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ORIGINAL PAPER

An inter-laboratory comparison demonstrates that [1H]-NMR metabolite fingerprinting is a robust technique for collaborative plant metabolomic data collection

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Abstract In any metabolomics experiment, robustness and reproducibility of data collection is of vital importance. These become more important in collaborative studies where data is to be collected on multiple instruments. With minimisation of variance in sample preparation and instrument performance it is possible to elucidate even subtle differences in metabolite fingerprints due to genotype or biological treatment. In this paper we report on an inter laboratory comparison of plant derived samples by [1H]-NMR spectroscopy across five different sites and within those sites utilising instruments with different probes and magnetic field strengths of 9.4 T (400 MHz), 11.7 T (500 MHz) and 14.1 T (600 MHz). Whilst the focus of the study is on consistent data collection across laboratories, aspects of sample stability and the requirement for sample rotation within the NMR magnet are also discussed. Comparability of the datasets from participating laboratories was exceptionally good and the data were amenable to comparative analysis by multivariate statistics. Field strength differences can be adjusted for in the data pre-processing and multivariate analysis demonstrating that [1H]-NMR fingerprinting is the ideal technique for large scale plant metabolomics data collection requiring the participation of multiple laboratories.

Keywords Metabolomics · Metabolite profiling · NMR spectroscopy · Inter-laboratory experiment · Experimental variability

1 Introduction

The impact and utility of metabolomics on the systematic study of biological systems will be much greater if data

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from many laboratories can be pooled in centralised searchable electronic resources. One of the main constraints to overcome, in working towards an international metabolomic database, is the standardisation and normalisation of data collected from different laboratories. Discussions aimed at harmonising methodologies and the setting of guidelines for the collection and reporting of metabolomics experiments have been published (Sumner et al. 2007; Fiehn et al. 2007). These discussions form a suitable framework for progression to standardised data, but the use of a number of different technologies for metabolite data collection will always require different parameters and techniques for automated data alignment and comparison. The various spectroscopic technologies utilised in metabolomics each present their own problems. The alignment of datasets from chromatography-linked mass spectroscopic methods presents the biggest problem although algorithms to align data within experiments have been developed (Lommen 2009). The technology has not yet however advanced to the extent that different laboratories can readily combine data in such a way to allow electronic searching and matching. The situation with metabolite fingerprint data, collected without chromatography is potentially less problematic and alignment tools for Nuclear Magnetic Resonance (NMR) spectra have been made available (Stoyanova et al. 2004; Dumas et al. 2006). High resolution NMR is the technique of choice for the organic chemist for metabolite structure determination, and has played a leading role in the development of metabolomics, particularly in medical and pharmaceutical sciences (Beckonert et al. 2007). [^{1}H]-NMR fingerprinting, in particular, has been applied extensively to biofluids and tissue extracts (Lindon et al. 2000) and to plant extracts (Ward et al. 2003, 2006; Biais et al. 2009; Moco et al. 2008). The NMR spectra contain much information about the chemical composition of these complex sample mixtures. The spectra are normally collected with a common internal standard, or possibly with an electronic reference (Moing et al. 2004, Martínez-Bisbal et al. 2009), and the instrumentation is generally highly stable and the data quantitative, regardless of metabolite chemistry and free from the baseline drift issues that plague other analytical techniques. Despite these positive attributes in data quality, the development of electronic searching and matching of NMR spectra has been hindered by the different spectral resolutions provided by the variety of magnetic field strength instruments in use for metabolomics. A recent paper has addressed this issue, using a standard mixture and a fish tissue extract, in an inter-laboratory study (Viant et al. 2009). In this paper we also demonstrate, using plant derived samples, that inter-laboratory data collection and electronic comparison of NMR data can be achieved, even when data is collected on a variety of different field strength instruments.

2 Materials and methods

2.1 Plant growth

Seeds of the broccoli (*Brassica oleracea* var. *italica*) cultivars, Monaco and Iron Man, each commercial F1 hybrids were obtained from Syngenta^{TM} and Seminis^{TM} respectively. Seedlings were transplanted, after germination, in open field in Brittany in France (48°48′15″ N × 03°09′14″ W) in early August 2007 and grown until end of October 2007 (harvest date). The soil type was eolian-silt (12% clay, 16% fine silt, 44% coarse silt, 24% fine sand) and the plant density was 27,500 plants/ha. Irrigation, watering, fertilisation and pathogen-pest control were performed according to organic commercial practices. For each cultivar, 36 broccoli heads were randomly harvested at commercial maturity on October 29th 2007. Samples were processed within 24 h.

2.2 Tissue processing

Harvested tissues were washed for 1 min with tap water at room temperature then wiped and used for sample preparation. For each cultivar, 36 broccoli heads were selected to make six homogeneous lots (biological replicates) of six heads each. For each biological replicate, in order to get a homogenous sampling, several florets were taken in the centre and on both sides of each broccoli head, corresponding to 130 ± 10 g_{FW} (Fresh Weight) per head. The florets were cut into two equal parts and one half was immediately deep frozen in liquid nitrogen and stored at −80°C. The frozen pieces were milled (UMC5 grinder, STEPHAN^{TM}, Lognes, France) for 1.5 min with liquid nitrogen in order to get a homogeneous fine powder. The resultant powdered samples were stored in 50 ml Falcon tubes at −80°C until analysis. For freeze-drying the tube tops were pierced and replaced with new screw tops after processing. Freeze-dried tissue was also stored at −80°C until NMR extracts were prepared (4 months after harvest). One biological replicate of the Monaco cultivar was randomly chosen for the selection of tissue type and sample stability test performed in one laboratory.

2.3 Solvent extraction and NMR sample preparation

Twelve individual extracts of each of the two different broccoli cultivars were prepared from a homogeneous freeze-dried batch of broccoli tissue in one laboratory utilising a standardised polar solvent extraction protocol described in Baker et al. (2006). Freeze-dried tissue (15 mg) was extracted in 80:20 D_2O:CD_3OD, containing 0.05% w/v TSP-d_4 (sodium salt of trimethylsilylpropionic acid) (1 ml), for 10 min at 50°C. After cooling (5 min) and

centrifugation, the supernatant was transferred to a new tube and subjected to a 90°C heat shock for 2 min. After a second round of cooling (30 min) and centrifugation, 850 µl of the supernatant was transferred to the final vial for sample pooling. The 12 replicate extracts from each cultivar were combined to give a homogeneous master extract. From this pooled sample, identical aliquots (750 µl), were transferred into 10 separate glass vials which were transported at room temperature to the individual partner laboratories for initial analysis within 48 h. At the receiving laboratory, 600 µl of each sample was transferred into 5 mm NMR tubes for analysis. The NMR tubes (5 mm economy NMR tubes WG-1226) were also supplied to ensure parity across laboratories in terms of tube quality.

2.4 NMR data collection

[¹H]-NMR spectra were acquired at 300 K on seven different Bruker Avance Spectrometers at five separate laboratories, operating at 400, 500 and 600 MHz, equipped with either 5 or 10 mm probes as detailed in Table 1. A water suppression pulse sequence was utilised employing a presaturation pulse during the relaxation delay of 5 s. 500 and 600 MHz data were acquired using either 128 or 256 scans of 65,536 data points. 400 MHz data were acquired using either 256 or 1024 scans of 32,768 data points across a sweep width of 12 ppm. Where available, gradient shimming was utilised, otherwise conventional automated lock signal shimming was carried out. All FIDs were zero filled to double their original size, and Fourier transformed with an exponential window function (0.5 Hz). Spectra were manually phased and automatically baseline corrected using a 2nd order polynomial. ¹H chemical shifts were referenced to d₄-TSP at δ 0.00.

2.5 Schedule of data collection

NMR spectra were recorded three times across a 6 day period according to the schedule outlined in Table 2, in both spinning and non-spinning mode. In each laboratory, data collection was performed on pre-defined days with the initial dataset collected within 24 h of sample receipt.

2.6 Data analysis

For multivariate analysis [¹H]-NMR spectra were automatically reduced, using AMIX (Analysis of MIXtures software, Bruker Biospin), to create two different ASCII files containing integrated regions or "buckets" of equal width (0.01 and 0.04 ppm). Spectral intensities were scaled to the d₄-TSP region (δ 0.05 to −0.05). The ASCII file was imported into Excel for the addition of sampling/treatment details. The regions for unsuppressed water (δ 4.865–4.775), d₄-MeOH (δ 3.335–3.285) and d₄-TSP (δ 0.05 to −0.05) were removed prior to importing the dataset into SIMCA-P 11.0 (Umetrics, Umea, Sweden) for multivariate analysis. Principal component analysis (PCA) was carried out using mean centred data for the full NMR dataset and with unit variance scaling for models constructed from calculated metabolite concentrations.

2.7 Calculation of metabolite concentrations

Concentrations of individual metabolites present in the solvent extract were calculated by comparison to the known concentration of d₄-TSP present in the solvent as follows: Moles metabolite per 100 ml = (area metabolite peak/area TSP peak) × (moles TSP per 100 ml) × (9/no of hydrogen atoms represented in metabolite peak). 15 mg of tissue was

Table 1 NMR parameters used for the inter-laboratory "Ring" Experiment

Lab codes	RRes		WU		Rikilt	INRA	Bruker
Field strength (MHz)	600	400	600	500	400	500	400
Time domain	64 k	32 k	64 k	64 k	32 k	64 k	32 k
Number of scans	128	1024	128	128	256	256	256
Lock signal	D₂O	D₂O	D₂O	D₂O	D₂O	D₂O	D₂O
Sweep width	12 ppm	12 ppm	12 ppm	12 ppm	12 ppm	12 ppm	12 ppm
Relaxation delay	5 s	5 s	5 s	5 s	5 s	5 s	5 s
Water suppression power level (db)	54.4	65.0	62.0	–	80.0	65.0	68.5
Size of the processed data	64 k	32 k	64 k	64 k	32 k	64 k	32 k
FID window multiplication mode	0.5 EM	0.5 EM	0.5 EM	0.5 EM	0.5 EM	0.5 EM	0.5 EM
Polynomial degree for baseline correction	2	2	2	2	2	2	2
Probe diameter and type	5 mm SEI	5 mm BBO	5 mm CtPCl	10 mm multinuclear	5 mm pabbi 1H-BB	5 mm BBI	5 mm BBI

Lab codes: *RRes* Rothamsted Research, *WU* Wageningen University, *Rikilt* Institute of Food Safety, Wageningen; *INRA* Centre INRA de Bordeaux, *Bruker* Bruker Biospin

Table 2 Schedule of dataset collection for the inter-laboratory "Ring" Experiment

Dataset number	Day	Broccoli cultivar	Rotation status
A1	1-Wednesday	Iron Man	No-spinning
A2	1-Wednesday	Iron Man	Spinning
B1	1-Wednesday	Monaco	No-spinning
B2	1-Wednesday	Monaco	Spinning
A3	3-Friday	Iron Man	No-spinning
A4	3-Friday	Iron Man	Spinning
B3	3-Friday	Monaco	No-spinning
B4	3-Friday	Monaco	Spinning
A5	6-Monday	Iron Man	No-spinning
A6	6-Monday	Iron Man	Spinning
B5	6-Monday	Monaco	No-spinning
B6	6-Monday	Monaco	Spinning

extracted with 1 ml of solvent. Thus the concentration (ug/mg) of metabolite in the tissue was derived from (moles/100 ml) × (molecular weight of metabolite) × 10,000/15).

For absolute quantification a correction must be applied to account for differences in T1 between different NMR spectrometers.

3 Results and discussion

3.1 Tissue selection and sample stability

In order to carry out the inter-laboratory comparison using NMR it was important to establish the stability of the "test" samples. In the proposed comparison study, whereby the technique of [^{1}H]-NMR fingerprinting was to be conducted at different European laboratories over a

7 day period it was imperative that no variance would be introduced by the deterioration of the test samples during this time period. The test samples consisted of extracts of broccoli florets and in order to ensure extract stability the chosen protocols were applied to generate NMR samples that were repeatedly analysed, at 600 MHz, on a single instrument. This initial study also included an assessment of the stability of extracts of both fresh and freeze-dried tissue. Figure 1 shows the typical NMR spectra obtained from a polar (80:20 D_2O:CD_3OD) solvent extraction on both fresh (Fig. 1a) and freeze-dried (Fig. 1b) broccoli tissue. As with many NMR spectra obtained from plants, the spectrum is dominated by primary metabolites such as carbohydrates, amino acids and organic acids. Although there are a few peaks present in the fresh tissue spectrum that are absent from the freeze-dried tissue spectrum, there is good comparability between the two tissue types for the majority of peaks with only small changes evident between samples (e.g. peak 13, Fig. 1b2 which corresponds to sucrose). Importantly there are no peaks in the spectra of freeze-dried tissues that may have arisen due to the freeze-drying process. The analytical reproducibility is demonstrated in Fig. 2 which shows data generated from three separate tissue aliquots. Two common regions of the spectrum have been selected and include the region corresponding to the anomeric proton of α-glucose (5.25–5.15 ppm; Fig. 2a1) and secondly the region containing characteristic valine, leucine and isoleucine peaks (0.9–1.1 ppm, Fig. 2a2). For both regions there is good reproducibility for the freeze-dried samples but it is evident that there was more variability in the peak intensity of samples derived from fresh material, particularly for the glucose region which is known to be problematic in plant derived samples due not only to its proximity to the water suppression region of the spectrum but also due to
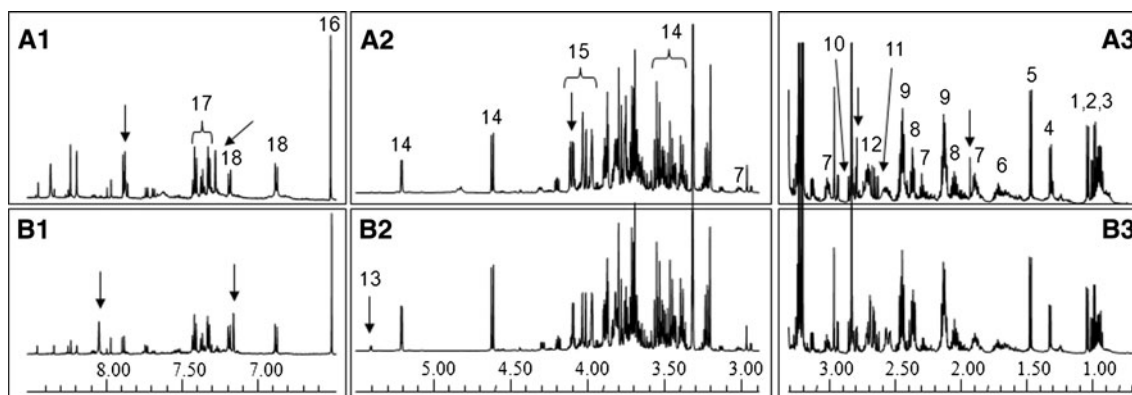

Fig. 1 Comparison of [^{1}H] NMR spectra obtained from fresh (**a**) and freeze-dried (**b**) broccoli tissue using an 80:20 D_2O:CD_3OD solvent extraction protocol. Sections 1, 2 and 3 relate to the aromatic region, the carbohydrate region and aliphatic region of the spectra respectively. Regions denoted by an *arrow* highlight possible differences between tissue types. Compounds with characteristic non-overlapping signals: 1: valine; 2: isoleucine; 3: leucine; 4: threonine; 5: alanine; 6: leucine; 7: GABA; 8: glutamate; 9: glutamine; 10: asparagine; 11: aspartate; 12: citrate & malate; 13: sucrose; 14: glucose; 15: fructose; 16: fumarate; 17: phenylalanine; 18: tyrosine
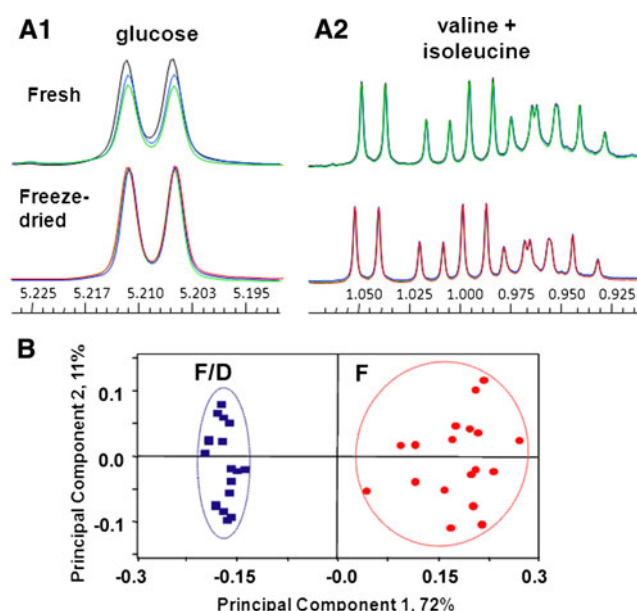
Fig. 2 Comparison of two regions (**a1** and **a2**) of 600 MHz NMR spectra of extracts of fresh and freeze dried broccoli tissue. Each tissue type contains three superimposed spectra. Higher variability observed in data when fresh tissue was extracted. (**b**) PCA analysis of full NMR dataset, binned to 0.01 ppm, from fresh (F) and freeze-dried (F/D) broccoli tissue

unwanted carbohydrate conversion if enzyme activity is not eliminated in the extraction procedure (Baker et al. 2006). What was clear, however, was that there was little drift in the recorded chemical shifts and that, as expected, the stability of the instrumentation was excellent across all samples studied. PCA was carried out on the full NMR datasets obtained from fresh and freeze-dried samples (Fig. 2b). Clearly sample types separated in the direction of PC1 which described 72% of the variance, and a tighter clustering was obtained with the freeze-dried samples. From these data it was concluded that for the inter-lab comparison, samples would be generated from aliquots of freeze-dried tissue. In order to assess the stability of the

solvent extracts over time, the same NMR samples were re-analysed 6 days later under identical NMR instrument conditions. Data from this comparison is shown in Fig. 3 and demonstrates that there has been no deterioration in sample quality and no qualitative or quantitative differences in peaks were observed. The datasets overlay perfectly when visualised together demonstrating that samples could be kept and re-analysed without the risk of deterioration during extended studies. Most importantly, with confidence in sample stability, any variation introduced during the inter-laboratory comparison could be ascribed to differences in magnetic field strength, instrument set-up and configuration or physical location differences.

3.2 Inter-laboratory comparison—instrument set-up and experimental schedule

Prior to the inter-laboratory comparison, details of the NMR parameter set were sent to each laboratory in order that contributing partners could set up their instruments to collect data in as near identical conditions as possible. Details of instrument configurations, probe specification and the final data collection parameters used by each laboratory are shown in Table 1. The inter-laboratory comparison comprised Bruker Avance NMR instruments of different magnetic field strength (400, 500 & 600 MHz), utilising a range of probe types including selective inverse, broadband observe and inverse, multinuclear and even a cryoprobe. In general the size of the probes used in this experiment were 5 mm although one 500 MHz instrument was configured with a 10 mm probe. Data was collected using a water suppression pulse sequence across an identical sweep width of 12 ppm in all cases. The number of scans utilised varied depending on the magnetic field strength and probe type. To avoid potential problems associated with temperature variation in different NMR laboratories, instruments were all set to 300 K, using a calibration sample of d_4 methanol (Findeisen et al. 2007).

Fig. 3 Results from stability test. Aliphatic and carbohydrate regions of NMR spectra of freshly prepared broccoli sample (A) aligned with that from 6-day old extract (B)
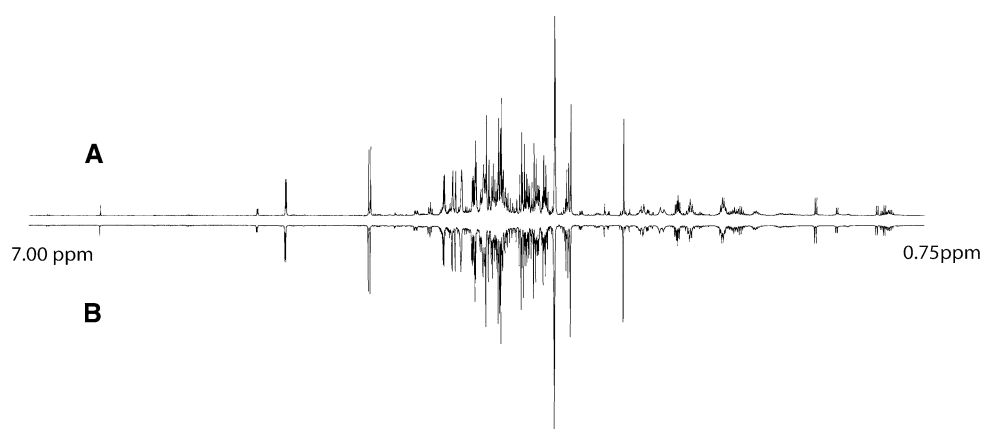
In addition to instrument set-up instructions, a schedule for data collection was given (Table 2). The schedule allowed collection of three datasets over 6 days for each of the two biological samples in the experiment. On each occasion data was collected with and without rotation of the sample. Two different broccoli cultivars, Iron Man and Monaco, were included in the experiment. On each occasion participants were asked to collect data in spinning and non spinning mode to assess the effect of sample rotation on the quality of the final dataset.

3.3 NMR stability—individual laboratories

Data from each laboratory was visually inspected by superimposing the three replicate datasets obtained from each biological sample. Figure 4 shows an expansion of the region between 5.22 and 5.19 ppm containing the anomeric hydrogen signal of α-glucose as an example. The data overlays exceptionally well in this region in terms of both chemical shift and intensity, demonstrating that within one laboratory there is very little, if any, variation in the NMR spectrum when the same sample is analysed over 6 days. Data from each laboratory and each separate NMR machine behaved in a similar manner (not shown). This result was entirely expected since it has previously been reported that drift in chemical shift in samples from green plant tissue is minimal (Ward and Beale 2006) and that with careful sample preparation, control of pH and temperature during data acquisition, signals arising from the same metabolite should occur at exactly the same chemical shift from sample to sample (Krishnan et al. 2005).

3.4 Spinning versus non spinning

Spinning an NMR sample within the magnet has the advantage of averaging field inhomogeneity and thus increases the resolution of the peaks observed in the final spectrum. Under some conditions however, spinning the sample produces spurious signals or "sidebands" that are a consequence of modulation of the magnetic field at spinning frequency. In instruments of lower magnetic field strength (e.g. 400 MHz) it may be necessary to rotate the sample in order to achieve as good resolution as possible but at higher magnetic field (e.g. 600 MHz) rotation of the sample is seen to be undesirable due to the increased risk of these spinning sidebands. The problem in a metabolomics experiment is, that when analysing a complex mixture, such as un-fractionated plant extracts, the final spectrum will contain a large number of overlapping peaks and spinning sidebands of large peaks could possibly interfere and thus skew the final dataset. In a metabolomics experiment, where the experimentalist is trying to determine differences between the datasets, these peaks should actually cause very little problem as although undesirable, they have a fixed intensity (usually 1–2%), proportional to that of the genuine peak and thus would not vary across the dataset. In order to test this theory and the consequences of sample rotation, partners in the inter-laboratory comparison study were requested to collect datasets in both spinning and non-spinning mode. To further standardise the experiment, partners were supplied with identical NMR tubes to use of a set grade to minimise any variation due to cylindrical symmetry or glass quality of the NMR tube itself.

Sample spectral data (from the characteristic aliphatic valine, isoleucine and leucine region (0.9–1.1 ppm)) obtained in spinning and non spinning mode is shown in Fig. 5 and includes that from 600 MHz (Fig. 5a) and 400 MHz (Fig. 5b) instruments. Resolution was found to be slightly decreased in the non-spinning samples at both 400 and 600 MHz (e.g. mean line width of TSP peak at half height in 600 MHz spinning samples was 0.90 Hz compared to 1.19 Hz for non-spinning samples). This is accompanied with an apparent increase in signal intensity in the non-spinning samples over those which had been rotated (due to similar resolution differences in TSP signal and subsequent scaling thereof). This is offset by a lower
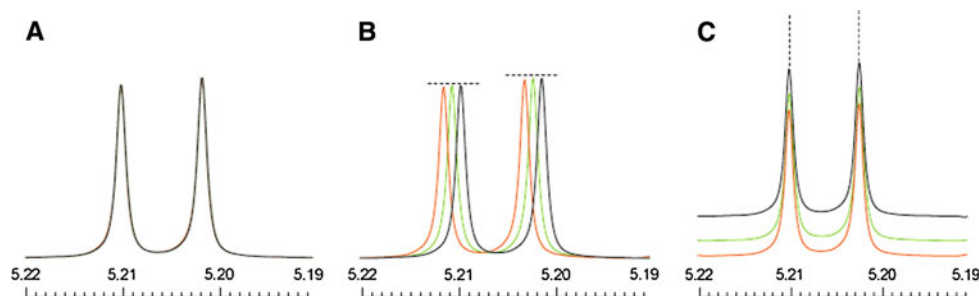

Fig. 4 Assessment of instrument stability. Results obtained from three successive runs of the same NMR sample at days 1, 3 and 6. **a** α-Glucose anomeric proton region (δ5.22–5.19) from three superimposed 600 MHz NMR spectra collected at one site. **b** Spectral region as **a** with x-axis offset, demonstrating intensity reproducibility. *Red* Day1, *Green* Day3, *Black* Day6. **c** Spectral region as **a** with y-axis offset, demonstrating chemical shift reproducibility. *Red* Day1, *Green* Day3, *Black* Day6
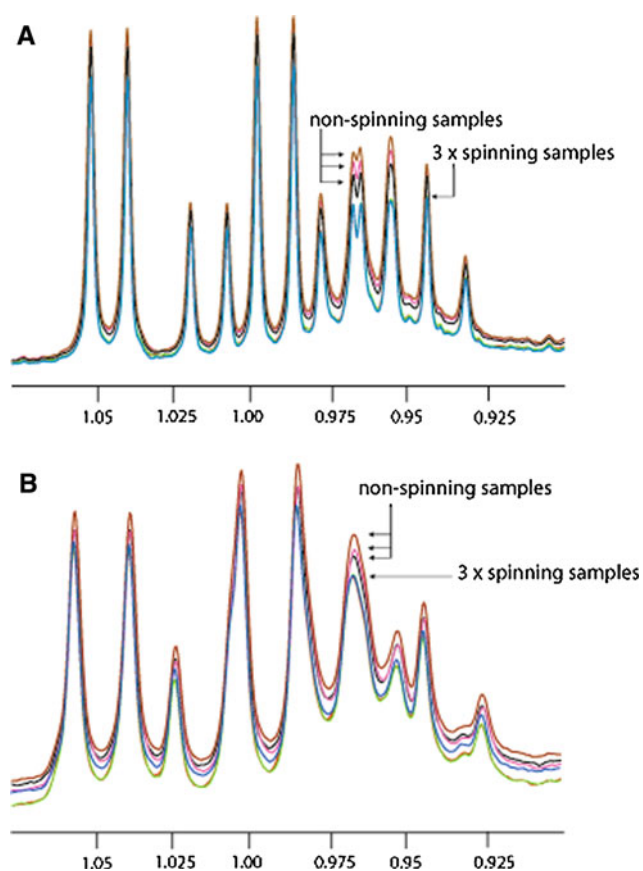
Fig. 5 Comparison of a broccoli extract run with and without rotation during spectra acquisition. **a** 600 MHz spectra region from 0.9 to 1.1 ppm. **b** 400 MHz spectra region from 0.9 to 1.1 ppm. Spectra have been scaled to the signal height of TSP. As a consequence, signals with linewidth broader that of the d_4-TSP peak show higher amplitude in the non-spinning case than in the spinning case. Since quantification evaluates signal area rather than signal height, determination of concentration values is not affected

resolution of the individual peaks within a particular signal and thus represents a broadening of the resonance due to errors in low order X/Y shims. At 600 MHz, the broadening of the peaks due to not rotating the sample is evident but in the case of most metabolomics studies where NMR data is employed, a bucketing or binning routine is employed to segment the dataset into equal size buckets and thus any small changes in peak width or slightly poorer resolution would be dealt with in post processing of the data.

3.5 Multivariate analysis of combined inter-laboratory dataset

Datasets from individual laboratories were processed using identical parameters by the lead laboratory (Table 1). A visual comparison of data obtained from instruments at *comparable* field strength showed little variation in

chemical shift, resolution or intensity of the peaks. This was the case for data collected at both 400 and 600 MHz. When comparisons were made across the *entire experiment*, however, it was clear to see that the spectral quality, in terms of resolution, increased with field strength. This was not unexpected and is the reason why higher strength instruments, albeit that they are more expensive, are used where possible, not only to shorten data acquisition times but also to improve separating power and resolution, especially when the need is to resolve overlapping peaks in a complex mixture.

Multivariate statistical approaches are often employed in metabolomics experiments to discriminate between samples of interest and to discern the metabolites responsible for the separation. Data for this inter-laboratory comparison study has been similarly modelled (Fig. 6) utilising PCA. Here the data from each partner laboratory, was reduced to bins of equal width (0.01 ppm) across the full spectral width and modelled together to try and establish if the two broccoli cultivars present in the study could be separated irrespective of the field strength of the instrument or location of the laboratory and associated instrument set-up. Figure 6a shows six clusters within the PCA scores plot and a separation in the direction of PC1, accounting for 61% of the total variance, according to magnetic field strength and thus resolution of the final spectra. Pleasingly, data from the instruments of the same magnetic field strength cluster very tightly irrespective of where they are located, whether the sample was rotated or not, and which probe was utilised for data collection. In the same PCA model, PC2, accounting for 35% of the variance, was able to separate the two broccoli cultivars. Analysis of the loadings plots (Fig. 6b) for this statistical model demonstrated this further showing that PC2 gives the true information on genotypic difference (carbohydrates, amino acids etc.) whilst that for PC1 contains a series of positive and negative peaks which resemble "noise". This effect is due to the fact that bucketing or binning to 0.01 ppm has divided the broader peaks obtained at 400 MHz and to a lesser extent, 500 MHz whereas the selection of 0.01 ppm binning width is entirely suitable for the more resolved 600 MHz data. Widening the bucket width to 0.04 ppm and re-building the PCA model (Fig. 6c) now shows the same six clusters as previously but the orientation has now changed such that PC1, accounting for 80% of the variance, now describes the difference between the two broccoli cultivars rather than the magnetic field strength which is now, at this higher bucket width, explained by PC2 (accounting for 18% of the variance). Thus the expansion of the width of the bin utilised in post acquisition processing has effectively reduced the resolution of a 600 MHz spectrum to that resembling data collection at 400 MHz. This is highlighted in Fig. 6d which represents the loadings plot of the second PCA model.

Fig. 6 Impact of bucketing width on Principal Component Analysis. **a** PC1 vs. PC2 scores plot resulting from model constructed with data binned to 0.01 ppm. **b** Loadings plots obtained from 0.01 ppm binned data. **c** PC1 vs. PC2 scores plot resulting from model constructed with data binned to 0.04 ppm. **d** Loadings plots obtained from 0.04 ppm binned data. In both cases X cluster relates to Iron Man samples and Y cluster relates to Monaco samples. Subclusters are coloured according to individual NMR instruments at participating laboratories
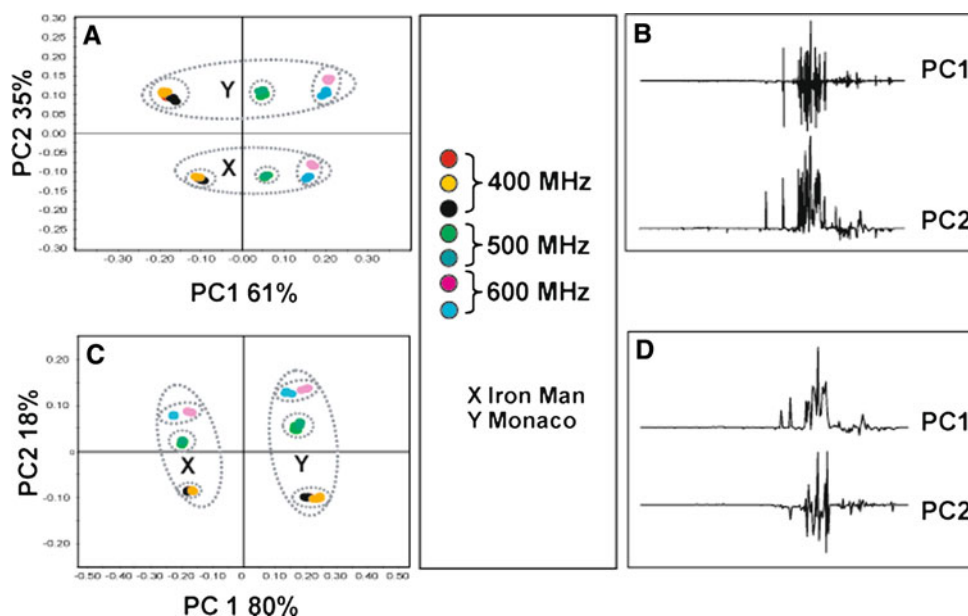
Comparison of the two loadings plots (Fig. 6b, d) clearly demonstrate the loss of resolution by choosing a higher bucket width but nonetheless show that metabolites responsible for the biological difference between samples are the same. Therefore when instruments of different field strength are used in the same study, selection of a larger bucketing width can "reduce" the contribution of technological variability to the first Principal Component in a PCA model.

3.6 Statistical comparison of quantitative data

The advantage of [^{1}H] NMR is that if certain NMR acquisition conditions are fulfilled (e.g. delay between scans (D1) > five times the relaxation time (T1)) it is a quantitative technique and with the addition of an internal standard, one can calculate concentrations of individual metabolites in the solvent extract if they display a clear non-overlapping peak. To illustrate the robustness of NMR and its quantitative ability, a selection of characteristic chemical shift regions which related to key metabolites found in typical broccoli polar solvent extracts were selected across the spectral width. These represent a range of metabolites of different concentrations from high (sucrose) to low (valine). Concentrations of these individual metabolites are given as histogram plots in Fig. 7. As can clearly be seen, irrespective of magnetic field strength, the concentrations of metabolites derived from the [^{1}H] NMR data are very reproducible, agree with expected literature values (Gomes and Rosa 2000) and demonstrate which metabolites contribute to the biological difference between the two broccoli cultivars.

Finally, by taking the matrix of selected metabolite concentrations derived, via integration of characteristic metabolite peaks, from the NMR spectra of participating laboratories, and modelling these using PCA, one can see that the subsequent scores plot (Fig. 8) now contains only two clusters which simply relate to the biological difference between the two samples. All variance due to field strength and set-up parameters has been minimised. It can be seen that there is more variance between the higher field instruments in this analysis, and this can be attributed to a greater sensitivity of these instruments to changes in ambient temperature. Obviously the approach we have taken would clearly be suitable for a multi-laboratory metabolomics study where all the project data may need to be modelled together or against other Y datasets, such as transcriptomics or proteomics results.

4 Concluding remarks

In this study we have demonstrated that, with attention to experimental design and careful set-up, data collection for large-scale plant metabolite fingerprinting using [^{1}H]-NMR can be carried out as a dispersed activity across laboratories using different NMR instruments. Data from the two cultivars provided a means to examine different data processing techniques to reveal the biochemical differences whilst minimising the effect of the different spectral resolutions. Thus, for plant [^{1}H]-NMR fingerprinting, multi-laboratory experiments with pooling of data are now perfectly feasible and the way forward for co-ordinated multi-national screens and databasing of large collections of genotypes is now open. Although we have concentrated
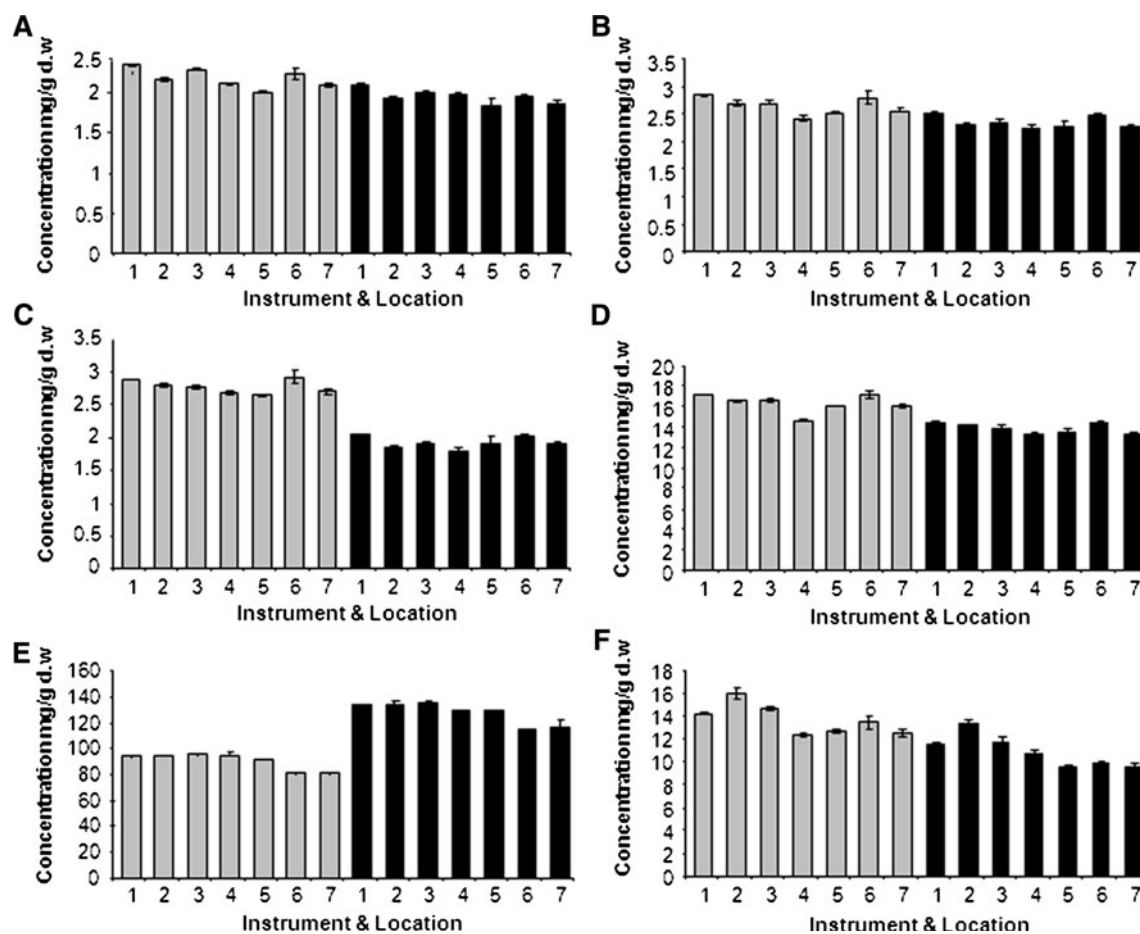
Fig. 7 Mean concentrations of six selected metabolites obtained via characteristic peak integration highlighting intra and inter-machine variation across NMR data (with and without rotation) obtained from two broccoli cultivars. Data collected on days 1, 3 and 6 ($n = 6$). *Black* Monaco, *Grey* Iron man. Partner/Instrument—1: Bruker 400 MHz; 2: Rothamsted 400 MHz; 3: Rikilt 400 MHz; 4: INRA 500 MHz; 5: Wageningen University 500 MHz; 6: Rothamsted 600 MHz; 7: Wageningen University 600 MHz. Metabolites—**a** valine (δ 1.07–1.03); **b** threonine (δ 1.35–1.29); **c** alanine (δ 1.51–1.44); **d** glutamine (δ 2.49–2.42); **e** glucose (δ 5.23–5.19 & δ 4.64–4.59); **f** sucrose (δ 5.44–5.39). *Error bars* represent standard deviation of mean values
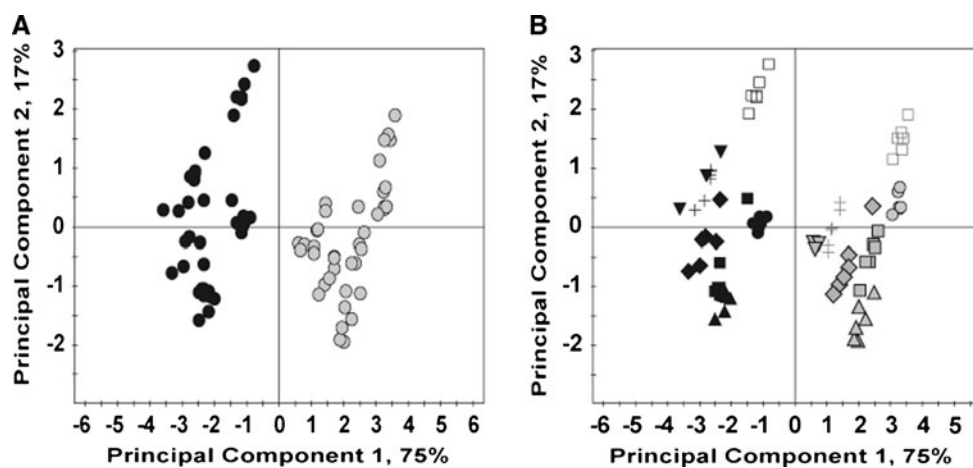

Fig. 8 Scores plot from PCA model constructed from actual metabolite concentrations derived via integration of characteristic chemical shifts from a subset of six primary metabolites found in broccoli NMR spectra (with and without rotation). **a** Data coloured according to cultivar (*Grey* Iron Man, *Black* Monaco); **b** data as **a**, depicted by symbols to illustrate different NMR field strength and location (*open box* Rothamsted 600 MHz, *dot* Bruker 400 MHz, *diamond* INRA 500 MHz, *solid box* RIKILT 400 MHz *triangle* Rothamsted 400 MHz, *cross* Wageningen University 600 MHz, *inverted triangle* Wageningen University 500 MHz NMR)

here on testing the ability to collect data from different instruments and batch process them together, as we envisage would happen in large multi-national screens where raw data from different instrumentation is deposited centrally, we realise that dispersal of the sample preparation to the different laboratories is another potential source of data variation. This has not been tested in this work. However, the experience of the Rothamsted laboratory, where more than 20,000 plant samples (yielding > 60,000 NMR) samples have been processed, in the past few years, indicates that this source of operator/wet laboratory variation can be eliminated with strict adherence to the standard operating procedure that was also used in the sample preparation in this work.

Standardisation in methodology and reporting of results is a focus of concern across all the 'omics' technologies (Sumner et al. 2007; Fiehn et al. 2007; Hardy and Taylor 2007) and other inter-laboratory studies have been reported. An inter-laboratory plant metabolomics study by GC–MS across three research groups has recently been reported (Allwood et al. 2009), This study, which utilised identical GC–MS instrumentation and samples prepared in a single laboratory, but derivatised locally, concluded that major metabolite features could be consistently measured across the partners, but that automated peak retrieval, mode of injection, chromatographic performance and data processing all contributed to variation and that more standardisation would be necessary for large-scale dispersed data collection by this technique. In contrast we have shown that NMR profiling is a technique that is much more robust and free from such machine variation.

References

Allwood, J. W., Erban, A., de Koning, S., Dun, W. B., Luedemann, A., Lommen, A., et al. (2009). Inter-laboratory reproducibility of fast gas chromatography-electron impact-time of flight mass spectrometry (GC-EI-TOF/MS) based plant metabolomics. *Metabolomics, 5*, 479–496.

Baker, J. M., Hawkins, N. D., Ward, J. L., Lovegrove, A., Napier, J. A., Shewry, P. R., et al. (2006). A metabolomic study of substantial equivalence of field-grown genetically modified wheat. *Plant Biotechnology Journal, 4*, 381–392.

Beckonert, O., Keun, H. C., Ebbels, T. M. D., Bundy, J. G., Holmes, E., Lindon, J. C., et al. (2007). Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nature Protocols, 2*, 2692–2703.

Biais, B., Allwood, W.J., Deborde, C., Xu, Y., Maucourt, M., Beauvoit, B., Dunn, W.B., Jacob, D., Goodacre, R., Rolin, D., & Moing, A. (2009). ^{1}H-NMR, GC-EI-TOF-MS and data set correlation for fruit metabolomics, application to spatial metabolite analysis in melon. *Analytical Chemistry*, 81, 2884–2894.

Dumas, M. E., Maibaum, E. C., Teague, C., Ueshima, H., Zhou, B. F., Lindon, J. C., et al. (2006). Assessment of analytical reproducibility of H-1 NMR spectroscopy based metabonomics for large-scale epidemiological research: The INTERMAP study. *Analytical Chemistry, 78*, 2199–2208.

Fiehn, O., Sumner, L. W., Rhee, S. Y., Ward, J. L., Dickerson, J., Lange, B. M., et al. (2007). Minimum reporting standards for plant biology context information in metabolomic studies. *Metabolomics, 3*, 195–201.

Findeisen, M., Brand, T., & Beger, S. (2007). A ^{1}H-NMR thermometer suitable for cryoprobes. *Magnetic Resonance in Chemistry, 45*, 175–178.

Gomes, M. H., & Rosa, E. (2000). Free amino acid composition in primary and secondary inflorescences of 11 broccoli (*Brassica oleracea* var. *italica*) cultivars and its variation between growing season. *Journal of the Science of Food and Agriculture, 81*, 295–299.

Hardy, N. W., & Taylor, C. F. (2007). A roadmap for the establishment of standard data exchange structures for metabolomics. *Metabolomics, 3*, 243–248.

Krishnan, P., Kruger, N. J., & Ratcliffe, R. G. (2005). Metabolite.fingerprinting and profiling in plants using NMR. *Journal of Experimental Botany, 56*, 255–265.

Lindon, J. C., Nicholson, J. K., Holmes, E., & Everett, J. R. (2000). Metabonomics: Metabolic processes studied by NMR spectroscopy of biofluids. *Concepts in Magnetic Resonance, 12*, 289–320.

Lommen, A. (2009). MetAlign: Interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. *Analytical Chemistry, 81*, 3079–3086.

Martínez-Bisbal, M. C., Monleon, D., Assemat, O., Piotto, M., Piquer, J., Llácer, J. L., et al. (2009). Determination of metabolite concentrations in human brain tumour biopsy samples using HR-MAS and ERETIC measurements. *NMR in Biomedicine, 22*, 199–206.

Moco, S., Forshed, J., De Vos, R. C. H., Bino, R. J., & Vervoort, J. (2008). Intra- and inter-metabolite correlation spectroscopy of tomato metabolomics data obtained by liquid chromatography-mass spectrometry and nuclear magnetic resonance. *Metabolomics, 4*, 202–215.

Moing, A., Maucourt, M., Renaud, C., Gaudillère, M., Brouquisse, R., Lebouteiller, B., et al. (2004). Quantitative metabolic profiling by 1-dimensional ^{1}H-NMR analyses: Application to plant genetics and functional genomics. *Functional Plant Biology, 31*, 889–902.

Stoyanova, R., Nicholls, A. W., Nicholoson, J. K., Lindon, J. C., & Brown, T. R. (2004). Automatic alignment of individual peaks in large high-resolution spectral data-sets. *Journal of Magnetic Resonance, 170*, 329–335.

Sumner, L. W., Amberg, A., Barrett, D., Beale, M. H., Beger, R., Daykin, C. A., et al. (2007). Proposed minimum reporting standards for chemical analysis: Chemical Analysis Working

Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics, 3*, 211–221.

Viant, M. R., Bearden, D. W., Bundy, J. G., Burton, I. W., Collette, T. W., Ekman, D. R., et al. (2009). International NMR-based Environmental Metabolomics Intercomparison Exercise. *Environmental Science and Technology, 43*, 219–225.

Ward, J., & Beale, M. H. (2006). NMR spectroscopy in plant metabolomics. In K. Saito, R. A. Dixon, & L. Willmitzer (Eds.), *Plant metabolomics, biotechnology in agriculture and forestry series* (Vol. 57, pp. 81–91). Springer.

Ward, J. L., Harris, C., Lewis, J., & Beale, M. H. (2003). Assessment of [^{1}H]-NMR spectroscopy and multivariate analysis as a technique for metabolite fingerprinting of *Arabidopsis thaliana*. *Phytochemistry, 62*, 949–957.