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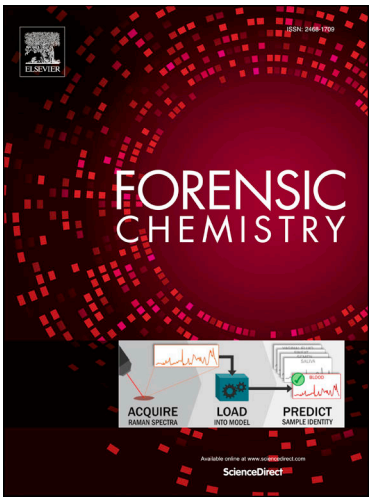

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Quantitative NMR as a tool for analysis of New Psychoactive Substances

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Abstract

Several New Psychoactive Substances (NPS) have appeared on the drug market, following a new trend of drug consumption. Nuclear Magnetic Resonance (NMR) has been used, especially in the case of forensic analysis, as a tool for unambiguous structure determination of unknown NPS. The quantification of NPS in complex mixtures is, however, a very challenging task, especially in the absence of certified reference materials (CRM). In this work, we applied a quantitative ^{1}H-NMR (^{1}H-qNMR) methodology performed without certified analytes for quantification of twelve NPS samples seized by the Brazilian Federal Police. The molecular structure of NPS samples were first confirmed by mono and bidimensional ^{1}H and ^{13}C NMR with unequivocally assigned signals, which allowed for the discrimination of constitutional isomers. A detailed compilation of NMR spectroscopic data showed that these NPS samples belong to cathinones, phenethylamines, and tryptamines groups. The quantitative analyses showed high precision (RSD = 2.67%) and low uncertainty (from 0.44% to 0.37%, with k=2, 95% confidence). The NPS samples exhibited sufficient stability for a period of 48 hours, which is longer than the experimental time frame and, therefore, assures the reliability and validity of the obtained results. Evaluation of some other figures of merit (selectivity, limits of quantification and detection) was also performed and confirmed the proposed method presents suitable and reproducible results. These achievements suggest the present methodology is highly adequate for forensic purposes, attaining excellent precision and accuracy, even in the absence of CRM.

Keywords: qNMR, NPS, forensic analysis, structural elucidation, reference material.

Abbreviations:

2C-B, 4-bromo-2,5-dimethoxyphenethylamine; 2C-I, 4-iodo-2,5-dimethoxyphenethylamine; 2-FA, 2-fluoroamphetamine; 2-MAPB, N,α-dimethyl-2-benzofuranethanamine; 4-CMC, 4-chloromethcathinone; 4-FA, 4-fluoroamphetamine; 4-FMC, 4-fluormethcathinone; 4-HO-MiPT, 4-hidroxy-N-methyl-N-isopropiltriptamine; 5-MAPB, N,α-dimethyl-5-benzofuranethanamine; 5-MeO-MiPT, 5-methoxy-N-methyl-N-isopropyltryptamine; 25B-NBOMe, 2-(4-bromo-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine; b, broad signal; CRM, certified reference materials; $CDCl_3$, chloroform-d; DMS, dimethyl sulfone; D_2O, deuterium oxide; DOC, 4-chloro-2,5-dimethoxyamphetamine; d, doublet; dd, doublet of doublets; dt, doublet of triplets; ethylone, N-ethyl-3,4-methylenedioxycathinone; ethylphenidate, ethylphenyl(piperidin-2-yl)acetate; FTIR, Fourier transform infrared; GC-MS, gas chromatography-mass spectrometry; GC-FID, gas chromatography with flame ionization detector; m, gravimetric mass; HPLC-MS, high-performance liquid chromatography-mass spectrometry; IR, infrared; I, integral; ICE, International Collaborative Exercise; JWH-073, 1-buthyl-3-(1-naphthoyl)indole, LOQ, limit of quantification; LOD, limit of detection, MA, maleic acid; MDDMA, N,N-dimethyl-3,4-methylenedioxyamphetamine; MDMA, methylenedioxy methamphetamine; MDPV, methylenedioxy pyrovalerone; MeOD, methanol-d_4; M, molecular weight; m, multiplet; MS, mass spectrometry; NPS, new psychoactive substances; NMR, Nuclear Magnetic Resonance; N, number of protons; P, purity; ^{1}H-qNMR, quantitative ^{1}H-NMR; q, quartet; RSD, relative standard deviation, s, singlet; S/N, signal-to-noise ratio, SD, standard deviation; std, internal standard; TFMPP, trifluoromethylpiperazine; t, triplet; td, triplet of doublets; TSP-d_4, 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt; TMS, tetramethylsilane; U, expanded uncertainty, UNODC, United Nation Office on Drug and Crime.

1. Introduction

In the last decade, the drug consumption has followed a new trend because several new psychoactive substances (NPS) have appeared in the drug market as non-illegal alternatives to common drugs of abuse. Nevertheless, the effects of most NPS are not fully known at the moment and little information is available about their pharmacology and potential toxic effects. In general, they act on the central nervous system by momentarily changing the perceptions, mood, and behavior. Meanwhile, dangerous effects that ultimately lead to hospitalizations and fatal intoxications have also been reported [1,2].

The United Nation Office on Drug and Crime (UNODC) defines NPS as drugs of abuse those

which have not been included in the list of illegal drugs controlled by the Convention on Narcotic Drug of 1961 and the Convention on Psychotropic Substances of 1971. Therefore, the term NPS has been used whenever these substances are found in the illegal market, and not necessarily because they are new drugs, either discovered or synthetized. In fact, some NPS, such as ketamine, are well known and have been stablished in illegal markets for decades. Since 2008, NPS have been under the scrutiny of the United Nations and, between 2009 and 2018, 892 different NPS have been reported and found in 119 countries worldwide [3-5].

The increase of NPS abuse has led law enforcement services and public health agencies in an attempt to understand and restrain this dangerous phenomenon. On the other hand, the analysis of an ever-increasing number of NPS by routine analytical techniques faces great challenges, owing to the unavailability of certified reference materials (CRM) for carrying out proper identification and quantification [6-9]. In the absence of CRM and standards containing the targeted analytes, Nuclear Magnetic Resonance (NMR) arises as a pivotal technique, since it allows for unambiguous structure determination of an unknown NPS, as well as quantification while using a few commonly available reference materials [10]. Quantification using NMR in the absence of a reference material containing a specific analyte is possible thanks to a basic principle, which establishes that the analyte to standard mole ratio depends on the number of nuclei contributing to each resonance signal. Moreover, the NMR analysis can be accomplished in a short-time and sample preparation is very simple [11,12].

In 2005, one of the first reports in which quantitative ^{1}H-NMR (^{1}H-qNMR) was used to determine the purity of heroin, methamphetamine, methylenedioxy methamphetamine (MDMA), and cocaine samples was reported by Hays [12]. An extensive list of compounds, their solubilities and the solvent(s) and internal standard used were also provided [12]. Since then, the use of NMR as a tool for forensic analysis has been increased, even though a few works have shown its practical use to quantify NPS samples [10, 11]. Recently, ^{1}H-qNMR using internal standard method was applied successfully to identify and quantify complex samples of MDMA and other NPS, e.g. ethylone, methylone, trifluoromethylpiperazine (TFMPP), N,N-dimethyl-3,4-methylenedioxyamphetamine (MDDMA), 4-bromo-2,5-dimethoxyphenethylamine (2C-B), and 4-iodo-2,5-dimethoxyphenethylamine (2C-I) [9]. Nonetheless, most of studies concerning quantification of NPS often make use of mass spectrometry (MS), which is much more sensitive but has a limited quantification performance and is destructive. Furthermore, hyphenated methods such as gas chromatography-mass spectrometry (GC-MS) or high-performance liquid chromatography-mass spectrometry (HPLC-MS) work properly when using certified reference standards, even

though NPS standards are rarely available [13-15].

Besides that, NMR is underutilized for the identification of drugs of abuse. In general, forensic laboratories use Fourier transform infrared (FTIR) or Raman spectroscopies to identify the seized samples. This is made possible only with the support of reference libraries, which help in rapid sample identification. Recently, Jones and co-workers [16] highlighted the importance of identification and characterization of NPS structures by NMR to extend these reference libraries. The potential of FTIR and Raman spectroscopies for rapid identification of NPS has been tested using a set of 221 seized samples. They showed that the percentage of samples identified by IR and Raman screening increased considerably when new compounds were previously identified by NMR and the reference library was updated. A similar conclusion was also reported by Antonides and co-workers [17]. In this work, however, authors employed ^{1}H NMR spectra registered with a low field benchtop NMR equipment to elucidate components present in seized drug, including NPS samples. This research was made possible only because the development of an algorithm that compared their data with a reference library composed by high field NMR spectra [17]. Ameline and co-workers [2] also demonstrated the importance of NMR to identification and analytical characterization of seven NPS, which allowed them to solve a complex toxicological fatal case. Therefore, NMR is proven to be necessary for maintaining libraries updated and assisting the identification of drugs samples, especially in the case of NPS.

In a previous work, our group has developed and validated a ^{1}H-qNMR method that uses the internal standard for quantification of methylenedioxy methamphetamine (MDMA) in ecstasy tablets [18]. MDMA.HCl CRM was used for validation purposes, whereas data acquired by gas chromatography with flame ionization detector (GC-FID) were utilized as reference to ascertain the ^{1}H-qNMR method performance when working with real samples. In this work, a discerning validation report was performed. The method was considered suitable for the purpose of routine forensic analysis and quantification of MDMA, providing excellent results of accuracy (relative error <5%) and precision (RSD <2%).

Herein, we applied the previously developed ^{1}H-qNMR methodology [18, 19] for structural identification and quantification of twelve different NPS seized between 2015 and 2018 by the Brazilian Federal Police, including eleven NPS for which quantification by ^{1}H-qNMR was not previously reported. In order to support our investigation, we have used structural and quantitative information on drug samples supplied by the United Nation Office on Drugs and Crime International Collaborative Exercise (ICE/UNODC). The proposed method presented over 99% of correlation

with the ICE/UNODC reference data. In addition, evaluation of some other figures of merit (selectivity, limits of quantification and detection) was also performed to validate the method and confirmed the proposed approach worked properly for NPS analyses in the absence of specific CRM, consolidating ^{1}H-qNMR as a reliable method for forensic purposes.

2. Material and Methods

2.1. Chemicals and Samples

All chemicals were of analytical grade or better and used without additional purification procedures. Maleic acid (MA, 99.99% ± 0,01%) and dimethyl sulfone (DMS, 99.73% ± 0,01%) traceCERT® Sigma-Aldrich were used as the only CRM. All samples were prepared with deuterium oxide (D_2O) and chloroform-d ($CDCl_3$) from CIL, or methanol–d_4 (MeOD) from Sigma-Aldrich. Also, 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP-d_4) from Sigma-Aldrich or tetramethylsilane (TMS) form CIL were used for frequency reference.

Samples provided by ICE/UNODC registered between 2015 and 2017 were stored under refrigeration prior to ^{1}H-qNMR analysis. A total of sixteen different samples, including nine different substances, were analyzed. Twelve different NPS samples were obtained from police seizures conducted by the National Institute of Criminalistics of the Brazilian Federal Police.

2.2. Sample Preparation

NPS samples were thoroughly grinded with mortar and pestle and mixed to ensure proper homogeneity. Samples were weighted in an XP 205 Mettler Toledo scale (0.01 mg) in eppendorff tubes. Solutions were prepared by weighing approximately 10 mg of NPS and approximately 8 mg of the internal standard, followed by addition of 700 μL of the appropriate solvent. To ensure complete dissolution and homogeneity, solutions were stirred in a vortex mixer for 1 min and were subsequently transferred to 5 mm NMR tubes. Samples with insoluble excipients were centrifuged and supernatants were transferred to 5 mm NMR tubes. For ^{1}H-qNMR experiments, all samples were running in triplicate, except for the Nimetazepam sample SM2/2/2015 that was analyzed in duplicate. Also, a reference solution was prepared by weighing approximately 10 mg of both reference materials, MA and DMS, and solubilized in 700 μL of D_2O. Since their purity is known, they were used to ensure proper equipment calibration and quantitative performance.

2.3. ^{1}H-qNMR experiments

The ^{1}H-qNMR experiments were performed in a Bruker Avance III HD spectrometer operating at ^{1}H frequency of 600.13 MHz and equipped with a broadband or a triple resonance probe. Lock and shimming were performed automatically for each experiment, whereas tuning and matching were performed manually. The 90° pulse was automatically calibrated with *pulsecal* routine. All spectra were obtained at 25°C, without sample spinning and with 30° pulse angle and ^{13}C decoupling during acquisition (zgig30 pulse sequence) to avoid sidebands and minimize eventual signal overlapping. All acquisitions were registered with a relaxation delay of at least 7 times the largest T_1 value (6.4 s for MA) to ensure complete relaxation. The relaxation time T_1 was measured using an inversion-recovery experiment. Acquisition parameters are provided in Table 1.

Table 1. NMR acquisition parameters used to obtain quantitative spectra.

Parameter (Bruker symbols)	Value
Pulse angle	30°
Relaxation delay (D1)	15 s
Number of dummy scans (DS)	4
Total number of scans (NS)	16
Specter Width (SW)	20 ppm
Receiver Gain (RG)	32
Digitization mode (DIGMOD)	baseopt
Pre-scan delay (DE)	10 µs
Filter correction (FILCOR)	1.5 µs
Total experiment time	5.55 min

2.4. NMR qualitative experiments

A set of 1D and 2D NMR experiments (^{1}H, ^{13}C, DEPT-135, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC and ^{1}H-^{1}H COSY) were routinely performed to each NPS to confirm their structure and fully attribute NMR signals. The experiments were carried out in a Bruker Avance III HD spectrometer operating at ^{1}H frequency of 600.13 MHz and equipped with a broadband or a triple resonance probe. Lock, shimming, tuning and matching were performed automatically for each experiment. The routine set of qualitative experiments were usually left overnight (8 to 12 hours) to be completed with adequate S/N ratio. All spectra were obtained at 25°C.

2.5. NMR data processing and purity calculation

Spectra were processed with the Bruker TopSpin 3.2 and ACD/NMR software. Fourier

transform was applied after zero filling the data to 64 k time domain points. The acquired NMR spectra were manually phase-corrected and baseline was automatically set with a fifth order polynomial function. The spectra were referenced using TMS or TSP-d_4 signal (0 ppm) when D_2O and organic solvents ($CDCl_3$ and MeOD) were used, respectively. Integrations were manually obtained using the Bruker Topspin 3.2 software. For quantification and determination of purity of an analyte (Px), equation 1 was applied [20], in which the molecular weight (M), area of relative integral (I), gravimetric mass (m) and number of protons (N) of an internal standard (std) and the analyte of interest (x), as well as the purity of the internal standard (Pstd), are used:

$$P_x = \frac{I_x \, N_{std} \, M_x \, m_{std}}{I_{std} \, N_x \, M_{std} \, m_x} P_{std} \quad \text{Equation 1}$$

3. Results and Discussion

3.1. ICE/UNODC exercise samples

Firstly, as a manner to validate the proposed methodology, we have evaluated sixteen ICE/UNODC samples and compared the analyte concentration obtained by our ^{1}H-qNMR method with the exercise final results [21].

As all the samples are soluble in water, D_2O was chosen as solvent and, accordingly, MA was used as the internal standard. In general, ^{1}H-NMR signal selected to quantify the analyte appeared as well-resolved peaks of methyl or methylene groups at spectral regions free from any interference of other signals. Chemical shifts values and multiplicity of the peaks used to quantify each analyte are available in the supplementary material (Figures S1-S8). Analysis were performed in triplicates during 2018 and 2019.

Experimental ^{1}H-qNMR purity compared to ICE/UNODC results are shown in Table 2. Accuracy was evaluated based on the relative error of the proposed method against exercise results. As shown in Table 2, the majority of the results was satisfactory, with errors values found below 9%, except for the ketamine sample SM3/2/2015, whose relative error was -25.27%. Nonetheless, ketamine analyte was also quantified in sample SM3/2/2016, but with a much smaller error (-0.46%), thus suggesting that the 2015 sample could be partially degraded after 3 years of storage, even though it was kept under refrigeration.

Table 2. Values of ^{1}H-qNMR experimental purity compared to the ICE/UNODC reference values and respective relative errors.

Sample	ICE/UNODC i.d./year	Analyte	^{1}H-qNMR		ICE/UNODC average purity % (w/w)	Relative Error %
			average purity % (w/w)	RSD %		
1	SM2/2/2015	Nimetazepam	3.71	0.30	3.70	0.30
2	SM3/2/2015	Ketamine	8.89	0.33	11.90	-25.27
3	SM4/2/2015	Amphetamine	21.81	0.73	23.90	-8.73
4	SM1/1/2016	Cocaine	52.01	0.60	51.70	0.59
5	SM2/1/2016	MDMA	13.39	0.69	13.80	-2.96
6	SM3/1/2016	Amphetamine	5.50	1.75	5.50	0.06
7	SM1/2/2016	Cocaine	79.70	0.00	77.30	3.10
8	SM2/2/2016	JWH-073	14.33	0.33	13.20	8.56
9	SM3/2/2016	Ketamine	10.45	0.33	10.50	-0.46
10	SM4/2/2016	Heroin	24.93	1.60	25.90	-3.74
11	SM1/1/2017	MDPV	45.01	0.80	45.30	-0.63
12	SM2/1/2017	Cocaine	26.45	0.35	25.10	5.37
13	SM3/1/2017	MDMA	54.91	0.55	56.00	-1.94
14	SM1/2/2017	MDPV	31.39	0.97	31.30	0.29
15	SM2/2/2017	Amphetamine	5.70	0.43	5.60	1.84
16	SM3/2/2017	Methamphetamine	39.20	0.08	39.90	-1.74

A t-test was applied and showed no significant differences (95% confidence) when comparing the proposed method to ICE/UNODC values. The mean value of purity determined by ^{1}H-qNMR was also compared to ICE/UNODC results by plotting both values for all sixteen samples. Linear regression adjustment presented a R^2 of 0.9968 with the slope value of 0.9792 and intercept of 0.7698 (Figure 1), suggesting consistent performance for different substances among a representative range of concentration. Again, this outcome shows the good correlation between the proposed ^{1}H-qNMR method and ICE/UNODC results.

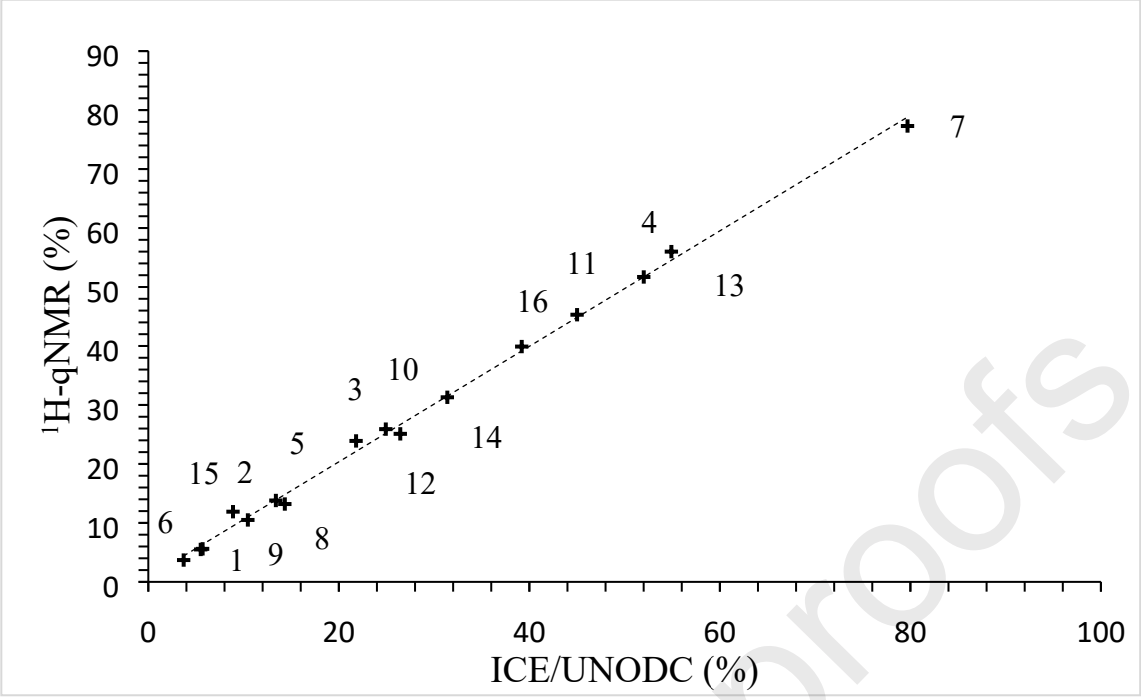

Figure 1. ^{1}H-qNMR experimental purity versus ICE/UNODC reference values. Data from Table 2.

The ability to quantify many substances using only one readily available and relatively inexpensive reference material, MA in this instance, is crucial to understand the potential of ^{1}H-qNMR in NPS analysis. The rising number of substances being reported, sometimes with unprecedent molecules with no CRM available to purchase, can represent a huge analytical challenge for most forensic laboratories.

3.2. NPS seized samples

Once the ^{1}H-qNMR method was applied to UNODC exercise samples and demonstrated reliable results, the same strategy was implemented to quantify NPS seized by the Brazilian Federal Police. Most of the NPS samples was seized as tablets or powders. Routine forensic chemistry methods, such as FTIR spectroscopy and MS spectrometry (data not shown), have classified the NPS samples as cathinones, phenethylamines, and tryptamines. The structures of all molecules were confirmed by careful analysis of 1D (^{1}H and ^{13}C) and 2D (^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC) NMR qualitative experiments. Complete assignment of ^{1}H and ^{13}C NMR spectra for all NPS analyzed in this study are presented in the supplementary material (Figures S9-S33 and Tables S1-S12).

Twelve NPS were analyzed and successfully identified as: 2-FA (2-fluoroamphetamine), 4-

FA (4-fluoroamphetamine), 2-MAPB (N,α-dimethyl-2-benzofuranethanamine), 5-MAPB (N,α-dimethyl-5-benzofuranethanamine), 4-HO-MiPT (4-hidroxy-N-methyl-N-isopropiltriptamine), 4-CMC (4-chloromethcathinone), 4-FMC (4-fluormethcathinone), DOC (4-chloro-2,5-dimethoxyamphetamine), 25B-NBOMe (2-(4-bromo-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine), 5-MeO-MiPT (5-methoxy-N-methyl-N-isopropyltryptamine), ethylone (N-ethyl-3,4-methylenedioxycathinone), ethylphenidate (ethylphenyl(piperidin-2-yl)acetate).

The NMR spectroscopy is only available for forensic chemistry laboratories in Brazil through collaborative initiatives with University Centers. Since forensic chemistry and structural analysis is mainly based on hyphenated methods, e.g. GC-MS, the absence of specific NPS reference materials does not either allow analysts to rely on retention times to ascertain NPS identity, or quantify the amount of that chemical in unknown samples. So, any conclusion regarding the identity of an NPS must entrust mostly on monography data, which are compiled by several crucial initiatives available online [22, 23]. The lack of NPS reference materials also demands for the utilization of an additional structural analysis as a confirmation step to conclude the forensic case, as FTIR or Raman spectroscopies or high-resolution MS spectrometry, overloading the laboratory analysis and increasing the response time, meanwhile not providing a quantitative and definitive answer. Furthermore, fabrication of NPS often involves the deliberate changes of their structure, by changing substituents and/or its positions, in order to create a "new" substance that may not be available in the list of illegal drugs. Also, as samples can be seized in many forms (powder, stamps or pills) and levels of purity, there are many situations in which MS or FTIR/Raman cannot be enough to reach an unequivocal conclusion.

To illustrate that point, one can refer to the MS spectra available to identify each of 5-MAPB or 2-MAPB isomers [24], whose MS data look virtually the same. Only small relative intensities changes on minor mass peaks can be used to differentiate between each isomer. In situations like that, the NMR spectral data is a key tool to unequivocally identify the correct structure of new seized samples. As shown in the ^{1}H NMR spectra of 2-MAPB and 5-MAPB (Figure 2), immediate analysis of signals in the aromatic region (6.50-8.00 ppm) can clearly distinguish their constitutional isomers. Therefore, the careful characterization of twelve NPS samples described in this work can be an opportunity to help forensic laboratories to firmly identify NPS. Moreover, it can be used to extend reference libraries, which assist the use of other techniques to identify and quantify abuse drugs, in particular, complex samples containing multiple components, as described elsewhere [2, 16, 17, 25].

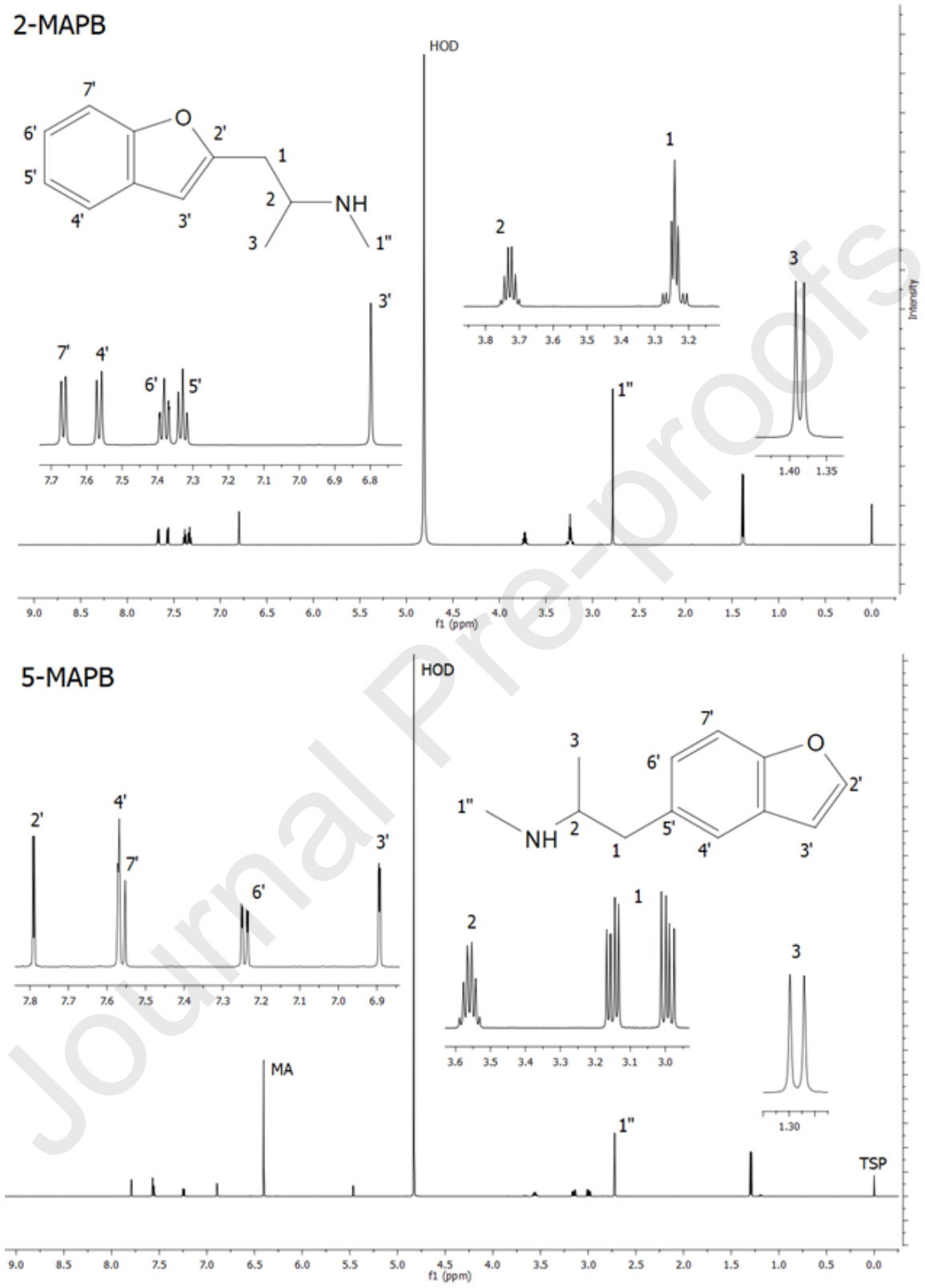

Figure 2. Comparison of ^{1}H-NMR spectra for 2-MAPB and 5-MAPB.

Selection of NMR signals to NPS quantification followed the same criteria used to ICE/UNODC exercise samples, i.e., lower multiplicity signals at spectral regions free of any overlapping signals. Since purification steps are avoided, diluents and adulterants can be present in NPS samples, but the use of high field spectrometers and a well-adjusted shim system provided at least one appropriate non-overlapping signal for quantification. Table 3 summarizes some of the data obtained for all the analyzed NPS samples. To the best of our knowledge, this is the first time that quantification by ^{1}H-qNMR is described for almost all NPS samples presented in this work, except for ethylone.

Quantitative results show that NPS purities ranged from 83.61% (2-FA) to 99.98% (ethylphenidate) (Table 3). The highest relative standard deviation (RSD) was 2.67% for 5-MeO-MiPT. In terms of precision assessment, RSD values below 10% are considered acceptable by the quality management system applied at forensic chemistry laboratory in the Brazilian Federal Police. Comparatively, Naqi and co-workers [9] reported the quantification of MDMA in the presence of other NPS (i.e. methylone, ethylone, 3-trifluoromethylpiperazine, among others) using ^{1}H-qNMR and MA as the internal standard. They demonstrated that MA is also suitable to quantify NPS even in complex mixtures. Good results were achieved, and RSD values varied from 0.9% to 11.0% [9]. The latter are, however, higher than the results presented herein and are likely due to the prevalence of NPS as minor components (<10%) in the complex mixture they analyzed.

It is interesting to note that MA was used as internal standard in most of the NPS analysis when using D_2O as a solvent (Table 3). The MA versatility to ^{1}H-qNMR is mainly due to its characteristic signal at 6.40 ppm, a spectral region that is usually free of signals. DMS was only used as an internal standard to quantify 25B-NBOMe in $CDCl_3$, since this compound was not soluble in water.

Even in studies in which the development of new methods does not use internal standards, such as electronic reference [26], external reference [16, 27] or low field benchtop NMR [25, 28] methods, the approach proposed herein can be suitable to the validation process. Recently, Hussain and co-workers [28] showed the quantification of MDMA in seized tablets using 60 MHz benchtop ^{1}H NMR spectroscopy performed without internal standards. However, authors synthesized MDMA to ensure the authenticity of the material and to explore the new methodology. In fact, the use of an internal standard approach to quantify a reference material can facilitate the development of a new method, becoming less laborious its assessment. For the development of new methods applied to NPS samples, CRM were most of the time not available, thereby making the use of internal standard

highly valuable.

Table 3. Solvent and internal standard used for analyzed NPS. Proton chemical shifts, mean purity and its relative standard deviation (RSD) are also shown. Signals used for quantification are highlighted in bold.

Substance	Solvent	Internal Standard	^{1}H chemical shifts (ppm)	Purity ± RSD (%)
2-FA	D_2O	Maleic Acid	1.31 (d), **3.00 (m)**, 3.68 (sextet), 7.17 (m), 7.21 (m), 7.34 (m), 7.38 (m)	83.61 ± 1.07
4-FA	D_2O	Maleic Acid	1.31 (d), **2.93 (m)**, 3.62 (sextet), 7.13 (m), 7.30 (m)	99.59 ± 1.13
2-MAPB	D_2O	Maleic Acid	1.40 (d), 2.78 (s), 3.24 (m), 3.73 (sextet), **6.80 (s)**, 7.33 (td), 7.38 (td), 7.56 (dd), 7.66 (dd)	97.28 ± 1.22
5-MAPB	D_2O	Maleic Acid	1.29 (d), 2.72 (s), 3.00 (dd), 3.16 (dd), 3.56 (sextet), 6.89 (d), **7.24 (dd)**, 7.56 (d), 7.57 (d), 7.79 (d)	95.16 ± 0.21
DOC	D_2O	Maleic Acid	1.32 (d), **2.93 (d)**, 3.66 (sextet), 3.83 (s), 3.88 (s), 7.02 (s), 7.16 (s)	97.77 ± 2.11
Ethylphenidate	D_2O	Maleic Acid	**1.17 (t)**, 1.39 (m), 1.47 (m), 1.63 (m), 1,80 (m), 1.88 (m), 3.08 (td), 3.46 (dt), 3.82 (td), 3.99 (d), 4.22 (m), 7.33 (m), 7.45 (m)	99.98 ± 1.26
25B-NBOMe	$CDCl_3$	Dimethyl sulfone	**3.09 (m)**, 3.63 (s), 3.76 (s), 3.84 (s), 4.13 (s), 6.83 (d), 6.87 (s), 6.93 (t), 6.96 (s), 7.31 (dt), 7.39 (d)	99.80 ± 1.83
4-CMC	D_2O	Maleic Acid	**1.63 (d)**, 2.83 (s), 5.10 (q), 7.65 (dt), 8.01 (m)	99.37 ± 1.15
4-FMC	D_2O	Maleic Acid	**1.63 (d)**, 2.83 (s), 5.11 (q), 7.36 (m), 8.11 (m)	98.94 ± 0.79
Ethylone	D_2O	Maleic Acid	1.36 (t), 1.61 (d), 3.12 (m), 3.21 (m), 5.06 (q), **6.14 (d), 6.15 (d)**, 7.06 (d), 7.50 (d), 7.70 (dd)	99.51 ± 0.32
5-MeO-MiPT	D_2O	Maleic Acid	1.27 (d), **2.81 (s)**, 3.18 (t), 3.39 (b), 3.61 (septet), 3.91 (s), 6.97 (dd), 7.19 (d), 7.32 (s), 7.47 (d)	96.86 ± 2.67
4-HO-MiPT	MeOD	Maleic Acid	1.28 (d), **2.78 (s)**, 3.24 (t), 3.39 (t), 3.56 (septet), 7.00 (s), 6.84 (dd), 6.89 (m), 6.37 (dd)	87.78 ± 0.62

s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets, m = multiplet (denotes complex pattern), b = broad signal.

In previous works, we developed and validated the ^{1}H-qNMR for quantification of cocaine[19] and MDMA[18] in seized samples, with suitable results for accuracy, precision, linearity, robustness, selectivity, and limits of detection and quantification. In this work, some figures of merit previously described were probed for the NPS.

Uncertainty of the proposed ^{1}H-qNMR method was assessed with control samples prepared with CRMs, MA and DMS. Five solutions were prepared and measured as described in section 2.2 in order to calculate the individual standard uncertainty contribution from each source, treating DMS as our analyte. Combined standard uncertainty [μ(Px)] was 0.0022 and expanded uncertainty [U(x)] was 0.44% (with k=2, 95% confidence). Experimental method uncertainty was then extrapolated to analyzed NPS (Table 4) and ranged from 0.44% to 0.37%, for ethylphenidate and 2-FA, respectively. Limit of quantification (LOQ) and limit of detection (LOD) were determined through the measurement of signal-to-noise ratio (S/N) of analyte signals involved in quantification, according to an approach already described [19, 20]. In this work, a S/N ratio of 30 and 10 was used to estimate LOQ and LOD, respectively. Results are shown in Table 4, with all LOQ and LOD values found below 2% and 0.8%, respectively. It is important to emphasize that these values are highly dependent on the signal linewidth, which can differ significantly due to instrument calibration (shim) and sample composition, for example. Thus, we encourage analysts to always check the S/N ratio of signals involved in quantification.

Stability of NPS and internal standard solutions were also checked (Table 4), demonstrating that all solutions are stable for at least 24 hours when stored at room temperature. The only sample that showed fair stability for solely 24 hours was the compound 25B-NBOMe in $CDCl_3$. Most NPS samples have shown sufficient stability for a period of 48 hours, thereby implying that the analyses performed in this time frame have provided reliable and valid results.

Table 4. Some figures of merit for the proposed ^{1}H-qNMR method: expanded uncertainty (U), limit of detection (LOD), limit of quantification (LOQ) and stability.

Substance	Expanded Uncertainty	Limits of Quantification and Detection		Stability		
	U(x)	LOQ (% w/w)	LOD (% w/w)	Mean purity t = 0 h (% w/w)	Mean purity t = 24h (% w/w)	Mean purity t = 48h (% w/w)
2-FA	0.37	0.46	0.15	83.91	83.90	83.90
4-FA	0.44	0.40	0.13	99.77	99.68	99.58
2-MAPB	0.43	0.56	0.19	96.36	96.58	96.54
5-MAPB	0.42	0.58	0.19	97.60	96.53	96.61
DOC	0.43	1.38	0.46	94.37	94.61	94.59
Ethylphenidate	0.44	0.42	0.14	99.92	100.48	99.61
25B-NBOMe	0.44	1.56	0.73	99.80	99.19	-
4-CMC	0.44	0.46	0.15	92.14	91.32	91.72
4-FMC	0.44	0.49	0.16	100.10	99.97	100.49
Ethylone	0.44	0.97	0.33	99.04	99.56	99.02
5-MeO-MiPT	0.43	0.35	0.11	96.59	95.88	95.68
4-HO-MiPT	0.39	1.38	0.46	87.35	87.95	88.70

The selectivity of ^{1}H-qNMR is demonstrated by the non-overlapping of signals used for quantification of NPS, the internal standard and for some common adulterants or diluents found in real samples (*e.g.* caffeine, procaine, sucrose, aminopyrine). As an example, Figure 3 displays the stacked spectra of these substances and 2-MAPB, showing that the signal used for quantification (indicated by an arrow) does not overlap with any other signal from common adulterants. Also, 2D experiments used for characterization revealed no signals of minor components "hidden" in larger signals (data not shown). Other NPS samples can also be checked in a similar way. The use of a high field spectrometer can help to find at least one analyte's signal free of overlapping and, so, suitable for quantification of each sample in this work. On the other hand, if the method is implemented in a lower field spectrometer, selectivity should be checked. If the analyst has access to a fairly set of reference materials for most common adulterants and diluents, it is possible to acquire a separate set

of spectra of these substances to build a small library.

It is important to emphasize that NPS samples analyzed in this work show high purity (> 80%). After characterized and quantified by NMR, these samples are now suitable for use as reference materials with routine techniques in a forensic laboratory, such as GC-MS and FTIR. Moreover, we encourage analysts to implement specific validation procedures regarding selectivity whether NMR will be used as routine method of analysis, such as the quantification of the analyte in the presence of possible matrix components, especially when dealing with a less pure ("street grade") pool of samples.

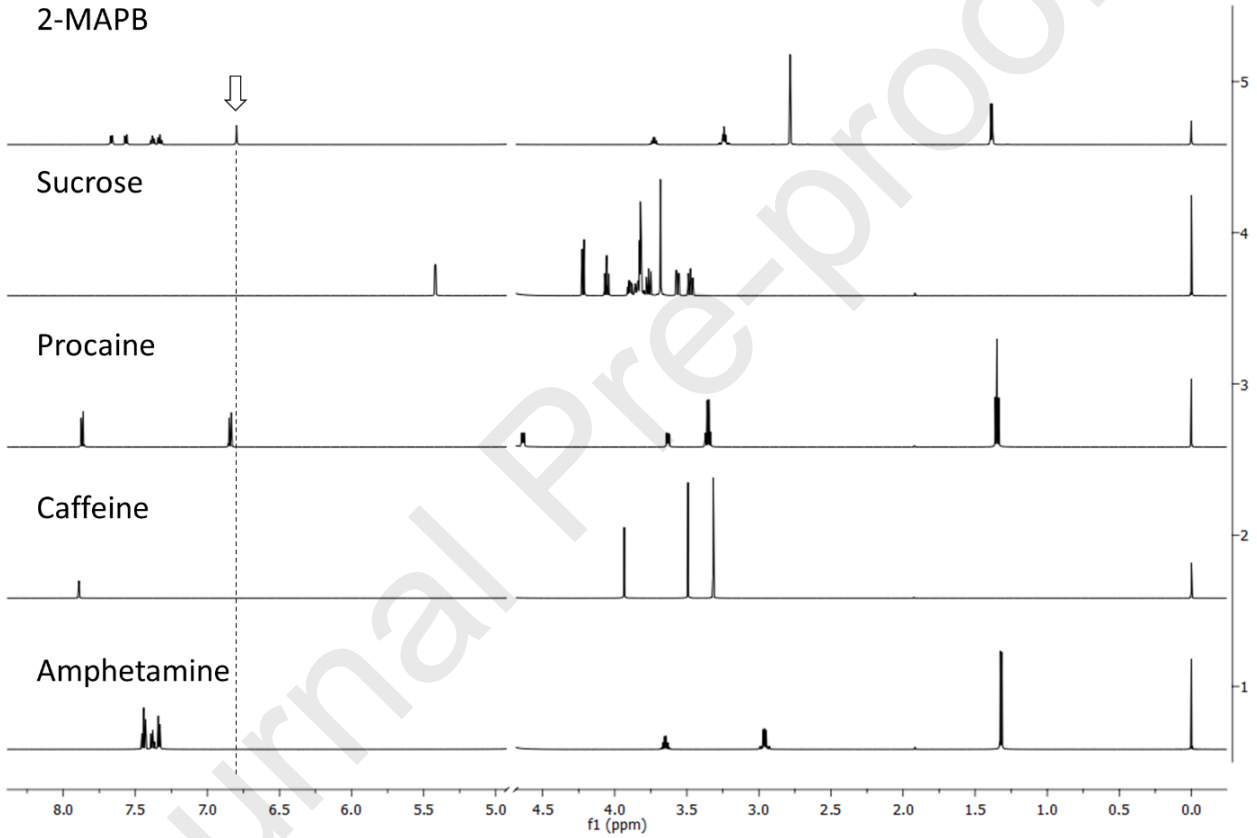

Figure 3. Stacked view of spectra of Aminopyrine, Sucrose, Procaine, Caffeine and 2-MAPB in the presence of MA. Signal used for quantification and MA signal are indicated by arrows. All spectra were obtained in D_2O at 25ºC.

4. Conclusion

In this work, we presented a detailed compilation of NMR spectroscopic data for determination of the molecular structure and proper quantification of twelve NPS found in samples seized by the Brazilian Federal Police. In particular, all the analysed NPS samples were identified

and the unidimensional and bidimensional ^{1}H and ^{13}C NMR data were completely assigned, allowing one for the determination of the molecular structure of all NPS present in samples. The careful characterization of NPS described in this work can be an opportunity to help forensic laboratories to unambiguously identify NPS, besides being useful to increase the data of reference libraries.

Afterward, a quantitative approach, namely ^{1}H-qNMR, was successfully applied to determine the composition of NPS samples, without needing a specific reference material containing those analytes. This is the first time that a ^{1}H-qNMR method is applied to quantify almost all NPS samples assessed in this work, except for ethylone. The accuracy of this approach was firstly demonstrated by applying the proposed ^{1}H-qNMR method to a set of sixteen samples from ICE/UNODC. Results were in accordance with the ICE/UNODC data. In our quantitative analyses of NPS, the highest RSD was 2.67% and highest estimated expanded uncertainty was 0.44%. Some other figures of merit were checked to ensure the method has suitable and reproducible results, namely selectivity, stability and limits of quantification and detection. Moreover, this methodology achieved an acceptable level of selectivity, since no overlapping could be observed for signals of interest.

In summary, the present ^{1}H-qNMR method allows for the absolute quantification of a representative set of different compounds in a straightforward manner and without needing a specific reference material provided that readily available substances, such as MA or DMS, are used as internal standards. It is not possible to ensure that this approach will be applicable to 100% of NPS, as dozens of new substances appear every year. Problems that arise from the use of internal standards (mainly solubility problems and interaction) and selectivity will always have to be studied individually. However, we demonstrate that validating an existing qNMR method applied to a new set of substances is a straightforward process and is made possible when using reference materials that are available at hand, without the need of acquiring specific reference materials for each new analyte.

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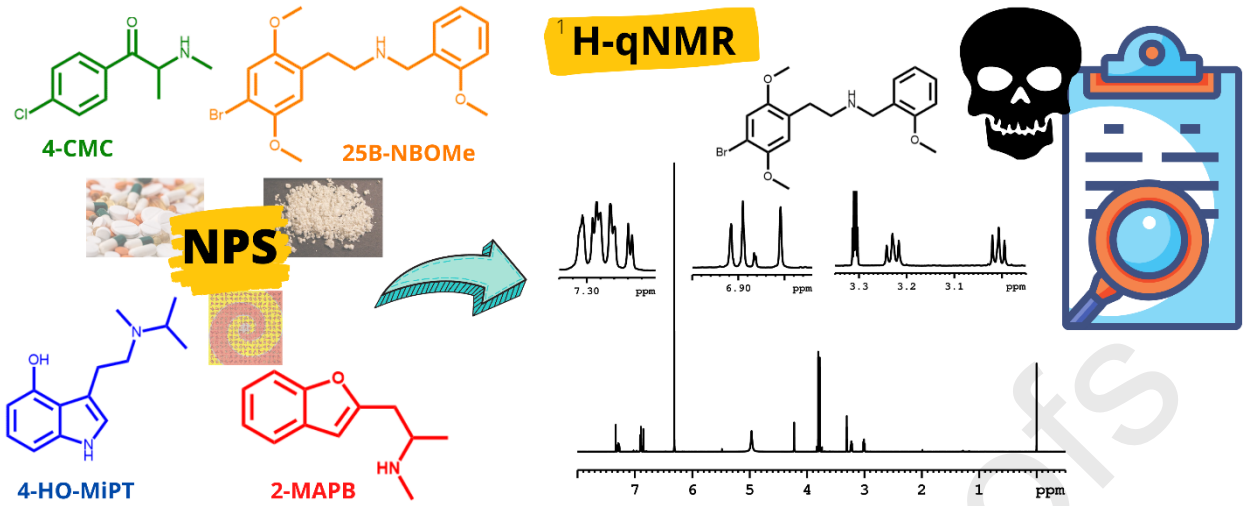

Highlights

- The molecular structure of twelve NPS is elucidated by NMR spectroscopy.
- The amount of each NPS is quantitatively determined by ^{1}H-qNMR.
- The quantification of NPS is performed without needing a certified reference material.
- The ^{1}H-qNMR method exhibits high precision and very low uncertainty.