VIP NMR Spectroscopy Very Important Paper

DOI: 10.1002/anie.201409795

Quantitative Trace Analysis of Complex Mixtures Using SABRE Hyperpolarization**

*Nan Eshuis, Bram J. A. van Weerdenburg, Martin C. Feiters, Floris P. J. T. Rutjes, Sybren S. Wijmenga, and Marco Tessari**

Abstract: *Signal amplification by reversible exchange (SABRE) is an emerging nuclear spin hyperpolarization technique that strongly enhances NMR signals of small molecules in solution. However, such signal enhancements have never been exploited for concentration determination, as the efficiency of SABRE can strongly vary between different substrates or even between nuclear spins in the same molecule. The first application of SABRE for the quantitative analysis of a complex mixture is now reported. Despite the inherent complexity of the system under investigation, which involves thousands of competing binding equilibria, analytes at concentrations in the low micromolar range could be quantified from single-scan SABRE spectra using a standard-addition approach.*

The last decade has witnessed a widespread interest in hyperpolarization techniques as methods to overcome the intrinsically low sensitivity of NMR spectroscopy. These include dynamic nuclear polarization (DNP),[1] spin exchange optical pumping (SEOP),[2] para-hydrogen-induced polarization (PHIP),[3] and, more recently, signal amplification by reversible exchange (SABRE).[4] However, the large signal increase that results from nuclear spin hyperpolarization unavoidably comes with some costs: Particularly, the assumption of a linear dependence of the nuclear magnetization on the concentration, as stated by Curie's law for samples at thermal equilibrium, is no longer necessarily correct. As a consequence, the quantification of analytes in solution from NMR spectra of hyperpolarized samples is not straightforward. Thus, although hyperpolarization has proven effective in significantly lowering NMR detection limits, its application to quantitative NMR analysis has thus far been rather modest.[5] However, in a recent investigation on the feasibility of SABRE for dilute solutions, we have found that under suitable conditions, the integrals of hyperpolarized signals depend linearly on the concentrations,[6] an essential requirement for quantitative NMR applications. Herein, we further explore this finding and show that it is indeed possible to detect and quantify analytes at low micromolar concentrations in complex mixtures using SABRE hyperpolarization.

SABRE is a technique with which nuclear spin hyperpolarization can be obtained for substrate molecules that weakly associate to a suitable metal complex together with *p*-H_2. This reversible association provides a transient scalar coupling network through which the spin order of *p*-H_2 can be transferred to the nuclear spins of the substrate.[7] Strongly enhanced NMR signals are then observed for substrate molecules free in solution owing to the reversible character of the interaction. A conventional quantitative analysis of such enhanced signals based on integral comparison with an internal or external standard is not applicable as the efficiency of SABRE depends on several molecular parameters (i.e., relaxation times, scalar coupling constants, dissociation rates) that can strongly vary for different proton spins.

However, for dilute substrates (referred to as analytes) in the presence of a large excess of a second ligand, a linear dependence of the SABRE signal integrals on the concentration can be obtained.[6] Herein, we show that such conditions can also be realized in far more complex mixtures, provided that the total substrate concentration (C_{sub}) largely exceeds the concentration of the analyte and of the metal complex:

$$[X] < [M] \sim [S_i] \ll \sum_{i=1}^{N} [S_i] = C_{sub} \quad (1)$$

where X, M, and S_i indicate the analyte, the metal complex, and the additional substrates present in the mixture. In this concentration regime, where a linear dependence between the SABRE signal and the concentration holds, calibration techniques, such as standard addition,[8] can be employed to quantitate dilute analytes in solution.

We have tested the validity of this approach on a dilute solution ($C_x = 8.5\ \mu M$) of nicotinamide in the presence of metal complex precursor **1** ($C_M = 333\ \mu M$) and a mixture of fifteen components with a total substrate concentration (C_{sub}) of 6 mM. Activation of complex precursor **1** by molecular hydrogen (5 bar) in the presence of this substrate mixture leads to the formation of thousands of possible metal complexes of the formula [Ir(IMes)$(H)_2(S_i)(S_j)(S_k)$]Cl (**2**; Figure 1).

A SABRE spectrum was acquired for such a mixture by applying a 90° read-out pulse shortly after dissolution of *p*-H_2 (5 bar) at a fixed position in the stray field of the NMR

[*] N. Eshuis, B. J. A. van Weerdenburg, Dr. M. C. Feiters, Prof. Dr. F. P. J. T. Rutjes, Prof. Dr. S. S. Wijmenga, Dr. M. Tessari
Institute for Molecules and Materials
Radboud University Nijmegen
Heyendaalseweg 135, 6525 AJ Nijmegen (The Netherlands)
E-mail: m.tessari@science.ru.nl

[**] We thank Ruud Aspers for his help with the experimental setup, NMR measurements, and p-H_2 production, and Dr. Hans Heus for his helpful comments. We thank the European Union and the provinces of Gelderland and Overijssel for support through the EFRO Ultrasense NMR project. SABRE = signal amplification by reversible exchange.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201409795.

 © 2015 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

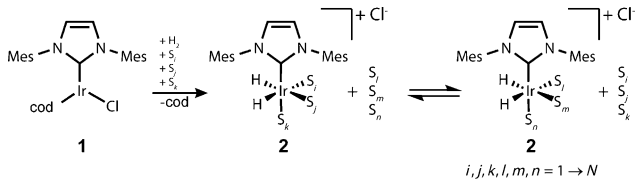

Figure 1. Formation of metal complexes **2** upon hydrogenation of the cod ligand of precursor **1** ($C_M = 333$ μM) in the presence of N substrates ($N = 16$, $C_{sub} = 6$ mM) that reversibly associate to **2**.

spectrometer (Figure 2, red trace). Increased signal intensities were observed in the aromatic region as well as for the methyl groups of acetonitrile and 1-methyl-1,2,3-triazole (see Figure S2B for signal enhancement factors). It is noteworthy that only the signals of the substrates free in solution were observed in the NMR spectra, as the bound form is distributed over the thousands of different complexes, each with concentrations in the sub-micromolar range.

For comparison, a conventional ^{1}H NMR spectrum of the original mixture was acquired with 256 scans and a long recovery delay ($5 \times T_1$) resulting in a total measuring time of approximately eleven hours (Figure 2, top). From this spectrum, a concentration of 8.8 μM was estimated for nicotinamide by integral comparison with the methylene signal of ethanol, which was added as an internal reference.

Standard addition was performed on the original mixture, increasing the analyte concentration in six consecutive steps. The single-scan SABRE spectra acquired after each addition of nicotinamide are displayed in Figure 2 (bottom). In the lower inset of Figure 2, the increasing SABRE signal of a well-resolved nicotinamide resonance is shown. Note that all other substrate signals in the SABRE standard-addition series remain substantially unperturbed (see also the Supporting Information). The corresponding linear plot of the SABRE signal integral versus the added analyte concentration is shown in Figure 3A (red squares). The extrapolated value of the concentration of nicotinamide (9.0 μM) is in good agreement with its nominal concentration.

Comparable results were obtained for the diluted substrates pyrazine, isoxazole, and quinazoline in similar mixtures (Figure 3B–D). The converging trend lines for the two nicotinamide resonances in Figure 3A and the two quinazoline resonances in Figure 3D are noteworthy. This nicely confirms the validity of the standard-addition technique in combination with SABRE: Different slopes are obtained because the enhancement factors for these signals are different; nevertheless, extrapolation of the curves leads to the same concentrations within experimental error.

As previously pointed out by Mewis et al.,[9] the reproducibility of SABRE performed by manually shaking the sample in the stray field of the magnet is hardly better than 10–20%. Variations of the polarization transfer field (PTF) and of the

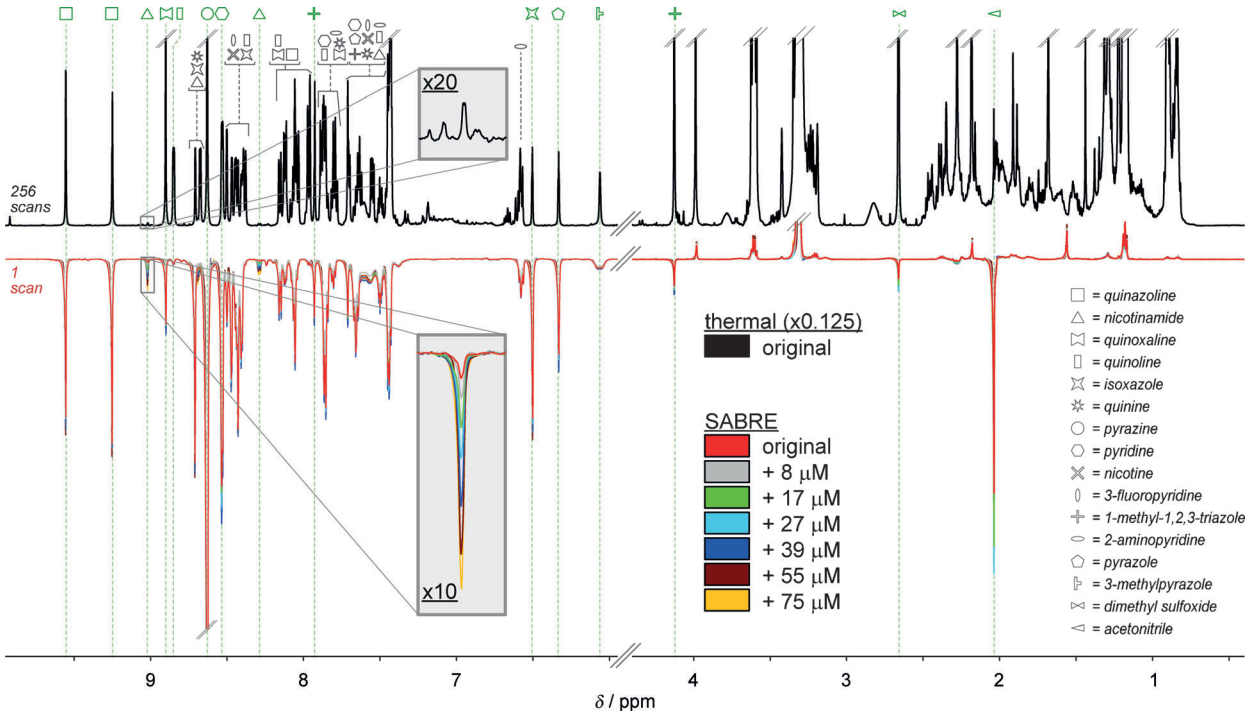

Figure 2. ^{1}H NMR spectra acquired at 600 MHz at thermal equilibrium (top, 256 scans, ×0.125 vertically scaled) or after SABRE hyperpolarization at 6.5 mT (bottom, red trace) on a sample consisting of nicotinamide as the analyte ($C_x = 8.5$ μM), together with complex precursor **1** and a mixture of fifteen substrates present in high micromolar amounts ($C_M = 333$ μM, $C_{sub} = 6$ mM). Individual substrate concentrations and signal enhancements (for peaks indicated in green) are provided in the Supporting Information. The SABRE spectra of samples after standard addition, with C_{add} between 8 and 75 μM (color-coded), showed increases in the nicotinamide signals, while the signal integrals of other substrates remained constant. Insets show the resonance of proton d in nicotinamide (see Table S1 for annotations) with a signal-to-noise ratio of 31:1 after hyperpolarization (red trace, ×10 vertically scaled), and 15:1 after 256 scans at thermal equilibrium (black trace, ×20 vertically scaled).

© 2015 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

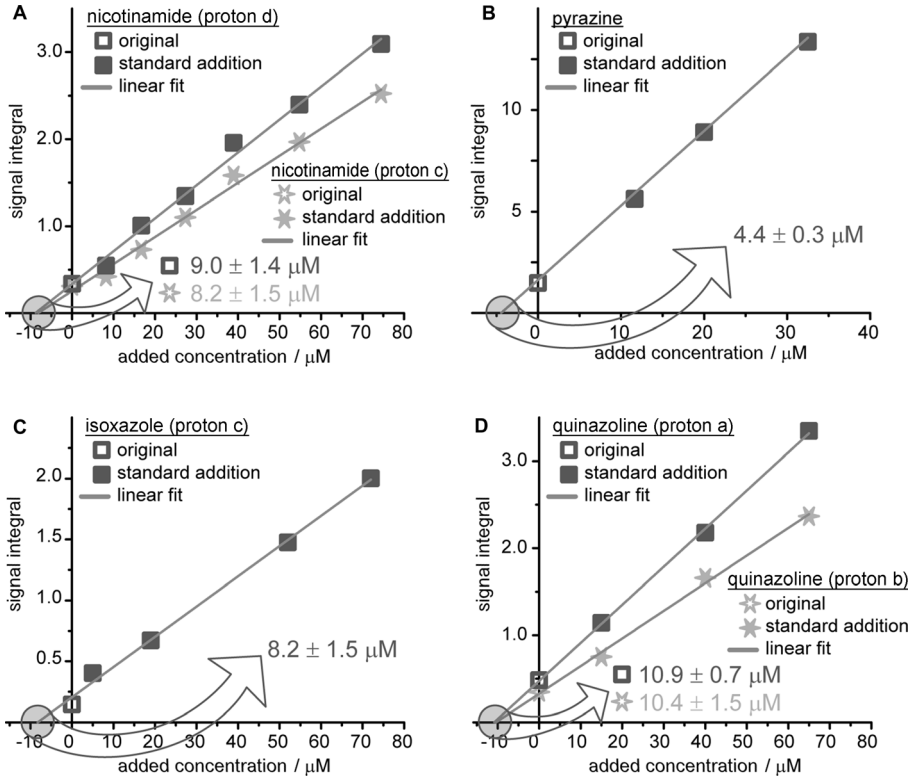

Figure 3. Standard-addition curves for proton resonances (see Table S1 for annotations) of nicotinamide (A), pyrazine (B), isoxazole (C), and quinazoline (D) with nominal substrate concentrations of 8.5, 5, 8, and 10 μM respectively. Concentrations were estimated from the abscissa intercepts (circled) of the standard-addition curves (grey lines). Experimental uncertainties were derived by error propagation.

sample insertion time into the magnet represent the main source of scatter of the measured integrals (see the Supporting Information) and a major contribution to the uncertainty in the extrapolated concentration values. A better precision should be achieved by performing SABRE with an automated polarization setup to improve experimental reproducibility.[4b]

In conclusion, we have demonstrated that SABRE nuclear spin hyperpolarization does not preclude a quantitative analysis of NMR spectra. The standard-addition method here employed is widely used for quantitative NMR applications; to the best of our knowledge, this is the first time that such an analytical approach is proposed in combination with hyperpolarization for the quantitative analysis of complex mixtures. By exploiting the signal enhancement provided by SABRE, we could determine the concentration of dilute species (in the low micromolar range) in solution with a precision of approximately 1 μM. Furthermore, by comparison with conventional NMR quantification methods, we have shown that the proposed approach results in a dramatic reduction (ca. 100-fold) of the measuring time. These results support possible applications of SABRE for the quantitative determination of dilute components in complex mixtures, such as natural product extracts, reaction mixtures, or biofluids.

Experimental Section

Complex precursor [Ir(cod)(IMes)Cl] [**1**; IMes = 1,3-bis(2,4,6-trimethylphenyl)imidazole-2-ylidene, cod = cyclooctadiene] was dissolved in a [D_4]MeOH solution containing 16 substrates, one of which was the analyte at a low micromolar concentration (obtained by gravimetric dilution). The analytical concentrations of the complex precursor (C_M) and all substrates together (C_{sub}) were maintained constant at a ratio of 1:18. Equal amounts of this mixture were diluted with solutions of increasing analyte concentrations to obtain samples for the standard-addition series. More details as well as experimental procedures, substrate structures, and NMR data are provided in the Supporting Information.

Received: October 6, 2014
Published online: December 2, 2014

Keywords: analytical chemistry · hyperpolarization · NMR spectroscopy · SABRE · trace analysis

[1] a) J. H. Ardenkjaer-Larsen, B. Fridlund, A. Gram, G. Hansson, L. Hansson, M. H. Lerche, R. Servin, M. Thaning, K. Golman, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 10158–10163; b) L. Frydman, D. Blazina, *Nat. Phys.* **2007**, *3*, 415–419.

[2] a) L. Schröder, T. J. Lowery, C. Hilty, D. E. Wemmer, A. Pines, *Science* **2006**, *314*, 446–449; b) P. Nikolaou, A. M. Coffey, L. L. Walkup, B. M. Gust, N. Whiting, H. Newton, S. Barcus, I. Muradyan, M. Dabaghyan, G. D. Moroz, M. S. Rosen, S. Patz, M. J. Barlow, E. Y. Chekmenev, B. M. Goodson, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 14150–14155.

[3] a) K. V. Kovtunov, I. E. Beck, V. I. Bukhtiyarov, I. V. Koptyug, *Angew. Chem. Int. Ed.* **2008**, *47*, 1492–1495; *Angew. Chem.* **2008**, *120*, 1514–1517; b) M. Roth, P. Kindervater, H.-P. Raich, J. Bargon, H. W. Spiess, K. Münnemann, *Angew. Chem. Int. Ed.* **2010**, *49*, 8358–8362; *Angew. Chem.* **2010**, *122*, 8536–8540.

[4] a) R. W. Adams, J. A. Aguilar, K. D. Atkinson, M. J. Cowley, P. I. P. Elliott, S. B. Duckett, G. G. R. Green, I. G. Khazal, J. Lopez-Serrano, D. C. Williamson, *Science* **2009**, *323*, 1708–1711; b) M. J. Cowley, R. W. Adams, K. D. Atkinson, M. C. R. Cockett, S. B. Duckett, G. G. R. Green, J. A. B. Lohman, R. Kerssebaum, D. Kilgour, R. E. Mewis, *J. Am. Chem. Soc.* **2011**, *133*, 6134–6137; c) S. Glöggler, M. Emondts, J. Colell, R. Müller, B. Blümich, S. Appelt, *Analyst* **2011**, *136*, 1566–1568; d) L. S. Lloyd, R. W. Adams, M. Bernstein, S. Coombes, S. B. Duckett, G. G. Green, R. J. Lewis, R. E. Mewis, C. J. Sleigh, *J. Am. Chem. Soc.* **2012**, *134*, 12904–12907; e) E. B. Dücker, L. T. Kuhn, K. Münnemann, C. Griesinger, *J. Magn. Reson.* **2012**, *214*, 159–165; f) J.-B. Hövener, N. Schwaderlapp, T. Lickert, S. B. Duckett, R. E. Mewis, L. A. Highton, S. M. Kenny, G. G. Green, D. Leibfritz, J. G. Korvink, J. Hennig, D. von Elverfeldt, *Nat. Commun.* **2013**, *4*, 2946–2950; g) D. A. Barskiy, K. V. Kovtunov, I. V. Koptyug, P. He, K. A. Groome, Q. A. Best, F. Shi, B. M. Goodson, R. V. Shchepin, A. M. Coffey, K. W. Waddell, E. Y. Chekmenev, *J. Am. Chem. Soc.* **2014**,

 © 2015 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

136, 3322 – 3325; h) F. Shi, A. M. Coffey, K. W. Waddell, E. Y. Chekmenev, B. M. Goodson, *Angew. Chem. Int. Ed.* **2014**, *53*, 7495 – 7498; *Angew. Chem.* **2014**, *126*, 7625 – 7628.

[5] a) M. H. Lerche, S. Meier, P. R. Jensen, S. O. Hustvedt, M. Karlsson, J. O. Duus, J. H. Ardenkjaer-Larsen, *NMR Biomed.* **2011**, *24*, 96 – 103; b) T. Xu, D. Mayer, M. Gu, Y.-F. Yen, S. Josan, J. Tropp, A. Pfefferbaum, R. Hurd, D. Spielman, *NMR Biomed.* **2011**, *24*, 997 – 1005.

[6] N. Eshuis, N. Hermkens, B. J. van Weerdenburg, M. C. Feiters, F. P. J. T. Rutjes, S. S. Wijmenga, M. Tessari, *J. Am. Chem. Soc.* **2014**, *136*, 2695 – 2698.

[7] R. W. Adams, S. B. Duckett, R. A. Green, D. C. Williamson, G. G. R. Green, *J. Chem. Phys.* **2009**, *131*, 194505.

[8] A.-L. Hauswaldt, O. Rienitz, R. Jährling, N. Fischer, D. Schiel, G. Labarraque, B. Magnusson, *Accredit. Qual. Assur.* **2012**, *17*, 129 – 138.

[9] R. E. Mewis, K. D. Atkinson, M. J. Cowley, S. B. Duckett, G. G. R. Green, R. A. Green, L. A. R. Highton, D. Kilgour, L. S. Lloyd, J. A. B. Lohman, D. C. Williamson, *Magn. Reson. Chem.* **2014**, *52*, 358 – 369.

 © 2015 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim