Assessment of a ^1H high-resolution magic angle spinning NMR spectroscopy procedure for free sugars quantification in intact plant tissue

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Abstract

In most plants, sucrose content is the primary product of photosynthesis, the transport form of assimilated carbon, and also one of the main factors determining sweetness in fresh fruits. Traditional methods for sugar quantification (mainly sucrose, glucose and fructose) require obtaining crude plant extracts, which sometimes involve substantial sample manipulation, making the process time-consuming and increasing the risk of sample degradation. Here we describe and validate a fast method to determine sugar content in intact plant tissue by using High Resolution Magic Angle Spinning Nuclear Magnetic Resonance spectroscopy (HR-MAS NMR). The HR-MAS NMR method was used for quantifying sucrose, glucose and fructose in mesocarp tissues from melon fruits (*Cucumis melo* var. *reticulatus* and *Cucumis melo* var. *cantalupensis*). The resulting sugar content varied among individual melons, ranging from 1.4–7.3 g of sucrose, 0.4–2.5 g of glucose; and 0.73–2.83 g of fructose (values per 100 g fw). These values were in agreement with those described in the literature for melon fruit tissue, and no significant differences were found when comparing them with those obtained using the traditional, enzymatic procedure, on melon tissue extracts. The HR-MAS NMR method offers a fast (usually <30 minutes) and sensitive method for sugar quantification in intact plant tissues; it requires a small amount of tissue (typically 50 mg fw) and avoids the interferences and risks associated to obtaining plant extracts. Furthermore, this method might also allow the quantification of additional metabolites detectable in the plant tissue NMR spectrum.

Keywords

HR-MAS NMR, sugar quantification, melon mesocarp, enzymatic analysis, differences vs averages method.

Abbreviations

CoR

Coefficients of Repeatability

CV	Coefficient of Variation
D ₂ O	Deuterium Oxide
FMF	Focused microwave fixation
fw	Fresh weight
GC	Gas Chromatography
¹ H NMR	Proton NMR
HPLC	High-Performance Liquid Chromatography
HR-MAS	High Resolution Magic Angle Spinning
LoA	limits of agreement
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
qNMR	quantitative NMR
SNR	Signal to Noise Ratio
T1	longitudinal relaxation time
TR	Recycling Time.

Introduction

The rapidly evolving field of metabolomics aims at a comprehensive measurement of all endogenous metabolites in biological systems, like sugars in plants. Unfortunately, the determination of soluble sugars (e.g., sucrose, fructose and glucose) in plants is somewhat cumbersome, due to diverse structural features and highly variable sugar concentration in plant tissues. This is rather complicated in woody plants and lignin-rich tissues (Chow and Landhausser 2004) as well as in tissues exhibiting high oil content, such as olive fruits (Mafra et al. 2001). There is thus a need to develop a fast and reliable method for accurate determination of soluble sugars in plants.

Melon (*Cucumis melo* L.) is an economically important species of the *Cucurbitaceae* family. It is grown in temperate and tropical regions worldwide and is one of the most important fruits for fresh consumption. The *Cucurbitaceae*

family is characterized by the translocation of sucrose and galactosyl-sucrose oligosaccharides (raffinose and stachyose) from the source leaves to the fruit sink. The metabolism of these photoassimilates during ripening controls fruit growth and development, as well as sweetness of melon fruits. Sweetness is primarily a function of the accumulated sucrose, while glucose and fructose levels fluctuate much less, if at all (Burger and Schaffer 2007). Metabolic changes associated with the accumulation of sucrose in melon mesocarp tissues involves the activities of sucrose synthesizing enzymes (i.e. sucrose phosphate synthase) and sucrose degrading enzymes (i.e. acid invertases) (Giaquinta 1979; Hubbard et al. 1989; Schaeffer et al. 1987). Thus, sugar content quantification in melon fruits is a very useful tool for determining the physiological state of the fruit (Hubbard et al. 1989) and detecting biotic and abiotic stress symptoms (disease, pathogen infections) (Chou et al. 2000; Fumagalli et al. 2009). Moreover, it can be used for quality control purposes, for example referred to new genetic varieties characterization (Eduardo et al. 2005).

Traditional approaches for assaying soluble sugar content (mainly sucrose, fructose and glucose) involve, as first step, the preparation of crude extracts from plant material. Moreover, an exquisite control over the extraction process is required to avoid the degradation of individual sugars and to attain a high reproducibility (Hendrix and Peelen 1987). Upon obtaining the extract, several analytical methods are available for sugar quantification. Among them, chromatographic analyses like High-Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) have a high sensitivity and specificity (Adams et al. 1999). The HPLC technique separates the constituent sugars of a sample and allows the identification and quantification based on previously obtained elution profiles of sugar standards (Wade and Morris 1982). For HPLC analysis, an intermediate sample preparation step is usually required to avoid interferences by coeluting compounds and to achieve an adequate chromatogram resolution (Lester 2008; Shin et al. 2007; Zhang and Li 2005). Although HPLC protocols have high levels of sensitivity and specificity, the time and labour costs needed for sample preparation and sequential analyses limit the use of this technique to the analyses of relatively short series. In GC analysis, substantial sample preparation (sugars must be transformed into volatile components by prior derivatization) and lengthy run-times per sample limit the high throughput use of

these techniques. Mass Spectrometry (MS) has also been used for sugar determination of plant-derived food products, e.g. processed saps, syrups and juices (Jahren et al. 2006; Patzold and Bruckner 2005). In other studies, matrix-assisted laser desorption/ionization Fourier transformation mass spectrometry (MALDI/FTMS) has been used to detect sugar alcohol borate complexes in plant extracts (Penn et al. 1997). Finally, several Capillary Electrophoresis methods for analysing sugars in plant extracts have also been described, however, most of them require prior sample derivatization (Warren and Adams 2000).

Sucrose content in plant tissues is most commonly determined by using enzymatic methods based on sucrose hydrolysis. These methods require plant tissue extraction generally performed with water/alcohol as solvent, most usually with a 70-80% of ethanol (Murillo et al. 2003). Enzymatic assays are usually performed without further purification of the ethanolic extracts. Additionally, commercial kits for the determination of soluble sugars are readily available. Because of the above reasons, the enzymatic method is widely used for sugar quantification in plant tissue extracts.

Colorimetric methods, such as the anthrone method (Mokrasch 1954), allow soluble sugars to be globally determined, but their use tends to decrease due to the cumbersome process required to eliminate phenols in the supernatants. Furthermore, results obtained by colorimetric methods are less accurate than those resulting from enzymatic methods or from time-consuming chromatographic methods (Borek et al. 2006; Burger and Schaffer 2007). Finally, both polarimetry and refractrometry are available for industrial sugar quantification but neither one is suitable for accurate analyses (Trebbe and McGrath 2004).

In summary, although there are several methods available for measuring sugar content in plants, there remains a need for a sensitive, reliable and fast procedure for the quantitative assessment of soluble sugars in intact plant tissues and/or plant extracts.

NMR spectroscopy is a powerful technique that allows the characterization of biological samples. *In vivo* NMR is ideally suited for the non-destructive and/or non-invasive study of animal tissues, although it is not as widely used for the analysis of plant tissues. Unfortunately, its spectral resolution is adversely affected

by excess resonance broadening, due to residual dipolar interactions and variations in the bulk magnetic susceptibility, that may hinder the analytic quality of the resulting spectra. Accordingly, *in vivo* NMR studies are complemented with results obtained by *ex vivo* and/or *in vitro* NMR surveys, or other *in vitro* methods such as those previously described.

The main advantage of *in vitro* NMR is the much improved sample homogeneity, which provides narrow and well resolved NMR spectra (Kruger et al. 2008). Nevertheless, the sample extraction process can be somewhat cumbersome and subject to sample losses. *In vitro* NMR studies have been carried out on extracts to obtain the metabolic profile of plant tissues (Biais et al. 2009; Kruger et al. 2008). For instance, NMR has been applied to the determination of sugars, organic acids, amino acids and phenolic compounds in extracts prepared from carrot roots (Cazor et al. 2006). The spatial variation of various metabolites in melon fruit extracts or juice (Biais et al. 2009), and the commercial fruit juice authentication were approached by ^1H NMR spectroscopy in combination with chemometric tools (Cuny et al. 2008). Also ^{13}C NMR has been used to quantify abundant metabolites in plant tissues, including sucrose, glucose and fructose (Ratcliffe 1994; Ratcliffe and Shachar-Hill 2001). For a review of NMR applications in plant metabolomics see references (Krishnan et al. 2005; Schripsema 2009).

High-Resolution Magic Angle Spinning (HR-MAS) NMR spectroscopy is an alternative technique that can evaluate intact tissues with a spectral resolution comparable with that of extract solutions (Broberg and Kenne 2000; Gil and Duarte 2008; Shintu et al. 2004). The lack of an extraction step strongly reduces the amount of time needed to obtain quantitative metabolic pattern information, and eliminates potential artefacts or losses arising from the extraction procedure. Experiments are performed using standard NMR spectrometers supplemented with a HR-MAS accessory (driving pneumatic unit and probe), and conventional high-resolution pulse sequences. These analyses require minimal amounts of tissue (e.g. few microliters or micrograms), and the results are spectra from intact tissue with high quality and resolution. Because of the above noted reasons, HR-MAS NMR is gaining favour as first step methodology for the analysis of intact tissues (Beckonert et al. 2012).

Early work on intact fruit tissues (grape, banana and apple) using low-speed MAS did show that various metabolites, sugar among them, could be readily quantified (Ni and Eads 1993). Furthermore, sugar compositional changes during ripening of mango fruits using HR-MAS have been described (Gil et al. 2000). More recently, changes in the metabolite pattern, including sugars, of plant vegetative tissues (shoot, root, stem) upon abiotic (Fumagalli et al. 2009) and biotic stress (Sidhu et al. 2010) have been measured using HR-MAS. Nevertheless, a full validation of the quantitative HR-MAS results with respect to traditional extraction techniques seems to be missing.

In this work, a ^1H HR-MAS NMR procedure has been developed and successfully used for quantification of soluble sugars (sucrose, glucose and fructose) in mesocarp tissues from two commercial varieties of melon fruit. Factors that might influence the determination of sugar content in the mesocarp tissue of melon fruits were investigated, namely sample preparation, tissue preservation and HR-MAS NMR data acquisition and processing protocols. Furthermore, the results obtained using HR-MAS NMR were validated by comparison with those obtained using the standard enzymatic procedure for determination of soluble sugars in melon fruit extracts. The detection of ^1H further improves the method performance because of its high intrinsic sensitivity and large natural abundance (99.985%) resulting in short experimental run times.

Finally, factors that might influence the determination of sugar content in the mesocarp tissue of melon fruits were investigated, namely sample preparation, tissue preservation, and HR-MAS NMR data acquisition and processing protocols.

Materials and methods

Chemicals

Deuterium Oxide (D_2O (99.97%) and 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid (TSP, were purchased to Sigma-Aldrich Química, S.A. (Madrid, Spain). N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was purchased to Duchefa Biochem (Haarlem, The Netherlands). The "Sucrose/D-Glucose/D-Fructose" enzymatic kit was purchased to Biopharm (Catalog reference no. 10

716 260 035, Darmstadt, Germany). HR-MAS NMR rotors of the BL4 type (3 mm internal diameter, 18 mm long, and 50 μ l of sample volume), the matching spherical Teflon spacers and caps, and the tools required to handle the rotors were purchased either to Bruker Española, S.A. (Madrid, Spain) or to CortectNet (Paris, France).

Plant material

Two different commercial melon varieties (3 fruits per variety) were analyzed: Galia (*C. melo* L. var *reticulatus*) and a *Charentais* melon type (*C. melo* var. *cantalupensis*). Fresh fruits from commercial crops were purchased at a local supermarket and they were selected of similar size and quality according to standard consumer criteria like external appearance and fruit firmness.

Sample collection and handling

For each melon fruit, a 3.5 cm thick equatorial slice was cut, and 4 cylinders (2 cm diameter) were obtained from equidistant regions of the mesocarp tissue (Figure 1). A 0.5 cm diameter cylinder was extracted from one portion and used for HR-MAS NMR analysis. The remaining material was stored in liquid nitrogen for enzymatic assays. The second portion was used to test the influence of tissue microwave fixation (Focused Microwave Fixation (FMF) System, Muromachi Kikai CO., LTD. Tokyo) on HR-MAS NMR and enzymatic assays. A one centimeter diameter cylinder was extracted from the center and was fixed by FMF (0.5 s, 5 kW). A small aliquot (2 mm thick slice) was used for HR-MAS NMR analysis and the rest was preserved in liquid nitrogen for enzymatic assays (Figure 1).

HR-MAS NMR

Samples were analysed with a Bruker Avance III 400 MHz NMR spectrometer operating at 9.4 T, using a ^1H - ^{13}C - ^{31}P HR-MAS probe (Bruker Española S.A., Madrid, Spain). The magnetic field homogeneity was maximized by adjusting the magnetic field manually (“shimming”) to compensate for distortions in the effective field caused by the sample. The probe resonant circuits were tuned and matched with the sample in place. The “magic angle” (54.7°) was adjusted using the ^{79}Br signal from powdered KBr as standard (Frye and Maciel 1982). A

spinning rate of 3000 Hz was chosen to move unwanted spinning sidebands outside the spectral range of interest, while minimizing the damage to the sample. The sample temperature was set to 297 K by properly adjusting the probe temperature using previously obtained calibration curves (Nicholls and Mortishire-Smith 2001; Raiford et al. 1979). The sample was locked on the deuterium signal from D₂O, and the magnetic field homogeneity was optimized. The receiver gain was checked prior to each acquisition to avoid overloading the spectrometer digitizer with a too intense water signal, which would alter the actual intensity of the signals and consequently the metabolite quantification. More detailed descriptions and advice on HR-MAS NMR experimental setup is available on the literature (Beckonert et al. 2012; Lindon et al. 2009).

Sample and rotor preparation

Samples were cut in small pieces until forming a homogeneous pulp. For each sample, an empty HR-MAS rotor, with a spherical teflon spacer inserted into it and a cap, were weighted in a four-digit analytical balance yielding the empty rotor assembly weight (m_0). Next, a sample weighting approximately 50 mg was introduced into the rotor, which was weighted again with its cap yielding the rotor assembly plus sample weight (m_1). Last, a small amount (approximately 10 μ l) of deuterium oxide for locking purposes was added into the rotor with a 1 ml syringe, and it was mixed with the tissue sample. The HR-MAS rotor was weighted again upon capping (m_2) and immediately before placing it into the spectrometer for the NMR analysis of the sample. The sample weight was calculated as $m_{\text{sample}} = m_1 - m_0$ and the amount of deuterium oxide added to the sample was measured by weight difference as $m_{D_2O} = m_2 - m_1$. These values were recorded and used later to calculate the absolute amount of water and sugars contained in the samples (see below).

1D-¹H Quantitative NMR

Monodimensional ¹H NMR HRMAS spectra intended for quantification purposes (qNMR) were acquired without water suppression using a pulse-and-acquire pulse sequence with parameters: 90° pulse angle (6 μ s), 12 ppm (4795.4 Hz) of spectral width, digitized with 32,768 datapoints, 3.4 s acquisition time, and 4 scans per sample. Based on the measured water and free sugars T₁ values, a pre-scan delay

of 25 s was chosen to acquire spectra with negligible saturation effects ($TR > 5T_1$). Additional water attenuated quantitative 1D ^1H NMR spectra were recorded exclusively for peak assignment purposes (Figure 2) by applying a water selective presaturation pulse (420 μW) during the 25 s pre-scan delay.

Calculation of T_1 relaxation times

The spin-lattice relaxation times (T_1) of the $\text{H}_2\text{O}/\text{HDO}$, sucrose, glucose and fructose resonances were measured to optimize the recycling time of quantitative NMR spectra. For this purpose, a set of fully relaxed ^1H NMR spectra were acquired using an inversion-recovery pulse sequence with 12 different interpulse delays (τ) in the range 0.01-10 s, where the longest τ is expected to be longer than $5T_1$, and the parameters reported above for 1D- ^1H qNMR. The areas of the resonances of interest were measured in each spectrum and they were used to calculate the relaxation time by fitting the Equation 1 to the series of areas,

$$A_\tau = A_0(1 - 2\exp(-\tau/T_1)) \quad (1)$$

where A_τ is the integrated area for a given interpulse delay (τ), A_0 is the area at equilibrium (i.e. area recorded with $\tau > 5T_1$) and T_1 is spin-lattice relaxation time (Claridge 2009).

Spectra processing

Spectra were processed using the manufacturer's software TOPSPIN v. 2.x (Bruker Biospin GmbH, Rheinstetten, Germany) and MestReNova v. 5.2.1 (Mestrelab Research, S.L., Santiago de Compostela, Spain). Briefly, an exponential multiplication resulting in a line broadening of 1 Hz was applied prior to the Fourier transformation, spectrum baseline was corrected by applying the Whittaker Smoother algorithm, and the chemical shift axis was calibrated by setting to 5.4 ppm the doublet arising from the protons attached to the C1 carbon of the glucose moiety in sucrose. To avoid saturating the resonances close to the water peak, only spectra acquired without water suppression were used for quantification purposes (Figure 2). Assignment of sugar resonances in the ^1H NMR spectra was made by comparison with values reported in the literature (Barclay et al. 2012; Biais et al. 2009; Fan 1996; Gil et al. 2000; Ni and Eads

1993; Sobolev et al. 2005), or available on-line from the Human Metabolome Database or the BioMagResBank (Biological Magnetic Resonance Data 2012; Human Metabolome Database 2012), and verified using our own sample solutions, and are summarized in Table 1. The peaks used to quantify the sugar compounds were: H-C2 of β -glucopyranose at 3.25 ppm, H-C4 of β -fructofuranose and H-C3 of α,β -fructofuranose at 4.11-4.13 ppm, and H-C1-glucopyranosyl in sucrose at 5.42 ppm. When required, signal multiplicity and molecule isomerism were taken into account. Peak areas were integrated, and sugar content was calculated by area comparison with respect to the water peak area.

Water content determination

The absolute mass of water in the sample ($m_{\text{H}_2\text{O}}$ in g) was accurately measured by weight difference between the original sample weight (fresh weight) and the sample weight upon freeze-drying (dry weight). The moles of water in the sample ($n_{\text{H}_2\text{O}}$ in mol) were calculated as the quotient of the measured mass of water ($m_{\text{H}_2\text{O}}$ in g) and the molar mass of water ($M_{\text{H}_2\text{O}} = 18.015 \text{ g/mol}$). The mass fraction of water in the sample ($w_{\text{H}_2\text{O}} = m_{\text{H}_2\text{O}} / m_{\text{sample}}$ in g/g fw) and the moles of water per gram of sample ($n_{\text{H}_2\text{O}} = n_{\text{H}_2\text{O}} / m_{\text{sample}}$ in mol/g fw) were calculated too, and they later were used as reference value in sugars quantification by qNMR.

Quantification of sugar content

The absolute quantification of sugars in samples was achieved by numerical integration of a sugar resonance in the quantitative NMR spectrum of the sample, and by comparison of the sugar area to the area of water, which was in turn related to the absolute content of water in the samples by using the general equation,

$$\beta_{\text{suc}} = \beta_{\text{H}_2\text{O}} \frac{a_{\text{suc}}}{a_{\text{H}_2\text{O}}} \frac{N_{\text{H}_2\text{O}}}{N_{\text{suc}}} \quad (2)$$

where a_{suc} and $a_{\text{H}_2\text{O}}$ are the integrated areas of the sugar and water peaks, N_{suc} and $N_{\text{H}_2\text{O}}$ are the number of protons contributing to the integrated sugar (e.g. suc for sucrose) and water peaks, and β_{suc} and $\beta_{\text{H}_2\text{O}}$ are the moles of sugar and water per gram of fresh sample (i.e. sample fresh weight). When needed, areas were

corrected to account for the number of nuclei originating the integrated peak and the proportion of conformers in the quantified sugar (Table 1).

For sucrose quantification, the doublet peak of the H-C1-glucopyranosyl in sucrose at 5.42 ppm was integrated, with one contributing proton and one conformer, and the integrated area (a_{suc}) was related to the water peak area ($a_{\text{H}_2\text{O}}$) using the equation,

$$\beta_{\text{suc}} = \beta_{\text{H}_2\text{O}} \frac{a_{\text{suc}}}{a_{\text{H}_2\text{O}}} \frac{N_{\text{H}_2\text{O}}}{N_{\text{suc}}} = 2\beta_{\text{H}_2\text{O}} \frac{a_{\text{suc}}}{a_{\text{H}_2\text{O}}} \quad (3)$$

If we normalize the areas such that the numerical value of the water peak area ($a_{\text{H}_2\text{O}}$) equals the number of moles of water per gram of sample, Equation 3 simplifies to $\beta_{\text{suc}} = 2 a_{\text{suc}}$ and the amount of sucrose can be directly obtained from

its integrated area in moles per gram of sample.

For glucose quantification, the triplet peak of the H-C2 of β -glucopyranose at 3.25 ppm was integrated, with one contributing proton, and accounting the β -tautomer for the 63.1% of the glucose (x_{glc}) (Angyal 1994), and the integrated area (a_{glc}) was related to the water peak area ($a_{\text{H}_2\text{O}}$) using the equation,

$$\beta_{\text{glc}} = \beta_{\text{H}_2\text{O}} \frac{a_{\text{glc}}}{a_{\text{H}_2\text{O}}} \frac{N_{\text{H}_2\text{O}}}{x_{\text{glc}} N_{\text{glc}}} = 3.170\beta_{\text{H}_2\text{O}} \frac{a_{\text{glc}}}{a_{\text{H}_2\text{O}}} \quad (4)$$

Again, if we normalize the areas such that the numerical value of the water peak area ($a_{\text{H}_2\text{O}}$) equals the number of moles of water per gram of sample, Equation 4 simplifies to $\beta_{\text{glc}} = 3.170 a_{\text{glc}}$ and the amount of glucose can be directly obtained

from its integrated area in moles per gram of sample.

For fructose quantification, the peak ensemble H-C4 of β -fructofuranose and H-C3 of α,β -fructofuranose at 4.11-4.13 ppm was integrated, with one contributing proton from the α -tautomer and two contributing protons from the β -tautomer. The

fructose conformer composition in water at 27°C is 71.7% beta-D-fructopyranose, 20.4% beta-D-fructofuranose, 5.4% alpha-D-fructofuranose, and 2.5% alpha-D-fructopyranose (Angyal 1994) and was assumed to remain essentially unchanged at 24°C. The integrated area (a_{fru}) was related to the water peak area ($a_{\text{H}_2\text{O}}$) using the equation,

$$\beta_{\text{fru}} = \beta_{\text{H}_2\text{O}} \frac{a_{\text{fru}}}{a_{\text{H}_2\text{O}}} \frac{N_{\text{H}_2\text{O}}}{x_{\alpha\text{-frufur}} N_{\alpha\text{-frufur}} + x_{\beta\text{-frufur}} N_{\beta\text{-frufur}}} = 4.329 \beta_{\text{H}_2\text{O}} \frac{a_{\text{fru}}}{a_{\text{H}_2\text{O}}}$$

(5)

As before, if we normalize the areas such that the numerical value of the water peak area ($a_{\text{H}_2\text{O}}$) equals the number of moles of water per gram of the sample,

Equation 5 simplifies to $\beta_{\text{fru}} = 4.329 a_{\text{fru}}$ and the amount of fructose can be

directly obtained from its integrated area in moles per gram of sample.

For an immediate estimate of the sugar content in the sample, the mass of water in the sample was replaced by an approximate value, calculated from the sample weight (m_{sample}) and a sensible percentage of water, either from the literature (Maestrelli et al. 2001) or from on-line databases (USDANDB 2011) or previously measured on equivalent samples. Final values were eventually obtained upon freeze-drying of each investigated sample. In case of analyzing fruits in different ripening state, a previous analysis of water content using control fruits should be performed at that stage of maturation, in order to establish a water content standard for immediate estimation (before obtaining the accurate water content by freeze-drying the investigated samples).

The amount of sugar in the sample (β_{sugar} in mol/g fw) was converted into mass percentage (m_{sugar} in g/100 g fw) using the Equation 6,

$$m_{\text{sugar}} = \left(\beta_{\text{sugar}} / M_{\text{sugar}} \right) \cdot 100 \quad (6)$$

where M_{sugar} is the molar mass of the sugar molecule (342.30 g/mol for sucrose and 180.16 g/mol for glucose and fructose).

Determination of HR-MAS method variability

To determine the coefficient of variation of the HR-MAS NMR method, ten independent samples from contiguous regions of the mesocarp of a single melon fruit were collected and analysed. To minimize the intra-fruit variability (Biais et al. 2009) the distance between sampling locations was made as short as possible (about 0.5 cm).

Enzymatic assays

The melon samples were freeze-dried and the dry weight determined. Tissue powder (50 mg) was incubated in 80% ethanol, 10 mM HEPES pH 7.4 (1 ml) at 70°C for 1 hour. Samples were centrifuged (16,000 g) for 30 min at room temperature and the supernatants were taken. The ethanolic extracts were immediately used for enzymatic assays using the “Sucrose/D-Glucose/D-Fructose” enzymatic kit (R-Biopharm AG). At least three technical quantification replicates of the same ethanolic extract, and two independent ethanolic extracts from the same biological sample were spectroscopically assayed, using a total of 4 samples per melon (totalling 8 extracts and 24 quantification data per fruit). The CV of the enzymatic method was calculated using all of the quantification data (in this case samples were not from equivalent locations, so the CV value obtained may be artefactually increased; see Results section).

Data statistical analysis

Data obtained from different fruits and varieties were statistically analyzed using the statistics software R v. 2.15.1 (R et al. 2012a) with the RStudio v. 0.96 interface (R et al. 2012b). Method comparison was performed using the MethComp package v. 2 (Carstensen et al. 2012). The significance level was set at 0.05 for all tests.

Comparison of sugar content determined by the HR-MAS NMR and enzymatic methods

The sugar content measurements by the HR-MAS NMR method were compared with the values obtained from the same sample by using the enzymatic analysis protocol. For this purpose, data were grouped in twelve datasets by combining three factors: sugar molecule (3 levels: sucrose, glucose and fructose), fruit

variety (2 levels: *cantalupensis* and *reticulatus*), and the analytical procedure (2 levels: enzymatic method and HR-MAS NMR); and comparisons between methods measurements were performed for each sugar at variety level. Next, the fruit variety factor was removed by merging the two levels, *cantalupensis* and *reticulatus*, and sugar content comparisons between methods were repeated. Data distribution in each group was inspected by calculating the skewness and kurtosis coefficients, and deviation from normality was assessed by visual inspection of the QQ-plots and by running the Shapiro-Wilk test of normality.

Statistical assessment of the agreement between HR-MAS NMR and enzymatic analysis methods

To assess the agreement between methods, we pooled the measurements made in both varieties by each method and compared them to assess the level of agreement between both techniques. The method of differences vs averages proposed by Bland and Altman (Altman and Bland 1983; Bland and Altman 1999; Bland and Altman 2007) according to the guidelines by Castersen (Carstensen 2010; Carstensen et al. 2012) was used on this purpose. The steps involved include: (1) plotting the measurements made by the new method vs the measurements by the method of reference, (2) to plot the differences vs averages for each pair and measurements and the average difference, and to estimate the limits of agreement, (3) to regress the differences on the averages to derive the approximate conversion equations between methods and to test the null hypothesis of constant bias over the range of measurements, (4) to regress the absolute values of the residuals obtained in the previous step on the averages, and to test the null hypothesis of constant error over the range of measurements, (5) to fit the appropriate linear mixed effect model to estimate the contributions of several sources of error to the overall variation, and to calculate the repeatability coefficient of each method. If the differences of measurements exhibit a non-constant error across the range of measurements, a suitable transformation of the data must be found that makes the relationship linear before proceeding further with the analysis.

Results and Discussion

The aim of this work was to establish a rapid and reliable quantification method of sugar content in plant intact tissues, particularly in melon fruits, by means of HR-

MAS NMR spectroscopy. For this study, we selected two common commercial varieties of melon: *Cucumis melo* var. *reticulatus* and *Cucumis melo* var. *cantalupensis*. Results obtained with the HR-MAS NMR method were compared to those obtained by using the standard enzymatic method on ethanolic extracts prepared from the same melon tissue.

HR-MAS NMR

HR-MAS NMR spectra obtained from melon mesocarp tissue from the two varieties assayed in this work show multiple signals corresponding mainly to sugars (sucrose, glucose, and fructose), folate and other compounds (Biais et al. 2009; Lester 2008), which are amenable to integration for quantification purposes (Figure 2). Spectra without water suppression acquired with only 4 scans had a signal-to-noise ratio (SNR) larger than 1141 in the *reticulatus* variety and 791 in the *cantalupensis* variety, as measured on the sugar signals of interest, and were suitable for numerical integration. Assignment of sugar resonances in the ^1H NMR spectra was made by comparison with values reported in the literature (Barclay et al. 2012; Biais et al. 2009; Fan 1996; Gil et al. 2000; Ni and Eads 1993; Sobolev et al. 2005) or available on-line from the Human Metabolome Database or the BioMagResBank (Barclay et al. 2012; Biais et al. 2009; Biological Magnetic Resonance Data 2012; Fan 1996; Gil et al. 2000; Human Metabolome Database 2012; Ni and Eads 1993; Sobolev et al. 2005), and are summarized in Table 1. The peaks chosen to quantify the sugar compounds were H-C2 of β -glucopyranose at 3.25 ppm, H-C4 of β -fructofuranose and H-C3 of α,β -fructofuranose at 4.11-4.13 ppm, and H-C1-glucopyranosyl in sucrose at 5.42 ppm. When required, signal multiplicity and molecule isomerism were taken into account (see Methods section for details).

T_1 relaxation time measurements

We measured the longitudinal relaxation time (T_1) of water in the intact tissue, which was 1.12 seconds, to set the minimum recycling time to be used in the NMR sequence to ensure proper quantification of total water. We also calculated T_1 values in the intact tissue for the resonances of interest corresponding to the sugars to be quantified: 1.014 ± 0.134 s for H-C1-glucopyranosil in sucrose, 0.731 ± 0.104 s for H-C2 of β -glucopyranose, and 0.770 ± 0.137 s for H-C4 of β -

fructofuranose. These values are similar to T_1 values reported for the same compounds in banana fruit at 4.7 T: water, 1.38 s; sucrose, 0.43 s; glucose, 0.68 s; and fructose, 0.49 s (Ni and Eads 1993).

Quantitative NMR

To obtain the absolute concentration of a compound in a sample by means of NMR spectroscopy, the sample spectrum must be recorded in quantitative experimental conditions. For 1D- ^1H NMR, spectra recorded using a simple pulse-and-acquire method, the most critical parameters are the pulse duration, that must result in a magnetization nutation angle of 90 degrees, and the recycling time, that must be longer than $5T_1$ to ensure that more than 99% of the equilibrium magnetization is recovered before each scan and that signal saturation is negligible (Boogaart 1995; Pauli et al. 2005; Pauli et al. 2012; Saito et al. 2004; Soininen 2008). Although our measured T_1 values would allow us to use a recycling time (TR) of approximately 6 s, the overall HR-MAS acquisition time with the initial TR of 28.4 s was so short (2.5 minutes) that we decided to maintain this long TR. Moreover, this long TR would allow to simultaneously quantify other metabolites with T_1 values much longer than those reported in this work, like fumarate which has a T_1 of approximately 10 s in some solutions and tissues (Candiota et al. 2004). Accordingly we performed all experiments with a longer than required TR value (28.4 s) to make our protocol amenable to future quantification studies of a larger number of metabolites.

In quantitative NMR (qNMR) spectra the resonance areas are directly proportional to the amount of compound in the sensitive sample volume, and compounds can be quantified by integrating at least the area of one of its resonances and comparing the integrated area to the area of an internal reference compound of known concentration, and correcting for the number of protons contributing to the integrated resonance peak, and accounting for any dilution factor (Boogaart 1995; Soininen 2008). In our experiments, the content of sucrose could be calculated by direct comparison of areas, the content of glucose had to be corrected because the integrated peak originates from one of the two glucose tautomers, and the fructose content had to be corrected because two of the four existing conformers contribute to the integrated spectral region.

Influence of sample collection and preservation on sugar quantification.

Sample collection and processing prior to analysis is a critical step for obtaining a meaningful and reproducible sugar quantification of plant tissue. Post sampling metabolic conversions by still active enzymes present in tissues can be drastically reduced by flash-freezing samples in liquid nitrogen immediately after harvesting. Nowadays, fast fixation by Focused Microwave Fixation (FMF) is an effective and practical alternative method of preserving the metabolite composition of a tissue. This method has been previously described for enzyme inactivation in mouse or rat brain (Davila et al. 2012; Kanamatsu and Tsukada 1999). To test whether sample fixation improved the results obtained by HR-MAS qNMR, backup samples (Figure 1b) were subject to FMF prior to HR-MAS qNMR, and their sugar content was compared with measurements obtained from non-fixed samples by the same analytical method. No differences in sugar content were found between FMF fixed and non-fixed samples when analysed by HR-MAS qNMR (see results and discussion in supplementary material), and non-fixed samples were used elsewhere in this study.

In addition to sample preservation, another factor that may influence any subsequent statistical analyses is the natural heterogeneity between different parts of the mesocarp, from the outermost subpeal mesocarp to the inner mesocarp, closest to the seed cavity. Moreover, sugar concentration in apical regions may differ from that in the equatorial mesocarp region of a fruit, and also there might be intra-fruit differences between the apical parts adjacent to the pedicel and mesocarp regions adjacent to the umbilicus (Biais et al. 2009; Lester 2008; Zhang and Li 2005). This is an important point in order to characterize sugar content spatial distribution in the whole fruit; a wide sampling should be performed, taking into account all the different regions present in the tissue, and avoiding collecting larger samples which would mean a mixture of regions with different sugar accumulation. On the other hand, when the goal is to compare data from different melon fruits, it is crucial to collect samples always from the same region. Also, it is highly advisable to collect four or more samples from the same individual fruit, so that intra-fruit variability can be taken into account. Similarly, when comparing different species, three or more individuals of each species must be analysed to consider the effect of intra-species variability. Consequently, in this

study we analyzed 4 samples per fruit, taken from the edible equatorial middle-mesocarp region of each melon fruit, and 3 fruits per variety were sampled. Samples were split in half and processed by the two analytical methods being compared, HR-MAS qNMR and enzymatic analysis, to minimize the influence of fruit heterogeneity on measurements by both methods (Figure 1).

Water and sugar content

The average water percentage in our melon mesocarp samples was $87 \pm 2.46\%$ (n=48), in good agreement with previously reported values, e.g. 84.35% and 87.35% in muskmelon pulp of Rony and Mirado cultivars respectively (Maestrelli et al. 2001), and 90.15%, 91.85% and 89.82% in cantaloupe, casaba and honeydew muskmelon varieties respectively (USDANDB 2011). Table 2 summarizes the content of sugar in six melon fruits of the varieties *reticulatus* and *cantalupensis* as determined by the HR-MAS NMR and enzymatic analysis methods, and the average content in each variety. Sugar content values varied amongst individual melons and were in the range: 1.4–7.3 g for sucrose, 0.4–2.5 g for glucose; and 0.73–2.83 g for fructose (values per 100 g of fresh tissue). Additionally, as summarized in Table 3, our values compare well with those in the previous literature (Biais et al. 2009; Hubbard et al. 1989; Kolayli et al. 2010; Lingle and Dunlap 1987; Schaeffer et al. 1987; Wang et al. 1996).

An inverse correlation was observed between sucrose and hexose accumulation. This observation reflects the existence of fine-tuning mechanisms for the control of sucrose metabolism in melon fruits Figure 3. In this respect, both acid invertase and sucrose phosphate synthase have been shown to be the key determinants of sucrose accumulation in melon fruit (Hubbard et al. 1989). An increase in sucrose concentration in melon fruits has been associated with a decline in acid invertase activity and an increase in sucrose phosphate synthase activity (Lingle and Dunlap 1987).

Sugar content in melon fruit determined by HR-MAS qNMR spectroscopy and enzymatic analysis methods

The sugar content results obtained by using the HR-MAS qNMR method were compared with the values obtained from the same sample by using the established enzymatic analysis protocol. For this purpose, data were grouped in twelve

datasets by combining three factors: sugar molecule, fruit variety, and the analytical method. Results are summarized in Table 2 and shown in Figure 4. The assessment of groups normality by visual inspection of the QQ-plots suggested that some may not follow a normal distribution but, only two datasets failed to pass the Shapiro-Wilk normality test (see supplementary material), a non-surprising result because for small samples even big departures from normality are not detected. The analysis of dataset skewness and kurtosis further evidenced a departure from normality (see supplementary material). Accordingly, non-parametric tests to compare the location and spread of datasets: a Levene's test was used to assess variance homogeneity, and a Wilcoxon signed rank test of paired data was performed to assess mean ranks differences.

The groups involved in the sugar content comparisons passed the Levene's test for homogeneity of variance across groups at level 0.05. They also passed the Wilcoxon signed rank test of paired data except for fructose in the *cantalupensis* ($V = 75$, $p = 0.002$) and *reticulatus* fruits ($V = 8$, $p = 0.012$), and of glucose in the *reticulatus* fruits ($V = 6$, $p = 0.007$), which showed significant difference between methods. This could be due to compound effect of the natural interfruit variability (Table 2), the short number of fruits in this study ($n=3$), and the bias between the two analytical methods (see below).

Comparison of HR-MAS NMR spectroscopy and enzymatic analysis methods of measurement

As mentioned in the previous section, a bias between the two analytical methods used in this study may be partly responsible for the disagreement found when comparing sugar content measurements made by such methods in two melon varieties, more specifically, glucose content in the *reticulatus* variety and fructose content in both varieties, *reticulatus* and *cantalupensis*. To assess the agreement between methods, we pooled the measurements made in both varieties by each method and compared them. We used well accepted statistical analysis procedures specifically developed for this purpose.

The statistical analysis of method comparison studies has been the subject of extensive research and is reviewed elsewhere (Altman and Bland 1983; Carstensen 2010; Dewe 2009). More specifically, in this study we have used the

method of differences vs averages proposed by Bland and Altman (Altman and Bland 1983; Bland and Altman 1999), according to the guidelines by Castersen (Carstensen 2010). Briefly, the model assumes that two equivalent methods should result in similar measurements and exhibit a similar error across the range of measurements, and deviations from these assumptions are regarded as an indication that methods are not equivalent. The plot and regression of differences vs averages of paired measurements by each method is a simple way to visually assess and numerically test the model assumptions of constant bias and constant error across the range of measurements for equivalent methods. Additionally, the standard deviation of the differences vs averages equation(s) and conversion equations between methods can be used to compare the relative merits of several model assumptions. Last, the comparison of the several sources of variability quantified by the model can further help to identify the largest sources of variation amongst methods.

The correlation plots of sugar content by the HR-MAS NMR and enzymatic methods, and the calculated conversion equations and prediction limits, are presented in Figures 5a, 5c and 5e, while Figures 5b, 5d and 5f show the difference vs average plot of paired measurements, the calculated average difference or bias (solid lines), and the 95% limits of agreement (LoA, dashed lines) with LoA values shown next to the lines. Red lines link replicate measurements from the same melon fruit (4 replicates per fruit).

Comparison of methods for sucrose quantification

Figure 5a shows that sucrose measurements by HR-MAS NMR correlate well with measurements made by the enzymatic method, with values laid along a line close to the 45° identity line and uniformly spread across the range of measurements. Likewise, the difference vs average plot in Figure 5b suggests a constant bias and constant error across the range of sucrose content, a fact statistically confirmed (see p-values in Table 4). For sucrose, the average difference between methods is much smaller than the smallest amount of sucrose measured (0.268 vs 1.331 g/100 g fw), but the limits of agreement between methods are rather large and could be affected by a suspected outlier measurement (sample R1.1, see below). Also, as reported in Table 4, the enzymatic method variability is the largest source of variability, while the HR-MAS qNMR method

plays a smaller role, albeit it contributes more than the variance between replicates and the matrix effect. Last, the methods residual variances are in agreement with the difference between the coefficients of repeatability of the enzymatic and HR-MAS NMR methods (see Table 4), a difference that could be attributable to the larger amount of manipulation required for the enzymatic method, although we cannot rule an artefactual contribution by the candidate outlier R1.1 (see below). In summary, these findings confirm that these methods can be regarded as equivalent when measuring sucrose content in this range of values.

Comparison of methods for glucose quantification

The scatter-plot of glucose measurements by the HR-MAS NMR and enzymatic methods shows a good agreement amongst methods, with data points arranged along a line close to the 45° identity line and uniformly spread across the range of measurements (Figure 5c). Likewise, Figure 5d shows a constant bias and constant error across the range of glucose content, with $P = 0.741$ and $P = 0.728$, respectively (Table 4). The average difference in glucose measurements between methods is 0.155 g/100 g fw, less than half the lowest amount of glucose measured (0.400 g/100 g fw), but the limits of agreement between methods (LoA: 0.742 and -0.433) are rather large and could be affected by a suspected outlier measurement (sample R1.1, read below). The sources of variance in the linear mixed effects model (see Table 4) show that the residual variance of the enzymatic method is the largest source of variability by large, and that all other sources of variability have a similar and much smaller contribution. This is in agreement with the coefficients of repeatability obtained for both analytical methods (Table 4). Accordingly, both methods can be regarded as equivalent for the measurement of glucose, although the enzymatic method has a much larger variability, maybe because the amount of manipulation it requires. Also, as stated before, we cannot rule an artefactual contribution by the candidate outlier R1.1 (see below).

Comparison of methods for fructose quantification

Figure 5e shows that fructose measurements by HR-MAS NMR correlate well with measurements made by the enzymatic method but that they deviate from the

identity line, yielding the HR-MAS NMR method smaller values when fructose content is below 2 g/100 g fw and conversely larger values when fructose content is above that point. Similarly, the differences vs averages plot (Figure 5f) shows a non-constant bias and constant error across the range of fructose content, with $P = 0.002$ and $P = 0.764$, respectively (Table 4). Accordingly, measurements were fitted again with a non-constant bias model, although we were forced to use a non-linked replicates model because the minimizing algorithm failed to converge when using a linked-replicates model. The consequences of this simplification of the model are slightly overestimated prediction intervals, and the impossibility to quantify the replicates variance contribution, which leaves the method residual variances as the only source of variance. The results of the fitting of a non-constant bias model confirm that measurements by the HR-MAS NMR method are linearly related to measurements by the enzymatic method, with a scale factor of 1.58 and an offset of -1.1, and that HR-MAS NMR would yield smaller values than the enzymatic method when fructose content is under 1.89 g/100 g fw, and larger values above that point. The fitting to a non-constant bias model does not yield limits of agreement in the sense defined by Bland and Altman for a constant-bias model, but the comparison of the prediction standard deviation of the regression equations of differences on averages shows that the non-constant bias model results in somewhat narrower prediction limits (SD = 4.071 vs 4.278). Finally, the HR-MAS NMR method residual variance is 30% smaller than the enzymatic method residual variance (SD = 0.212 vs 0.304). These values show that, in our fructose measurements, the enzymatic method is the largest source of variability, maybe because the amount of manipulation it requires. As stated before, we cannot rule an artefactual contribution by the candidate outlier R1.1 (see below).

Assessment of R1.1 candidate outlier effect

In spite of the good agreement between methods, in all cases the prediction limits of the conversion equations and the limits of agreement are rather large, and the enzymatic method residual variance is much larger than the HR-MAS NMR method residual variance. This could be related to the sugar content values reported by the enzymatic method for replicate no 1 from fruit no 1 of the *reticulatus* variety (sample labelled as R1.1) which shows a large discrepant value

for all three sugars when compared to the other three replicate measurements by the same method and to the four replicate measurements by the HR-MAS NMR method. Since these values originate from the same sample, we can suspect that it was accidentally altered during its manipulation before the enzymatic assay, which would allow us to disregard its values as outliers (Finney 2006; Ludbrook 2008). Accordingly, we have redone the method comparison analysis upon discarding these anomalous values.

Comparison of methods for sucrose quantification with outliers excluded

The reanalysis of sucrose measurements upon removal of the R1.1 outlier results in a better agreement between analytical methods: the average difference (bias) and the error remain constant over the range of measurements; the average difference is smaller (0.136 vs 0.268 g/100 g), and the limits of agreement are closer, yielding a smaller prediction interval ($SD = 0.587$ vs 0.914). Furthermore, both analytical methods have now similar residual variances ($SD = 0.392$ and 0.362), on the same scale of the replicates variance component ($SD = 0.419$), while random interactions between methods and items (matrix effect) contribute the least to data variability ($SD = 0.174$). Last, both analytical methods exhibit now similar coefficients of repeatability (Table 4). In consequence, both methods can be regarded as equivalent for the measurement of sucrose.

Comparison of methods for glucose quantification with outliers excluded

The reanalysis of glucose measurements upon removal of the R1.1 outlier results in a better agreement between analytical methods: the bias and error remain constant over the range of measurements; the average difference is smaller (0.107 vs 0.155 g/100 g), the limits of agreement between methods are much closer, and the prediction interval narrows (Figures 5c and 5d, and Table 4). Nonetheless, the enzymatic method residual variance remains much larger than the HR-MAS NMR method residual variance ($SD = 0.156$ vs 0.060), which is on the scale of the replicates variance component ($SD = 0.087$) and matrix effect variance ($SD = 0.041$). That difference between analytical methods can be seen too in their coefficients of repeatability: $CoR = 0.505$ g/100 g fw and 0.300 g/100 g fw, respectively for the enzymatic and HR-MAS qNMR methods. Thus, we conclude that both methods can be regarded as equivalent for the measurement of glucose,

yielding the HR-MAS NMR method values slightly higher on average, and having it a better repeatability.

Comparison of methods for fructose quantification with outliers excluded

The reanalysis of fructose measurements upon removal of the R1.1 outlier results in a better agreement between analytical methods. As before, the null hypothesis of constant bias must be rejected with $P = 0.001$ and the error remains constant with $P = 0.543$, and the fitting of a non-constant bias model (now with linked measurements) confirms that measurements by both methods are linearly related, and that HR-MAS NMR yields smaller values when fructose content is under 2.06 g/100 g fw and larger values above that point (Figures 5e and 5f, and Table 4). Furthermore, the prediction intervals of the regression equations are narrower, and the enzymatic method residual variance is now much smaller ($SD = 0.180$ vs 0.304) and similar to the HR-MAS NMR method residual variance ($SD = 0.213$). Thus, the exclusion of the outlier has clearly improved the agreement between methods, and further supports our conclusion that both methods can be regarded as equivalent for the measurement of fructose.

Coefficient of variation and repeatability

The coefficient of variation (CV) was measured to evaluate the method repeatability and to allow for the comparison of our method with other previously reported. Ten sampled aliquots from the equatorial middle-mesocarp region of a single fruit (var. *reticulatus*) were directly analysed by HR-MAS qNMR. The areas of selected resonances at 5.4, 3.2 and 4.12 ppm were used as described in methods to determine sucrose, glucose and fructose concentrations respectively. The CV for the selected resonances was 10.2% for sucrose, 5.6% for glucose and 7.2% for fructose. These CV are somewhat higher than values reported on 1D ^1H -NMR analysis of juice samples (range 1-6%) (Moing et al. 2004) and on HPLC analysis of ethanolic extracts from fresh tissues (2.1-4.8%) (Hubbard et al. 1989), but are similar to values reported for other analytical methods, (CV in the range 3.7-15.6%) (Biais et al. 2009). Furthermore, because of the described sugar gradient concentration with sampling position (Biais et al. 2009), intra-fruit variability could account for some of the higher variability in our measured data.

The CV obtained for the enzymatic analysis of ethanolic extracts were 10.5% for sucrose, 11.4% for glucose, and 9.4% for fructose, and were similar to the CV values obtained for HR-MAS qNMR. Intrinsic heterogeneity due to individual characteristics of each fruit was observed in both melon varieties and affected all three quantified metabolites (Biais et al. 2009). Due to the important heterogeneity between fruits of the same variety, it would not be really sensible to compare sugar concentrations from the two different varieties without accounting for biological heterogeneity. Accordingly, the relevant information that should be here considered is the one from the single fruit data. For comparison, Figure 6 shows representative spectra from fruits of the same variety but with different sugar content, while Figure 4 compares the sugar content determined by both methods in the two melon varieties studied. In this respect, our results fully agree with the variability of sucrose concentration previously observed by ¹H-NMR of melon fruit extracts (Biais et al. 2009). In line with results obtained with other fruits (Gil et al. 2000), differences in ripening stage could well account for the sucrose content heterogeneity observed in the individual fruits studied here.

Interest of HR-MAS NMR for sugar determination in plant tissues.

Sugar quantification provides useful information for a wide range of plant studies. For instance, alterations in sugar content might reflect important features of the plant physiological state, such as photoassimilate content and partitioning or fruit maturation (Gil et al. 2000; Hubbard et al. 1989; Monforte et al. 2004). Moreover, melon cultivars accumulate different levels of sucrose which correlate with an important phenotypic and genetic variability. In addition to the natural variability, variations in sucrose levels also occur within a particular cultivar as a result of environmental and developmental factors. Thus, the HR-MAS NMR method could be beneficial for studying the ripening process of melon fruits as well as for evaluating their sweetness level.

In melon, genomic studies, genetic mapping and Quantitative Trait Locus (QTL) analyses, have substantially contributed to a better understanding of the regions of the genome involved in fruit quality traits (Gonzalez-Ibeas et al. 2007; Gonzalez et al. 2010; Monforte et al. 2004). Very recently, the genome sequence of melon has been reported (Garcia-Mas et al. 2012). New genetic resources for melon genetics and genomics, such as a collection of near isogenic lines (NILs) have

also been developed, and can be used in fruit quality improvement programs (Eduardo et al. 2005). HR-MAS NMR spectroscopy clearly has a potential application in melon breeding programs designed for the selection of sucrose-accumulating genotypes focusing on consumer interests, as well as to determine the postharvest behaviour and the effect of post-harvest storage conditions of this fruit.

Finally, the HR-MAS NMR technique can be useful to detect physiological changes in plants in response to different environmental conditions, including biotic and abiotic stress (Fumagalli et al. 2009; Sidhu et al. 2010). In this respect, several studies support a link between sugar metabolism and disease resistance in plants, the susceptibility of plants to a number of diseases being often related to sucrose levels of plant tissues (Herbers et al. 1996; Murillo et al. 2003; Whipps and Lewis 1981). Pathogen infection might also result in an increase in sucrose levels in those plant tissues (Chou et al. 2000). Thus, in addition to the above mentioned studies, HR-MAS NMR might be a convenient technique for fast analytical sampling in plant-pathogen research programmes.

Conclusions

We show here that the HR-MAS qNMR technique provides a reproducible and reliable method for quantifying sucrose, glucose and fructose content in melon fruits. When comparing measurements obtained by HR-MAS NMR with those obtained by the traditional, time-consuming enzymatic assay on ethanolic extracts, we observe that the results are statistically equivalent and conversion equations between methods are derived. Most importantly, the results are obtained much faster by HR-MAS than with the enzymatic method or by any other presently available method. Furthermore, the repeatability of the HR-MAS NMR methods equals or is better than that of the enzymatic method. Traditional strategies for quantifying these compounds require a considerable amount of time (usually more than 48 h) and rely on the need of extract preparation. Contrary to this, HR-MAS NMR spectroscopy of fresh tissue only takes about 20-25 minutes, including sample preparation, and offers first-pass quantitative results in less than 1 hour and precise results in 12 hours, after sample freeze drying for actual water content determination. Furthermore, although we focused on the content of sugars in this study, it is obvious that any other metabolite of interest present in the NMR

spectra could be similarly quantified without requiring additional sample manipulation, which will substantially aid in a wide range of plant studies.

Competing interests. The authors declare that they have no competing interests

Authors' contributions. TD-G and SC carried out the experimental work with support from JM-S and MEC. CA and BSS conceived the study and devised the experimental design. TD-G, MEC, BSS and CA wrote the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1 - Diagram of the sampling procedure. **(a)** An equatorial slice (3.5 cm thick) was cut from each melon and 4 cylinders of 2 cm diameter were obtained from equidistant regions of the mesocarp region. **(b)** Cylinders were cut in half, and one of the halves was stored in liquid nitrogen as backup sample (e.g. for the focused-microwave tests), and the other one was used for HR-MAS qNMR and enzymatic analysis. **(c)** The inner core (0.5 cm diameter) of the cylinder was

removed and used for the immediate analysis of fresh tissue by HR-MAS qNMR. The outer part of the cylinder was stored in liquid nitrogen and later analysed by the enzymatic procedure.

Figure 2 - Water suppressed HR-MAS ^1H NMR (9.4 T spectrum from *Cucumis melo* var. *reticulatus* intact mesocarp. **(a)** Full range of the spectral profile recorded from 55.1 mg of fruit tissue is shown at the top. **(b)** Expansion of the region of interest (3.0 to 5.5 ppm containing the sugars quantified in this work) is shown below it. Major resonances of interest are numbered: 1, H-C1-glucopyranosyl in sucrose at 5.42 ppm; 2, H-C1 α -D glucopyranose at 5.24 ppm; 3, H-C1 α -D-glucopyranose at 4.65 ppm; 4, H-C4 β -fructofuranose and H-C3 α,β -fructofuranose at 4.11-4.13 ppm; 5, H-C6-glucopyranosyl in sucrose at 3.84 ppm; 6, H-C4 D-glucopyranose at 3.25 ppm. Peaks labelled with numbers 1,4 and 6 were used to quantify sucrose, fructose and glucose content, respectively.

Figure 3 - Correlation plots of sugar content. Correlation plots between free sugar compounds for all samples from ripe melons analyzed in this study by HR-MAS qNMR and enzymatic analysis. As expected, the free sugars equilibrium through sucrose phosphate synthase and acid invertases enzymes causes glucose and fructose to be positively correlated, and both sugars to be negatively correlated with sucrose.

Figure 4 – Comparison of **(a)** sucrose, **(b)** glucose and **(c)** fructose content determined by HR-MAS qNMR (mas) vs enzymatic analysis (enz) for each melon variety. The analytical methods yielded a highly significant difference in fructose content in the *cantalupensis* (cantal, $V = 75$, $p = 0.002$) and *reticulatus* (retic, $V = 8$, $p = 0.012$) varieties, and in the content of glucose in *reticulatus* fruits ($V = 6$, $p = 0.007$). Box plots show the median (horizontal line) within a box with boundaries indicating the 1st and 3rd quartiles, and containing the central 50% of the observations. Whiskers mark the lowest datum within 1.5 IQR of the lower

quartile and the highest datum within 1.5 IQR of the upper quartile. *Statistically significant difference at the 0.05 level,

Figure 5 - Comparison of HR-MAS qNMR and enzymatic analysis methods for measuring the amount of free sugars in melon fruits. **(a, c, e)** Correlation plots and **(b, d, f)** Bland-Altman plots of paired values of the amount of **(a, b)** sucrose, **(c, d)** glucose and **(e, f)** fructose in the mesocarp of *Cucumis melo* fruits measured by the HR-MAS qNMR and enzymatic analysis procedures. Measurements by both methods show good agreement, with constant bias between methods for sucrose and glucose measurements, and non-constant bias for fructose measurements, and constant error across the range of sugar content in all cases. All measurements in replicate no 1 from fruit no 1 of the *reticulatus* variety (red open circle, labeled “R1.1.”) differ from measurements in all other replicates from the same fruit, and are candidate outliers. Accordingly, regressions were performed for all measurements (light blue lines) and upon excluding the R1.1 outlier (dark blue lines). Correlation plots show the conversion equation between methods (solid lines) and the prediction limits (dashed lines), while Bland-Altman plots show the average difference (solid line) and the 95% limits of agreement (dashed lines, LoA values are shown next to the lines). Red lines link replicate measurements from the same melon fruit (4 replicates per fruit). Although fructose measurements by both methods exhibit a non-constant bias, the average difference and limits of agreement of a constant bias model are shown for comparison purposes (light green lines).

Figure 6 - 9.4 T HR-MAS NMR spectra from *Cucumis melo* var. *cantalupensis* without water suppression illustrates the large variability in sugar composition that can be found amongst fruits of the same variety. Quantification by HR-MAS qNMR and enzymatic assays demonstrated **(a)** high levels of sucrose and low levels of glucose and fructose in melon 4 and **(b)** conversely low levels of sucrose and high levels of glucose and fructose in melon 6. Peak assignments (numbered as in Figure 2): 1, H-C1-glucopyranosyl in sucrose at 5.42 ppm; 4, H-C3 fructose (fructofuranose and fructopyranose forms) at 4.12 ppm; 6, H-C4 D-glucopyranose at 3.25 ppm.

a **b** **c**

a **b** **c** **d** **e** **f**

Supplementary content - Assessment of a ¹H high-resolution magic angle spinning NMR spectroscopy procedure for free sugars quantification in intact plant tissue

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Summary of sugar content data

The tables below summarize the sugar content in individual melon fruits and in the several subsets resulting from the combination at different levels of the variety, sugar and method factors.

TABLE S1 - Average amount of free sugars (in g / 100 g fw) in each fruit used in this study (mean $\pm$ sd, of n = 4 replicates)

melon	variety	sugar	method	amount	
				mean	sd
1	retic	fru	enzym	2.3775	0.65769
1	retic	fru	hrmas	2.7896	0.41833
1	retic	glc	enzym	1.9500	0.64895
1	retic	glc	hrmas	2.3730	0.06389
1	retic	suc	enzym	4.1525	1.46133
1	retic	suc	hrmas	5.2160	0.91024
2	retic	fru	enzym	2.2325	0.17443
2	retic	fru	hrmas	2.7182	0.10838
2	retic	glc	enzym	2.4400	0.13342
2	retic	glc	hrmas	2.5762	0.13780
2	retic	suc	enzym	1.7050	0.13964
2	retic	suc	hrmas	1.5099	0.24431
3	retic	fru	enzym	2.5050	0.28769
3	retic	fru	hrmas	2.5600	0.15886
3	retic	glc	enzym	1.7850	0.08505

TABLE S1 - Average amount of free sugars (in g / 100 g fw) in each fruit used in this study (mean $\pm$ sd, of n = 4 replicates)

melon	variety	sugar	method	amount	
				mean	sd
3	retic	glc	hrmas	1.9587	0.13384
3	retic	suc	enzym	4.0925	0.43904
3	retic	suc	hrmas	4.5779	0.64835
4	cantal	fru	enzym	1.8825	0.14523
4	cantal	fru	hrmas	1.5940	0.11343
4	cantal	glc	enzym	1.5875	0.22021
4	cantal	glc	hrmas	1.5318	0.11555
4	cantal	suc	enzym	2.8225	0.29658
4	cantal	suc	hrmas	2.4410	0.08745
5	cantal	fru	enzym	1.3800	0.09626
5	cantal	fru	hrmas	1.0528	0.06890
5	cantal	glc	enzym	1.0250	0.08963
5	cantal	glc	hrmas	1.2432	0.06055
5	cantal	suc	enzym	5.5150	0.50017
5	cantal	suc	hrmas	6.0050	0.59048
6	cantal	fru	enzym	1.1050	0.12715
6	cantal	fru	hrmas	0.8554	0.20768
6	cantal	glc	enzym	0.5075	0.09878
6	cantal	glc	hrmas	0.5405	0.09720
6	cantal	suc	enzym	7.7225	0.96979
6	cantal	suc	hrmas	7.8669	0.41703

TABLE S2 - Data summary for subsets resulting from the combination of variety, sugar and method factors

Melon	Variety	Sugar	Method	Amount of sugar													
				Mean	SD	n	Min	1st. Qu	Median	Trimm Median	MAD	3rd Qu.	Max	Range	Skew	Kurt	SE
1 to 3	retic	fru	enzym	2.372	0.4029	12	1.4200	2.210	2.430	2.412	0.2743	2.58	2.920	1.5000	-0.72973	0.2087	0.11632
1 to 3	retic	fru	hrmas	2.689	0.2605	12	2.1796	2.530	2.769	2.703	0.3118	2.80	3.067	0.8870	-0.25589	-0.9243	0.07519
1 to 3	retic	glc	enzym	2.058	0.4540	12	1.0900	1.760	2.025	2.095	0.4818	2.37	2.660	1.5700	-0.44386	-0.6750	0.13105
1 to 3	retic	glc	hrmas	2.303	0.2885	12	1.8453	2.100	2.355	2.309	0.3250	2.49	2.699	0.8535	-0.30305	-1.3399	0.08327
1 to 3	retic	suc	enzym	3.317	1.4345	12	1.6000	1.850	3.770	3.281	2.1646	4.30	5.390	3.7900	-0.02287	-1.7909	0.41410
1 to 3	retic	suc	hrmas	3.768	1.7922	12	1.3309	1.770	4.365	3.804	1.7814	5.07	5.847	4.5158	-0.31641	-1.7472	0.51737
4 to 6	cantal	fru	enzym	1.456	0.3546	12	0.9900	1.220	1.410	1.444	0.3929	1.75	2.040	1.0500	0.29582	-1.4024	0.10236
4 to 6	cantal	fru	hrmas	1.167	0.3506	12	0.6198	0.942	1.107	1.165	0.3960	1.49	1.742	1.1226	0.21881	-1.3758	0.10121
4 to 6	cantal	glc	enzym	1.040	0.4799	12	0.4000	0.600	1.055	1.026	0.6005	1.39	1.820	1.4200	0.18392	-1.4231	0.13853
4 to 6	cantal	glc	hrmas	1.105	0.4430	12	0.4040	0.617	1.229	1.122	0.4782	1.41	1.642	1.2381	-0.37798	-1.6109	0.12789
4 to 6	cantal	suc	enzym	5.353	2.1745	12	2.4500	3.080	5.290	5.325	3.1728	6.72	8.540	6.0900	0.05070	-1.5427	0.62772
4 to 6	cantal	suc	hrmas	5.438	2.3818	12	2.3118	2.500	5.949	5.455	2.7849	7.49	8.390	6.0785	-0.27174	-1.7438	0.68758

1st.Qu: first quartile. 3rd.Qu: third quartile

Trimm Median: trimmed median. MAD: Median absolute deviation.

Skew: skewness coefficient. Kurt: kurtosis coefficient

SE: standard error

TABLE S3 - Data summary for subsets resulting from the combination of sugar and method factors

Melon	Variety	Sugar	Method	Amount of sugar													
				Mean	SD	n	Min	1st. Qu	Median	Trimm Median	MAD	3rd Qu.	Max	Range	Skew	Kurt	SE
1 to 6	r & c	fru	enzym	1.914	0.5972	24	0.9900	1.41	2.000	1.905	0.8228	2.43	2.920	1.930	0.027167	-1.3546	0.1219
1 to 6	r & c	fru	hrmas	1.928	0.8339	24	0.6198	1.11	1.961	1.937	1.2169	2.77	3.067	2.447	-0.095132	-1.6873	0.1702
1 to 6	r & c	glc	enzym	1.549	0.6923	24	0.4000	1.07	1.725	1.552	0.9415	1.99	2.660	2.260	-0.082997	-1.2299	0.1413
1 to 6	r & c	glc	hrmas	1.704	0.7126	24	0.4040	1.24	1.744	1.730	0.8371	2.35	2.699	2.295	-0.312764	-1.1878	0.1455
1 to 6	r & c	suc	enzym	4.335	2.0803	24	1.6000	2.65	4.115	4.189	1.9793	5.36	8.540	6.940	0.440571	-0.8387	0.4246
1 to 6	r & c	suc	hrmas	4.603	2.2309	24	1.3309	2.48	4.928	4.571	3.0878	5.97	8.390	7.059	0.007067	-1.3404	0.4554

r & c: both varieties, reticulatus and cantalupensis

1st.Qu: first quartile. 3rd.Qu: third quartile

Trimm Median: timmed median. MAD: Median absolute deviation.

Skew: skewness coefficient. Kurt: kurtosis coefficient

SE: standard error

Normality assessment of sugar content subsets

The sugar content results obtained by using the HR-MAS qNMR method were grouped in twelve datasets by combining three factors: sugar molecule, fruit variety, and the analytical method. The assessment of groups normality by visual inspection of the QQ-plots suggested that some may not follow a normal distribution. In spite of this, only two datasets fail to pass the Shapiro-Wilk normality test: sucrose content by HR-MAS NMR in *cantalupensis* fruits ($p = 0.040$) and in *reticulatus* fruits ($p = 0.039$), although two additional datasets had p-values close to significance: glucose content by HR-MAS NMR in *cantalupensis* fruits ($p = 0.085$) and sucrose content by enzymatic analysis in *reticulatus* fruits ($p = 0.079$). These results are not surprising as it is well known that for samples of small size ($n = 12$ in our case) even big departures from normality are not detected by the normality test. Also, the fact that our 12 measurements come from a set of 4 replicates obtained from each of 3 different fruits, and that intrafruit variability (inter-replicates) is expected to be smaller than interfruit variability, may further contribute to this situation. The skewness and kurtosis coefficients of the groups were also inspected as they provide certain evidence of deviations from normality. No group showed a skewness significantly different than zero, and only one data set showed a moderate skewness to the left (fructose content by enzymatic analysis in *reticulatus* fruits, $g_1 = -0.730$), and all the other data sets had

skewness values in the range [-0.444, 0.296]. Similarly, no group showed a kurtosis significantly different than zero, but nine data sets were clearly platykurtic with kurtosis coefficients in the range [-1.791, -1.340], two were moderately platykurtic ($g_2 = -0.924$ and -0.675) and one sample showed a slightly leptokurtic distribution ($g_2 = 0.209$). Because of these indications of non-normality, non-parametric tests were used to compare the location and spread of datasets: a Levene's test was used to assess variance homogeneity, and a Wilcoxon signed rank test of paired data was performed to assess mean ranks differences.

Comparison of datasets obtained by the two analytical methods

The datasets obtained by the two analytical methods were compared by using the non-parametric Levene's test to assess variance homogeneity, and the non-parametric Wilcoxon signed rank test of paired data to assess mean ranks differences.

TABLE S4 - Comparison of sugar content measured by HR-MAS NMR and Enzymatic Analysis

Melon	Variety	Sugar	Levene's Test for Homogeneity of Variance (center = median)	Wilcoxon signed rank test
1 to 6	cantal	fru	F value = 0.01, Pr(>F) = 0.91	V = 75, p-value = 0.002441 *
1 to 6	cantal	glc	F value = 0.1, Pr(>F) = 0.75	V = 23, p-value = 0.2334
1 to 6	cantal	suc	F value = 0.22, Pr(>F) = 0.64	V = 32, p-value = 0.6221
1 to 6	retic	fru	F value = 0.64, Pr(>F) = 0.43	V = 8, p-value = 0.01221
1 to 6	retic	glc	F value = 2.4, Pr(>F) = 0.14	V = 6, p-value = 0.006836 *
1 to 6	retic	suc	F value = 0.55, Pr(>F) = 0.47	V = 25, p-value = 0.3013
1 to 6	r & c	fru	F value = 8.16, Pr(>F) = 0.0064 *	V = 148, p-value = 0.9664
1 to 6	r & c	glc	F value = 0.06, Pr(>F) = 0.81	V = 56, p-value = 0.005898 *
1 to 6	r & c	suc	F value = 0.23, Pr(>F) = 0.63	V = 111, p-value = 0.2768

r & c: both varieties, *reticulatus* and *cantalupensis*

* statistically significant differences

All groups involved in the sugar content comparisons passed the Levene's test for homogeneity of variance across groups at level 0.05 except for fructose in the dataset combining *reticulatus* and *cantalupensis* fruits ($p = 0.0064$). The comparison of the sugar content determined by the enzymatic and HR-MAS NMR methods using the Wilcoxon signed rank test of paired data resulted in no statistically significant differences at level 0.05 for sucrose and glucose content in the *cantalupensis* variety, and sucrose in the *reticulatus* fruits. Conversely, the content of fructose in the *cantalupensis* ($V = 75$, $p = 0.002$) and *reticulatus* fruits ($V = 8$, $p =$

0.012), and the content of glucose in the *reticulatus* fruits ($V = 6$, $p = 0.007$) showed a significant difference between methods. This could be due to compound effect of the natural interfruit variability, the short number of fruits used in this study, and the bias between the two analytical methods.

Comparison of HR-MAS NMR spectroscopy measurements in focused-microwave denaturated samples and intact samples

To assess whether the high rate sample spinning and the room temperature acquisition times required by the HR-MAS NMR method caused any artefactual alteration of free sugar levels, we measured the content of sucrose, glucose and fructose by recording 1D ^1H HR-MAS NMR spectra from paired samples: an untreated sample (control) and its focused microwave denaturated counterpart (FMF), and we compared the sugar content by using the method of differences vs averages proposed by Bland and Altman (1, 2), according to the guidelines by Castersen (3). A more detailed explanation of the method and the interpretation of its results is given in the article.

Comparison of sucrose measurements

As shown in the Figure S1, sucrose measurements in paired control and treated samples show a good agreement, with constant bias and constant error across the range of measurements ($P = 0.571$ and $P = 0.430$, respectively), a small average difference (-0.0515), and close limits of agreement (-1.0972 , 0.9942). The sources of variance in decreasing order are: the replicates variance component ($SD = 0.472$), the residual variance for the FMF samples ($SD = 0.344$), the residual variance for the control samples ($SD = 0.289$), and the random interactions between treatments and items (matrix effect) variance ($SD = 0.189$). The coefficients of repeatability obtained for the irradiated and non-irradiated samples ($\text{CoR} = 1.652 \text{ g}/100 \text{ g fw}$ and $1.566 \text{ g}/100 \text{ g fw}$, respectively) are in good agreement with the residual variances. Furthermore, HR-MAS NMR sucrose content measurements from FMF and control samples are in better agreement than sucrose content values obtained by using the enzymatic and HR-MAS NMR methods, and result in a smaller average difference (-0.051 vs $0.136 \text{ g}/100 \text{ g}$) and closer limits of agreement (2.091 vs 2.347). Thus, FMF has a negligible effect upon sucrose content measured values without FMF. Accordingly, we conclude that the HR-MAS NMR acquisition conditions used in fresh samples are not able to influence the sucrose content measurement in the melon tissue due to potential activation or release of enzymatic activities.

Figure S1: Comparison of sucrose content measurements by HR-MAS qNMR in FMF (fmf) and control (hrmas) samples.

Comparison of glucose measurements

As shown in the Figure S2, glucose measurements in paired control and treated samples show a good agreement, with a slightly significant non-constant bias and constant error across the range of measurements ($P = 0.045$ and $P = 0.462$, respectively), and small prediction SD (0.198 and 0.220). The sources of variance in decreasing order are: the residual variance for the FMF samples ($SD = 0.173$), the control samples residual variance and the FMF replicates variance component (both with $SD = 0.079$), the matrix effects variance from FMF measurements ($SD = 0.073$) and from control measurements ($SD = 0.066$). Since we have a slightly significant non-constant bias ($P = 0.045$), for convenience we will use the average difference and limits of agreement from the constant bias model to compare the agreement between FMF and control samples, with the agreement between HR-MAS NMR and enzymatic analysis. The former show a much smaller average difference (-0.051 vs 0.136 g/100 g) and closer limits of agreement (2.091 vs 2.347). Thus, FMF has a negligible effect upon glucose content measured values without FMF. Accordingly, we conclude that the HR-MAS NMR acquisition conditions used in fresh samples are not able to influence the glucose content measurement in the melon tissue due to potential activation or release of enzymatic activities.

Figure S2: Comparison of glucose content measurements by HR-MAS qNMR in FMF (fmf) and control (hrmas) samples.

Fructose

As shown in the Figure S3, fructose measurements in paired control and treated samples show a good agreement, with a clearly significant non-constant bias and constant error across the range of measurements ($P = 0.017$ and $P = 0.922$, respectively), and small prediction SD (0.256 and 0.310). The sources of variance in decreasing order are: the residual variance for the FMF samples ($SD = 0.199$) and the control samples residual variance ($SD = 0.196$), while the replicates variance component and the matrix effects variance, both with $SD = 0$, have a negligible contribution. Since we have a clearly significant non-constant bias in the FMF vs control samples comparison ($P = 0.017$) and the HR-MAS NMR vs enzymatic analysis comparison ($P = 0.001$), we must rely on the prediction SD to assess the relevance of the FMF step on the fructose measurements variability, and the prediction SD (fmf vs control: 0.256 and 0.310; HR-MAS NMR vs

enzymatic analysis: 0.390 and 0.281) indicate that *fmf* causes a smaller variability than differences amongst fructose content measurements made by the two analytical methods. Thus, FMF has a negligible effect upon fructose content measured values without FMF. Accordingly, we conclude that the HR-MAS NMR acquisition conditions used in fresh samples are not able to influence the fructose content measurement in the melon tissue due to potential activation or release of enzymatic activities.

Figure S3: Comparison of fructose content measurements by HR-MAS qNMR in FMF (*fmf*) and control (*hrmas*) samples.

Overall conclusion about the FMF-step

The denaturation of samples by means of focused-microwave irradiation prior to the HR-MAS NMR experiments did not improve the results obtained by direct analysis of the samples, quite possibly because of the comparatively slow metabolism of sugars in the melon mesocarp. On the other side, the cause of the non-constant bias between control and denaturated samples in the case of glucose and fructose measurements

remains unknown. These conclusions fully support our decision to only compare the HR-MAS NMR results obtained from intact samples with the results obtained by the enzymatic analysis method.

Comparison of HR-MAS NMR spectroscopy and enzymatic analysis methods of measurement

These are the results of the initial comparison of the full datasets prior to excluding an outlier sample. The final statistical analysis of the datasets upon excluding the outlier point is reported in the Results & Discussion section of the manuscript.

Comparison of methods for sucrose quantification

Figure S4a shows that sucrose measurements by HR-MAS NMR correlate well with measurements made by the enzymatic method, with values laid along a line close to the 45° identity line and uniformly spread across the range of measurements. Likewise, the difference vs average plot in Figure S4b suggests a constant bias and constant error across the range of sucrose content, a fact statistically confirmed (see p-values in Table S5). For sucrose, the average difference between methods is much smaller than the smallest amount of sucrose measured (0.268 vs 1.331 g/100 g fw), but the limits of agreement between methods are rather large and could be affected by a suspected outlier measurement. Also, as reported in Table S5, the enzymatic method variability is the largest source of variability, while the HR-MAS qNMR method plays a smaller role, albeit it contributes more than the variance between replicates and the matrix effect. Last, the methods residual variances are in agreement with the difference between the coefficients of repeatability of the enzymatic and HR-MAS NMR methods (see Table S5), a difference that could be attributable to the larger amount of manipulation required for the enzymatic method and to the contribution by one suspected outlier value. These findings confirm that both methods can be regarded as equivalent when measuring sucrose content in this range of values.

Figure S4: Comparison of HR-MAS qNMR and enzymatic analysis methods for measuring the amount of free sugars in melon fruits. (a, c, e) Correlation plots and (b, d, f) Bland-Altman plots of paired values of the amount of (a, b) sucrose, (c, d) glucose and (e, f) fructose in the mesocarp of *Cucumis melo* fruits. Methods are in good agreement, with constant bias between methods for sucrose and glucose measurements, and non-constant bias for fructose measurements, and constant error across the range of sugar content in all cases. Correlation plots show the conversion between methods and the prediction limits (solid and dashed lines respectively), while the Bland-Altman plots show the average

difference and the 95% limits of agreement (solid and dashed lines respectively, LoA values are shown next to the lines). Red lines link replicate measurements from the same melon fruit (4 replicates per fruit). Although fructose measurements by both methods exhibit a non-constant bias, the average difference and limits of agreement of a constant bias model are shown for comparison purposes (light green lines).

Comparison of methods for glucose quantification

The scatter-plot of glucose measurements by the HR-MAS NMR and enzymatic methods shows a good agreement amongst methods, with data points arranged along a line close to the 45° identity line and uniformly spread across the range of measurements (Figure S4c). Likewise, Figure S4d shows a constant bias and constant error across the range of glucose content, with $P = 0.741$ and $P = 0.728$, respectively (Table S5). The average difference in glucose measurements between methods is 0.155 g/100 g fw, less than half the lowest amount of glucose measured (0.400 g/100 g fw), but the limits of agreement between methods (LoA: 0.742 and -0.433) are rather large and could be affected by a suspected outlier measurement. The sources of variance in the linear mixed effects model (see Table S5) show that the residual variance of the enzymatic method is the largest source of variability by large, and that all other sources of variability have a similar and much smaller contribution. This is in agreement with the coefficients of repeatability obtained for both analytical methods (Table S5). Accordingly, both methods can be regarded as equivalent for the measurement of glucose, although the enzymatic method has a much larger variability, maybe because the amount of manipulation it requires, although we cannot exclude an artefactual contribution by a candidate outlier value.

Comparison of methods for fructose quantification

Figure S4e shows that fructose measurements by HR-MAS NMR correlate well with measurements made by the enzymatic method but that they deviate from the identity line, yielding the HR-MAS NMR method smaller values when fructose content is below 2 g/100 g fw and conversely larger values when fructose content is above that point. This is confirmed by the differences vs averages plot (Figure S4f) that shows a non-constant bias and constant error across the range of fructose content, with $P = 0.002$ and $P = 0.764$, respectively (Table S5). Accordingly, measurements were fitted again with a non-constant bias model,

although we were forced to use a non-linked replicates model because the minimizing algorithm failed to converge when using a linked-replicates model. The consequences of this simplification of the model are slightly overestimated prediction intervals, and the impossibility to quantify the replicates variance contribution, which leaves the method residual variances as the only source of variance. The results of the fitting of a non-constant bias model confirm that measurements by the HR-MAS NMR method are linearly related to measurements by the enzymatic method, with a scale factor of 1.58 and an offset of -1.1, and that HR-MAS NMR would yield smaller values than the enzymatic method when fructose content is under 1.89 g/100 g fw, and larger values above that point. The fitting to a non-constant bias model does not yield limits of agreement in the sense defined by Bland and Altman for a constant-bias model, but the comparison of the prediction standard deviation of the regression equations of differences on averages shows that the non-constant bias model results in somewhat narrower prediction limits (SD = 4.071 vs 4.278). Finally, the HR-MAS NMR method residual variance is 30% smaller than the enzymatic method residual variance (SD = 0.212 vs 0.304). These values show that, in our fructose measurements, the enzymatic method is the largest source of variability, maybe because the amount of manipulation it requires, although we cannot discard an artefactual contribution by one candidate outlier measurement.

Table S5 – Summary of the statistical comparison of the HR-MAS NMR (h) and enzymatic analysis (e) methods for the full datasets. Methods were compared by using the difference vs averages approach (see text for details), and testing the assumptions of constant bias and constant error across the range of measurements. The conversion equations between methods, the corresponding prediction SD, the limits of agreement (LoA), the sources of variability, and the coefficient of repeatability (CoR) are reported. If the constant bias assumption holds true, the conversion equation yields the average difference or bias.

Sugar	n	Range		Model assumption	Conversion equations between methods (prediction SD)	P of constant bias	LoA	P of constant error	Sources of variability ^{a,b}			CoR ^b
		Min	Max						IxR	MxI	Resid	
sucrose	48	1.331	8.54	const bias	h - e = 0.2678 (0.9145)	0.429	-1.561 2.097	0.955	0.286	0.212	0.474 (h) 0.723 (e)	1.566 (h) 2.199 (e)
glucose	48	0.400	2.70	const bias	h - e = 0.1547 (0.2938)	0.741	-0.433 0.742	0.728	0.094	0.061	0.049 (h) 0.277 (e)	0.300 (h) 0.827 (e)
fructose	48	0.620	3.07	const bias	h - e = 0.0146 (0.4278)	0.002	-0.841 0.870	0.764	0.195	0.240	0.086 (h) 0.246 (e)	0.603 (h) 0.889 (e)
	48	0.620	3.07	non-const bias	h = -1.0939+1.5792 e (0.5249) e = 0.6927+0.6332 h (0.3324)	n.a. ^c	n.a. ^c	n.a. ^c	0	0	0.212 (h) 0.304 (e)	n.a. ^c

^a Sources of variance can be: IxR, item by replicate variance; MxI, method by item (matrix effect) variance; Resid, residual variance of the method. ^b h: HR-MAS

qNMR method, e: enzymatic analysis method. ^c n.a., not available in the non-constant bias model.

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