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# Non-Uniform Sampling to enhance the performance of compact NMR for characterizing New Psychoactive Substances

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Abstract :

Efficient and robust analytical methods are needed to improve the identification and subsequent regulation of New Psychoactive Substances (NPS). NMR spectroscopy is a unique method able to determine the structure of small molecules such as NPS even in mixtures. However, high-field NMR analysis is associated with expensive purchase and maintenance costs. For more than a decade, compact NMR spectrometers have changed this paradigm. It was recently shown that a dedicated analytical workflow combining compact NMR and databases could identify the molecular structure of NPS, in spite of the lower spectral dispersion and sensitivity of compact spectrometers. This approach relies on <sup>1</sup>H-<sup>13</sup>C HSQC to both recognize NPS and elucidate the structure of unknown substances. Still, its performance is limited by the need to compromise between resolution and experiment time. Here, we show that this strategy can be significantly improved by implementing non-uniform sampling (NUS) to improve spectral resolution in the <sup>13</sup>C dimension of HSQC at no cost in terms of experiment time. Gains in the range of 3 to 4 in resolution are achieved for pure NPS and for a mixture. Finally, 2D HSQC with NUS was applied to improve the identification of NPS with the assistance of databases. The resulting method appears as a useful tool for the characterization of NPS in mixtures, which is essential for forensic laboratories.

## 1. Introduction

In the field of forensic analysis, high field (HF) NMR is one of the most useful analytical techniques able to identify, elucidate, and quantify illicit substances [1]. However, HF NMR suffers from high purchase and maintenance costs, and requires dedicated staff and laboratory space. More than a decade ago, miniaturized NMR instruments became available. They are transportable, more affordable and can be installed on a table or in a fume hood. However, such systems are characterized by a lower field (LF) leading to a lack of spectral dispersion and a lower sensitivity. Under such conditions, LF NMR spectra suffer from ubiquitous peak overlap

making spectral analysis a challenging task, even for relatively simple molecules. Thus, there is a need to implement advanced NMR methods and/or to integrate complementary analytical approaches to circumvent the limits of LF NMR. In a recent paper, we reported the development of an analytical platform including compact NMR, Infra-Red spectroscopy, and databases for identifying and elucidating new psychoactive substances (NPS) on samples seized by the Law Enforcement [2]. The cornerstone of this analytical platform is the well-known  $^1\text{H}$ - $^{13}\text{C}$  HSQC method with multiplicity editing (ME) or HSQC ME, which includes an echo block for labelling correlation signals with the CH parity in order to differentiate  $\text{CH}_3/\text{CH}$  from  $\text{CH}_2$ . HSQC ME appeared as a key experiment in combination with databases to improve the spectral dispersion and increase the information available from 2D maps for identifying and elucidating six seizures embedding unknown NPS. Despite the efficiency of this approach, the determination of NPS structures appeared challenging in some cases: one compound from a seizure could not be easily identified, and in the case of a mixture, only the major compound was identified [2]. Such mitigated results arose from the limited sensitivity and resolution of HSQC at low field. The purpose of the present paper is to investigate if the performance of this analytical approach can be improved by Non-Uniform Sampling (NUS), which has the potential to enhance resolution at no cost in terms of experiment time.

NUS [3,4] was initially introduced to reduce the number of acquired points in indirect dimensions of multidimensional maps in order to speed up their recording time [5] thanks to the reconstruction of non-acquired points by using signal reconstruction algorithms. The NUS approach was also implemented to enhance the spectral resolution of multidimensional maps 3D or higher dimensions [6]. In addition, for the same amount of time as a conventional 2D map, NUS improved the separation of correlation peaks for complex mixtures [7,8].

In this article, the contribution of NUS is explored to improve the characterization of NPS on a compact NMR instrument, by improving the spectral resolution of HSQC spectra, with no penalty in terms of experiment time. Applications to NPS samples as a pure compound or in a mixture are presented. Finally, NUS 2D HSQC is combined to our previously developed database to better identify NPS in a mixture.

## 2. Materials and Methods

### 2.1. Sample preparation

Two samples were prepared from seizures obtained from the Police. The first was made of AB-FUBINACA at 300 mM in  $\text{DMSO-d}_6$ . The second was composed on 2-CI at 179 mM and 2-CE at 41.4 mM solubilized in  $\text{DMSO-d}_6$  (see S6).

$\text{DMSO-d}_6$  purchased from Eurisotop with a purity of 99.8% was used as NMR solvent. TMS from Acros Organics with a purity of 99.9% and TSP from Eurisotop with a purity of 99.8% were used as chemical shift and concentration references, respectively.

### 2.2. NMR

All the spectra were recorded at 26.5 °C using a  $^1\text{H}$ ,  $^{19}\text{F}$ ,  $^{13}\text{C}$  60 MHz Spinsolve spectrometer from Magritek equipped with a 20-position autosampler and a gradient coil in the direction of the static magnetic field  $B_0$ . The duration of 90° hard pulses was 14  $\mu\text{s}$  at 0 dBW for  $^1\text{H}$  and 65.9  $\mu\text{s}$  at -14 dBW for  $^{13}\text{C}$ . All acquisitions were made with the Spinsolve Expert software (v.1.41.07).

## 1D NMR acquisition parameters

The length of 90° hard pulses was 14.5  $\mu$ s at 0 dBW. 8 scans were collected for each 32k FID, with a spectral width of 81 ppm and an acquisition time of 1.6 s.

## 2D NMR acquisition parameters

HSQC experiments were recorded using a 2 s repetition time and a WALTZ-16  $^{13}\text{C}$  decoupling scheme during the 0.41 s acquisition. The inter-pulse INEPT delay was set to 1.8 ms, corresponding to an average value of  $1/(4 \cdot ^1J_{\text{CH}})$  with  $^1J_{\text{CH}} = 140$  Hz. The spectral width in the  $^1\text{H}$  dimension was 81 ppm, whereas the spectral width on the  $^{13}\text{C}$  dimension was optimized to the minimum value allowing the observation of relevant peaks, *ie.* 140 ppm.

HSQC multiplicity edited (ME) spectra were acquired with different values of NI (Number of Increments) in the  $^{13}\text{C}$  dimension, either with a uniform sampling (US) or with a flat random non-uniform sampling (NUS) scheme. Either TANGO (*Testing for Adjacent Nuclei with a Gyration Operator*) or BIRD (*Bilinear Rotation Decoupling*) blocks were also added prior to the HSQC-ME for the sake of comparison (see S1, for the related pulse sequences and S2 for the associated 2D spectra).

For NUS-based experiments, a given number of increments (NI) was acquired as a part of the full number of points ( $N_{\text{tot}}$ ) corresponding to a given NUS rate (%). The remaining number of points were reconstructed through a Modified Iterative Soft Threshold (MIST) algorithm with the MestReNova software. Different NUS rates were tested (see Table I) and compared in terms of peak resolution at 5% height for the same experimental time. All experiments were performed with the same overall duration.

Table 1

## Processing parameters

The NMR processing was performed with MestReNova<sup>TM</sup> software (version 14.1).  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts were referenced to TMS. For 1D spectra, free induction decays (FID) were first multiplied by an exponential apodization function (0.3 Hz line broadening) and zero-filled to 64k points for improving the digital resolution, then manually phased. For 2D HSQC, a cosine apodization was applied along the 2 dimensions. A States method was used to obtain a phase sensitive map. A zero-filling to 16k x 1k was applied for all acquired 2D HSQCs. After Fourier transform, all 1D and 2D spectra were manually phased in pure absorption along all dimensions. Moreover, a Whittaker Smoother baseline correction was applied for both 1D and 2D spectra.

In the case of 2D HSQC, the  $^{13}\text{C}$  signal resolution was obtained by extracting the column corresponding to the maximum of the  $^{13}\text{C}$  considered signals (see S4).

### 2.3. ACD/Labs software

All analytical data were gathered in the ACD/Labs<sup>TM</sup> software (version 2020.1.0 of July 15, 2020) from Advanced Chemistry Development, Inc (ACD/Labs). For making our NPS database, a project with all NMR experiments and the dedicated structure was created for each molecule and added to the database after peak picking and assigning all signals and  $^1\text{H}$ - $^{13}\text{C}$  correlations.

## 2.4. Database parameters

Our **in-house** database [2] was created with the ACD/Labs software able to compare an unknown spectrum with one from the database. The “peak searching” method is used for 2D maps and compares the position of NMR signals determined through peak-picking, an approach which can be significantly operator-dependent. The comparison between the experimental spectrum and the database yields a result called the Hit Quality Index (HQI) which quantifies the agreement between the database spectrums the most closely matching and the spectrum of the unknown compound [2]. The level of error between a spectrum to identify and those of the database is the Looseness Factor (LF) that allows considering the variability in the chemical shifts of the different peaks obtained in NMR. LF corresponds to the highest difference in ppm which can be accepted to provide a match between the experimental spectra and the database.

## 3. Results and discussion

### 3.1 Optimization of NUS HSQC parameters on a pure NPS sample

To evaluate the potential of NUS HSQC, a pure sample of AB-FUBINACA (see Section 2.1 and S6) was used as a model. First, different 2D HSQC ME pulse sequences without NUS were compared, starting with an optional BIRD scheme to filter out unwanted  $^1\text{H}$ - $^{12}\text{C}$  coherences, or with an optional TANGO scheme to get rid of long range  $^1\text{H}$ - $^{13}\text{C}$  signals (see S1 for pulse sequences). As shown in Figure 1, SNRs are not significantly different for the basic HSQC ME and for the BIRD HSQC ME experiments. Moreover, the TANGO version induces an average sensitivity loss of 18%. Based on these results, the standard HSQC was chosen as a compromise, also being a more compact pulse sequence that is more favorable for NPS with potentially short  $^1\text{H}$   $T_2$  values.

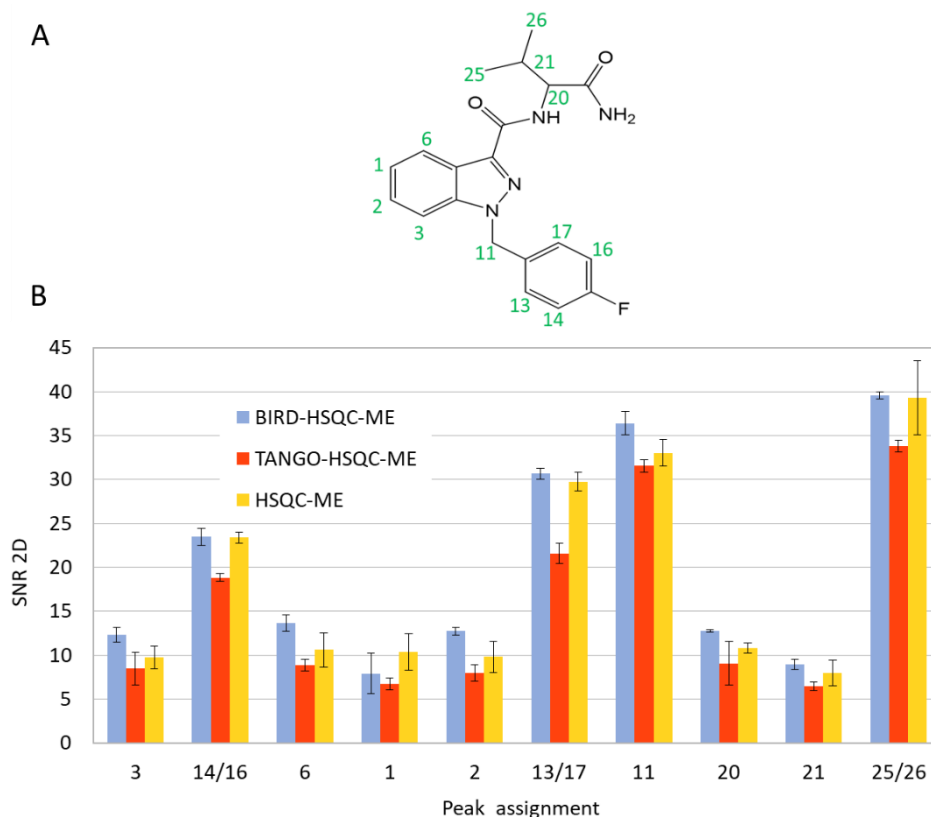


Figure 1 : 2D SNR measured on different 2D HSQCs on (A) AB-FUBINACA : with (B) a BIRD (in blue), a TANGO (in red) scheme or the standard one (in yellow). Error bars were obtained through a triplicate. SNRs were calculated by dividing the intensity of 2D peaks by a 2D noise region devoid of any peak.

The optimization of NUS along the  $^{13}\text{C}$  dimension was carried out to find the best compromise between signal separation and sensitivity. In the whole study, resolution was assessed through the determination of linewidths at 5% height, since the more classical linewidth at half-height could be significantly impacted by reconstruction algorithms like MIST [9-11]. Different NUS rates were tested by setting the experimental time to 204 min. The time-saving allowed by NUS was either used to increase  $N_{\text{tot}}$  in order to improve the spectral resolution (see Table 1: entries 2 to 8), or to increase the number of scans to improve the detection of peaks with low intensity (see Table 1, entries 4, 6, 7, and 8). For AB-FUBINACA, the main goal was to determine which combination of parameters could lead to the detection of the maximum of  $^1\text{H}$ - $^{13}\text{C}$  correlation peaks on 2D maps. In the case of AB-FUBINACA, 11 peaks are expected (see S3) and Table 2 summarizes the results obtained with different NUS conditions. If the correlation peak is present « 1 » is noted for the considered peak and the associated experimental condition in the corresponding cell. When the peak is absent « 0 » is mentioned. The total number of detected peaks is given in the rightmost column of Table 2. Under such requirements, only NUS schemes with 50% NUS and  $N_{\text{tot}} = 256$  and 25% NUS and  $N_{\text{tot}} = 512$  allowed to detect at least 10 of the 11 correlation peaks. Thus, in the following, only these conditions were considered for further optimizations.

Table 2

By focusing on the more crowded aromatics region of 2D HSQC spectra for AB-FUBINACA (see Figure 1A), one notices unresolved signals for signals assigned to 13/17 and 14/16 sites

(see Figure 2B). When using NUS at 50% along the  $^{13}\text{C}$  dimension (see Figure 2C), such correlation signals become better resolved thanks to the subsequent resolution improvement in the  $^{13}\text{C}$  dimension. This resolution enhancement is further improved with a NUS rate of 25% (see Figure 2D) where the  $^{13}\text{C}$  number of points is reaching 512. Under these conditions, it is now possible to clearly reveal  $^{19}\text{F}$ - $^{13}\text{C}$  as well as  $^{19}\text{F}$ - $^1\text{H}$  J-couplings that can be used to position the  $^{19}\text{F}$  on the phenyl moiety.

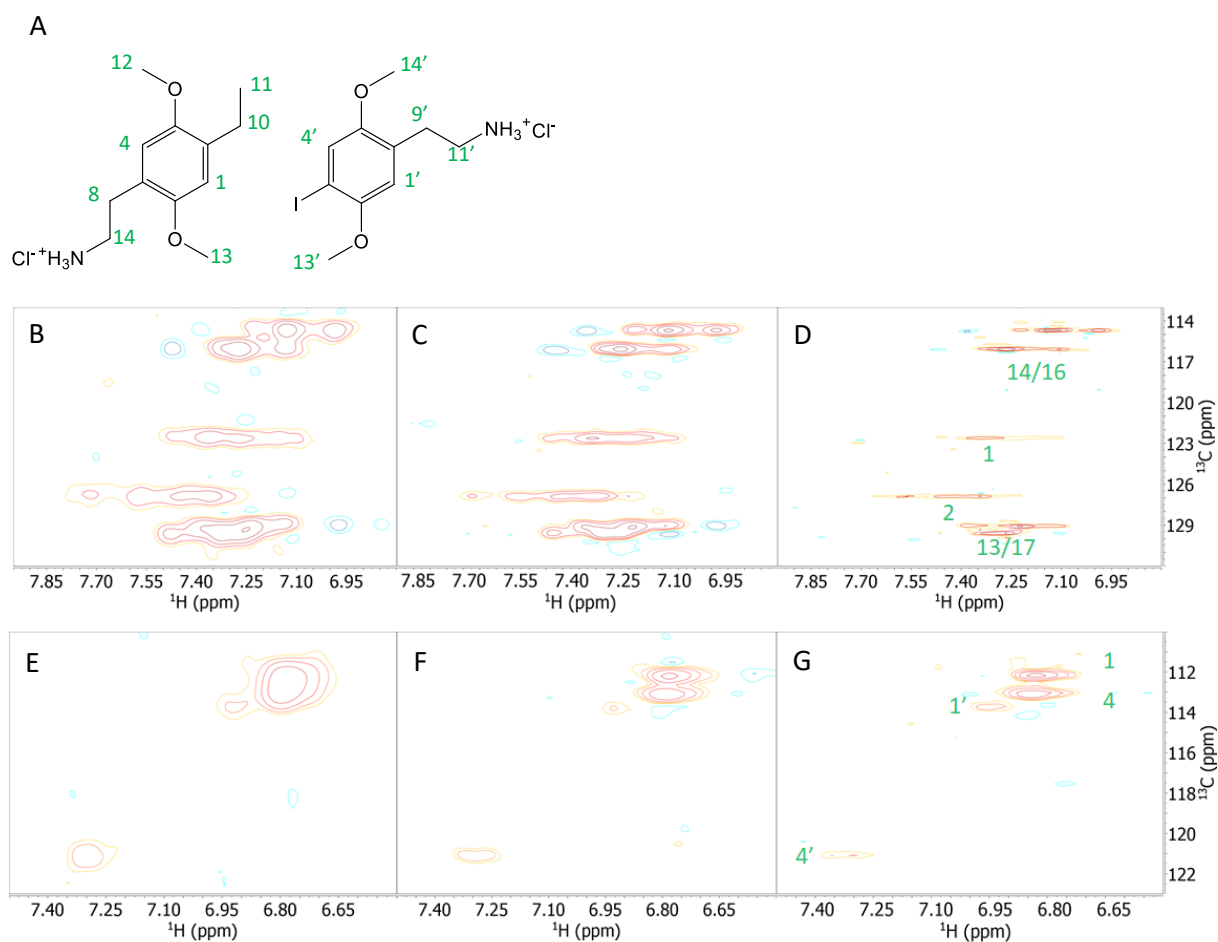


Figure 2 : (A) Molecular structure of the NPS model mixture of 2-CE (on the left)/ 2-CI (on the right) used in this study. For AB-FUBINACA, see Figure 1A . Aromatic region of the 2D HSQC maps recorded in 3h 24 min on AB-FUBINACA (B,C,D) and on the mixture of 2-CE/2-CI (E, F, G). Uniformly sampled spectra (B,E) with  $N_{\text{tot}}=128$  and 24 scans are compared to spectra recorded with the following NUS conditions: (C) 50% NUS with  $N_{\text{tot}} = 256$  and  $NS = 24$ ; (D,G) 25% NUS with  $N_{\text{tot}} = 512$  and  $NS = 24$  and (F) 25% NUS with  $N_{\text{tot}} = 256$  and  $NS = 48$ .

In order to quantify the impact of NUS on resolution, the  $^{13}\text{C}$  linewidths at 5% height were measured in comparison with those obtained with uniform sampling (see Figure 3).

For NUS 50% and  $N_{\text{tot}} = 256$ , most of  $^1\text{H}$ - $^{13}\text{C}$  correlation peaks show an average reduction of the linewidth by  $\sim 2$  (see green bars, Figure 3 and S4.1) for most of AB-FUBINACA sites, as expected due to the doubling of the number of points. Similar results are obtained for NUS = 25% and  $N_{\text{tot}} = 256$  (see yellow bars), since the time saving is mainly used to increase the number of scans. Further reduction of the linewidth is obtained for NUS 25% and  $N_{\text{tot}}=512$

(blue bars), with an average linewidth reduction of a factor 3 compared to the initial condition, and up to 5 for some peaks, e.g., 14/16.

To summarize, for AB-FUBINACA aromatics (see Figure 2), 512 points are needed to reveal all 6 expected correlation peaks. It is important to mention that 256 points are not sufficient to clearly distinguish all correlation peaks (see S4.1). Thus, optimizing the number of points to get the best spectral resolution after reconstruction is essential [11].

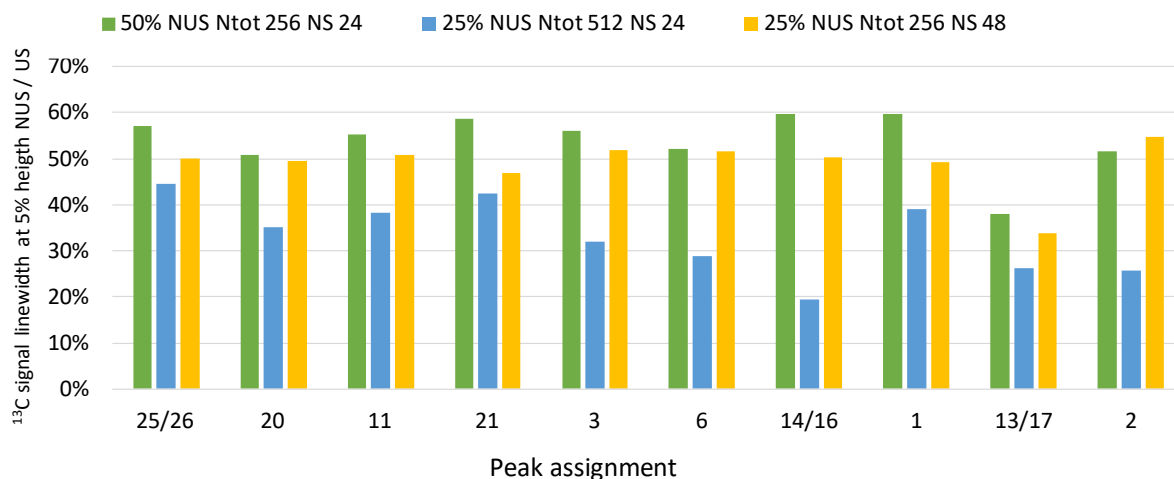


Figure 3 : Effect of NUS rates on the resolution of  $^1\text{H}$ - $^{13}\text{C}$  HSQC for the AB-FUBINACA sample  $^{13}\text{C}$  linewidth as compared to US for a constant experimental time (204 min) for the conditions indicated in the color code.

### 3.2 Application to a mixture of NPS : 2-CE/2-CI

NPS are often seized in the form of mixtures, which poses a challenge for their analysis at low field, even with databases, as illustrated in our previous study [2]. Here, the performance of the 2D NUS HSQC experiment was tested on a mixture of 2-CE and 2-CI (Figure 2A). For this mixture, the analysis appeared really difficult with the initial US conditions because only 2 correlation peaks could be detected on the US 2D HSQC map [2] amongst the 4 expected correlations (see Figure 2E). We focused on the NUS 25% condition which provided optimum results in the case of AB-FUBINACA. Using 25% NUS with  $N_{\text{tot}} = 512$  does increase the resolution (Figure 2G) and reveals the four expected aromatic peaks, thus making their assignment easier. An intermediate parameter choice (NUS 25% with 256 points and 48 scans) is sufficient for assigning all aromatic sites with improved intensity (see Figure 2F) since a maximum of 2 peaks are expected along  $^{13}\text{C}$  columns (Figure S4.2). The evolution of resolution with NUS parameters shows the same trend as for the AB-FUBINACA sample (Figure 4) and the linewidth can be reduced by a factor 3.5 in average compared to US HSQC.



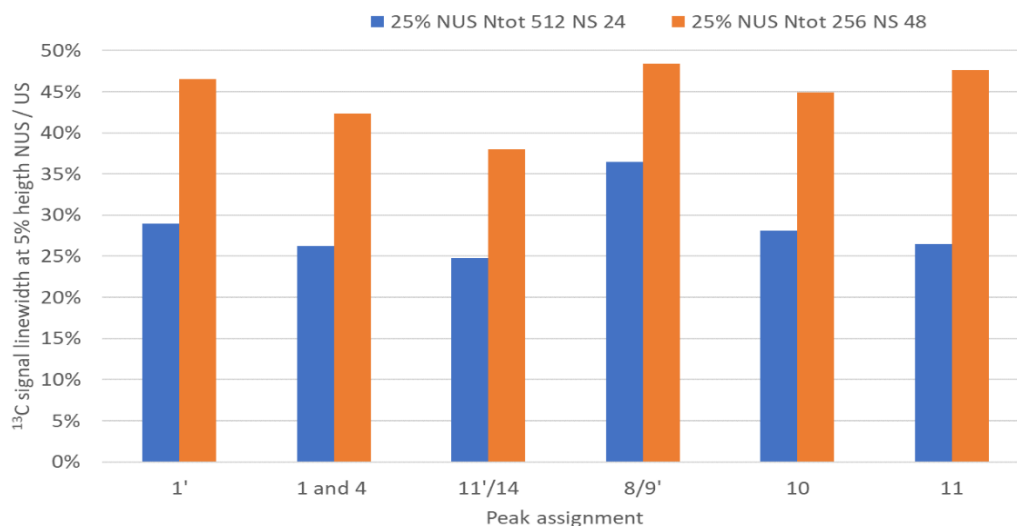


Figure 4 : Relative improvement of the  $^{13}\text{C}$  linewidth at 5% height for 2D HSQC with 25% NUS (two different choices of  $N_{\text{tot}}$  and  $NS$ ) versus US 2D HSQC, for spectra recorded in 204 min on a mixture of 2-CE and 2-CI.

### 3.3 Combination of NUS HSQC with a database to improve NPS recognition

As previously mentioned, we recently developed a combined analytical workflow relying on benchtop NMR and databases [2], but this approach appeared to be limited by the low resolution of US HSQC. In this context, we evaluated how this limitation could be circumvented by incorporating NUS HSQC in this workflow, using the mixture of 2-CE and 2-CI as a test case.

In order to identify NPS relying on our database in the ACD/Labs software, spectra are compared by calculating two essential parameters. On the one hand, a scoring factor called HQI (see Section 2.4) quantifies the agreement between the spectrum of the unknown compound and those most closely matching in the database. On the other hand, for the “peak searching” method, LF (see Section 2.4) accounts for the variability between 2D HSQC chemical shifts ( $^1\text{H}$ ,  $^{13}\text{C}$ ) of the unknown spectrum versus those of the database.

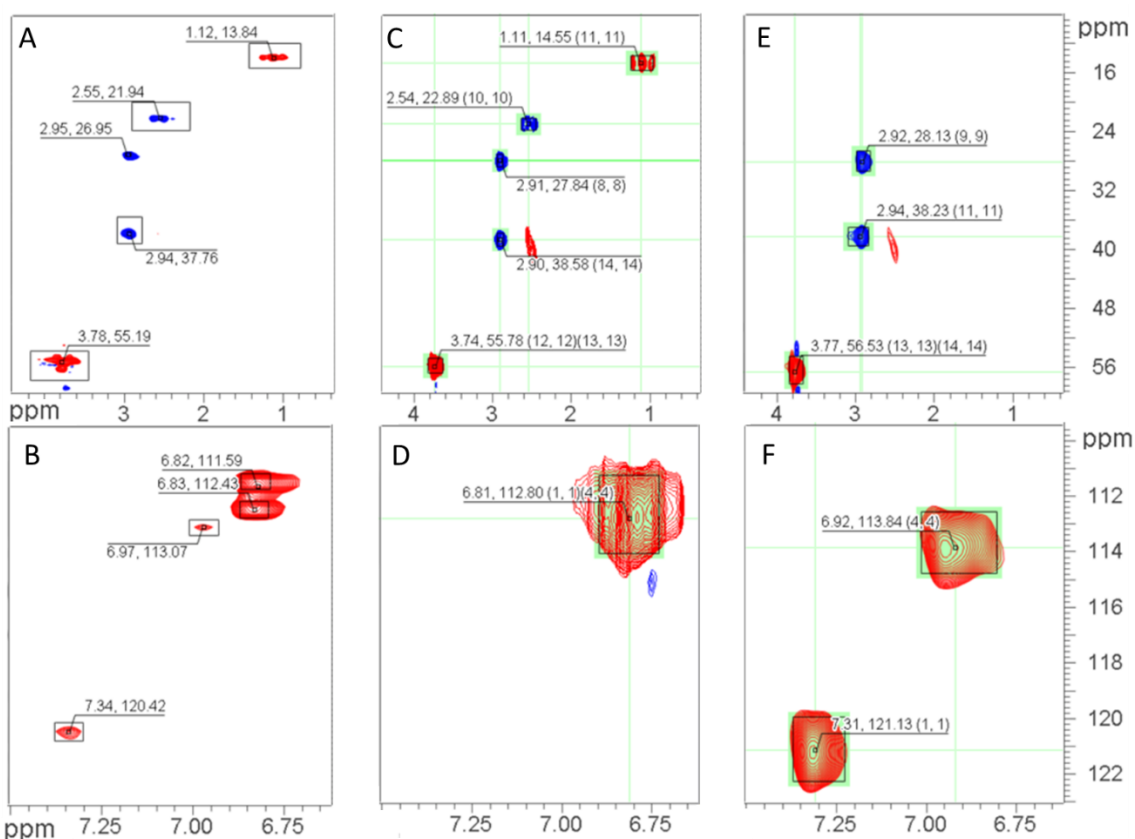


Figure 5 : 2-CE/2-CI recognition with ACD/Labs assistance. A, C, E show the aliphatic area and B, D, F the aromatic area of 2D HSQC maps. A and B are experimental 2D HSQC spectra acquired with 25% NUS on the mixture of 2-CE and 2-CI. NPS US HSQC spectra of 2-CE (C, D) and 2-CI (E, F) previously incorporated in our **in-house** database. Green colored correlation peaks put into relief a good match between the experimental and database spectra. Looseness factor of 0.2 ppm in  $^1\text{H}$  and 2 ppm in  $^{13}\text{C}$  were used. Peaks were assigned and related chemical shifts are mentioned in spectra.

By comparing the experimental 2D NUS 25% HSQC (see Figure 5A, B) recorded on a 2-CE/2-CI mixture with the 2D US HSQC spectra of our database, 2-CE, the major compound in the mixture, is ranked first with an HQI = 66.67 (see Figure 5C, D). In addition, 2-CI (minor compound in the mixture) is second-ranked, and is recognized thanks to its aliphatic (Figure 5E) and aromatic (Figure 5F) fingerprints, with an HQI = 60. In contrast, with a conventional US 2D HSQC the search procedure was only able to find 2C-E with a HQI = 100 and 2C-I could not be found (see S5). This result clearly illustrates the potential of NUS HSQC to improve the previously described NPS identification workflow. In addition, it shows that the previously built database can be interrogated with NUS HSQC spectra, although the database itself only contains US HSQC spectra at this stage.

#### 4. Conclusions

The potential of 2D NUS HSQC on compact NMR spectrometers was evaluated in the context of the characterization of NPS. Results highlight significant improvement in terms of resolution by a factor 3 to 4 in the  $^{13}\text{C}$  dimension for the studied samples as compared to US HSQC. Thanks to such improved performance, 2D NUS HSQC was able to identify the 2 components of a 2-CE/2-CI NPS mixture using our **in-house** database, a result that could not be reached without NUS. This result paves the way towards the analysis of a variety of NPS samples, in

forensic labs for routine use under regulatory context where the need for accessible and performant analytical methods is increasing.

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## Tables

Table 1: HSQC-ME-NUS sequence acquisition parameters. NI = number of increments used for  $^{13}\text{C}$  experiments,  $T_{\text{exp}}$  = experiment time, TR = repetition time and NS = number of scans

Entry	1	2	3	4	5	6	7	8
NUS (%)	*	75	50	50	25	25	25	10
Total NI :Ntot	128	170	256	64	512	128	256	128
Acquired NI	128	128	128	128	128	32	64	13
Reconstructed NI	0	32	128	64	384	384	192	115
$T_{\text{exp}}$ (min)	204	204	204	204	204	204	204	204
TR (s)	2	2	2	2	2	2	2	2
NS	24	24	24	48	24	96	48	240

\*The full number of points was acquired. It corresponds to a Uniform Sampling (US).

Table 2: NUS optimizations in HSQC-ME for AB-FUBINACA

NUS conditions			AB-FUBINACA peak assignment											
NUS (%)	Ntot	NS	3	14/16	6	1	2	13/17	11	20	21	25	26	SUM
75	170	24	1	1	1	1	0	1	1	1	1	1	0	9
50	256	24	1	1	1	1	1	1	1	1	1	1	0	10
25	512	24	1	1	1	1	1	1	1	1	1	1	1	11
50	128	48	1	1	1	1	0	1	1	1	1	1	0	9
25	128	96	1	1	1	1	0	1	1	1	1	1	0	9
10	128	240	1	1	1	1	1	1	1	1	1	1	0	10
25	256	48	1	1	1	1	1	1	1	1	1	1	1	11