

^{13}C SPE MAS measurement of ligand concentration in compressible chromatographic beads

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A method for measuring the ligand concentration in heterogeneous materials like chromatography media is described. In this method, ^{13}C single pulse excitation magic angle spinning NMR experiment with broadband ^1H decoupling is used to determine the peak integrals for a butyl ligand in the spectrum of a dried chromatography medium. Within a carefully controlled protocol, those integrals compared with that of the internal reference compound dimethyl sulfone provide the required volume concentration with an accuracy of ca 2%. The effects of temperature, degree of hydration, and other experimental parameters are discussed. Copyright © 2015 The Authors. *Magnetic Resonance in Chemistry* published by John Wiley & Sons Ltd.

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Introduction

Chromatography is a crucial and indispensable tool in modern biotechnology, where it has a double role of being both a purification and a characterization method of proteins and other biomolecules. In low or ambient pressure liquid chromatography, synthetic or biopolymers in a liquid (called the mobile phase) are separated in columns packed with porous beads (called the stationary phase). The beads are soft, typically 5–200 μm in diameter, and their internal porous network has pore dimensions ranging approximately 10–200 nm. The resins (here and henceforth the medium before chemical coupling of linker and ligand is called resin) of the beads can be based not only on cross-linked agarose (such as here) or dextran but also on other polymeric or inorganic materials. In gel filtration (also known as size exclusion chromatography), the molecules are separated according to differences in their size; molecules that cannot enter the porous network experience a less tortuous flow and pass the column containing the beads faster. In other types of chromatography, the separation is based on having slightly different interactions between different biomolecules and the stationary phase.^[1,2]

Interactions exploited include electrostatic interactions in ion exchange chromatography and hydrophobic interactions in hydrophobic interaction chromatography (HIC). In the corresponding chromatography medium, the pore walls in the beads provide a large surface area at which interactions between molecules in the mobile phase and the medium can be manifested. Typically, the interactions required are mediated by ligands with specific properties that are grafted onto the resin pore wall. In HIC, examples of ligands are butyl, phenyl, and octyl moieties. Besides their chemical nature, the concentration of ligands is the other important factor for the chromatographic performance which then demands suitable analytical methods for determining ligand concentration in chromatography media.

There are several ways to measure ligand concentration such as spectrophotometric, radiolabel, and chemical analysis.^[3–10] They all suffer from different shortcomings including lack of general

applicability, time-consuming protocols, often poor accuracy, and precision. For HIC media, it is often necessary to chemically cleave the ligand from the resin prior to the analysis which prolongs time, increases cost, decreases accuracy and, in addition, requires the use of hazardous chemicals.^[11–15] This creates substantial logistic and other challenges with regard to quality control and limits the applicability of those methods in high-throughput mode.

In this article, we develop a protocol, based on a ^{13}C single pulse excitation (SPE) magic angle spinning (MAS) NMR experiment, for measuring the concentration of butyl ligands in the HIC medium Butyl Sepharose™ High Performance (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Such an NMR approach could complement or altogether replace other analytical methods for ligand concentration measurements. Because of the chemical generality and chemical selectivity of NMR spectroscopy, it should be possible to use this technique for different ligand types (e.g. phenyl, alkyl, and mixed mode), different ligand linking chemistries, and different base matrices. This would make NMR particularly useful for a variety of different needs related to HIC, reversed-phase chromatography, and other more specific affinity media. In liquid samples, quantitative analysis based on high-resolution NMR spectra is a well-established technique with a sub-percent accuracy readily achievable with carefully designed experimental protocols.^[16,17] For solid or semi-solid samples, quantitative measurements are indeed

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possible but are subject to several complications. Apart from ^1H high-resolution magic angle spinning NMR, that with few exceptions^[18] requires highly hydrated samples with well-resolved peaks, the most usual approach relies on detecting the low-abundance ^{13}C nuclei for which MAS and strong radiofrequency decoupling are typically sufficient to suppress the dominant ^{13}C - ^1H dipole-dipole coupling and ^{13}C chemical shift anisotropy.^[19] An obvious advantage of ^{13}C is its high spectral resolution, which means that ^{13}C detection is more generally applicable for quantitative analysis. While typical ^{13}C MAS NMR studies rely on cross-polarization (CP) to increase the signal, this latter approach is less suitable for quantitative studies even if one employs suitable calibration protocols or experiments at different contact times accompanied by model-dependent fitting.^[20–27] In any of those methods, the increased (by CP) precision costs accuracy. The SPE MAS NMR experiment, where the ^{13}C nuclei are excited by a 90° pulse and the signal is acquired under high power ^1H decoupling, preserves accuracy, and must be preferred in analytical applications. Inspired by a previous study, where resin loading in solvent swollen polymer resins for solid-phase organic synthesis was estimated by ^{13}C SPE MAS NMR,^[28] we investigate here if the lower signal-to-noise^[29] remains sufficient or not and if accuracy can be retained in a system with soft and thereby, under MAS conditions, deformable components. We also establish and communicate protocols that may allow one to achieve experimental errors that are assumedly sufficient for a range of materials in this analytical application of solid-state NMR spectroscopy.

Our study also connects to characterization of silica-based chromatography media with solid-state NMR,^[30–47] a field that has been active for many years. Besides investigating the molecular dynamics of components immobilized on silica^[18,32,39–41,44,46,48] or on polymeric resins^[49,50] and the interactions between bound ligands and analytes in the mobile phase,^[46,48,50–53] a few MAS NMR studies concerned the ligand concentration in stationary phases such as chromatography media^[54] or resins for solid-phase organic synthesis.^[28,55,56] Here, we present for the first time quantitative MAS NMR to measure ligand concentration in a chromatography medium based on a soft polysaccharide hydrogel resin.

Experimental

Sample preparation

Preparation of chromatography medium

The Butyl Sepharose High Performance chromatography medium consisting of the porous beads of *ca* 35 μm size was from GE Healthcare. A schematic structure of the butyl ligand coupled to Sepharose High Performance resin is shown in Fig. 1. This commercial product was chosen for evaluation because it has been in use for a long time and thereby is very well characterized, yet it represents a broad class of similar chromatography media that are soft, highly porous, and compressible hydrogels.

The medium was repeatedly washed with distilled water on a glass filter to remove the storage solution, and *ca* 33 volume%

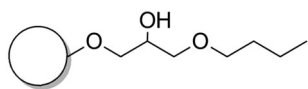


Figure 1. The ligand and linker to the agarose resin in the Butyl Sepharose High Performance chromatography medium.

slurry of beads in distilled water was prepared. Then, a special sample preparation tool manufactured for this purpose (Supporting information, SI) was used to compact a tightly packed bed of wet beads to a well-defined volume of 3.000 (± 0.0026) ml. After having packed the bed, most water was removed by vacuum suction, and the pack was then dried at 105 $^\circ\text{C}$ during 2 h. The dry resin content *R* (mg/ml bead pack) was determined by a weight measurement to be 70.0 (± 0.35) mg/ml. We note here that chromatographic applications typically call for concentrations expressed in $\mu\text{mol/ml}$ unit with the volume referring to that of the bead pack.

The reference materials dimethyl sulfone (DMS; purity 99.73% according to the supplier's certificate of analysis, Sigma-Aldrich) and 3-(trimethylsilyl)propionic-2,2,3,3- D_4 acid sodium salt (TMSP, 98.8% D, chemical purity >99.9% determined by ^1H NMR, Cambridge Isotope Laboratories) were used as received.

Preparation of samples for ligand concentration measurements

Two sample preparation pathways were followed. In the first pathway, the dried bead pack was simply exposed to ambient atmosphere for 2 h. The resulting bead pack was weighed to provide the standard volume weight M_1 (mg/ml bead pack) of the resulting material.

In the second pathway, the dried bead pack was equilibrated for 2 h in an exicator over a saturated solution of potassium nitrate (*pro analysi*, purity $\geq 99.0\%$, Merck) in distilled water corresponding to a the relative humidity (RH) of *ca* 95%.^[57] The moist bead pack was then weighed to provide its standard volume weight M_2 .

The medium such prepared via either of the pathways and the selected internal reference compound were both ground to fine powders and the two powders were mixed at *ca* 10 w% reference; the exact masses of reference and medium in the mixture were noted. The mixture was then carefully mixed and ground, and then tightly compressed to a density of *ca* 700 mg/ml into a MAS sample rotor. Here, we note that B_1 field strength and receptivity varies over the rotor volume.^[58–61] It is therefore essential to get a spatially homogenous mixture of reference and analyte. In addition, our prepared mixtures are somewhat compressible and can therefore compact toward the rotor wall upon fast spinning. Because that may vary by humidity, temperature, and sample handling, electronic signal referencing like that by ERETICTM^[62] (that has been shown to work well for incompressible materials^[58,63]) may have limited applicability here.

Preparation of accuracy test samples

The accuracy was tested by two different samples. In the first sample, TMSP and DMS were ground to fine powders, and the two powders were mixed at *ca* 50 w% each; the exact masses of the two compounds in the mixture were noted. The mixture was carefully mixed and ground and then tightly packed into a MAS sample rotor. The second sample was prepared by the same procedure, but chromatography medium (dried, moisturized at ambient humidity, and ground) was also added to the mixture before the final mixing. The proportions of the different compounds in this sample were *ca* 25 w% medium, 34 w% DMS and 41 w% TMSP.

NMR measurements

All experiments were performed at 75 MHz on a Bruker Avance II spectrometer equipped with a 4 mm broadband cross-polarization magic angle spinning probe. The spin rate was set to 10 kHz that was sufficient to remove spinning side bands. ^{13}C SPE MAS experiments were performed with SPINAL-64^[64] heteronuclear ^1H decoupling with decoupling field strength at 77 kHz and optimized

offset and pulse length during the acquisition. The ^{13}C 90° pulse length was $4.6\ \mu\text{s}$. The acquisition time was 60 ms, and the spectral width was set to 660 ppm.

In the NMR experiments for measuring the ligand concentration, at least 1700 scans were acquired for each sample. The recycle delay was set to at least five times the longitudinal relaxation time, T_1 , for the slowest relaxing signal estimated by inversion recovery. Hence, at 20°C the recycle delay was 4 s (yielding a total experimental time of ca 2 h) while at 80°C , it had to be set to 9 s. In the NMR experiments performed with two reference compounds in order to test accuracy, the number of scans was 4300 and the recycle delay set by TMSP (T_1 ca 4 s).

Exponential apodization by a line broadening factor of 10 Hz was performed prior to Fourier transformation. The ^{13}C background signal was invariant over the experiment and was recorded with empty rotor with a large number (15 000) of scans. The resulting spectrum (Fig. 2) was subtracted from the analyte spectrum before phase correction. Prior to integration, the baseline of the obtained difference spectrum was subjected to automatic baseline correction using a fifth-order polynomial. We note that attempts to remove the effect of the background signal not by reference subtraction but by any form of baseline correction proved to add significant systematic errors. We assume that the primary reason for this is that defining correct (that is, not influenced by the ligand signal) baseline correction reference points in the ligand spectral region (Fig. 2) is difficult or impossible.

The concentration of butyl ligands, c_{butyl} was calculated as

$$c_{\text{butyl}} = \frac{N_{\text{ref}}}{N_{\text{butyl}}} \frac{n_{\text{ref}}}{V_{\text{medium}}} \frac{I_{\text{butyl}}}{I_{\text{ref}}} [\mu\text{mol/ml}] \quad (1)$$

where I_{butyl} and I_{ref} are the peak integrals for butyl and reference signals, respectively, N_{butyl} and N_{ref} are the number of carbon atoms

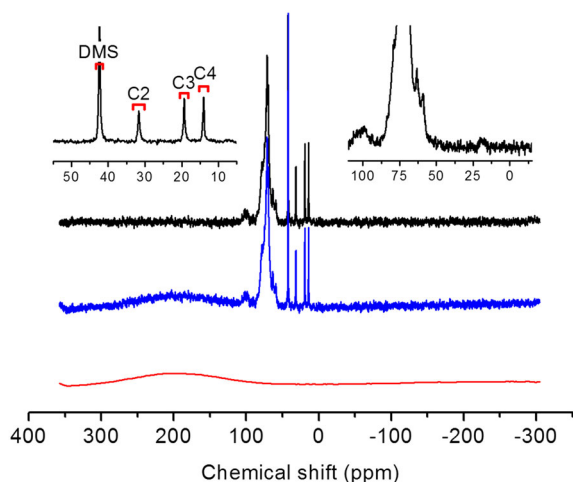


Figure 2. ^{13}C spectra for the medium hydrated at 95% relative humidity with the internal reference DMS. From bottom to top: background spectrum with empty rotor (red), spectrum of medium and DMS (blue), and spectrum of medium and DMS after background subtraction and baseline correction (black). The broad peak in the 60–120 ppm range arises from the agarose resin and also contains the butyl ligand C1 (carbon closest to the ether linker) peak. Left inset: magnification showing the spectral region with the DMS and butyl peaks in the background and baseline-corrected spectrum. The DMS (42.5 ppm), butyl C2 (32 ppm), C3 (19 ppm) and C4 (14 ppm) peaks were integrated in the ranges shown. Right inset: spectrum for resin before ligand attachment. The small peaks between 14–23 ppm are due to moieties on the resin that contribute to the C3/C4 integrals in the ligand-coupled resin.

in the moieties corresponding to the respective signals selected for integration, and n_{ref} is the amount of reference compound (in moles) in the mixture of reference and medium. V_{medium} is the medium volume calculated from the standard volume weight (M_1 or M_2 for the two different sample preparation pathways) and the known mass of moist medium in the mixture. It should be emphasized that n_{ref} and V_{medium} refer to the amount of reference respective the volume of medium in the prepared mixture and not to the amount and volume in the MAS rotor. In passing, it is noted that, if that would be required, the ligand concentration expressed per unit mass of medium can be obtained simply by dividing c_{butyl} with the dry content R . The integration interval was set to ± 3 times the line width at half maximum for each peak.^[65]

Results

Choice of internal reference for ligand concentration measurements

A reference compound must provide a strong and well-resolved peak. In addition, it should exhibit a longitudinal relaxation rate sufficiently high to allow for minimum (that is, set by the analyte) recycle delay at room temperature. For comparison, the T_1 for the observed butyl signals (Fig. 2) at 14, 19, and 32 ppm were 0.83, 0.58, and 0.37 s, respectively. In addition, the reference signal should not be at a widely different chemical shift (in order to avoid first-order phasing) and the compound should be of high purity, easy to process into fine powder and not hygroscopic. Based on these criteria, possible compounds were screened. Substances that provided the right spectral characteristics but a too long T_1 included adamantane ($T_1 = 1.9$ s), L-alanine ($T_1 > 3.5$ s), sodium acetate ($T_1 = 11.4$ s), TMSP ($T_1 = 4$ s) and sodium polystyrenesulfonate ($T_1 > 1.4$ s).

Dimethyl sulfone (DMS) with $T_1 = 0.17$ s, a well-resolved signal (Fig. 2) and with other criteria fulfilled, was selected as our internal reference compound for ligand concentration measurements. In our accuracy tests, the signals of two reference compounds, DMS and TMSP, were explored.

Effect of molecular mobility

^1H broadband decoupling is less efficient in case of slow motions.^[66] To investigate and suppress error arising from this effect, experiments were performed at three different temperatures, 20, 60, and 80°C and in a sample left hydrated at ambient humidity. The background corrected integrals of the various butyl peaks are shown in Table 1.

Within precision, the integrals for C3 and C4 are equal at each temperature and they are also temperature independent. However, the integral for C2 increases with temperature and approaches that

Table 1. Integrals of the C2, C3, and C4 carbons in the butyl ligand at different temperatures, normalized by the integral of the DMS peak

T ($^\circ\text{C}$)	C2 integral	C3 integral	C4 integral
20	0.28 (70%)	0.41	0.39
60	0.33 (82%)	0.40	0.40
80	0.36 (87%)	0.42	0.41

The integral value of C2 is also expressed as the fraction of the average value of the C3/C4 integrals.

of the other two carbons. This demonstrates that the decoupling efficiency is sufficient for C3 and C4, and these signals can thus be used for determination of ligand concentration. Because C2 is situated nearer to the resin-bound end of the ligand, the C–H bond vector in that moiety exhibits slower mobility than that for C3 and C4; this type of behavior has been indicated for other ligands and resins.^[67–71] Increasing temperature leads to faster mobility for the C2 moiety, although not sufficiently fast to enable efficient decoupling even at the highest explored temperature. Hence, we rely on C3 and C4 spectral integrals for our concentration measurement.

Another way to increase molecular mobility is to hydrate the sample. Figure 3 shows the spectra for samples hydrated either at ambient humidity or at 95% RH. The sample with higher moisture content has about half the peak widths compared with the less hydrated sample. More importantly, the spectral integral of the C2 peak is, at 20 °C, 88% of that for the C3 and C4 peaks in the more hydrated sample. Hence, any systematic error arising from spectral overlap and slow motions should be reduced for experiments performed in highly hydrated samples.

In connection, we note that we found far better quantitative performance of ¹³C SPE MAS when working with samples that were (partially) hydrated but not wet. The trivial reason was that resins without liquid water filling the pore space and the interbead volume can be compressed (Sample Preparation), and the increased ligand density for compressed samples dominates the change in signal-to-noise ratio over any spectral or relaxation effect. Moreover, fully hydrated samples exhibit systematic errors presumably caused by spatial re-distribution of medium and reference. Another possible issue with hydrated samples is that the decoupling can be less effective because of molecular motional correlation times with unfavorable values.^[66]

Accuracy and precision of the developed method

The accuracy in solid-state NMR experiments of homogeneous incompressible mixtures packed in MAS rotors has earlier been shown to be very good.^[72,73] In those studies, cross-polarization magic angle spinning experiments were used, and hence, the

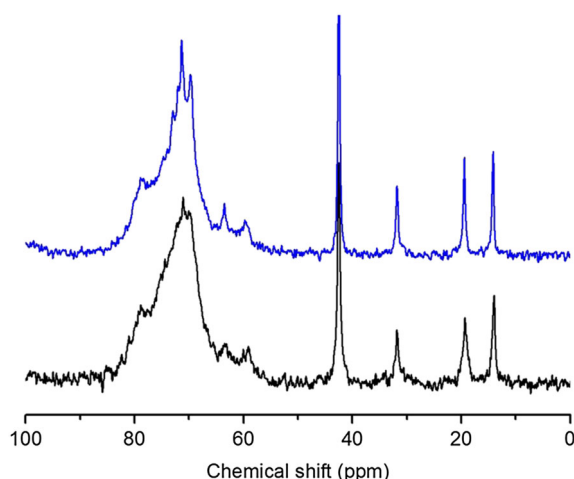


Figure 3. The spectral effect of moisture content variation in the medium. ¹³C spectra of the medium with the internal reference DMS for samples hydrated at ambient humidity (bottom, black) and at RH = 95% RH (top, blue). RH, relative humidity.

Table 2. Reproducibility in determination of butyl ligand volume concentration in the chromatographic medium

Hydration	C3	C4	C3 + C4-Resin ^a
Ambient	60.2±2.2	62.5±4.7	59.3±1.5
RH = 95%	60.8±2.1	60.3±1.9	58.6±1.2

All units are $\mu\text{mol/ml}$ and the error indicates the calculated standard deviation from five individual experiments. Ligand concentrations obtained from the integral intensities of butyl carbons C3 and C4 measured at 20 °C are shown. The last column shows the ligand concentration obtained from the sum of the C3 and C4 integrals, corrected for the signal contribution from the non-functionalized resin. RH, relative humidity.

^aCompounded standard deviation from c_{resin} and the sample-to-sample variation.

accuracy was estimated through calibration curves. Here, we assessed accuracy by weighing in two reference compounds and comparing the obtained peak integrals with the known amounts of those compounds. Measurements were performed on two different samples, the first one a mixture of TMSP and DMS and the second one with added dry chromatography medium (Preparation of Accuracy Test Samples). The accuracy was tested by dividing the ratio of TMSP/DMS integral intensities by the TMSP/DMS ¹³C molar ratio. Hence, the value of that indicator is 1 in case of accurate and precise NMR representation of concentration. The results (with details given in SI) are 0.998 ± 0.006 for the two-component sample and 1.009 ± 0.006 for the three-component sample. In other words, there is no unaccounted spectroscopic source of >1% systematic error.

The reproducibility of the ligand concentration was tested by replicating the analysis procedure five times, both for samples hydrated at ambient humidity and at 95% RH (Table 2) (individual results for the replicas are given in SI). The results are consistent with each other, with slightly better (2%) accuracy and precision for the sample hydrated at RH = 95%.

As shown in Fig. 2 (right inset), there is a small peak in the spectra that (as indicated by spectra recorded in the non-functionalized resin) arises from moieties that are originally in the resin. Being at approximately 18 ppm, this peak contributes to spectral integrals over the C3 and C4 peaks. From experiments performed in the non-functionalized resin, the corresponding spectral intensity contributes to the estimated ligand concentration by about $c_{\text{resin}} = 2 \pm 0.5 \mu\text{mol/ml}$. The final ligand concentration in Table 2 was corrected for this effect.

By integrating regions without signals in the spectrum and estimating the variation in the integrals we found that the final error manifested in Table 2 (see last column) can largely be accounted for by random spectral noise. Hence, the systematic errors are much smaller than the standard variation figures given in Table 2. That also indicates that reproducibility can, if so wished, be further improved by longer signal averaging.

Conclusions

We have presented here a robust method for measuring the ligand concentration in Butyl Sepharose High Performance chromatography media with ¹³C solid-state NMR spectroscopy. This NMR-based concentration determination method has very significant advantages over other available methods currently applied in

chromatography media; increased accuracy is achieved during a typically shorter time (in the order of 7 h including sample preparation) for analysis that, moreover, requires no hazardous chemicals. In addition, the method has general applicability that is in stark contrast to other, previously applied analytical methods that have steps that must be adjusted, if possible at all, to the specific chromatography medium.

A significant part of the protocol presented is not novel even though connecting all required steps must be helpful for others who would like to pursue quantitative NMR-based analysis in heterogeneous media. In general, high accuracy is rare in quantitative solid-state NMR. In particular, quantitative experiments in soft, porous, and compressible hydrogels like the current chromatographic medium present challenges, some detailed previously, additional to those in conventional quantitative NMR studies. We present two novel elements. First, we investigate the effect of those factors, like hydration degree and temperature that influence the molecular dynamics and therefore the NMR parameters here and, presumably, in many other soft matrices. We find that higher molecular mobility improves accuracy and precision. The other novel element applies for those systems, like chromatographic, ion exchange, and certain catalytic ones, where the concentration of certain moieties within a fixed volume (that is, in contrast to mass fraction) is of interest. We expect many forthcoming applications.

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