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Jonathan Farjon. Intrinsically quantitative 2D HSQC NMR: a tool for deciphering complex mixtures. Annual reports in NMR, 2023. hal-04242500

**HAL Id: hal-04242500**

**<https://hal.science/hal-04242500>**

Submitted on 15 Oct 2023

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# Intrinsically quantitative 2D HSQC NMR: a tool for deciphering complex mixtures

Jonathan Farjon<sup>a\*</sup>

<sup>a</sup>Nantes Université, CNRS, CEISAM UMR 6230, F-44000 Nantes, France

\*Corresponding author: [jonathan.farjon@univ-nantes.fr](mailto:jonathan.farjon@univ-nantes.fr)

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**Number of words including Figures and References: 10285**

## Abstract:

Analysis of complex mixtures is of main interest since they are ubiquitous in the nature. Thus, it becomes challenging to decipher their complexity by developing NMR methods able to identify but also to quantify molecules in the most accurate way. 1D <sup>1</sup>H NMR is highly utilized for quantifying molecules but suffers from many overlaps making this approach limited. 2D NMR is a great manner to improve the signal

dispersion over 2 dimensions and the  $^1\text{H}$ - $^{13}\text{C}$  HSQC experiment is the best choice to improve the spectral resolution despite a lower sensitivity. However, due to its multiple pulse nature, the quantitativity of HSQC is impacted and biased absolute concentrations are derived. In this chapter, we highlight the potential of a class of methods called intrinsically quantitative 2D HSQC. They are generated by designing pulse sequences able for each correlation peak to maintain constant or to compensate  $J_{\text{HH}}$ ,  $J_{\text{CH}}$ , off resonances and  $T_2$  effects responsible for the degradation of the quantitativity. Pioneer experiments as Q-HSQC and HSQC<sub>0</sub> are detailed as well as their improved versions and applicative examples are given. Then, these major techniques are compared to show their performances and limits. Finally, recent methodological developments are proposed and discussed to enhance the potential of future intrinsically quantitative 2D-based complex mixture analyses.

Keywords: Complex mixtures, Intrinsically Quantitative NMR, bidimensional NMR, fast NMR, pure shift NMR, Q-HSQC, HSQC<sub>0</sub>, QUIPU HSQC

## Abbreviations

<b>ASAP</b>	Acceleration by Sharing Adjacent Polarization
<b>BIRD</b>	Bilinear Rotation Decoupling
<b>COSY</b>	Correlation Spectroscopy
<b>CPMG</b>	Carr-Purcell-Meiboom-Gill
<b>D-DNP</b>	Dissolution Dynamic Nuclear Polarization
<b>FMLR</b>	Fast Maximum Likelihood Reconstruction
<b>HSQC</b>	Heteronuclear Single Quantum Correlation
<b>HSQC<sub>0</sub></b>	Heteronuclear Single Quantum Correlation zero
<b>INEPT</b>	Insensitive Nuclei Enhancement Polarization transfer
<b>NUS</b>	Non Uniform Sampling
<b>PHIP</b>	Parahydrogen Induced Polarisation
<b>QEC-HSQC</b>	Quantitative Equal Carbon HSQC
<b>Q-HSQC</b>	Quantitative HSQC
<b>Q-OCCAHSQC</b>	Quantitative, offset-compensated, CPMG-adjusted HSQC
<b>QQ-HSQC</b>	Quick Quantitative HSQC
<b>Q QUIPU HSQC</b>	Quick QUantitative Perfected and pUre shifted
<b>QUIPU HSQC</b>	QUantitative Perfected and pUre shifted
<b>SABRE</b>	Signal Amplification By Reversible Exchange

**SENSASS HSQC** SENSitivity increAsed and resolution enhanced by Signal  
Suppression

**SNRs** Signal-to-Noise Ratios

**ST2-PT** Single Transition-to-single Transition Polarization Transfer

$T_1$  Longitudinal relaxation time

$T_2$  Transverse relaxation time

**TAF HSQC** Tolerant of Any Factor HSQC

**TOCSY** Total Correlation Spectroscopy

**UFCOSY** Ultra Fast Correlation Spectroscopy

**UHPLC** Ultra High Performance Liquid Chromatography

## 1. Introduction

NMR is a unique ensemble of methods able to give diverse information on molecules even in complex mixtures, their identity and quantity can jointly be accessed in addition to dynamic data related to exchange and conformations. The determination of the molecular quantity per unit of matter or the related concentration is becoming essential in various fields including pharmaceutical analysis [1], food sciences [2], plants [3] and environmental investigations [4]. Even though the quantitativity by NMR is suitable for a wide range of applications like accessing the number of nuclei per site, the relative and absolute concentration but also isotopic ratios, the review will be dedicated to the determination of concentrations.

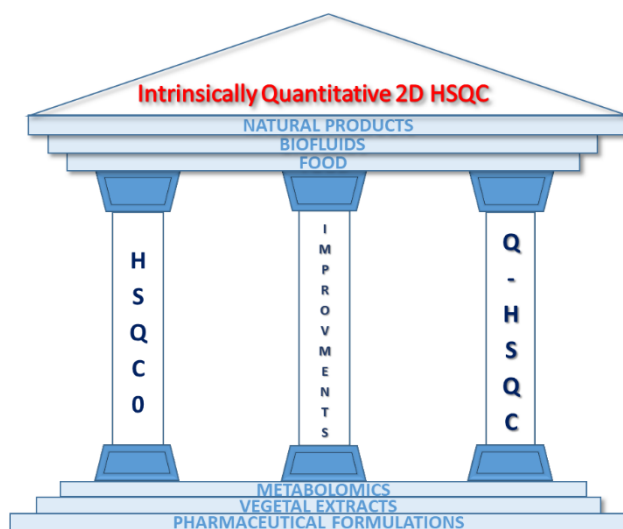
Thus, 1D quantitative NMR or so called as qNMR has been highly exploited using well established protocols [5]. Although  $^1\text{H}$  1D NMR is a highly repeatable method its

application to complex mixture is limited due to many peak overlaps making quantification inaccurate or impossible. As a good alternative 2D NMR has the main advantage to extend the spectral dispersion over 2 dimensions. Among them homonuclear like COSY with its ultra-fast version: UFCOSY have been intensively used [6] but also TOCSY [7]. Heteronuclear  $^1\text{H}$ - $^{13}\text{C}$  HSQC [8] is recognized as one of the most resolutive approach due to the larger  $^{13}\text{C}$  spectral range for improving correlations separation despite the lower intrinsic sensitivity of  $^{13}\text{C}$ . Moreover, 2D HSQC experiments are made of multiple pulses schemes embedding delays with a peak-dependent analytical response of the nuclei. This is due to numerous different modulations occurring during the pulse sequence either coming from homonuclear / heteronuclear scalar couplings, offsets effects and from transverse relaxation evolutions impeding the quantitative potential of NMR. Under these conditions, how could it be possible to make intrinsically 2D NMR quantitative?

An intrinsically quantitative 2D HSQC is self-sufficient for quantifying any molecule having correlation signals on 2D maps. The goal to tackle with intrinsically quantitative 2D HSQC is to reach like with 1D  $^1\text{H}$  NMR the possibility to derive quantities from the integration of signals by using either an internal or an external reference.

In this chapter, the main purpose is to show and explain the different HSQC-based strategies by designing pulse sequences for turning standard 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC into an “intrinsically” quantitative version. In this framework, Q-HSQC and HSQC<sub>0</sub> as pioneering experiments will be compared as well as their improved versions making them more performant and robust to tackle analytical challenges met in various fields of application (Figure 1). Moreover, some interesting recent methodological advances

will also be added to the main discussion to extend the potential of future intrinsically quantitative experiments.



**Fig. 1.** Q-HSQC and HSQC<sub>0</sub> and their improved versions are the pillars of the intrinsically 2D quantitative NMR temple.

## 2. Different strategies for quantifying with 2D NMR

Quantitative NMR (qNMR) is suitable to complex mixture analysis and has main applications for food, metabolomics, natural products, plants, herbs, forensics and pharmaceuticals. 1D <sup>1</sup>H NMR is recognized as intrinsically quantitative and is the most sensitive and fast strategy to access concentrations [5]. The most particular property of qNMR comes from the proportionality between the signal integral (Int<sup>1D</sup>), the number of nuclei (p), the sensitive volume of the coil (V<sub>s</sub>) and the concentration [c],

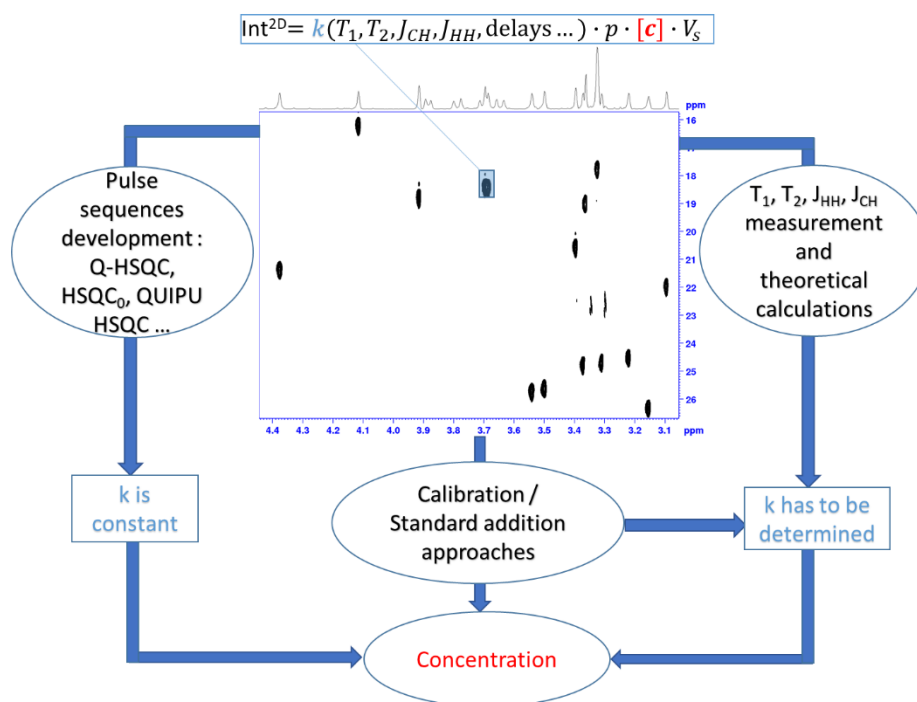
$$\text{Int}^{\text{1D}} = p \cdot [c] \cdot V_s \quad (\text{Eq. 1})$$

When overlaps are impeding 1D NMR, one good alternative is HSQC offering an enhanced spectral dispersion. HSQC can be used for quantifying complex mixtures and integral for correlation peaks (Int<sup>2D</sup>) are depending, like 1D, on relaxation  $T_1$  and

$T_2$  times but also all  $J_{HH}$ ,  $J_{CH}$  and chemical shifts are evolving during the whole HSQC pulse sequence duration. These additional modulations could be gathered as a k factor, a site-specific coefficient of response for each correlation peak (see Fig.2):

$$\text{Int}^{2D} = k \cdot p \cdot [c] \cdot V_s \quad (\text{Eq. 2})$$

Figure 2 shows the different approaches that have been implemented with this aim. The first one is based on the determination, for each peak, of the exact value of k, supported by theoretical considerations [9] where all scalar couplings, relaxation times shall be precisely determined (see right part of Figure 2). This approach is time-consuming since it requires all NMR interactions to be accurately measured to enable determination of the concentration in the best manner.



**Fig. 2.** Different approaches to extract concentrations data from 2D NMR maps like HSQC.



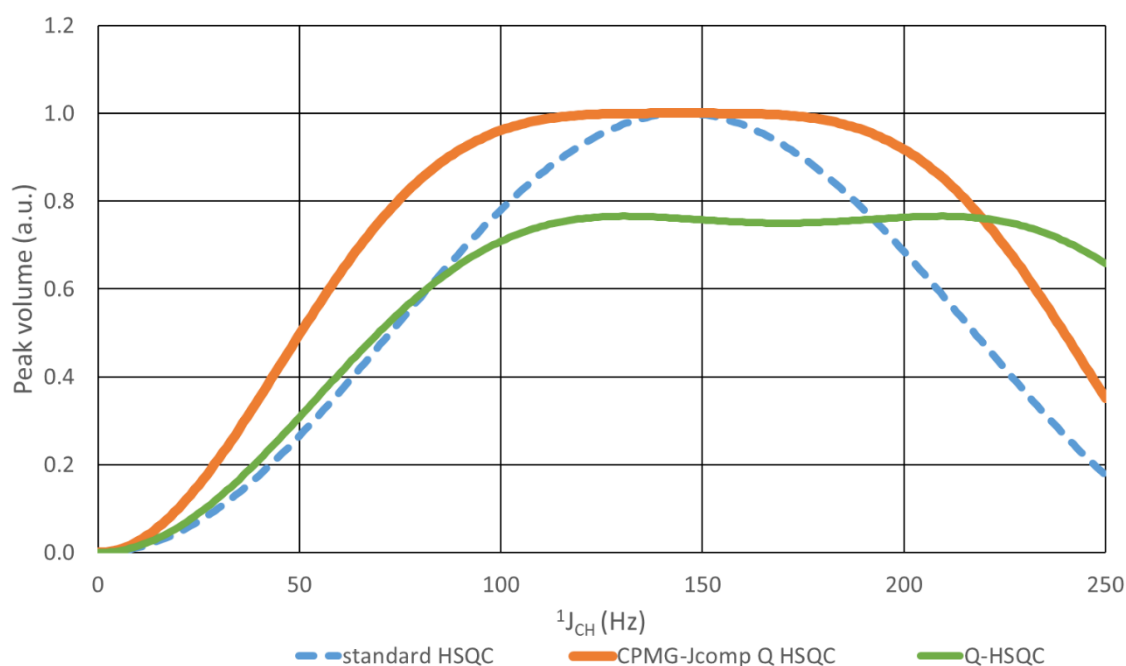
A second type of approach gives access to k values through conventional analytical methods such as calibration or standard additions [10,11,12] (bottom arrow under the spectrum on Figure 2). These strategies are not different from those used in previous decades in other analytical techniques such as gas and liquid chromatography and Infra-Red / Raman spectroscopies. In the external calibration approach, calibration curves are obtained by plotting the 2D peak volumes versus the corresponding concentration. Then, a 2D spectrum is recorded on the sample under study containing the analyte of interest to quantify. Its concentration is determined with the help of the regression curve equation. This approach offers a great solution to the problem of accuracy in 2D NMR but there is a need to commercially access or to synthesize each molecule to quantify with a known purity.

A third approach is based on intrinsically quantitative 2D NMR and related pulse sequence adaptations and developments to make them usable for reaching concentrations (see the left part of Figure 2). Among such intrinsically quantitative 2D NMR methods Q-HSQC and HSQC<sub>0</sub> were the pioneers to be implemented.

### **3. Intrinsically quantitative 2D NMR: Q-HSQC**

Making 2D experiments a quantitative tool needs modifying and/or combining several NMR pulse schemes, and such developments have been mainly focussed on <sup>1</sup>H-<sup>13</sup>C HSQC pulse sequences since they are easier to manipulate for compensating site-specific interactions. They were firstly developed by Heikkinen and coworkers, who proposed a method to eliminate the effects of heteronuclear <sup>1</sup>J<sub>CH</sub> couplings in the <sup>1</sup>H-<sup>13</sup>C HSQC pulse sequence [13]. In the conventional HSQC experiment, the 2D peak volumes are proportional to  $\sin^2(\pi\Delta^1J_{CH})$  where  $\Delta$  is the duration of the INEPT polarization transfer delay [14]. In 2003, Heikkinen *et al.* developed for compensating

$^1J_{\text{CH}}$  to sum four HSQC spectra with different and suitably selected  $\Delta$ : three with a smaller and one with a bigger value [15]. As a result, in the combined spectrum, 2D peak volumes are uniformly affected by  $^1J_{\text{CH}}$  couplings from 125 to 225 Hz range (see Figure 3) with only 2% of variation (bottom green curve in Figure 3) as compared to 50% variation with the standard HSQC (dashed blue curve in Figure 3). Moreover, due to  $^1J_{\text{CH}}$  compensation the sensitivity of signal is 25% lower (see Figure 3) as compared to the standard HSQC.



**Fig.3.**  $^1J_{\text{CH}}$  dependence on different HSQC schemes impeding the quantitativity.

The approach proposed by Heikkinen *et al.* [16,17] requires four times more scans than the standard HSQC for compensating  $^1J_{\text{CH}}$  evolutions. In 2007 this drawback was compensated for by Peterson and Loening, who proposed to acquire from different slices of the sample the signals corresponding to the different INEPT delays [18]. This approach called quick, quantitative HSQC (QQ HSQC) is 4 times faster than the one described by Heikkinen for the same result.

However, the 2D peak volumes in the HSQC spectra are also modulated by homonuclear  $J_{HH}$  couplings [19]. As described in theory, the cross-peak intensity can be affected by a factor:

$$\prod_{i=1}^k \left( \frac{1}{2} (1 + \cos(2\pi J_{HHi}\Delta)) \right)^2 \quad (\text{Eq. 3})$$

In 2005, Heikkinen *et al.* implemented a compensation for the effect of  $J_{HH}$  in the Carr–Purcell–Meiboom–Gill (CPMG) pulse trains in the INEPT and retro-INEPT schemes (see Figure 4 C) of the HSQC pulse sequence [19]. They also included composite CPMG pulse trains to reduce off-resonance effects that are more pronounced especially on the edge of the  $^{13}\text{C}$  chemical shift range. More recently, they introduced adiabatic pulses (see Figure 4 H) in the evolution period of their pulse sequence to improve the quantitativity of the experiment [19]. The resulting pulse sequence, called quantitative, offset-compensated, CPMG-adjusted HSQC (Q-OCCAHSQC), including all these improvements.

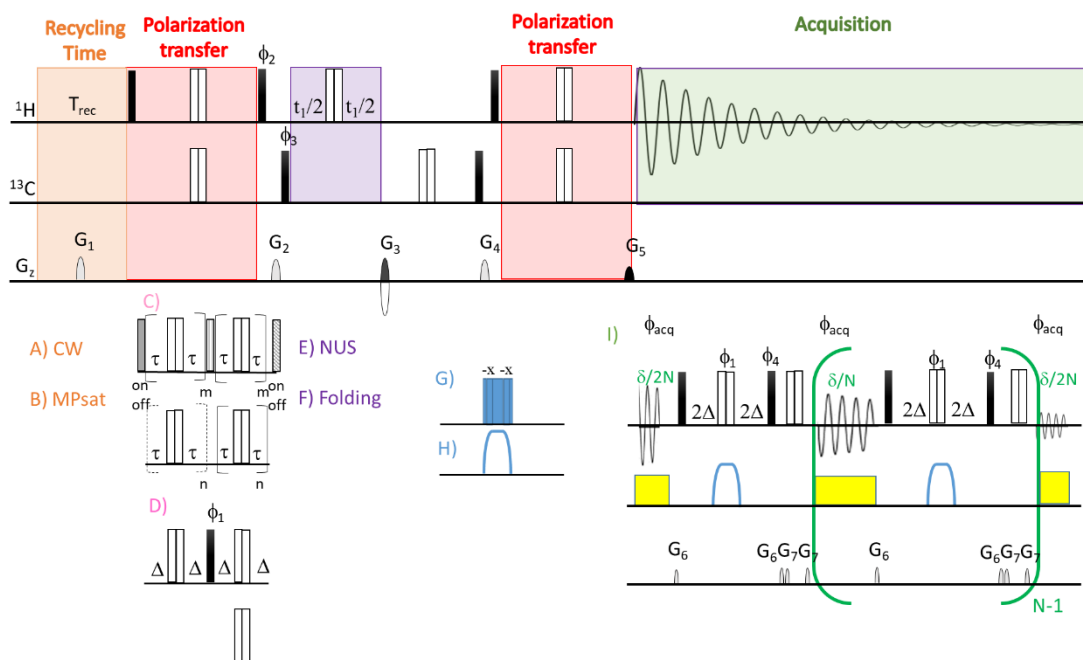
Lowered performances could be obtained since peak volumes also depend on  $T_2$  transverse relaxation times, that is not considered in the Q-HSQC protocol. This means that the Q-OCCAHSQC protocol is more convenient to quantify molecules with close or similar  $T_2$  values, as reported by Zhang and Gellerstedt who developed a protocol where cross peaks with the same  $T_2$  relaxation profiles were analyzed as individual groups [20]. The analytical performance was evaluated for this method, and they reported errors of up to 17% on a strychnine sample, with a 7.3% standard deviation.

More recently Heikkinen *et al.* were improving the Q-HSQC experiment by developing the Quantitative Equal Carbon or QEC HSQC [21]. QEC HSQC like Q

HSQC is requiring 4 times more scans to average out  $^1J_{\text{CH}}$  over different  $\text{CH}_n$  sites of the studied molecules. This approach is allowing for integrating any correlation peak to derive quantitative data with no need of normalizing integrals by the number of  $^1\text{H}$  (see p in Figure 2). A great advantage of equalizing  $\text{CH}_n$  responses allows for increasing the dynamic range and reducing  $t_1$  noise especially for methyl groups. With QEC HSQC, it was possible to obtain relative quantification of all  $^1\text{H}$  sites for brucine and also cinchonidine with a good 5 to 7% accuracy range.

In 2020, Zhang and coworkers were proposing another version of the Q-HSQC that is  $J$ -compensated and using a CPMG-based Levitt's composite echo-based HSQC. This Q-HSQC version is more sensitive than the first one published by Heikkinen [15] since having the same sensitivity than the standard HSQC in a  $^1J_{\text{CH}}$  range from 120 to 180 Hz (see Figure 3, the top orange curve). As previously pointed out, Q-HSQC is dependent on different  $T_2$  values varying from site to site and that can be different from the internal / external used reference. This approach in combination with a  $T_2$  normalization protocol was valorized for quantifying some drugs in cream formulations to determine their absolute concentration as well as their enantiomeric excess with the help of a chiral solvating agent [22].

In the family of intrinsically quantitative HSQC experiments, Guerrini *et al.* succeeded in obtaining the relative composition of different monosaccharidic units of heparines [23,24]. They showed that finely tuning INEPT delays in standard HSQC, with respect to specific sites like anomers allowed accurate relative quantities to be obtained.



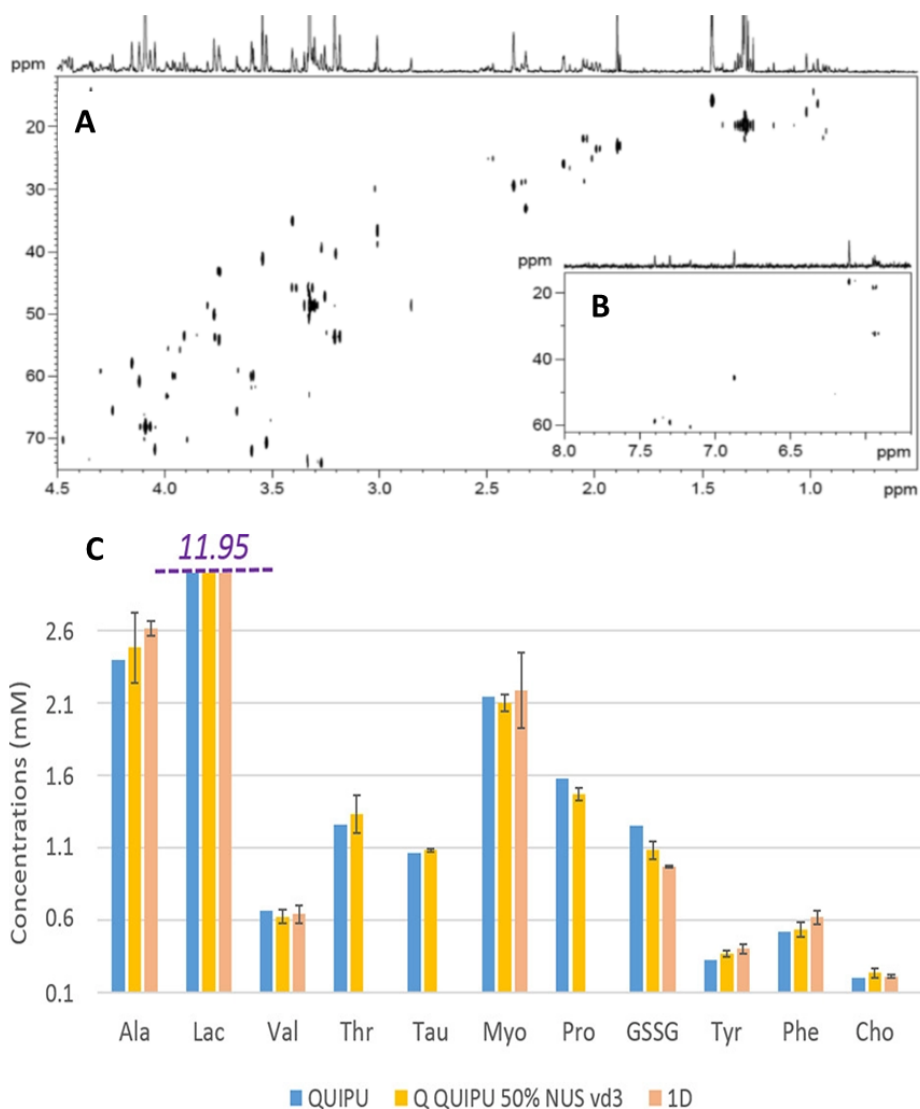
**Fig. 4.** Improvements of Q-HSQC by combining different schemes of interest. Prior to the excitation for the elimination of solvent signal(s) like A) presaturation with a continuous wave (CW) or B) multiple selective schemes (MPsat).  $J_{HH}$  are eliminated during INEPTs with C) CPMG or D) perfect echoes. The indirect dimension could be accelerated by using Non Uniform Sampling (NUS) and / or spectral aliasing. For preventing frequency offset effects, hard  $180^\circ$   $^{13}\text{C}$  inversion / refocussing pulses could be replaced by G) composite and H) adiabatic pulses. The acquisition could be a standard full FID or a pure-shift homodecoupled one by concatenated BIRD and chunks of FID.

#### 4. Improving sensitivity and resolution: QUIPU HSQC

One main limitation of  $^1\text{H}$ - $^{13}\text{C}$  HSQC is due to its lower sensitivity intrinsically resulting from the low natural abundance of  $^{13}\text{C}$ . Moreover, for highly complex mixture having structurally close molecules, there is a need of much more resolute methods. Under this context Farjon and coworkers designed in 2016 the QUIPU

HSQC experiment able to give access to absolute quantification like for 1D  $^1\text{H}$  qNMR by using an internal / external – reference with an accuracy that could reach 10%. This technique includes  $^1J_{\text{CH}}$  compensation like in Q-HSQC (see Section 3.) but with perfect INEPT (see Figure 4 D) and retro-INEPT [25] to replace CPMG-INEPT schemes (see Figure 3 C) to remove  $J_{\text{HH}}$  modulations. In addition, the acquisition is homodecoupled thanks to a BIRD based pure shift scheme [26] for highly disperse correlations over the 2D map. Thanks to  $J_{\text{HH}}$  collapsing, sensitivity gains up to 2.6 were obtained on vegetal extracts allowing for quantifying metabolites in the range of 100  $\mu\text{M}$  [27].

However, quantitative 2D NMR is time-consuming due to its 2D and quantitative nature. This experiment was accelerated in 2017 by Farjon *et al.* as Q QUIPU HSQC [28]. This approach combines Non Uniform Sampling (NUS) (see Figure 4 E), spectral aliasing (see Figure 4 F) [29] and variable recycling time [30] to speed up by a factor from 6 to 9, in comparison with the QUIPU version and allowing for quantification of metabolites in the 100  $\mu\text{M}$  range from breast cancer cell extracts (Figure 5) in 5 h [28]. In addition, Q QUIPU HSQC was successful in quantifying natural products in food supplements. In this application, quantifications were obtained in very good agreement with data derived from UHPLC-based methods [31].



**Fig. 5.** QQUIPU HSQC used to quantify cell breast extracts A) Aliphatics, B) aromatics. C) Diagram bars for quantifying different metabolites within cell breast extract by using lactate as an internal reference. Reprinted with permissions from [28] Copyright (2018) Elsevier

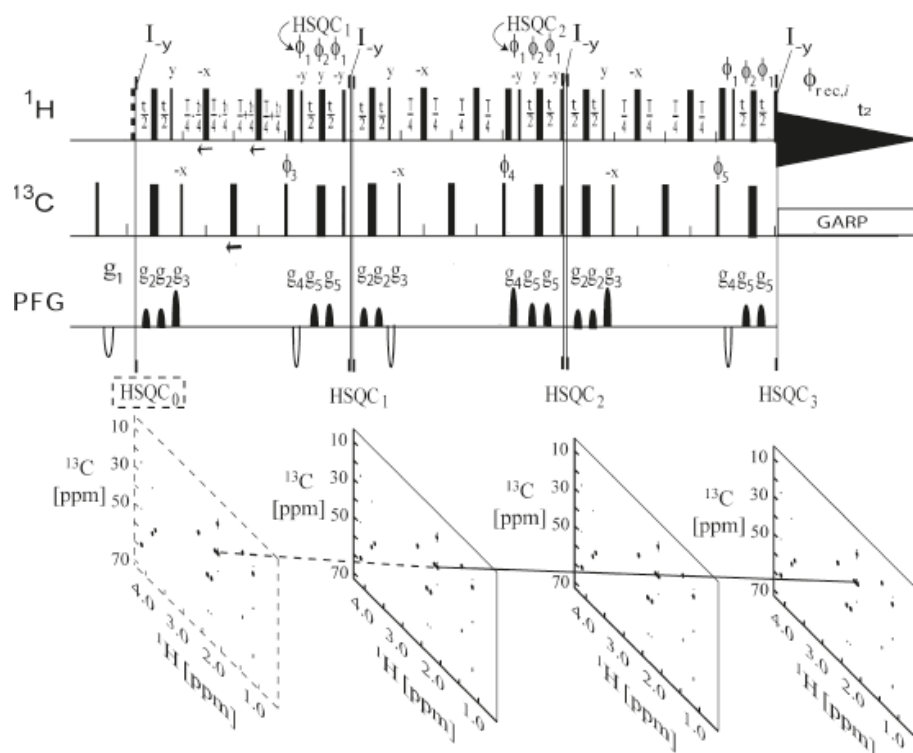
### 5. Mimicking 1D $^1\text{H}$ NMR with 2D HSQC<sub>0</sub>

Another strategy was proposed by Markley and his group to obtain k values (see Figure 2) that are constant whatever the site [32] This approach relies on obtaining an HSQC spectrum where correlation signals are not dependent on the molecular site. To obtain such a spectrum called HSQC<sub>0</sub> there is a need to acquire 3 successive HSQC

pulse sequences having 1, 2 or 3 HSQC blocks (Figure 6). With the hypothesis that the signal reduction is a function of  $J$ -couplings, relaxation times and off-resonance effects (see Figure 2) proportional to the number of HSQC $i$  with  $i=1,2,3$  block, the signal volume  $V_{i,m}$  for a given peak  $m$  follows the equation :

$$\ln(V_{i,m}) = \ln(V_{0,m}) + i \ln(k_{A,m}) \quad (\text{Eq. 4})$$

where  $k_{A,m}$  is the amplitude reduction factor for a 2D signal  $m$ . By plotting, for each 2D signal, the logarithm of the 2D volume  $\ln(V_{i,m})$  as a function of  $i$ , it is possible to calculate a 2D volume signal as though there was no HSQC or zero HSQC: HSQC $_0$ ,  $V_{0,m}$  considering no evolutions at all in the HSQC like a 1D 90° pulse. The HSQC $_0$  2D signal volume is consequently not dependent on evolution parameters like  $T_2$ ,  $J$ -couplings, delays, thus leading to absolute concentrations needing a reference signal. Even if the trueness of this technique was not highlighted, a good precision was reported, with a standard deviation close to 7%.





**Fig. 6.** (Top) Pulse sequence used for extrapolating HSQC<sub>0</sub> from 1, 2 or 3 consecutive 2D <sup>1</sup>H-<sup>13</sup>C HSQC. Narrow and wide black bars indicate 90° and 180° pulses, respectively. The delays are  $\tau = 3.3$  (or 3.4) ms ;  $T = 5.3$  (or 5.8) ms. The phase cycling is as follows :  $\phi_1 = -y, y$  ;  $\phi_2 = y, -y$  ;  $\phi_3 = 2[x], 2[-x]$  ;  $\phi_{\text{rec}} = x, -x, -x, x$ . All other radio frequency pulses are applied with phase x, except as indicated. Quadrature detection in the <sup>13</sup>C (t<sub>1</sub>) dimension is achieved using States-TPPI applied to the phase  $\phi_3$ . The duration and strength of the pulsed field gradients applied along the z-axis are as follows : (g<sub>1</sub>) 3 ms, 15.9 G/cm ; (g<sub>2</sub>) 0.4 ms, 10.6 G/cm ; (g<sub>3</sub>) 1 ms, 18.6 G/cm ; (g<sub>4</sub>) 1 ms, -26.5 G/cm, and (g<sub>5</sub>) 0.6 ms, 13.25 G/cm, followed by a gradient recovery period of 200  $\mu$ s. The 180° pulse marked with an asterisk serves to refocus proton chemical shift during the gradient delays for g<sub>3</sub> and g<sub>4</sub> to achieve better t<sub>1</sub> noise suppression. Pulse sequence proposed here for time-zero extrapolated HSQC (HSQC<sub>0</sub>) spectroscopy. Three data sets are collected: the first at the position indicated by HSQC<sub>1</sub>, the second at the position indicated by HSQC<sub>2</sub>, and the third at the position indicated by HSQC<sub>3</sub>; each data collection proceeds as shown for HSQC<sub>3</sub>. In all three pulse sequences, composite phase cycling of  $\phi_1$  and  $\phi_2$  is carried out in the HSQC unit that precedes data acquisition ; otherwise,  $\phi_1 = -y$  and  $\phi_2 = y$ .  $\phi_3 = 2[x], 2[-x]$  ;  $\phi_{\text{rec},1} = x, -x, -x, x$ . The constant time chemical shift evolution period T is replaced by the invariant constant time T in the second and third HSQC units, respectively for HSQC<sub>2</sub> and HSQC<sub>3</sub>. Additional phase cycling for HSQC<sub>2</sub> is  $\phi_4 = 4[x], 4[-x]$  ;  $\phi_{\text{rec},2} = x, -x, -x, x, -x, x, x, -x$ . Additional phase cycling for HSQC<sub>3</sub> is  $\phi_5 = 8[x], 8[-x]$  ;  $\phi_{\text{rec},3} = x, -x, -x, x, x, -x, x, x, -x, -x, x, x, -x, x, -x, -x, x$ . (Bottom) Shown are examples of HSQC<sub>1</sub>, HSQC<sub>2</sub>, and HSQC<sub>3</sub> spectra following Fourier transformation to yield frequency domain spectra. Peak intensities in the virtual HSQC<sub>0</sub> spectrum (bordered by the dashed line) are derived from linear extrapolation of the peak intensities of the

corresponding peaks in the HSQC<sub>1</sub>, HSQC<sub>2</sub>, and HSQC<sub>3</sub> spectra. Reprinted and adapted with permission from [32] Copyright (2011) American Chemical Society

This approach was improved in 2011 by incorporating suitably chosen coherence selection gradients yielding cleaner spectra [33]. It was successfully applied to quantify molecules in model samples [34], complex mixtures of natural products [35] in a targeted metabolomics study [36] as well as mixtures of lignins [37]. In 2022, HSQC<sub>0</sub> was even combined with the EXSY experiment and kinetic studies to better understand conformational changes in a peptide called Nukacin ISK-1 [38].

## 6. Global comparison between HSQC<sub>0</sub> and Q-HSQC

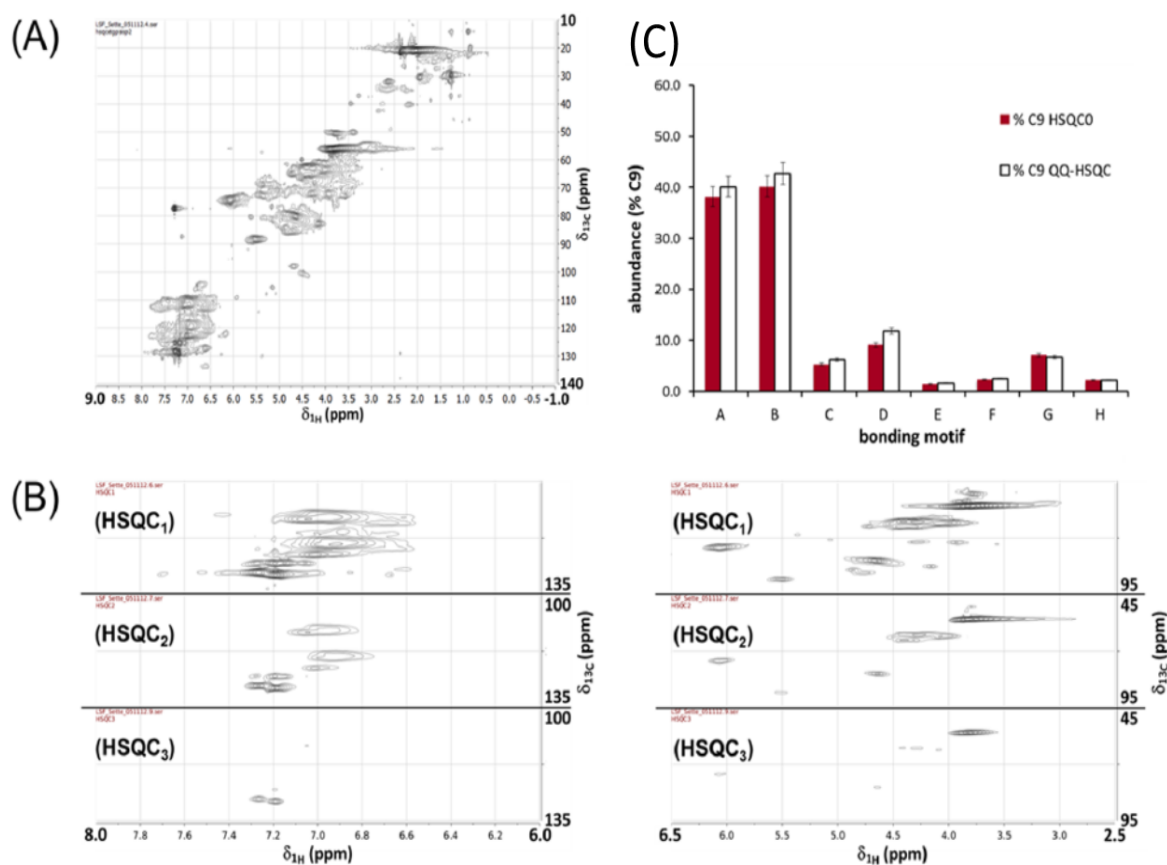
As previously described, in the class of intrinsically 2D HSQC, the best candidates are HSQC<sub>0</sub> and Q-HSQC that have advantages and drawbacks (see Table 1).

**Table 1.** Detailed comparison for intrinsically quantitative HSQCs and performed improvements

	<b>HSQC<sub>0</sub></b>	<b>Improvements</b>	<b>Q-HSQC</b>	<b>Improvements</b>
Acquisition time	+++ - At least 3x more time - HSQC <sub>3</sub> could reacquire more scans	Use of gradients to select coherences to reduce t <sub>1</sub> noise	++++ 4x more scans / <sup>1</sup> J <sub>CH</sub> compensation	+ * Q-OCCAHSQC J <sub>HH</sub> compensation More tolerant to offset effects * QQ HSQC Time /4 * QUIPU HSQC More sensitive and resolved * QQUIPU HSQC Faster
Easiness to implement	++++	/	++	/
Accuracy of concentrations	++++ <5%	++++ <5%	++ 5 to 10% Due to uncompensated	T <sub>2</sub> normalization

			$T_2$ effect	
$T_2$ effects	$T_2$ are compensated by extrapolation to zero HSQC but for HSQC <sub>2</sub> and more for HSQC <sub>3</sub> $T_2$ is affecting a lot SNRs, especially for bigger-sized compounds	/	Not compensated	$T_2$ normalization
Sensitivity considerations	++++HSQC <sub>1</sub> ++HSQC <sub>2</sub> +HSQC <sub>3</sub>		+++ -25% due to $^1J_{CH}$ compensation	++++ - QUIPU with improved SNRs +20 to 180% / no pure-shift version ++++ -CPMG-Jcomp the same sensitivity than standard HSQC
Postprocessing	Longer since 3 maps to process +		+++	/

Variations of HSQC<sub>0</sub> seem more worthwhile to consider than Q-HSQC, as it considers the effects of transverse relaxation and leads to more accurate quantitation, but it is not always easy to get a good sensitivity especially for HSQC<sub>3</sub> due to  $T_2$  evolution and the associated loss in sensitivity. Moreover, for HSQC<sub>0</sub>, the hypothesis is made that the NMR signal intensity is attenuated linearly as a function of the number of repetitions. Q-HSQC and HSQC<sub>0</sub> were jointly implemented to determine very similar accurate absolute concentrations for organic acids in mixture [27] as well as the relative amount of different structural units in wood lignin, a complex polymer (see Figure 7) [39].



**Fig. 7.** (A) Full range HSQC<sub>1</sub> obtained for acetylated Norway spruce MWL using a 600 MHz spectrometer equipped with a cryoprobe. (B) Zoom-ins for the aromatic region, and the aliphatic region of contour-diagrams obtained for interesting regions for the HSQC<sub>1</sub>, HSQC<sub>2</sub>, and HSQC<sub>3</sub>. (D) Graphical comparison of the intensities of signals corresponding to the main interunit bonding motifs found in the Norway spruce MWL sample (as % C9); legend: **A** –  $\beta$ -O-4' (H $\alpha$ ), **B** –  $\beta$ -O-4' (H $\beta$  including oxidised derivative), **C** –  $\beta$ -O-4' (C2-H' of oxidised derivative), **D** –  $\beta$ -5' (H $\alpha$ ), **E** –  $\beta$ -1' (H $\beta$ ), **F** – 5,5'- $\alpha,\beta$ -O-4' (H'), **G** –  $\beta$ - $\beta'$  (H $\alpha$ ), **H** – cinnamyl alcohol (H $\Gamma$ ). Figure adapted and reprinted from [39] under the terms of the Creative Commons CC-BY license.

These techniques do not need knowledge on the targeted analytes. They must be used together with a reference method either internal or external, as in 1D  $^1\text{H}$  qNMR

experiments. Still, some development will be essential in the future to make these methods more performant.

## 7. Extending the toolbox for intrinsically 2D quantitative NMR

Some improvements were already published to compensate some weaknesses of Q-HSQC and HSQC<sub>0</sub> (see Section 6.). However, there is still a need for some improvements for enhancing accuracy and precision on concentrations by better compensating for all biasing quantitativity sources. In addition, the quest to improve the sensitivity and speed of acquisitions for high throughput studies remain essential for complex mixture analyses. Further, making intrinsically 2D quantitative NMR usable on compact NMR systems is also highly desirable when there is a need of more accessibility.

In this part, recent developments taken from literature are discussed and proposed to reach more performant intrinsically quantitative 2D HSQCs.

### 7.1 Better solvent elimination

For biological samples, which are mainly made of water, it remains essential to have good water elimination especially to improve the sensitivity through the optimisation of receiver gain. Moreover, the choice of the elimination scheme and its position within the HSQC is of particular interest. In this field, Gronwald *et al.* [40] compared the presaturation-based (see Figure 4 A) Q-HSQC with the QUIPU HSQC and showing that such water vanishing methods did not sufficiently remove the residual water signal and its sidebands due to the homodecoupled acquisition. Thus, authors showed that the trueness for signals closer to the water was worse with QUIPU HSQC as compared to the Q-HSQC. Under these conditions, there is a need of a better global

water elimination technique in particular during the pure-shift scheme such as using weak gradients during BIRD and 180° hard pulse as proposed by Luy *et al.* [41].

## 7.2 $^1J_{\text{CH}}$ compensation

In order to further compensate for  $^1J_{\text{CH}}$  couplings in INEPT blocks (see Sections 3. and 4.), other strategies of interest are using  $J$ -compensation through perfect-echo in combination with optimally controlled shaped pulse sandwiches [42]. In addition, swept coherence transfer based on the co-incrementation of  $J$ -evolution delay in INEPT with 2D incremental time by using the time-resolved NUS method allowed for obtaining quantitative data [43]. More recently, spatially encoded INEPT using a frequency swept broadband pulse for homogeneously transferring  $^1J_{\text{CH}}$  seems to give access to 2D integrals that are more usable for quantitative studies especially when combined with a perfect INEPT [44], it allows for refocusing  $J_{\text{HH}}$  over the INEPT time [45]. These different approaches to compensate for  $^1J_{\text{CH}}$  evolutions deserve to be implemented and tested in intrinsically quantitative 2D HSQC in the near future.

## 7.3 Tolerant of any factor (TAF) HSQC

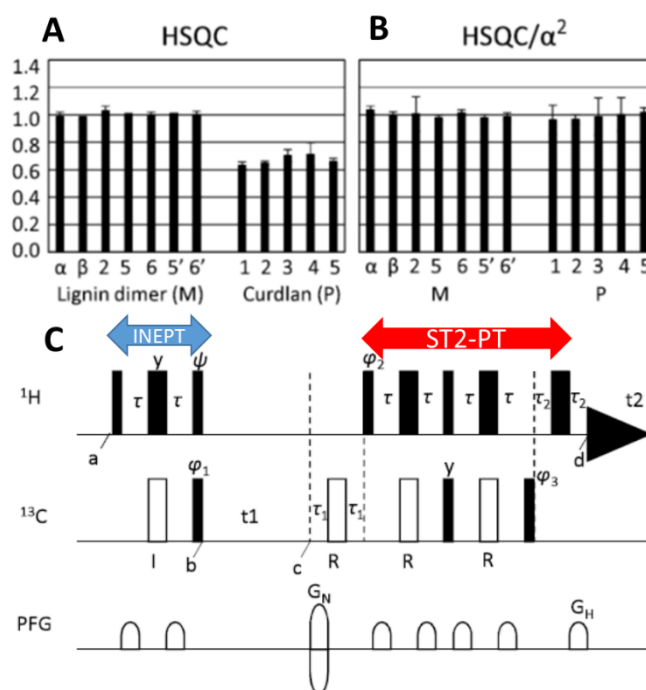
In the field of compensating for any source of mis-quantification (see Figure 2), to push over the possibilities of quantitative HSQC for larger molecules, Okamura *et al.* [46] have developed a family of HSQC that could implement a TROSY scheme as intrinsically quantitative. In order to compensate for the different modulations especially due to  $T_2$  evolutions, the authors combined different HSQC allowing for normalizing all volumes within the 2D map. They analyzed a mixture of lignin dimer and curdlan to test their approach. Under these conditions, it was possible to reach in a relative quantitative manner all  $T_2$  compensated volumes (see Figure 8). By

considering the transverse relaxation during INEPT (see Figure 8 C): The magnetization decreases to  $\delta \cdot 2H_z C_x$  (b) with  $0 < \delta < 1$ , and become  $\delta \cdot \epsilon \cdot H_\gamma C^\beta + \delta \cdot \epsilon \cdot H_\gamma C^\alpha$  (d) with  $0 < \epsilon < 1$  the reduction of the magnetization due to Single Transition-to-single Transition Polarization Transfer or ST2-PT (see Figure 8 C). Inverting the phase of the second  $90^\circ$   $^1\text{H}$  pulse:  $\psi$  and combining spectra is leading to differentiate different  $T_2$  evolutions  $\epsilon$  and  $\delta$  through the  $\alpha$  factor:

$$\alpha = [\text{Peak volume } (\psi = -y) + \text{Peak volume } (\psi = y)] /$$

$$[\text{Peak volume } (\psi = -y) - \text{Peak volume } (\psi = y)]$$

$\alpha$  is proportional to  $\delta \cdot (u/v)$ . In order to consider the different  $T_2$  evolutions along with HSQC sequences (see Figure 8 C), peaks volume shall be corrected by dividing them by  $\alpha^2$ . Under these conditions signal M from lignin and P from curdlan are reaching a ratio 1 : 1 for M/P (Figure 8 B) instead of 0.6 : 1 for the conventional HSQC (Figure 8 A).



**Fig. 8.** Calibration of volumes of HSQC correlation peaks to correctly determine the amount of each component. The volumes of each correlation peak of the  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectrum recorded with either A) the standard HSQC or B) the TROSY-based HSQC (B). The volume of the peak corresponding to each CH moiety was obtained. The volumes are normalized so that the average of the volumes for the peaks of the lignin dimer is 1. Calibrated volumes were calculated and plotted for HSQC/ $\alpha^2$ . Error bars represent standard deviations of four independent experimental data sets. Adapted and reprinted from ref [46] under the terms of the Creative Commons CC-BY license.

#### 7.4 Faster acquisitions

As previously reported (see Sections 3 for QQ HSQC and 4 for QQUIPU HSQC) for some applications especially for high throughput studies, requiring to analyze a high number of samples in a short time, there is a need of faster NMR methods. For speeding up 2D HSQC, different approaches were proposed in the literature like fast repetition NMR [47] especially SENSASS HSQC [48] and ASAP HSQC [49]. Moreover, NUS [50] is nowadays an easy way to accelerate the acquisition of 2D matrices. More recently, the Time-Resolved NUS version was able to monitor quicker events with 2D experiments [51].

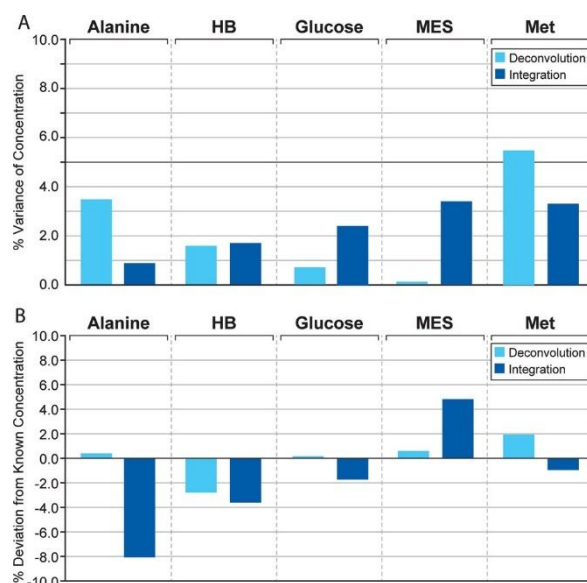
#### 7.5 Improving processing procedures for more accurate quantities

Designing sequences for intrinsically quantitative 2D HSQC is one key to reach the most accurate concentration. However, this is essential to be able to process spectra with the most reliable procedures for reaching the most accurate concentrations. Thus, 2D signal volumes apodization and integration have to follow some requirements.

Thus, preserving the 2D peak volume determined by the maximum amplitude of the FID is crucial [52]. Thus, the weighting function and the number of points along  $F_1$



have to be finely chosen. They have to follow the complete decrease to zero at the end of the FID sampling and its maximum has to fit with the maximum of the NMR signal on the beginning of the FID. In addition, baseline distortions could also impact peak volumes and related concentration accuracy [53]. For 2D NMR two baseline corrections along each dimension can be applied to peak volumes. Baseline correction are most of the time applied in the frequency domain as a polynomial function with a degree lower than 3 [54,55]. When the polynomial order is higher certain peaks could be mixed with the baseline leading to distortions biasing the quantitative analysis. The most interesting approaches to perform a reproducible analysis of 2D NMR spectra are with a parametric model fitting to spectral deconvolution [56]. Some papers reported the use of such techniques to determine peak volumes from 2D NMR spectra. Chylla *et al.* [57] for a protein showed the application of a hybrid time-domain, frequency-domain maximum likelihood fitting. They developed an algorithm called fast maximum likelihood reconstruction (FMLR) that performs spectral deconvolution of 2D NMR spectra for the purpose of accurate signal quantification [58]. FMLR is very promising and allowed reaching greater accuracy (0.5– 5.0% error) than peak height analysis and peak integral analysis with an operator work (Figure 9).



**Fig. 9.** A) Evaluation of the precision (% variance) and B) accuracy (% variation from a known concentration) of the fast maximum likelihood reconstruction method (light blue bars) in comparison with manual peak integration (dark blue bars), for accessing absolute concentrations in a mixture. Reprinted with permission from [58] R.A. Chylla, K. Hu, J.J. Ellinger, J.L. Markley, Deconvolution of Two-Dimensional NMR Spectra by Fast Maximum Likelihood Reconstruction: Application to Quantitative Metabolomics, *Anal. Chem.* 83 (2011) 4871–4880. Copyright (2011) American Chemical Society.

### 7.6 Making quantitative analysis more accessible

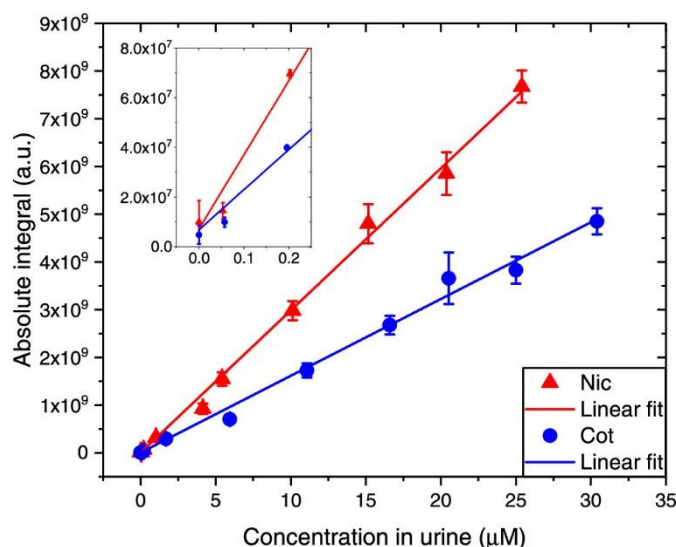
For more than a decade, compact mobile NMR apparatus have emerged, they don not require any cryogenic fluids, are cheaper and have reduced running and maintenance costs [59]. The previous sections show the interest in methodological developments to overcome the general limitations of NMR in the context of quantitative investigations. These techniques (see Sections 7.1 to 7.5) could become highly beneficial when implemented on compact NMR spectrometers since leading to peak overlap with lower sensitivities. Benchtop spectrometers have many advantages for quantitative investigations, for quality control of food [60], in forensic sciences [61] for monitoring a bioprocess [62] and for point-of-care test capabilities [63].

### 7.7 Boosting sensitivity

A great challenge to face in quantitative NMR for complex mixtures is its intrinsically low sensitivity. There is a need to improve it to tackle new challenges in chemistry and biology. Among the different solutions, hyperpolarization methods such as dissolution

dynamic nuclear polarization (D-DNP) and parahydrogen induced polarization (PHIP) are certainly the most promising.

D-DNP holds the promise to amplify the sensitivity of liquid state NMR by several orders of magnitude [64]. However, due to the short decay time of the generated hyperpolarized signal, D-DNP has been mainly applied to  $^{13}\text{C}$  NMR, opening the boulevard to sensitive  $^{13}\text{C}$  NMR detection in complex mixtures of molecules. Even if D-DNP has complex instrumental sources of biasing, the D-DNP experiment has recently shown to be repeatable at 5% on complex metabolic mixtures [65, 66]. A second class of hyperpolarization methods allowing to increase the sensitivity of quantitative NMR are based on the incorporation of para-hydrogen (p-H<sub>2</sub>) into a targeted analyte by a catalytic hydrogenation reaction [67]. This approach was initially limited to analytes that could be hydrogenated, but it has been extended to a higher number of molecules through the signal amplification by reversible exchange or SABRE [68]. In the SABRE approach, the hyperpolarization is transferred from p-H<sub>2</sub> to the substrate via the coordination sphere of a metal complex, extending the field of application for this hyperpolarization method. In this strategy, the reversible binding of p-H<sub>2</sub> and of the analytes to a metal complex is exploited to transfer hyperpolarization from p-H<sub>2</sub> to compounds under study. Combined with 2D NMR acquisition schemes, this technique can track metabolites at sub-micromolar concentrations [69]. Tessari's team showed that this approach could be applied to targeted quantitative analysis of nicotine and its metabolite like cotinine in urine by using a standard addition workflow (see Figure 10) [70,71]. Despite the fact that PHIP allows the detection of specific metabolites binding to the metal catalyst, it provides a very sensitive approach for quantitative investigations.



**Fig. 10.** Calibration curves for Nicotine (red triangles) and Cotinine (blue circles) based on the linear fitting of 10 data points (2D spectrum signal integrals). Three parallels were measured and averaged for every point. Error bars represent the standard deviation of the three parallels. Calibration curves are analyte-specific due to different iridium binding affinities. Reproduced with permission from Ref. [70], Copyright (2021) American Chemical Society

## 8. Concluding remarks

Up to now, the main experiments of the intrinsic quantitative 2D NMR toolbox are based on Q-HSQC and HSQC<sub>0</sub>. The methods described in this chapter are very promising from the quantitative point of view because they give access to 2D peak volumes directly reflecting the concentration with accuracies lower than 10%. The main point of improvement is relying on the compensation of  $T_2$  effects mainly responsible for the lack of accuracy and loss of sensitivity. Moreover, they suffer from other limits impeding their applications.

On one hand, they need specific acquisition and/or processing procedures that are not commercially available, which could limit their general use. On the other hand, they are focussed to the HSQC experiment, which could limit their application to more concentrated samples.

In order to improve the quantitative performances of Q-HSQC and HSQC<sub>0</sub> different newly reported *J*-compensated schemes could be compared in terms of accuracy of derived concentrations. Moreover, different approaches like fast repetition NMR and pure-shift acquisition schemes could be combined to improve the performances of 2D HSQC in terms of sensitivity and also spectral dispersion.

In addition, there is a need of quantitative homonuclear based 2D methods. Despite more difficulties to make them quantitative due to multiple  $J_{HH}$  modulations requiring compensation, homonuclear 2D NMR is more sensitive than HSQC-based approaches.

Achieving more sensitivity could also be accessed with the help of dedicated instrumentation. Of course, increasing the magnetic field of the spectrometer could be a solution but using micro probes and cryoprobe are more affordable for magnifying the sensitivity. To go on boosting the sensitivity, hyperpolarization techniques like Parahydrogen based methods are of primary interest to enhance SNRs by several orders of magnitudes. The combination of hyperpolarized methods and microcoil through a microfluidic system was a recent major evolution to make lie on the low sensitivity of NMR [72].

These improvements should open new applicative perspectives for intrinsically quantitative 2D NMR, in particular in fields such as metabolomics, forensics and environmental sciences, where the complexity of samples and their low concentrated analytes are really challenging.

## **Acknowledgments**

The author acknowledges the CNRS, Universities of Paris-South, Paris-Saclay, and INRA of Versailles for funding. J. F. warmly thanks the INRA for funding the internship of Soumaya Khlifi in the master formation context. This study benefited from a grant (LabEx Saclay Plant Sciences-SPS, reference ANR-10-LABX-0040-SPS), managed by the French National Research Agency under an Investments for the Future program (reference no. ANR-11-IDEX-0003-02). The authors from the CEISAM lab acknowledge the French National Infrastructure for Metabolomics and Fluxomics MetaboHUB-ANR-11-INBS-0010 ([www.metabohub.fr](http://www.metabohub.fr)) and the Corsaire metabolomics core facility (Biogenouest). J.F. thanks Sandrine Bouchet for an unfailing assistance.

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