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Fast and precise quantitative analysis of metabolic mixtures by 2D ^{1}H INADEQUATE NMR

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ABSTRACT

Quantitative analysis of metabolic mixtures by ^{1}H 1D NMR offers a limited potential for precise quantification of biomarkers, due to strong overlap between the peaks. Two-dimensional spectroscopy is a powerful tool to unambiguously and simultaneously measure a larger number of metabolite contributions. However, it is still rarely used for quantification, first because quantitative analysis by 2D NMR requires a calibration procedure due to the multi-impulsional nature of 2D NMR experiments, and above all because of the prohibitive experiment duration that is necessary to obtain such a calibration curve. In this work, we develop and evaluate a 2D ^{1}H INADEQUATE protocol for a fast determination of metabolite concentrations in complex mixtures. The 2D pulse sequence is carefully optimized and evaluated in terms of precision and linearity. Quantitative ^{1}H INADEQUATE 2D spectra of metabolic mixtures are obtained in 7 min with a repeatability better than 2% for metabolite concentrations as small as 100 μM and an excellent linearity. The method described in this work allows a fast and precise quantification of metabolic mixtures, and it forms a promising tool for metabonomic studies.

1. Introduction

The role of nuclear magnetic resonance (NMR) spectroscopy as a quantitative tool [1] is well established in a variety of domains, such as pharmaceutical applications, natural product authentication or metabolome analysis. Recently, the application of NMR spectroscopy in metabolomic and metabonomic studies has been increasing significantly [2]. In the vast majority of studies reported so far, its use for such purpose consists of a very standardized 1D ^{1}H NMR protocol associated with statistical analysis in order to perform efficient fingerprinting [3]. However, in most cases, this method does not allow a separate quantification of relevant biomarkers, as required for a full metabonomic treatment. This is mainly due to the very substantial overlap of resonances characterizing 1D proton NMR spectra of complex metabolic mixtures. Quantitative ^{13}C NMR [4] provides an interesting alternative, as it leads to a better discrimination of resonances because of its much larger chemical shift range. However, its use in metabolomics is generally limited to ^{13}C-labeled metabolites [5] because of its inherent low sensitivity. In order to circumvent these limitations, the use of bi-dimensional (2D) NMR [6] for quantitative analysis was recently proposed [7–10], as it performs a very efficient separation of signals originating from various compounds in a complex mixture. However, quantitative 2D NMR is associated with a number of drawbacks that restrain its use for quantitative purposes [9]. First, 2D NMR pulse sequences are multi-impulsional. Consequently a number of factors (*J*-couplings, relaxation times, etc.) influence peak volumes [9]. Moreover, multi-impulsional NMR experiments are very sensitive to pulse imperfections [11]. For these reasons, a calibration curve is indispensable to perform precise and accurate quantitative analysis by 2D NMR [7,10]. Unfortunately, obtaining such a curve is made difficult by the long experiment durations (up to several hours) of 2D NMR experiments, due to the necessity of collecting an array of transients to sample the indirect dimension.

Because of these various limitations, the use of 2D NMR for studying complex metabolic mixtures has been generally limited to metabolite identification [12], and only a few recent publications have reported a quantitative analysis of metabolite mixtures by 2D NMR [10,13–16]. A very recent study [17] suggested to bypass the calibration procedure by measuring all the relaxation times and *J*-couplings for a given sample in order to obtain, after mathematical correction, 2D peak volumes reflecting the exact metabolite concentration. However, it appears to be a very long and tedious procedure, which is probably difficult to generalize to a large number of metabolic samples. Therefore, the approach relying on a calibration curve [7,10] seems more appropriate for robust and precise quantitative analysis.

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Particularly interesting is the optimization of the 2D HSQC sequence for fast metabolite quantification developed by Lewis et al. [10] and leading to an experimental duration of 12 min per spectrum. However, in spite of its high potentialities, heteronuclear 2D NMR is limited by its inherent sensitivity due to the low natural abundance of ^{13}C. Therefore, homonuclear 2D NMR could form an interesting, complementary approach for fast quantitative analysis. A few studies have reported the use of homonuclear 2D NMR for quantification [7,18], including the quantitative analysis of metabolic fluxes by combining 2D zTOCSY NMR with ^{13}C-labeling experiments [18], proposed by Massou et al. However, all these methods are characterized by very long experiment times, and to our knowledge, no homonuclear 2D NMR method has been optimized for fast quantitative analysis of complex metabolic mixtures.

The purpose of this work is to develop and evaluate a 2D homonuclear NMR approach for a fast determination of metabolite concentrations in complex mixtures. Here, after considering different homonuclear strategies, a ^1H INADEQUATE [19,20] pulse sequence is carefully optimized and evaluated in terms of precision and linearity. The potentialities of this approach for analyzing biological samples are discussed.

2. Material and methods

2.1. Sample preparation

A phosphate buffer solution (pH = 2, 0.1 M) in D_2O was prepared. A low pH value was chosen on purpose, as it minimizes intermolecular interactions between metabolites due to the protonation of acid and amine functions. It has been shown that such interactions could alter the accuracy of quantitative NMR measurements [21]. A reference sample containing six metabolites (proline, alanine, lactic acid, glutathione, taurine (Sigma–Aldrich), and myo-inositol (Prolabo)) was dissolved in 1 mL of this buffer solution to obtain a sample with identical metabolite concentrations (50 mM) to optimize two-dimensional NMR experiments. Six test mixtures of 1 mL were prepared to study linearity. In each mixture, alanine concentration was kept constant at 50 mM to set the alanine signal as an internal reference. The five other metabolites were dissolved in the buffer solution in order to obtain concentrations between 0.08 mM and 50 mM. After homogenization, each sample was filtered and analyzed in a 5 mm tube.

2.2. Preparation of intracellular extracts

Cells from breast cancer MDA-MB-468 line were grown as monolayer cultures (2.49×10^6 cells/75 cm^2 flask in two identical flasks) in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% of heat-inactivated foetal bovine serum (FBS) and 1% of 10,000 units/mL penicillin–10,000 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. All the cell culture media and chemicals were obtained from Invitrogen. The culture medium was removed from the culture flask (75 cm^2), and then, cells were washed twice with phosphate-buffer saline (PBS, pH = 7.4). Afterwards, they were quenched using 2.7 mL HPLC-grade methanol (VWR) and detached from the culture flask using a cell lifter (VWR). The methanol solution containing quenched cells was pipetted into a 15 mL centrifuge tube for extraction. A mixture of methanol, chloroform and water (4:4:2.85, v/v/v) was used to extract intracellular metabolites. The aqueous phase contained water soluble metabolites while non-polar metabolites stayed in the organic phase. Between these two phases, proteins and other biological macromolecules were trapped by precipitation because of the addition of methanol and chloroform. After centrifugation (300 *g*, 5 min, 20 °C), the aqueous phase was used for analysis. Solvents were removed using a rotary evaporator. Then, the sample was dissolved in 1 mL of phosphate buffer solution (pH = 2, 0.1 M) in D_2O, homogenized, filtered and analyzed using a 5 mm tube.

2.3. NMR spectroscopy experiments

For the preliminary comparison between 2D pulse sequences, all the NMR data were recorded at 298 K on a Bruker Avance 400 DPX spectrometer, at a frequency of 400.13 MHz with a 5 mm dual probe equipped with *z*-axis gradients and PW_{90} = 9.5 μs. For all the spectra, the residual water signal was suppressed by applying a continuous, low power RF field during recovery delays and during the double-quantum evolution period for the INADEQUATE pulse sequence. Free induction decays (FIDs) were recorded with 4788 data points and an acquisition time of 0.5 s. 2D spectra were acquired with four dummy scans, 128 t_1 increments and a recovery delay of 5 s. For TOCSY, a Zero-Quantum-Filtered TOCSY pulse sequence was used, as described in Ref. [18], including a 80 ms DIPSI-2 mixing period (8.5 kHz). Zero-quantum filters were formed by adiabatic 180° smoothed chirp pulses (20 kHz bandwith) applied during 50 ms (before spin-lock) and 30 ms (after spin-lock), simultaneously with 2.5 G/cm gradients along the *z*-axis. The second filter was preceded by a 48.5 G/cm z-gradient applied during 3 ms. For the DQF-COSY experiment, we relied on the pulse sequence previously optimized for quantitative analysis, with additional coherence-selection gradients along the *z* axis (2 ms duration, 38.8 G/cm during the double-quantum evolution and 77.6 G/cm just before detection). For ^1H INADEQUATE, 2 ms coherence-selection gradients were applied in order to select double quantum coherences as indicated in Fig. 1(a), with 38.8 G/cm and 77.6 G/cm gradient strengths. The double-quantum evolution delay Δ was calculated from the average ^1H–^1H scalar coupling constant (J_{av}): $\Delta = 1/(4J_{av})$, with J_{av} = 7 Hz.

For the optimization of the 2D INADEQUATE pulse sequence (Fig. 1(a)), the analytical evaluation and the application to metabolic samples, ^1H INADEQUATE NMR data were recorded at 298 K on a Bruker Avance III 500 DRX spectrometer, at a frequency of 500.13 MHz, with a cryogenic probe including *z*-axis gradients and PW_{90} = 10 μs. For all the spectra, the residual water signal was suppressed by applying a continuous RF field during the recovery delay and the double-quantum evolution delay. 2D ^1H INADEQUATE spectra were acquired with 4 dummy scans and 1 transient, 64 t_1 increments and with a recovery delay of 5.45 s. FIDs were recorded with 6008 data points, an acquisition time of 0.5 s and a spectral width of 6010 Hz.

2.4. Data processing

All the apodization functions were optimized to choose the one leading to the best precision for a given method. For zTOCSY spectra, an exponential apodization function of 0.3 Hz was applied in each dimension before Fourier transform. For DQF-COSY spectra, a π/24 shifted square sine-bell function and a π/8 shifted sine-bell function were applied in F_2 and F_1 dimensions respectively. For ^1H INADEQUATE spectra, a Lorentzian–Gaussian apodization (LB = −1 Hz, GB = 0.15) was applied in each dimension before Fourier transform. zTOCSY spectra were phased in order to obtain optimized quantitative conditions. DQF-COSY and INADEQUATE spectra could not be phased due to the complex phase patterns characterizing these experiments; therefore they were processed in magnitude mode, even though such a procedure is not conventional for DQF-COSY spectra, as the coherence-selection gradient scheme should in principle lead to phased 2D spectra even in one scan. For all the 2D spectra, data matrices were zero-filled to 16k and to 512 points in F_2 and F_1 dimensions respectively. Automatic polynomial baseline corrections (n = 3) were applied in each

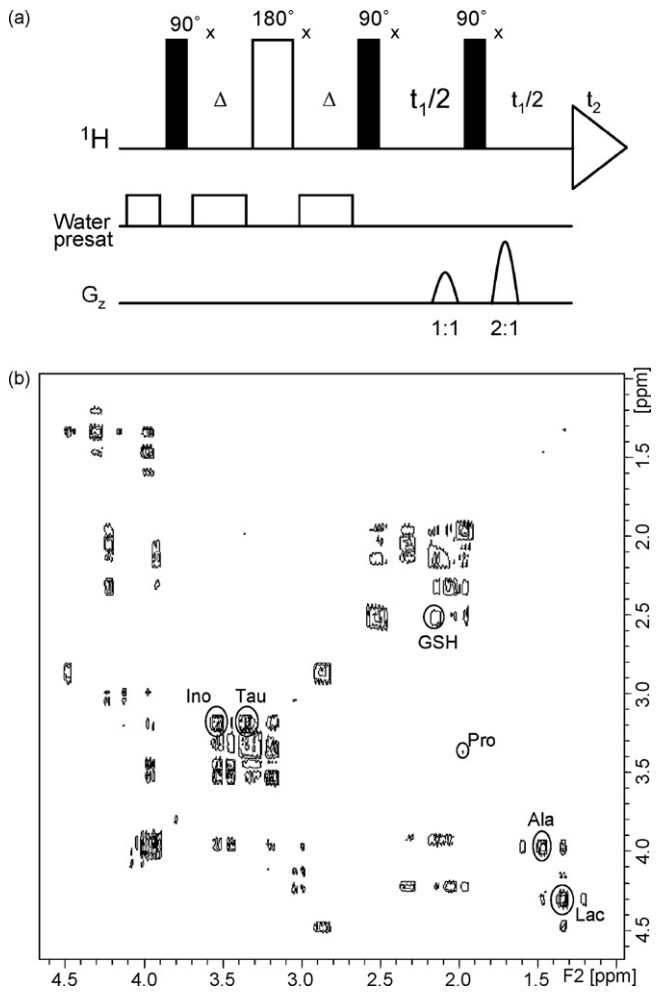

Fig. 1. (a) Pulse sequence for the acquisition of 2D ^{1}H INADEQUATE spectra and (b) corresponding 500 MHz proton INADEQUATE spectrum of a 50 mM model metabolic mixture. Spectrum acquired in 7 min with this pulse sequence, at 298 K on a 500 MHz spectrometer equipped with a cryogenic probe, with 1 transient and 64 t_1 increments. Peaks chosen for integration are indicated for each metabolite. Ala: alanine; GSH: glutathione; Ino: myo-inositol; Lac: lactic acid; Pro: proline; Tau: taurine.

dimension. INADEQUATE spectra were first symmetrised to facilitate peak identification, and then reprocessed without symmetrisation for quantification.

The integration of 2D peak volumes was performed using the integration routine in Bruker Topspin 2.1. All the integration results are the average of five experiments. Integration box widths were adapted for each pulse sequence, as 2D peak widths slightly differ from one experiment to another.

2.5. Statistical analysis

Repeatability was evaluated by calculating the relative standard deviation (RSD) on five successive experiments. Linearity was evaluated by plotting 2D peak volume ratios versus gravimetric concentration ratios and by calculating the linear regression parameters (slope and y-intercept) and the coefficient of determination r^2.

3. Results and discussion

3.1. Choice of the pulse sequence and NMR optimization

In a first step, we compared several ^{1}H homonuclear 2D pulse sequences in order to assess their analytical potentialities. Three pulse sequences were chosen and compared in terms of precision. Zero-Quantum Filtered TOCSY was first considered, as it has been reported in several studies as a quantitative tool for complex mixtures [18]. DQF-COSY was also evaluated, as we have recently highlighted its quantitative potentialities for fast quantitative analysis [7]. Finally, we also evaluated ^{1}H 2D INADEQUATE, a method that is well known for its capacity to establish ^{13}C–^{13}C connectivities [22], but whose application to study ^{1}H–^{1}H correlations is limited to a very small number of structural studies [19], probably because of the prevalence of COSY and DQF-COSY as structural elucidation tools. The pulse sequence employed (Fig. 1(a)) was described by Turner [20]. It consists in a modified INADEQUATE pulse sequence, where the t_1 evolution period is split by a 90° hard pulse, giving rise to a 2D spectrum with symmetric frequency axis. However, contrary to COSY or TOCSY spectra, the ^{1}H–^{1}H INADEQUATE spectrum contains additional, non-symmetric peaks that disappear when a symmetrisation procedure is applied. These non-symmetric peaks probably arise from strong coupling effects and cannot be suppressed by coherence order selection, as their coherence order is the same as for the desired peaks. Moreover, the symmetrisation procedure could create small artificial cross-peaks. As a consequence, it is important that the quantification procedure is carried out on the non-symmetrised spectrum. Here, spectra were first symmetrised to facilitate the visualisation and the identification of peaks arising from the target metabolites. Then, spectra were reprocessed without symmetrisation for quantification. All the signals that were used for quantification appeared at the same position on both symmetrised and non-symmetrised spectra.

In order to compare the precision of the three precited pulse sequences, each experiment was repeated five times successively on the same model mixture containing 6 representative metabolites, and for different experiment durations. Fig. 2 shows the relative standard deviation (RSD) for the relative lactic acid/alanine 2D peak volumes. Similar curves were obtained for the other 2D peak ratios. The graph shows the superiority of INADEQUATE versus the two other pulse sequences in terms of repeatability. The reasons explaining this relative superiority might be complex to determine,

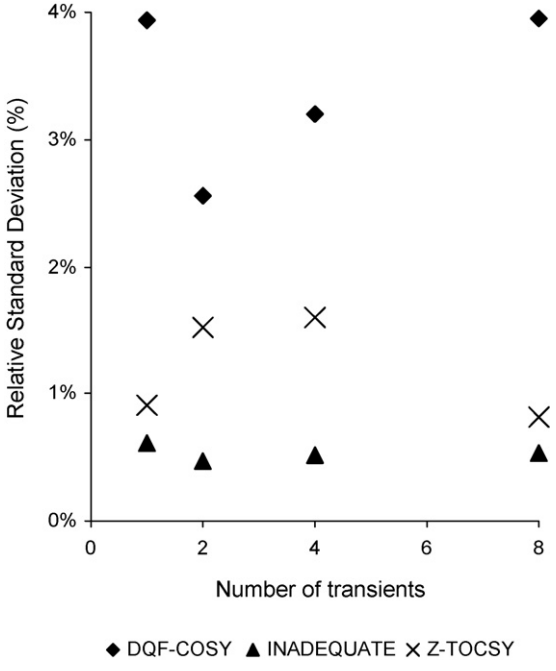

Fig. 2. Influence of the number of transients on the relative standard deviation (lactic acid/alanine relative 2D peak volumes) for DQF-COSY, zTOCSY and ^{1}H INADEQUATE recorded with 128 F_1 data points, for a standard 50 mM metabolite mixture.

but we believe that it could be attributed to a cleaner diagonal in the case of INADEQUATE. This can be observed in Fig. 3, showing 2D spectra acquired in fast conditions, corresponding to the data points of Fig. 2 obtained for NS = 1. The INADEQUATE spectrum (Fig. 3(a)) obtained on the model metabolite mixture highlights the cleanliness of the diagonal, and shows more intense correlation peaks versus diagonal peaks, contrary to zTOCSY and DQF-COSY spectra (Fig. 3(b) and (c), respectively) It can be noticed, however, that INADEQUATE diagonal signals do not perfectly cancel out. This would require longer and/or more intense coherence-selection gradients, a choice that we decided to bypass in order to avoid sensitivity losses due to molecular diffusion effects.

Another feature arising from Fig. 2 is a tendency towards correlation between precision and experimental duration (a notable exception is the value measured for zTOCSY and 8 scans). This evolution is particularly observable for DQF-COSY (for 8, 4 and 2 scans) where RSD values are higher. As already noticed in previous studies [7,23], long experiments are more sensitive to spectrometer instabilities in the course of time, leading to a degradation of precision. On the contrary, short experiments are less sensitive to such instabilities and show a better repeatability, as long as the signal-to-noise ratio (SNR) and the resolution are sufficient to quantify relevant peaks with the target precision. When concentration is sufficient, the acquisition can be reduced to one scan per t_1 increment, but this is true only if coherence-selection gradients efficiently replace phase cycling. This was apparently not the case for DQF-COSY, where RSD notably increases for NS = 1.

With these results at hand, we optimized the INADEQUATE pulse sequence in order to quantify all the metabolites with an optimum repeatability, in the shortest possible time. The total experiment time T_{exp} can be expressed as $T_{exp} = TR \cdot NS \cdot N_1 + TR \cdot DS$, where TR is the pulse sequence duration (including the recovery delay), NS is the number of transients, DS is the number of dummy scans and N_1 is the number of increments in the indirect F_1 dimension. We carefully optimized these parameters to reduce T_{exp} as much as possible.

In order to determine the optimum TR, we measured the longitudinal relaxation times (T_1) for the relevant ^{1}H signals and for various metabolite concentrations. It was observed that T_1's do not vary significantly within the concentration range explored (results not shown), therefore, it was chosen to work under partial saturation conditions, and a recovery delay of 5.5 s (leading to TR = 6 s) was found to be a good compromise, which was justified *a posteriori* by the excellent linearity of our method (see below).

As for the number of transients, we completely suppressed the 64-step original phase cycle of the INADEQUATE pulse sequence by incorporating suitably calibrated coherence-selection gradients in the pulse sequence. As a consequence, NS was reduced to 1, as the sensitivity of our method was sufficient to detect in one scan all the target metabolites at a 0.1 mM concentration. It should be emphasized that for samples in H_2O, two scans would be necessary to obtain optimum solvent signal suppression. The subsequent longer experiment time could be compensated by using a shorter recovery delay to reach the same SNR as in one scan. However, working on such partial saturation conditions could be problematic for quantitative purposes.

Finally, 64 t_1 increments were employed in the indirect dimension. Higher N_1 values did not significantly improve SNR and resolution, whereas for N_1 = 32, a significant alteration of the resolution along F_1 was observed. Lower N_1 values could potentially be applied by using linear prediction methods. Thanks to this optimization, the total experiment time was reduced to 7 min, a duration that makes it possible to obtain a complete calibration curve in a reasonable time. The corresponding optimized spectrum is presented in Fig. 1(b), highlighting the possibility to identify and quantify metabolites whose signals are close to diagonal peaks, such as taurine and myo-inositol.

3.2. Analytical evaluation

In order to assess our optimized method for quantitative analysis of metabolic mixtures, we chose a mixture of six metabolites reflecting real samples such as those obtained after extraction from cellular cultures [24]. We evaluated the repeatability for each metabolite signal at various concentrations by repeating five successive experiments. For each relevant signal, 2D peak volumes were measured relatively to a reference peak (alanine) whose concentration was kept constant from one sample to another. The reference concentration was kept at a maximum value even for weak metabolite concentrations, to ensure that the method would give quantitative results even in unfavourable conditions where the relevant metabolite peak intensities would be of the same order of magnitude as the t_1 noise arising from an intense signal. Table 1 summarizes the results obtained in terms of repeatability (RSD) and signal-to-noise ratio (SNR).

In this study, SNR values were used to assess the sensitivity of our methods. According to the IUPAC recommendations [25], this choice is valuable only if the calibration curves are straight lines. In our study, this choice was justified *a posteriori* by the excellent linearity characterizing our method, as indicated below. Moreover, in order to correctly measure SNR, one should keep in mind that the origin and the nature of noise is very different in each dimension of the 2D spectrum [26]. Along the direct F_2 dimension, noise arises mainly from thermal noise, like in 1D NMR. On the contrary, the indirect F_1 dimension is characterized by the so-called t_1-noise generated mainly by instrumental instabilities [27]. t_1-noise is always

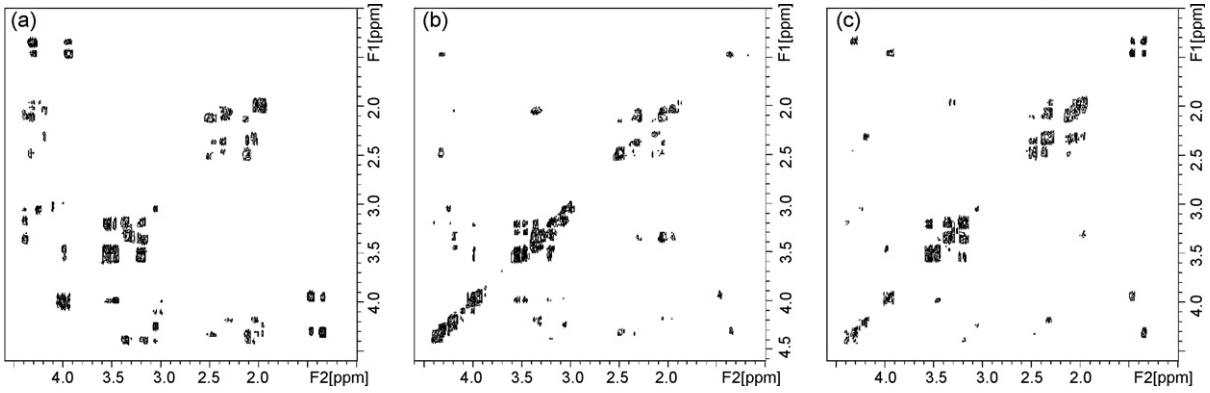

Fig. 3. Comparison between INADEQUATE (a), zTOCSY (b) and DQF-COSY (c) spectra of a 50 mM metabolic mixture, recorded at 298 K on a 400 MHz spectrometer with 128 F_1 data points and NS = 1. Spectra correspond to the leftmost data points of Fig. 2.

Table 1
Minimum and maximum signal-to-noise ratio (SNR) and relative standard deviation (RSD) obtained on ^{1}H INADEQUATE spectra using six different test mixtures (with concentrations ranging from 0.1 mM to 50 mM approximately).

	S/N for highest concentration	S/N for lower concentration	RSD (min)	RSD (max)
Lactic acid	657 (47.88 mM)	283 (0.41 mM)	0.04%	1.63%
Proline	33 (47.73 mM)	7 (0.09 mM)	0.83%	7.25%
Myo-inositol	599 (48.04 mM)	280 (0.28 mM)	0.42%	1.70%
Taurine	182 (49.44 mM)	136 (0.08 mM)	0.48%	1.32%
GSH	48 (47.98 mM)	2 (0.10 mM)	0.90%	8.06%

S/N were measured along F_1 dimension to account for the higher, so-called "t_1 noise" characterizing this dimension. RSD were determined on relative peak volume ratios (reference: alanine 50 mM) for five successive experiments. Minimum RSD is observed for maximum SNR and vice-versa.

higher than thermal noise, therefore SNR values are only relevant if they are measured along the indirect dimension. Consequently, SNR values indicated in Table 1 were measured along columns for the relevant chemical shifts on the non-symmetrized spectra. Moreover, it should be noticed that t_1-noise is concentration-dependant, consequently SNR along the indirect dimension does not vary linearly with concentration, thus explaining the somewhat surprising SNR values indicated in Table 1.

Still, Table 1 shows an expected correlation between SNR and RSD: minimum RSD is observed for maximum SNR and vice-versa. Repeatability remains under 2%, except for two metabolites at the lowest concentration (proline and glutathione). The two RSD values above 2% correspond to cases where metabolite concentrations are under the limit of quantification (LOQ), which is defined by a SNR of 10 according to the ICH guidance [28]. However, the corresponding peak volumes can still be measured with a precision better than 8%. Finally, our fast optimized INADEQUATE method appears well suited for quantifying metabolites above the LOQ with a precision better than 2%. Sub-millimolar concentrations can be characterized with a very good precision in 7 min. When necessary, several scans could be accumulated in order to reach lower concentrations.

For identical concentrations, 2D peak volumes are very different from one metabolite to another, because 2D peak volumes depend on a number of factors (number of nuclei, J-couplings, T_2, overlap and relative phases inside a multiplet). As a consequence, a calibration procedure is necessary to obtain accurate results, like in many other spectroscopic techniques such as UV–vis or IR spectroscopy. In the case of quantitative INADEQUATE 2D NMR, the short experiment duration now makes it possible to obtain a calibration plot in a very short time. Accurate results can therefore be obtained after calibration if a good linearity is achieved. We evaluated the linearity of the fast 2D INADEQUATE protocol by plotting calibration curves of 2D peak volume ratios as a function of gravimetric concentration ratios. Calibration curve parameters are shown in Table 2. The very high coefficients of determination show the excellent linearity of the fast INADEQUATE 2D method, thus proving that accurate measurements can be obtained after preliminary calibration. It can be noticed, however, that the linearity is lower for proline, a result that can be correlated with the lower SNR characterizing the 2D peak for this metabolite. Also noticeable are the differences between slope values, highlighting the heterogeneity of 2D NMR response coefficients. Finally, y-intercept values are not significantly different from zero, showing the absence of bias in the method.

Table 2
Calibration curve parameters obtained when plotting the dependence of metabolite peak volume ratios on the gravimetric concentration ratio for optimized ^{1}H INADEQUATE.

	Slope	y-Intercept	r^2
Lactic acid/alanine	1.060 ± 0.020	−0.0053 ± 0.096	0.9986
Proline/alanine	0.007 ± 0.001	0.0006 ± 0.005	0.9232
Myo-inositol/alanine	0.652 ± 0.011	0.0008 ± 0.0056	0.9988
Taurine/alanine	0.215 ± 0.002	0.0013 ± 0.0008	0.9998
GSH/alanine	0.023 ± 0.001	−0.0007 ± 0.0005	0.9932

Each calibration curve was obtained using six different test mixtures with concentrations ranging from 0.1 mM to 50 mM approximately. Alanine was used as a reference to calculate relative peak volume ratios and concentration ratios.

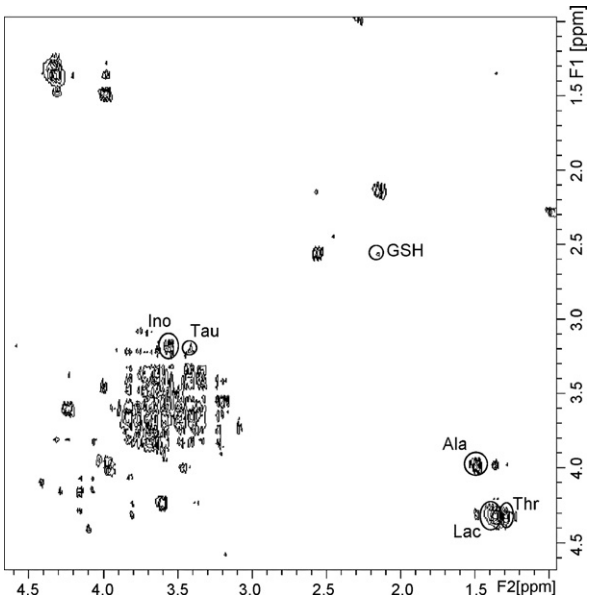

Fig. 4. 2D ^{1}H INADEQUATE spectrum of a MDA-MB-468 line cellular extract, obtained by $CHCl_3$/MeOH/H_2O extraction of breast cancer cells. Spectrum acquired in 7 min at 298 K on a 500 MHz spectrometer equipped with a cryogenic probe, with 1 transient and 64 t_1 increments. Relevant metabolite 2D peaks are indicated. Ala: alanine; GSH: glutathione; Ino: myo-inositol; Lac: lactic acid; Tau: taurine; Thr: threonine.

3.3. Applicability to biological samples

The fast INADEQUATE protocol described above was designed as a tool for studying real metabolic samples. In order to assess its capacity to identify and quantify relevant biomarkers in biological samples, we applied it to a breast cancer cell extract. The corresponding INADEQUATE spectrum, acquired in 7 min, is presented in Fig. 4. It is important to notice that the extraction procedure was not completely optimized here, and is the purpose of current research. Still, we were able to identify a number of metabolites above the quantification limit, including some of the metabolites that were present in the model mixture. This result highlights the appropriateness of our fast method for studying biological samples. The quantification of metabolites in such samples to differentiate various cancer cell lines using the fast 2D INADEQUATE protocol will be presented in a further publication.

A noticeable feature of Fig. 4 is the numerous peak overlaps characterizing the 3–4 ppm region. This is the main limit of the INADEQUATE method versus heteronuclear correlations: the discrimination of resonances is not as efficient as for heteronuclear techniques, such as the fast HSQC protocol described by Lewis et al. [10] However, the fast INADEQUATE experiment offers a higher inherent sensitivity and is still able to quantify a number of relevant metabolites. Therefore, the two fast methods could be used in a complementary manner for complete metabonomic studies.

4. Conclusion

In this work, quantitative ^{1}H INADEQUATE 2D spectra of metabolic mixtures were obtained in 7 min with a repeatability bet-

ter than 2% for metabolite concentrations as small as 100 μM and an excellent linearity. The 2D ^{1}H INADEQUATE protocol described in this work is an efficient method for fast and precise quantification of metabolic mixtures, and it forms a promising tool for metabonomic studies. In order to improve its precision, further NMR developments will consider the implementation of 180° adiabatic pulses and the investigation of other coherence-selection gradient schemes. From the analytical point of view, our method only allows the determination of relative concentrations, and thus requires adding an internal reference. The next step will be the implementation of the ERETIC™ method (electronic reference to access *in vivo* concentrations) that allows the determination of absolute concentrations [29]. ERETIC™ has already been successfully implemented in 2D pulse sequences [30].

Thanks to the optimization presented above, quantitative 2D spectra were obtained in 7 min. However, this value constitutes the lower limit for the experiment duration, as a number of t_1 increments will always be required to obtain a 2D spectrum with a sufficient resolution. In order to decrease this limit by several orders of magnitude, we have recently proposed an analytical evaluation of ultrafast 2D NMR [23], showing promising analytical results for 2D spectra acquired in a fraction of a second on model compounds mixtures. We are currently improving this methodology in terms of resolution and sensitivity in order to make it suitable for quantitative analysis of metabolic mixtures such as those described in this study.

In terms of application, various cancer cell lines will be analyzed by our fast INADEQUATE protocol and subjected to statistical analysis in order to find relevant biomarkers for differentiating them. These results will be presented in further, biological-oriented publications.

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