid, but also epicatechin and chlorogenic acid, among other variables, allowed differentiating cider apple juices from different cultivars by discriminant analysis.[5,6] These compounds also contributed

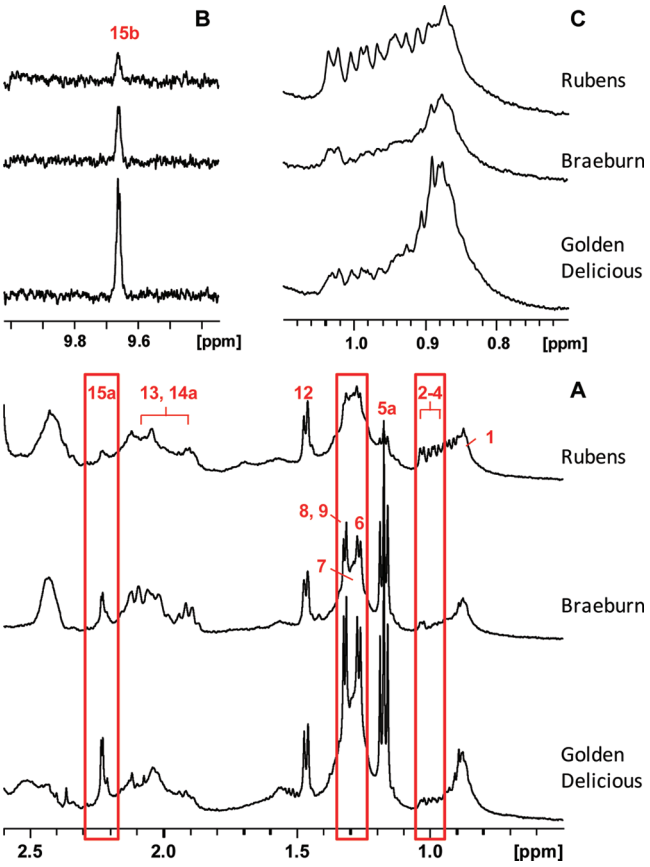

Figure 8. Comparison of ^{1}H HR-MAS NMR spectra from Golden Delicious, Braeburn, and Rubens cultivars. (A) 0−2.5 ppm. Spectral regions, which contribute to discrimination of cultivars are marked with a red box. (B) 9.35−10 ppm, AeCHO peak. (C) 0.7−1.1 ppm, Leu, Ile,Val peaks.

to cultivar separation in our study. Likewise in agreement with our study, Belton et al. have reported for apples juices that there is also a great variation in the patterns of amino acids for different cultivars, which however are also influenced by the ripening stage of the apple.[26]

Comparison of ^{1}H NMR Spectra of Apple Pulp and Juice. For a direct comparison of the metabolic profile, the averaged HR-MAS NMR spectrum of the pulp and the corresponding solution HR NMR spectrum of the juice deriving from the same apple, as an example of the Braeburn cultivar, are shown in Figure 9. While the sugar region between 3 and 5.6 ppm appeared mostly the same (not shown), there are some obvious differences in the spectral region between 0 and 3.1 ppm (Figure 9A) and the aromatic region between 5.6 and 10 ppm (Figure 9B). First, in the high-field region (0−3 ppm) of the HR-MAS spectrum there are some broad resonances (around 0.9, 1.3, 2.45, and 2.85 ppm) most likely deriving from macromolecules such as lipids or pectins, which do not occur in the juice spectrum (Figure 9A). Second, the ethanol and acetaldehyde resonances, both present in the HR-MAS spectrum (peaks 5a and 15a,b), do not appear in the juice spectrum. This is most likely due to oxidative processes taking place during juice preparation. Accordingly, the decrease of ethanol in apple juice exposed to air and the simultaneous increase of formic and acetic acid has been previously reported.[26] While lactic acid could not be detected in the apple pulp samples, it could clearly be detected in the juice as a doublet at 1.39 ppm

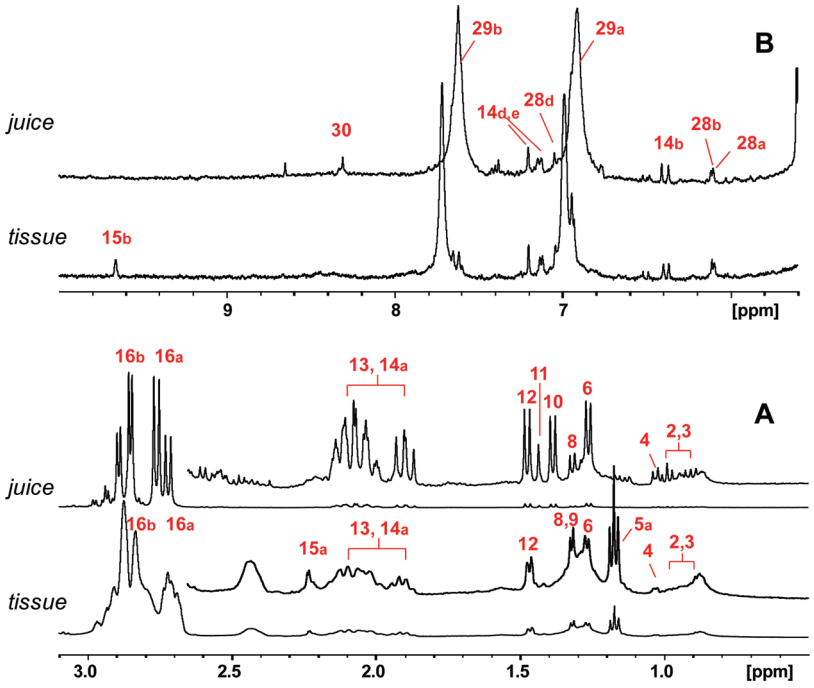

Figure 9. [1]H HR-MAS (500 MHz, 8 °C) and static liquid-state (400 MHz, 25 °C) NMR spectra of apple tissue (bottom) and juice (top) from the same apple (Braeburn cultivar): (A) amino acid region (0.5−3.1 ppm); (B) aromatic region (5.6−10 ppm).

(peak 10) suggesting lactic acid formation during juice preparation, which may most likely be a fermentation product of naturally occurring microorganisms. On the other hand, the yet unidentified compound with its proton signal overlapping with that of threonine at 1.32 ppm and a COSY cross peak at 5.24 ppm (peak 9) only appeared in the pulp but not in the juice. As already mentioned above, this peak may be assigned to paraldehyde, which most likely correlates to the presence of acetaldehyde. Therefore, the assignment is supported by the observation that both compounds, the unknown (peak 9) and AcCHO (peaks 15a,b), are present in apple pulp but not in the juice anymore. For the aromatic region, the decrease of monomeric phenolic compounds and the simultaneous increase of signals assigned to condensed polyphenols has been reported in apple juice over time (several hours) as result of the polyphenoloxidase activity and exposure to air.[26] The sharp signals of monomeric phenolic compounds (epicatechin and chlorogenic acid, peaks 28a−d and 14a−e) are still present in the juice. While for the condensed polyphenol signals (peaks 29a,b) the observed upfield shift in the juice is mainly a result of the increased temperature, they have become somewhat larger and broader (Figure 9B). This may indicate a progressive condensation of the polyphenol fraction probably mainly due to nonenzymatic oxygenation. The peak at 8.3 ppm (peak 30) can be assigned to formic acid,[26,35] which is visible in the juice spectrum but not in any of the apple pulp spectra. These results demonstrate that it may not be sufficient and representative to investigate the fruit juice instead of the unaffected fruit tissue, if the natural fruit itself or changes within the fruit are in the focus of investigation.

In conclusion, NMR studies on intact apple tissue demonstrate, due to their inherent sample heterogeneity, greater variability than on apple juices, which generally represent a homogeneous mixture of numerous apples. However, the results of the current study demonstrate the feasibility of HR-MAS NMR-based chemometric studies applied to apple tissue. First, the intra-apple variability was found to be significantly lower than the inter-apple variability within one cultivar. This is an important prerequisite for any subsequent studies investigating discriminant properties based on the metabolic profile. Second, a clear separation of the three different apple cultivars could already be obtained by basic unsupervised multivariate analysis (PCA) as well as by PLS−DA using prior knowledge, i.e. the cultivar assignments. While other factors than cultivar differences like ripening status may contribute to such separation, the clear clustering of HR-MAS NMR based data observed for each cultivar is an important prerequisite for studies addressing questions like the impact of different growth or storage conditions or physiological defects of apples. To further support the conclusion that different apple cultivars can be distinguished based on their characteristic discriminating metabolic profiles, a standardized history of fruit development would be required and potentially a higher number of apple samples.

Direct comparison of the NMR spectra obtained from apple tissue (with HR-MAS) and juice (with liquid-state HR NMR) has shown that there are distinct differences in some metabolites which must be due to changes induced by juice preparation. Thus, application of the HR-MAS technique has the advantage that it provides insight into the native mostly unaffected chemical components. Regarding the fact that minimal sample preparation is required and that high resolution [1]H spectra can be obtained in a short amount of time (minutes), HR-MAS NMR combined with multivariate analysis has great potential as a tool for chemometric studies applied to intact fruit and vegetable samples in general.

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