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Optimized slice-selective ^{1}H NMR experiments combined with highly accurate quantitative ^{13}C NMR using an internal reference method

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ABSTRACT

Isotope ratio monitoring by ^{13}C NMR spectrometry (irm-^{13}C NMR) provides the complete ^{13}C intramolecular position-specific composition at natural abundance. It represents a powerful tool to track the (bio)-chemical pathway which has led to the synthesis of targeted molecules, since it allows Position-specific Isotope Analysis (PSIA). Due to the very small composition range (which represents the range of variation of the isotopic composition of a given nuclei) of ^{13}C natural abundance values (50‰), irm-^{13}C NMR requires a 1‰ accuracy and thus highly quantitative analysis by ^{13}C NMR. Until now, the conventional strategy to determine the position-specific abundance x_i relies on the combination of irm-MS (isotopic ratio monitoring Mass Spectrometry) and ^{13}C quantitative NMR. However this approach presents a serious drawback since it relies on two different techniques and requires to measure separately the signal of all the carbons of the analyzed compound, which is not always possible. To circumvent this constraint, we recently proposed a new methodology to perform ^{13}C isotopic analysis using an internal reference method and relying on NMR only. The method combines a highly quantitative ^{1}H NMR pulse sequence (named DWET) with a ^{13}C isotopic NMR measurement. However, the recently published DWET sequence is unsuited for samples with short T_1, which forms a serious limitation for irm-^{13}C NMR experiments where a relaxing agent is added. In this context, we suggest two variants of the DWET called Multi-WET and Profiled-WET, developed and optimized to reach the same accuracy of 1‰ with a better immunity towards T_1 variations. Their performance is evaluated on the determination of the ^{13}C isotopic profile of vanillin. Both pulse sequences show a 1‰ accuracy with an increased robustness to pulse miscalibrations compared to the initial DWET method. This constitutes a major advance in the context of irm-^{13}C NMR since it is now possible to perform isotopic analysis with high relaxing agent concentrations, leading to a strong reduction of the overall experiment time.

1. Introduction

Isotopic analysis at natural abundance is a powerful tool to track the origin of biological or synthetic molecules by determining their isotopic profile. Indeed, the isotope composition of molecules is directly dependent on their manufacturing process which may induce isotopic fractionation which in turn induces variations in the isotope composition. Several techniques are already used to determine the isotopic content of a given molecule. The most commonly used method is the isotope ratio monitoring by Mass Spectrometry (irm-MS) that provides access to the bulk isotopic abundance x_g (the average value) [1–3] in ^{2}H, ^{13}C, ^{18}O, ^{14}N or ^{34}S. However, this information is not always sufficient to discriminate molecules as a function of their geographical and/or chemical origin. Additional information can be obtained by measuring the isotope composition on each molecular position by performing Position-Specific Isotope Analysis (PSIA) [4]. Although some techniques such as isotope ratio infrared spectroscopy (IRIS) [5,6] or multiple collector-inductively coupled plasma-mass spectrometry (MC-ICP-MS) [7] can yield such information, they require a preliminary degradation of the sample which can be very time consuming and not always desirable. A third analytical technique based on pyrolysis fragmentation performed on-line coupled to GC-irm-MS (Py-GC/irm-MS) [8] provides the position-specific isotopic abundance (x_i) but only for four C-atoms of the analyzed molecule at most. In 1981, a method based on Nuclear Magnetic Resonance (NMR) spectrometry was proposed by Martin et al. [9] to measure the position-specific ^{2}H isotope composition. Since NMR has the advantages of being a quantitative, non-destructive as well as a

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non-specific analytical technique, isotope ratio monitoring by NMR spectrometry (irm-NMR) has been recognized as a powerful and general method to perform PSIA. The irm-^{2}H NMR is based on the use of chemical internal references with known isotopic composition. A comparison with the area of the molecule of interest makes it possible to determine the position-specific isotopic abundance x_i from peak areas thanks to Eq. (1).

$$x_i = \frac{n^{ref}}{n_i} \cdot \frac{m^{ref} \times P_m^{ref}}{m \times P_m} \cdot \frac{M}{M^{ref}} \cdot \frac{S_i^{comp}}{S^{ref}} \cdot x^{ref} \quad (1)$$

Here, x_{ref} is the isotopic abundance of the internal reference; S_i^{comp} and S^{ref} represent the area of the signal of interest at position i and the area of the reference signal respectively; n_i and n_{ref} are the number of equivalent nuclei associated to the analyte and to the reference; m and m_{ref} are the weighed mass of the analyte and of the internal reference respectively; M and M_{ref} are the molar mass of the analyte and of the internal reference; P_m and P_m^{ref} represent the purity of the analyte and of the internal reference.

Since the composition range of ^{2}H is equal to 500‰ [10], the required accuracy (defined as the contribution of both precision and trueness [11]) for irm-^{2}H NMR is typically equal to 1% which means that the weighed mass and the purity of both compounds have to be known with both a precision and trueness of 1%. Here, precision is defined as *the closeness of agreement between independent test results obtained under stipulated conditions* while trueness is defined as *the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value* [11]. Nowadays, irm-^{2}H NMR is widely used in the authentication of natural products [12,13]. However, its scope of application is limited to small molecules (M < 300 g/mol) because of the small frequency range of ^{2}H (12 ppm, *i.e.* 736 Hz at 400 MHz), leading to signal overlap when larger molecules or complex mixtures are studied. Moreover, the low sensitivity of ^{2}H leads to very large experiment times. In this context, irm-^{13}C has been developed in the 2000 s since ^{13}C nuclei provide: a larger frequency range (250 ppm, *i.e.* 25 kHz at 400 MHz) than ^{2}H, a better sensitivity and because ^{13}C offers additional information about the molecules of interest [14]. At first sight, the internal reference method used to perform irm-^{2}H NMR could have been transposed to irm-^{13}C NMR. However, since the ^{13}C isotope composition range is about 50‰ [10], the required accuracy to perform irm-^{13}C NMR becomes 1‰ (0.1%). As regards sample preparation, both the weighed mass and the purity of the internal chemical reference and of the analyte have to be known with a precision and a trueness of 1‰. Unfortunately, it is very difficult to know the purity of an analyte with such an accuracy, especially in the case of natural extracts. Moreover, weighing a compound with a 1‰ accuracy is not very suitable in the framework of routine analysis. Therefore, up to now, the conventional method to determine *xi* relies on the combination of irm-MS and ^{13}C NMR. The position-specific isotopic abundance *xi* is calculated using Eq. (2):

$$xi = x_b \times \frac{fi}{Fi} \quad (2)$$

Here, x_b represents the bulk isotopic abundance calculated by irm-MS. Then, ^{13}C NMR is used to calculate the reduced molar fraction $f_i = S_i/\sum_i^S$ (where S_i represents peak areas) for each carbon position. F_i is the statistic molar fraction $F_i = p_i/\sum_i^p$ where p_i corresponds to the number of nuclei which are excited at the frequency of position *i*.

In addition to these considerations, irm-^{13}C NMR requires specific acquisition conditions. Usually, irm-^{13}C NMR analyses are performed thanks to an inverse-gated decoupling pulse sequence [15], since it is easier to measure with a good precision and trueness the area of singlets instead of potential multiplets and also because the ^{1}H-decoupled spectrum offers a greater Signal-to-Noise Ratio (SNR). In order to reach the targeted accuracy of 1‰, several studies have been conducted. In 2004, it was demonstrated that a 1‰ accuracy could be reached only if the repetition time *TR* was equal to ten times the longest T_1 (^{13}C) [15] of the analyzed molecule, referred to as $T_{1_{max}}$, in order to get rid of both longitudinal relaxation effects and NOE. Regarding the decoupling scheme, methodological developments were also necessary to avoid a non-uniform ^{1}H decoupling and thus a loss of accuracy. In 2007, it was demonstrated that ^{1}H decoupling using tailored adiabatic pulses allowed to reach a precision and a trueness of 1‰ [16].

The combination of irm-MS and irm-^{13}C NMR, which currently represents the conventional method, has been used to study the isotopic composition of pharmaceutical ingredients [17] with the aim of fighting against pharmaceutical counterfeiting. Another application is the characterization of reaction mechanisms to discriminate biosynthetic pathways [18,19]. It is also used to discriminate the geographic origin of natural products [20] or in forensic environmental investigations to determine the source of a pollution [21]. However this analytical method has a serious drawback as it requires to accurately measure the signals from all the carbons of the analyzed compound, which is not always possible. Indeed, the determination of absolute values of the position-specific isotopic abundance (x_i) by irm-^{13}C NMR is limited to samples whose spectrum do not present peak overlap which thus prevents the analysis of some molecules or complex mixtures. Moreover, multi-pulse sequences based on polarization transfer from ^{1}H to ^{13}C nuclei, such as INEPT [22–25] or HSQC [26], which can greatly improve sensitivity and/or peak separation, cannot be used since the isotopic information given by quaternary carbons is lost, thus preventing the calculation of the reduced molar fraction f_i. The best way to overcome this drawback is the internal reference method. However, as explained previously, this reference method cannot be simply transferred to irm-^{13}C NMR since it is very difficult to measure the concentration ratio of the internal reference over the compound of interest with a 1‰ accuracy using the gravimetric method. An alternative way to determine x_i is to quantify *in situ* the concentration ratio of both compounds by quantitative ^{1}H NMR with a 1‰ accuracy. Therefore, the determination of x_i is independent from the mass and the purity of the analyzed compounds. Such an *in situ* method has already been performed in irm-^{2}H analysis in order to make the sample preparation easier for routine analysis [27]. Position-specific isotopic abundances are thus obtained using Eq. (3):

$$x_i^{comp} = \frac{N^{ref}}{N_i^{comp}} \cdot K \cdot \frac{S_i^{comp}}{S^{ref}} \cdot x_g^{ref} \quad (3)$$

The concentration ratio ($K = \frac{S_{1_H}^{ref} \times n_H^{comp}}{S_{1_H}^{comp} \times n_H^{ref}}$) is here determined by ^{1}H NMR. $S_{1_H}^{ref}$ and $S_{1_H}^{comp}$ are the signal areas of the reference and of a given signal of the analyte respectively, n_H^{comp} is the number of equivalent ^{1}H of the analyte and n_H^{ref} the number of equivalent ^{1}H of the reference. Then, an irm-NMR analysis is performed on the same sample to determine the position-specific isotopic abundance x_i. In irm-NMR, the signal-to-noise ratio (SNR) has a direct impact on the measurement precision of the peak area. Hence, to reach a precision of an order of magnitude of 1‰, a SNR >500 [28] is typically required. In order to reach the required ^{13}C NMR SNR in a realistic time, the analyzed sample should therefore be highly concentrated. Unfortunately, the analysis of highly concentrated samples by ^{1}H NMR induces the radiation damping (RD) [29–31] phenomenon which severely hampers the accuracy of quantitative ^{1}H NMR. The visible consequences of RD on the NMR spectrum are (i) a broadening of the shape of peaks, (ii) a line