^{1}H NMR for the Accurate Quantification of Analytical Reference Standards.

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Introduction

NMR is generally considered to be a relatively insensitive technique. However, the signal intensity is essentially only dependent on the absolute quantity of hydrogen atoms causing the signal and this very feature makes it the technique of choice for certain applications, e.g. for the quantification of minute quantities of standard compounds. The high specificity of NMR signals permits absolute quantifications without interference of impurities present in the sample. Furthermore no specific pure reference compounds are necessary. We here demonstrate the feasibility and limits of NMR application, by employing the analysis of algal toxins and polar substances in butter as examples.

Quantification of Anatoxin-a

A method was developed for the quantification of microgram quantities of compound based on ^{1}H NMR. In this method an exact quantity of pyridine was added to an anatoxin-a solution, and the amount of anatoxin-a in the solution was calculated from the integrals of the ^{1}H NMR signals. **Fig. 1** shows the NMR spectrum of the anatoxin-a and pyridine solution. The signals chosen for integration did not suffer from interference from other signals. It was possible to quantify accurately (±2 %, Table 1) the amount of 22.4 µg of anatoxin-a present in the solution using 1000 scans on a 400 MHz NMR spectrometer. By increasing the number of scans, by using a higher field NMR spectrometer or with special probes (cryoprobes), even much smaller quantities can be quantified accurately by ^{1}H-NMR (up to sub-microgram levels).

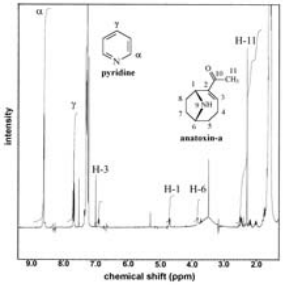

Fig. 1. ^{1}H NMR spectrum of the $CDCl_3$ solution containing pyridine and anatoxin-a. The amount of anatoxin-a was calculated as indicated in **Table 1**.

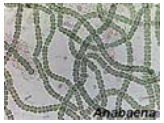

Table 1: Quantification of anatoxin-a by ^{1}H-NMR. Integrals of the signals were divided by the number of protons to calculate the average integral for a single proton. The amount of anatoxin-a was calculated by comparison with the average integral for a single proton of a known amount of pyridine.

1H-NMR (ppm)	no. of protons	integral	
6.90 dd (7.0; 4.4)	1	2.129	**Anatoxin-a**
4.69 d (9.9)	1	2.400	
3.81 m	1	2.098	
2.29 s	3	6.764	(average integral: 2.232 per proton)
8.62 m	2	20.989	**Pyridine**
7.68 m	1	10.634	(average integral: 10.541 per proton)

Quantification of [D-Leu^1]-Microcystine-LR

[D-Leu^1]-microcystin-LR was isolated from *Microcystis aeruginosa* (Schripsema & Dagnino, Magn. Reson. Chem. 2002, 40, 614-17). To determine the accurate quantity the sample was dissolved in MeOH-d4 containing an exactly known quantity of pyridine. By comparison of the integrals of selected signals of the microcystin with signals from pyridine (**Fig. 2**), the accurate quantity was calculated to be 2.95 mg. In this case 100 scans were obtained.

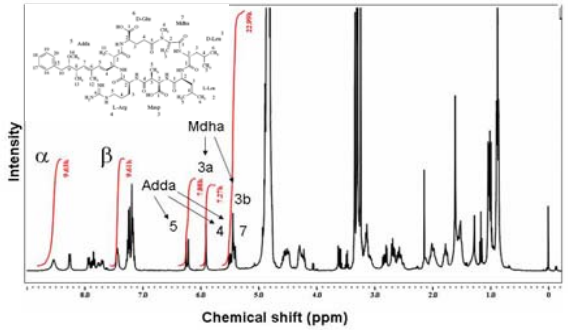

Fig. 2. ^{1}H NMR spectrum of the MeOH-d4 solution containing [D-Leu^1]-microcystin-LR and pyridine. The amount of the microcystin was calculated from the integrals of the indicated signals.

Quantification of polar constituents in butter

Likewise, in a polar extract of butter a series of compounds can be accurately quantified. These include the conservants sorbic acid and benzoic acid, the organic acids lactic, citric, acetic and formic acid and the sugar lactose (**Fig. 3**). For the quantification the D_2O contains an exactly known quantity of internal standard, tetradeuterotrimethylsilylproprionate. The limit of detection of this method is dependent on the compound and varies between 1-10 µg/g of butter.

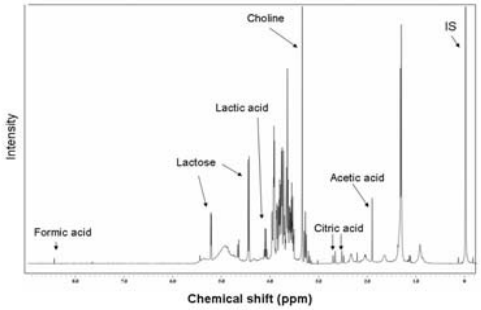

Fig. 3. ^{1}H NMR spectrum of the D_2O extract of butter, showing the presence of formic acid (0.035 mg/g), lactic acid (1.02 mg/g), citric acid (0.44 mg/g), acetic acid (0.16 mg/g) and lactose (12.5 mg/g).

Conclusions

Most compounds can be accurately quantified by ^{1}H NMR spectroscopy using a suitable internal standard. NMR offers a series of unique advantages, which turns it into the method of choice for the accurate quantitation of analytical reference standards.
A major advantage is the fact that there is no need for a specific reference compound in the quantitation. Moreover, quantitation of samples containing impurities is very well possible. Finally, the method is non-destructive, since no sample is consumed during the analysis.

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