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Quantitative NMR spectroscopy of biologically active substances and excipients

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Abstract Biologically active ingredients and excipients are the essentials of a drug formulation, such as a tablet, dragee, solution, etc. Quality control of such substances thus plays a pivotal role in the production process of pharmaceutical drugs. Since these agents often exhibit complex structures, consist of multiple components, or lack of a chromophore, traditional means of characterization are often not feasible. Furthermore, substances of small molecular weight or strong polar character generally exhibit poor chromatographic properties, thus, conventional procedures such as high-performance liquid chromatography are often not applicable. Instead, quantitative nuclear magnetic resonance (qNMR) spectroscopy has emerged as an alternative or orthogonal method in drug analysis. In this review, we elaborate on the application of qNMR to three important classes of biological substances, namely polysaccharides, amino acids, and lipids, and demonstrate the benefits of this modern tool in contrast to traditional techniques.

Keywords Quantitative NMR spectroscopy · European pharmacopoeias · Active pharmaceutical ingredients · Heparin · Amino acid · Lipid

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Introduction

The quality control of the components of a drug, i.e., the pharmacologically active pharmaceutical ingredient(s) (API) and the excipients, are of major importance in order to prevent patients from damages. The methods of quality control are regulated in different international pharmacopoeias, the major being the European Pharmacopoeia (PhEur), the US Pharmacopoeia, and the Japanese Pharmacopoeia as well as the International Pharmacopoeia elaborated by the World Health Organization (WHO). All of them are continuously revised in order to keep methods, drugs and excipients updated.

In 1955, the WHO has given the following definition: Pharmacopoeias are "Pharmaceutical norms intended to ensure, within a given political entity the uniformity of quality, nature, composition and concentration of medicines approved by medical representatives. More specifically, these norms are being compulsory for pharmacists by the competent authorities". In other words, a pharmacopoeia is a collection of norms for quality control. In the case of APIs, e.g. the PhEur controls and limits the impurities related to the production process and possible degradation reactions, i.e. so-called related substances (synthesis starting products, by-products, reagents, and degradation products), heavy metals, residual solvents, etc. In the case of excipients which are often a mixture of structurally related compounds, the kind and amount of the components, i.e. the composition of the mixture, is typically characterized.

Related substances of APIs are mostly controlled by high-performance liquid chromatography (HPLC) connected to an UV-detector, and in a decreasing number of cases by thin layer chromatography (TLC) or other chromatographic methods. Even though, quantitative nuclear magnetic resonance (qNMR) spectroscopy can be

considered as a *primary ratio method of measurement* [1, 2], it is rarely applied in all international pharmacopoeias.

Since the ratio of substances in a mixture can be determined directly from the nuclear magnetic resonance (NMR) measurement *without* referencing to another substance and the absolute amount of substances can be determined by using simple reference substances, qNMR can be principally applied to different fields of quality control:

1. Identification of an API or an excipient and its related impurities.
2. Quantification of the content of an API (assay) and impurities in presence of the major component (i.e. impurity profiling, related substances).
3. Characterization of the composition of a multi-component API or excipient.
4. Evaluation of the content of residual solvents.
5. Determination of the isomeric composition of APIs, i.e. the enantiomeric excess (using a chiral additive) or the ratio of diastereomers.

Innumerable qNMR examples for the aforementioned fields are described in the literature (for reviews see ref. [3, 4]). As can be seen from Table 1, the PhEur makes mostly use of NMR spectroscopy for identification purposes. Beside the NMR identification of single drugs, the PhEur is going to introduce a general chapter (PhEur 2.2.64) on "peptide identification" by means of both ^{1}H and ^{13}C NMR spectroscopy [5]. Here, the identity of small peptides with up to 15 amino acids, such as buserelin, goserelin, protirelin, and gonadorelin, can be confirmed by their typical fingerprint. The general chapter will give the acquisition conditions, sample size, sample preparation as well as spectral key factors. However, the NMR spectra are mostly used as the IR spectra for identification and therefore they will be compared with the spectrum of a reference substance of a defined quality. Thus, occurring additional impurities or impurities of a higher content may be likely visible to a certain extent and thus, concomitantly limited. In this context, the laboratories of the PhEur (i.e. the European Directorate for the Quality or Medicines and HealthCare (EDQM)) apply qNMR for the establishment of reference substances since a couple of years, beside mass spectrometry and the typical corresponding separation techniques used in the corresponding monographs.

Interestingly, since vaccines, especially conjugated vaccines, can easily be identified and distinguished from each other by NMR spectroscopy, a couple of monographs already consist of an NMR identification (see Table 1); additional monographs employing a similar NMR method are in preparation [6]. Furthermore, qNMR is highly appropriate to characterize the composition of excipients (see Table 1).

In this review the power of qNMR will be demonstrated by means of three different biologically relevant groups or compounds, i.e. heparin and its derivatives, amino acids, and lipids, belonging to both APIs and excipients.

A guided approach to qNMR spectroscopy

A prerequisite for quantitative NMR spectroscopy are clearly separated signals which can unambiguously be assigned. By utilizing supplementary one- (1D) and two-dimensional (2D) NMR experiments, it is possible to assure that the resonances to be integrated do not interfere or superimpose with those of either the analyte itself or potential related substances, i.e. impurities or the internal standard utilized. Using qNMR spectroscopy it is sufficient to find, out of the multitude of NMR signals, a single baseline separated resonance for each component of interest. Besides the application of elaborative pulse sequences and line shape fitting techniques [7] an optimal separation of overlapping resonances can be achieved by changing the kind of solvent [8–14] or the pH value of the test solution [15–19], by applying different sample concentrations [20–22], or by adding auxiliary reagents such as cyclodextrins [23–25] or lanthanide shift reagents [23, 26]. In some cases, the temperature also affects the signal separation [27, 28]. Additionally, NMR spectrometers operating at higher magnetic field strength can be advantageous for signal separation due to an enhanced resolution and increased chemical shift dispersion [29]. An overall discussion of these parameters can be found in the literature [30, 31].

The implementation of quantitative experiments by means of NMR spectroscopy is quite uncomplicated. However, to achieve accurate and reproducible results through integration-based determination certain key factors concerning both the acquisition parameter settings and the post-acquisition processing have to be considered and carefully adjusted. All crucial parameters which have to be taken into account for qNMR experiments are discussed in detail in many papers and NMR textbooks [30, 32–34]; thus, in this paper these decisive parameters are only summarized and briefly commented in Table 2.

In NMR spectroscopy, the intensity of a signal (I) is directly proportional to the number of nuclei (N) being observed and the quantitative information can be obtained via signal integration from the corresponding NMR spectrum (see Eq. 1).

$$I_A = k_S \cdot N_A \tag{1}$$

Under well-defined experimental conditions the spectrometer constant k_S remains constant for all signals within a NMR spectrum.

Table 1 The application of NMR spectroscopy in monographs of International Pharmacopoeias

Identification Test	
Tobramycin, Buserelin, Haemophilus type B conjugate vaccine, Meningococcal group C conjugate vaccine, pneumococcal polysaccharide conjugate vaccine, heparin sodium and calcium, amyl nitrite, hydrocortisone sodium phosphate	^{1}H NMR
Goserelin, low molecular weight heparins, Salmon oil farmed, cod-liver oil farmed	^{13}C NMR
Purity control	
Poloxamer	Ratio of oxypropylene/oxyethylene
Hydroxypropylbetadex	Molar substitution
Lauromacrogol 400	Average chain length of the fatty alcohol and average number of moles of ethylene oxide
Medronic acid for radiopharmaceutical preparation	Purity control: impurity A/B
Tetra-*O*-acetyl-mannose triflate for radiopharmaceutical preparation	Purity control: impurity B
Cod-liver oil farmed	Positional distribution (β(2)-acyl) of fatty acids
Orphenadrine citrate	Ratio of meta/para isomers
Assay	
Amyl nitrite	Internal standard method (benzyl benzoate)
Salmon oil farmed	Positional distribution (β(2)-acyl) of fatty acids

Table 2 Guided approach to quantitative ^{1}H NMR spectroscopy for the choice of optimal acquisition and processing parameter settings

Parameter	Suggested value	Comments
Acquisition		
Pulse width (flip angle)	Between 30° and 90°	90° pulses result in maximum signal intensity; in practice, pulse widths of less than 90° are often used, in order to acquire good qNMR data in a reasonable time
Acquisition time		The duration is related to the spectral width and the required digital resolution (≤ 0.25 Hz)
Relaxation delay	30° pulse: at least 3 times the longest T_1 90° pulse: at least 5 times the longest T_1	Necessary to ensure full relaxation of excited nuclei and to avoid distortion of integrated signal intensity
Spectral width	Sample spectral window±2 ppm on each end	
Transmitter offset	Centre of the spectral width	
Time domain	64 k	Should be zero-filled by a factor of two
Number of scans	Depends on sample concentration, desired S/N ratio and LOQ to be achieved	Quantitative determination with accuracy and precision significant better than 1% with S/N≥250:1 for ^{1}H NMR
Dummy scans	Variable; generally 2–8 dummy scans	
Receiver gain	Closely below the highest possible setting	Automatically set by NMR spectrometer
Spin rotation	Non-spinning	Elimination of residual spinning sidebands
^{13}C satellites	^{13}C decoupling pulse sequences, i.e. Waltz-16, GARP	Removal of ^{13}C satellites which interfere with signals of interest
Processing		
Exponential multiplication	lb=0.3 Hz (^{1}H NMR)	
Zero-filling	Generally by a factor of two	Improves digital resolution
Phasing	Manual	Manual phasing is preferred over automatic phase correction routines to obtain spectra with minimal distortion
Baseline correction	Automatic; polynomial *n*th order	If applicable, manual optimization over entire spectral range or integrated regions
Integration limits	64×FWHM Inclusion or exclusion of ^{13}C satellites for both the analyte and the internal standard	Integration of more than 99% of the entire signal intensity

Relative qNMR method

Quantitative results can be easily obtained using the so-called relative method, i.e. the calculation of the ratio of two compounds using peak areas. In particular, this normalization procedure is suitable for the determination of the quantitative composition of multi-component drugs [8] and isomers [25, 35, 36], i.e. diastereomers and enantiomers. In this case, the sample preparation is very simple and fast; the analyte has to be weighed and dissolved in an adequate deuterated solvent only, without knowledge of the molecular weight of the analyzed components and the definite weighted sample, and no expensive chemical reference substances are required.

Internal standard method

For the determination of absolute amounts of major component(s) or related substances a reference material of definite purity which is unrelated to the target analyte has to be added to the analyte solution and measured simultaneously; this procedure is called internal standard method. In this case the ratio of peak areas (I) of the analyte and the reference substance (IS) can directly be used to calculate contents under consideration of the molecular weights (M), the weights (m), the number of nuclei evoking the integrated resonances (N) of the analyte and the standard, and the purity of the standard (P_{IS}), respectively.

$$P_{analyte} = \frac{I_{analyte}}{I_{IS}} \cdot \frac{N_{IS}}{N_{analyte}} \cdot \frac{M_{analyte}}{M_{IS}} \cdot \frac{m_{IS}}{m_{analyte}} \cdot P_{IS} \quad (2)$$

The uncertainty of the internal standard method depends on (a) the purity of the reference material, (b) the uncertainties in the weights of standard and sample, and (c) the uncertainties in the signal areas which will be considerably affected by the operator precision of phasing, baseline correction and integration [37, 38].

Purity of the internal standard In order to get quantitative results with a high accuracy it is important to know the current purity of the applied internal standard. A study of the Swedish medical products agency pointed out, that after 3 years storage in a dry environment and in their original containers with tightly closed caps lower contents of frequently used reference substances were obtained than at the time of delivery [39]; thus, it is necessary to verify the purity of these compounds in routine work on a regular basis.

Weighted sample In order to decrease the contributions to the uncertainty of sample preparation greater masses of the analyte and the internal standard should be weighed or the use of stock solutions is recommended, even though an additional preparation/dilution step is necessary then [38]. The internal standard should be easily weighable; therefore, solid compounds are preferred, because liquid compounds like 2,5-dimethylfuran or 2,4,5-trichloropyrimidine require a careful handling during the weighing process. The ideal molecular weight of an internal standard should be similar to the analyte's, because when utilizing small molecular weight compounds, such as sodium acetate and dimethylsulfone, only small amounts of the reference substance has to be weighed to ensure that the NMR signals of both the analyte and the internal standard will be of similar intensity.

Processing parameters To simplify the integration procedure sharp signals, mainly a singlet, are preferred; in turn, the integration of broad resonances, i.e. carboxylic acid or hydroxyl protons, should be avoided. In order to ensure that more than 99% of the peak area has been measured an integration interval of 32 times the signal full width at half height on both sides of the resonance is recommended [40]. In many cases, it is not possible to meet these integral limits due to complex NMR spectra. However, in order to obtain reliable results, a meaningful integration procedure should involve integration intervals of equal relative size for analyte and internal standard. Appropriate standardized integration protocols of complex ^{1}H NMR spectra are currently missing in literature.

Internal standard substances According to many papers a suitable internal standard should meet the following criteria: (1) readily available in a highly pure form, (2) inexpensive, (3) stable and chemical inert, (4) nonvolatile and nonhygroscopic, (5) soluble in most of the NMR solvents, (6) simple ^{1}H NMR spectra are preferred, (7) easily weighable, and (8) optimal molecular weight [32, 34, 39]; the spin–lattice relaxation time is usually disregarded, even though the measurement time of a ^{1}H NMR experiment depends on the longest T_1 relaxation time of resonances of interest. In order to avoid distortion of integrated signal intensity due to relaxation effects, it is essential to delay the application of the next pulse by five times the longest T_1 relaxation time (90° pulse) to ensure full relaxation of the excited nuclei (99.3% of the equilibrium magnetization is measured after $5\times T_1$ [41]). The experiment time may increase considerably when the T_1 relaxation time of the internal standard deviates from the spin–lattice relaxation time of the analyte (T_1(analyte)<<T_1(IS)). The internal standard compounds reported in the literature [32, 39] exhibit simple ^{1}H NMR spectra, but the T_1 relaxation times are in the range up to approximately 10 s, which will cause very long measurement times. For example, maleic acid is one of the most widely used reference compounds in qNMR [32, 42–44], even though its large T_1 relaxation time (up to 9 s) excludes a short measurement time [30]. In contrast, our

group favours internal standards with a short T_1 relaxation time containing several signals in the NMR spectrum such as ethyl-4-(dimethylamino) benzoate, thymol, and nicotine amide, which are qualified for routine work because these compounds enable short measurement times [8].

One hundred percent method

A second method, which is also based on the comparison of peak areas in the NMR spectrum, is the so-called 100% method. This method has the advantage that the major component can be quantified without the use of a reference substance and without the necessity to weigh the sample. All organic impurities present in the sample are quantified by ^{1}H NMR spectroscopy relative to the target analyte and the sum of the organic and non-organic impurities such as inorganic salts, moisture, and ash (determined separately) are subtracted from 100% to establish the purity of the active ingredient. A prerequisite for this method are clearly separated signals of all potential impurities which can be reliably interpreted. The primary method is qualified for the quantitative analysis of fairly simple high-purity compounds which have only few signals in their ^{1}H NMR spectra. For example, the 100% method is an excellent method for the quantitative analysis of commercially available sodium acetate [45]. However, the quantitative results of this method do often not comply with corresponding results of the internal standard method. When more complex samples are analyzed, the identification and quantification of related substances becomes more difficult.

Standard addition method

Another possibility to quantify the content of a major component and/or related substances is the so-called standard addition method [46]. Known amounts of the pure target analyte or the isolated impurity are gradually spiked to the sample analyzed. In standard addition one plots the intensity of a certain baseline separated resonance against the amount added to the sample. From the regression line $y = mx + n$ one can calculate the initial amount of the analyte and/or the impurity. By dividing the intercept n by the slope m the total amount of the component can be calculated, independently whether the component is a defined chemical substance or a complex mixture. For example, the percentage of dermatan sulfate in a heparin sample can be calculated accurately by adding known amounts of a dermatan sulfate reference standard (with a defined content) to the heparin sample analyzed [28]. Utilizing the increasing *N*-acetyl signal at $\delta = 2.08$ ppm, a linear regression ($y = 1.0832x + 4.4586$) enables the calculation of the initial amount of dermatan sulfate (DS = 4.1% (*w*/*w*)) in the heparin sample (Fig. 1).

External standard method

When the contamination of the sample by the internal standard utilized has to be avoided, the application of an external standard is favoured. Either one can make use of a special NMR tube containing a sealed capillary, filled with the dissolved standard substance or two separate NMR tubes, one filled with the analyte and the other with the standard solution can be employed. The analyte and the external standard should be dissolved in the same solvent and the volume of each tube has to be calibrated previously to the NMR measurement. The external standard method applying an extra-capillary is, e.g. commonly used to determine the water content of solvents [34]. An alternative to the aforementioned external standard methods is a new technique which is called ERETIC (electronic reference to access in vivo concentrations) where an electronic reference signal, which can be shifted to a signal free region in the NMR spectrum by the operator, is used for quantitative purposes [47, 48].

Application to unfractionated heparin

Heparin is a complex mixture of highly sulfated glycosaminoglycans containing a disaccharide repeat unit of uronic acid linked to glucosamine which is widely used as an anticoagulant agent in the prevention and initial treatment of thrombosis as well as haemodialysis. In 2008, an increased number of serious side effects after intravenous application of heparin has been reported, mainly concerning anaphylactoid reactions caused by contaminated batches of the anticoagulant [49]. Using two-dimensional NMR techniques such as heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple-bond correlation experiments the contaminant was identified as oversulfated chondroitin sulfate (OSCS) [50]. In addition, considerable amounts of dermatan sulfate (DS) were found in heparin active pharmaceutical ingredients (API) and final heparin products; among other related glycosaminoglycans including chondroitin sulfate (CS), heparan sulfate (HS), and hyaluronic acid (HA), DS is a native impurity of heparin and its content is an indicator of the quality of the purification process of crude heparin. Until then, contaminants like OSCS could not be identified using conventional screening tests described in various pharmacopoeias. Therefore, new methods such as NMR spectroscopy [28, 51–54], capillary electrophoresis [55–57], strong-anion-exchange high-performance liquid chromatography [58], infrared (IR), near-infrared reflectance (NIR), and Raman spectroscopy [59–61], one-dimensional

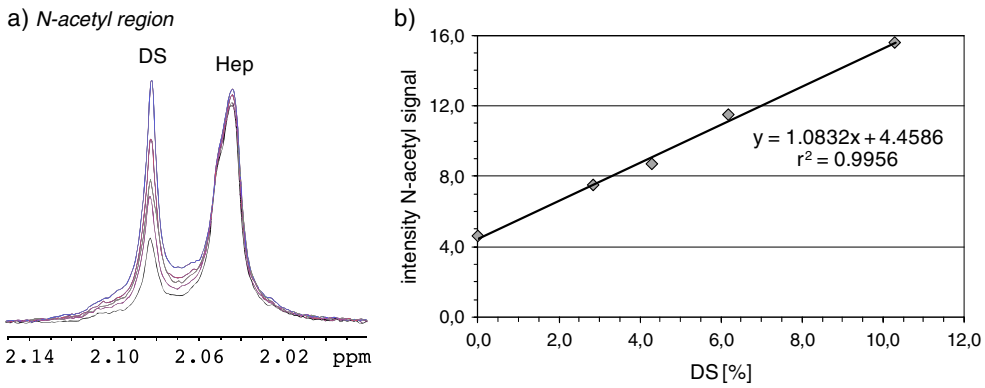

Fig. 1 **a** *N*-acetyl region of the ^{1}H NMR spectrum (300 MHz, 353 K, D_2O) of heparin for different amounts of DS (2%, 4%, 6%, and 10% (*w*/*w*)), **b** regression analysis of the initial amount of DS

cellulose acetate plate electrophoresis and polyacrylamide gel electrophoresis [62, 63], potentiometric polyanion sensors [64], and anticoagulant time assays [65–67] were developed and partly included in the international pharmacopoeias for identification and quantification purposes of potential contaminants in heparin.

Identification of potential contaminants in unfractionated heparin

With the ad hoc revision of the monographs "heparin sodium" and "heparin calcium" in the European Pharmacopoeia in June 2008, ^{1}H NMR spectroscopy was implemented as mandatory identity test which simultaneously identifies the heparin sample and evaluates its quality. This technique provides simple differentiation between heparin and its impurities OSCS, DS, and chondroitin sulfate CS A/C by analyzing the chemical shifts of the *N*-acetyl resonances. The corresponding signals in the ^{1}H NMR spectrum appear at δ=2.05 ppm for heparin sodium, δ=2.04 ppm for CS A/C, δ=2.08 ppm for DS, and δ=2.15 ppm for OSCS, respectively (Fig. 2). Slight variations of the exact positions (approximately ±0.01 ppm) can be observed in mixtures of these four components due to the anisotropy induced by sulfation. The fingerprint region provides a second distinguishing feature for the identification of drug impurities especially when operating at higher temperatures, e.g. at 315 K [28]. However, low concentrations of contaminants can only be detected via the *N*-acetyl signal with a limit of detection of approximately 0.1% for OSCS and 0.5% for DS, respectively [28, 51].

In addition, NMR spectroscopy is a powerful tool for the identification and quantification of additional impurities, e.g. components originated from the extraction and purification processes of heparin [68]. Preparation by-products such as ethanol, sodium acetate, and acetone are the most common impurities identified in heparin preparations by ^{1}H and ^{13}C NMR spectroscopy [68, 72]. Additionally residual solvents like methanol, formic acid [68], and butanol (δ= 0.91, 1.35, 1.53, and 3.61 ppm) [69] as well as the chelating agent ethylenediaminetetraacetic acid (EDTA) (δ~3.5 ppm) [70] were found in heparin samples. These contaminants are easy to detect and quantify because most of their characteristic resonances are outside the heparin regions (Fig. 3). The quantitative analysis of the potential by-products in 145 heparin batches analyzed was carried out

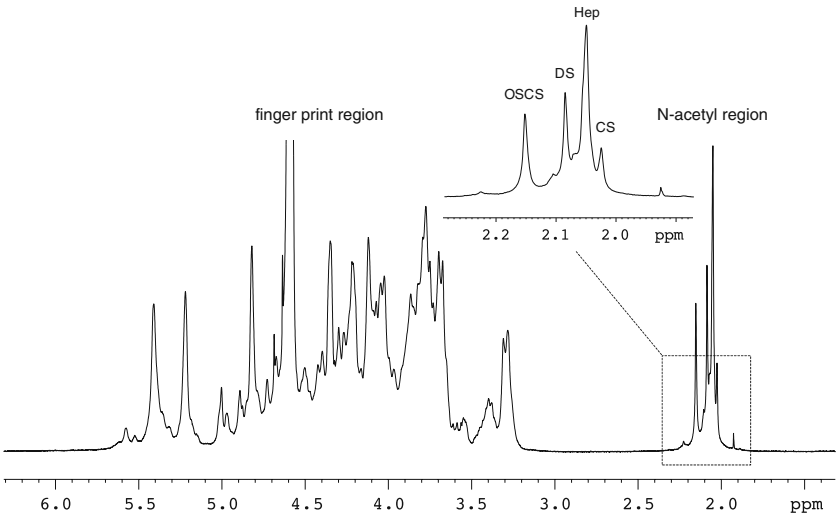

Fig. 2 ^{1}H NMR spectrum (400 MHz, 315 K, D_2O) of heparin (*Hep*) containing oversulfated chondroitin sulfate (*OSCS*), dermatan sulfate (*DS*), and chondroitin sulfate A/C (*CS*)

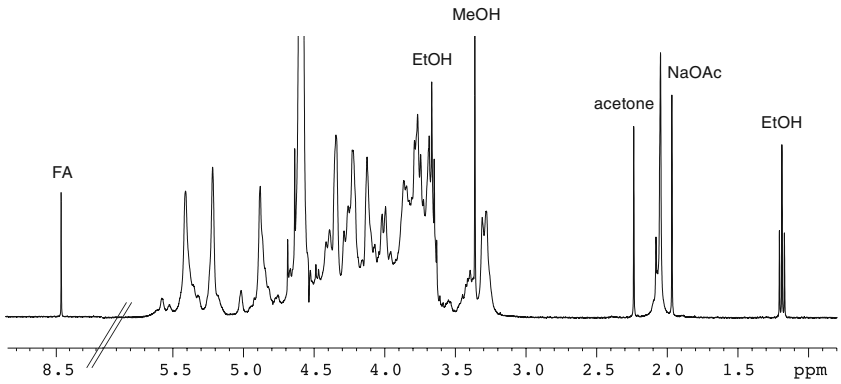

Fig. 3 ^{1}H NMR spectrum (400 MHz, 315 K, D_2O) of heparin containing preparation by-products ethanol (*EtOH*), sodium acetate (*NaOAc*), acetone, methanol (*MeOH*), and formic acid (*FA*)

using the internal standard method; the contaminants were found in strongly varying amounts [68]. For the identification and quantification of traces of contaminants, the application of ^{1}H NMR spectroscopy is preferable to ^{13}C NMR spectroscopy due to its higher sensitivity.

Determination of origin and composition of unfractionated heparin salts

In accordance with the monograph in the PhEur heparin preparations are sodium and calcium salts of a sulfated glycosaminoglycan present in mammalian tissues. Heparin can be prepared either from the lungs of cattle or from the intestinal mucosae of pigs, cattle, or sheep [71]. However, most of the pharmaceutical heparin products available on the European market are heparin sodium salts extracted from porcine intestinal mucosa [70]. Using monodimensional techniques such as ^{1}H and ^{13}C NMR spectroscopy a differentiation between both heparin sodium and calcium as well as heparin of porcine and bovine origin is possible [72].

The fingerprint region of the ^{1}H NMR spectrum of heparin calcium mainly exhibits significant differences compared with that of heparin sodium [73]; the resonances of the protons H1 and H5 of the iduronic acid (Id) residues are remarkably downfield shifted (Fig. 4).

Due to different sulfation patterns in heparin samples extracted from pig or cattle tissue, heparin preparations with different origin can easily be distinguished by means of ^{1}H and ^{13}C NMR spectroscopy. By integration of typical signals in the corresponding spectra a quantitative determination of the level of sulfation at different positions of the uronic acid and the glucosamine residues is possible [72]. Significant differences were found between porcine and bovine heparin samples for example in the degree of sulfation in position 3 and 6 of the glucosamine moiety [72]. The level of *N*-acetylation of the glucosamine residues affords a further possibility to distinguish between bovine and porcine heparin preparations [72, 74]. Whereas in the latter roughly one out of five nitrogen atoms is acetylated, heparin from cattle contains fewer *N*-acetylated glucosamine moieties as is shown by the presence of only a minor *N*-acetyl signal at δ=2.05 ppm in the ^{1}H NMR spectra compared with the corresponding signal in porcine heparin (Fig. 5).

Cation influence on chemical shift and line shape

The chemical shift of the *N*-acetyl signal of OSCS depends on the counter ion present in heparin and its concentration. Whereas the characteristic *N*-acetyl signal of OSCS in heparin sodium appears at δ=2.15±0.01 ppm in the ^{1}H NMR spectrum, the corresponding resonance is downfield shifted to δ=2.18±0.01 ppm in heparin calcium (Fig. 6).

By increasing the concentration of Ca^{2+} gradually the chemical shift of the N-acetyl signal of pure OSCS moves linearly downfield from δ=2.13 ppm to 2.18 ppm until a "saturation point" is reached; here each of the four sulfate groups in the OSCS disaccharide unit has formed a complex with a single Ca^{2+} ion [75]. The downfield effect can be explained by charge withdrawing from the carbonyl and *N*-acetyl groups or conformational changes in the polysaccharide.

By using a 600 MHz NMR spectrometer the detection of low amounts of OSCS in heparin sodium will be handicapped due to the interference of the *N*-acetyl signal of OSCS with the ^{13}C satellite of the corresponding heparin resonance. However, the addition of Ca^{2+} to the heparin sodium sample (~2 mg $CaCl\times H_2O$/10 mg heparin sodium) can avoid this signal overlap as a consequence of the aforementioned downfield shift [75]. Alternatively, after removal of the ^{13}C satellites by using ^{13}C decoupling pulse sequences an unambiguous identification of OSCS is possible.

For the purity test of heparin calcium or low molecular weight heparin containing Ca^{2+} such as nadroparin and calciparin the downfield shift of the *N*-acetyl signal of OSCS to δ=2.18 ppm has to be regarded. However, it has to be kept in mind that slight variations in the chemical shift of the OSCS signal can be observed depending on the level

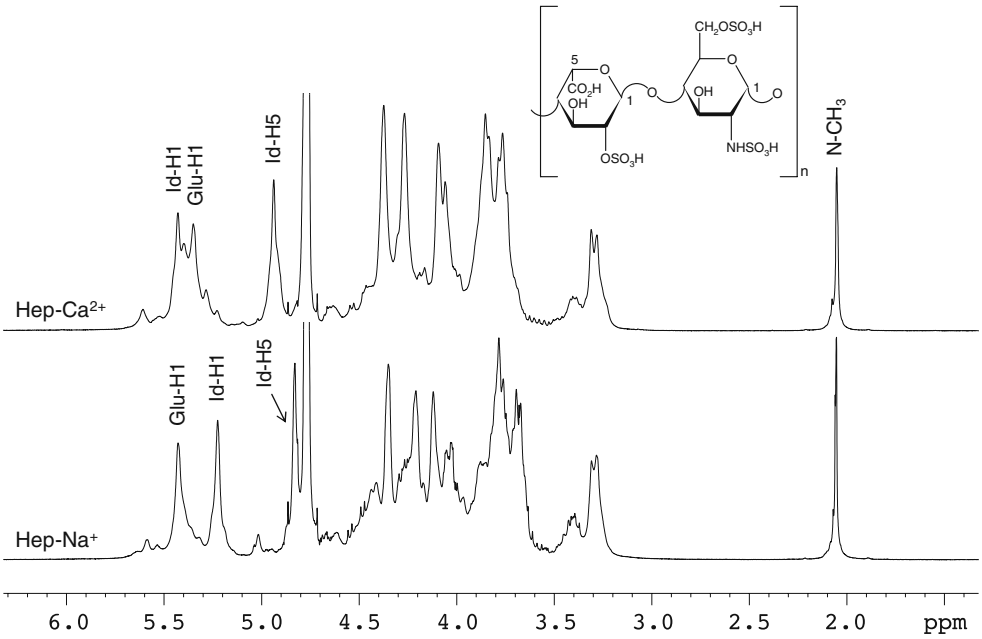

Fig. 4 ^{1}H NMR spectra (400 MHz, 300 K, D_2O) of heparin sodium (*Hep-Na*$^{+}$) and heparin calcium (*Hep-Ca*$^{2+}$). The spectra are best distinguished by the signals of glucosamine (*Glu-H1*) and iduronic acid (*Id-H1, Id-H5*)

of complexation with Ca^{2+}. When analyzing heparin calcium samples with high amounts of OSCS not all of the sulfate groups can be coordinated to the counter ion because the so-called saturation point is not reached yet; therefore, the methyl resonance of OSCS in heparin calcium can be consistent with the chemical shift in heparin sodium [75]. Additionally, a NMR signal appearing at δ= 2.18 ppm in a heparin sample does not necessarily indicate that the corresponding batch is contaminated by OSCS. In some unfractionated heparin batches, a signal at δ= 2.18 ppm appeared in the NMR spectrum which could not be assigned to OSCS by spiking experiments (Fig. 7). This unknown impurity was identified by the company Sanofi-Aventis as an *O*-acetylated heparin derivative which is a by-product of the treatment of bleaching with peracetic acid [76].

In the presence of small amounts of metal ions such as manganese (Mn^{2+}), actually below the maximum allowable concentration levels prescribed in the PhEur [77], a broadening effect of some signals of heparin and OSCS in the ^{1}H NMR spectrum can be observed which is due to paramagnetic relaxation mechanism reducing the relaxation time of the observed nuclei; thus, the shorter the relaxation time, the broader the signals. The heparin signals H1 and H5 of the iduronic acid moiety, which appear in the ^{1}H NMR spectrum at δ=5.22 ppm and δ=4.82 ppm, are mostly affected by the contamination of Mn^{2+} [70, 78]; an indicator for this contamination is a distinctive decrease of

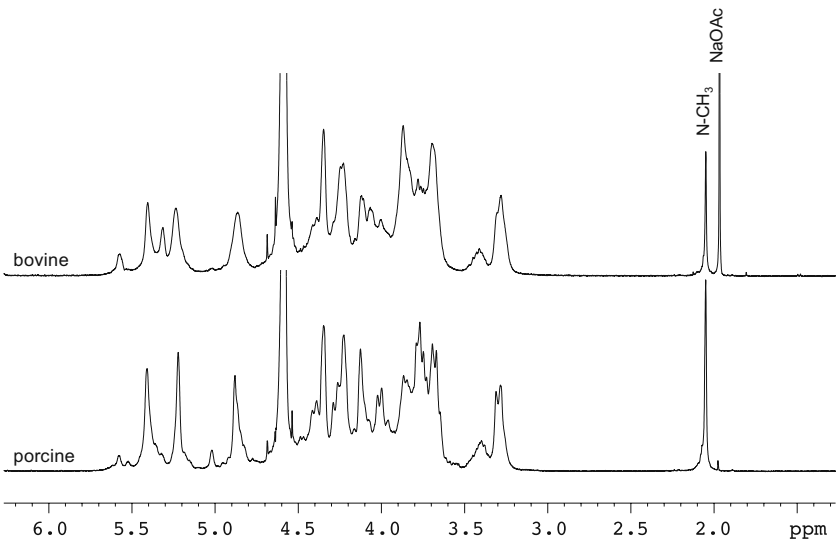

Fig. 5 ^{1}H NMR spectra (400 MHz, 315 K, D_2O) of porcine mucosal heparin and bovine mucosal heparin

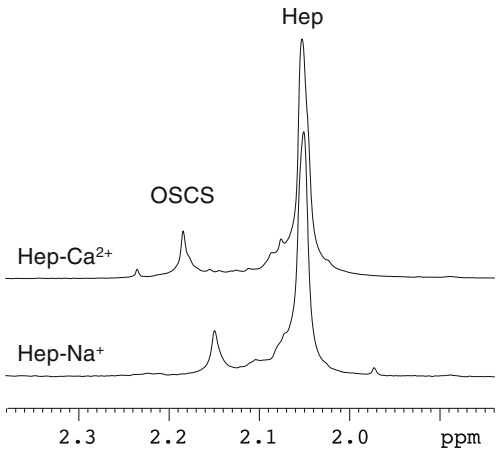

Fig. 6 *N*-acetyl region of the ^{1}H NMR spectra (400 MHz, 300 K, D_2O) of heparin sodium (*Hep-Na*$^{+}$) and heparin calcium (*Hep-Ca*$^{2+}$) contaminated with OSCS

the signal height of the corresponding H1 signal (δ= 5.22 ppm) compared with the adjacent H1 resonance of the glucosamine (Glu) moiety at δ=5.42 ppm (Fig. 8). The *N*-acetyl signal of OSCS which is used for its identification and quantification in heparin samples is broadening owing to the presence of Mn^{2+} (Fig. 8). Therefore, small amounts of OSCS cannot be detected by means of NMR spectroscopy because the broad OSCS signal overlaps with the large methyl resonance of heparin; thus the quantitative analysis of OSCS using the signal heights provides incorrect results.

To obtain "normal looking" ^{1}H NMR spectra small amounts of chelating agents such as EDTA have to be added to the heparin sample [70]. EDTA is used in the NMR analysis of glycosaminoglycans for a long time in order to complex possible traces of metal or bivalent ions, because these ions can modify both the line width and the chemical shift of signals. To avoid the interactions between

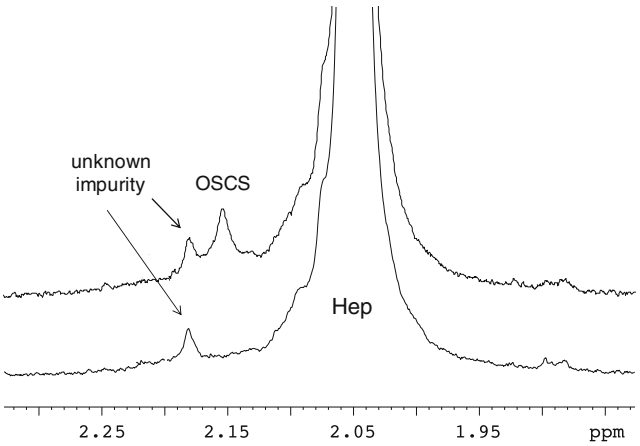

Fig. 7 *N*-acetyl region of the ^{1}H NMR spectrum (400 MHz, 300 K, D_2O) of heparin containing an unknown impurity at 2.18 ppm, shown to be different from OSCS by a corresponding spiking experiment

Mn^{2+} and the negatively charged sulfate groups in polysaccharides the addition of 100 μg up to 1,000 μg EDTA per gramme heparin (depending on the amount of Mn^{2+} present) is recommended [78]. In the PhEur the addition of 12 μg/ml EDTA sodium to the heparin test solution is prescribed when the signal at δ=5.22 ppm (Id-H1) is smaller than 80% of the signal at δ=5.44 ppm (Glu-H1) [71]. However, a priori addition of EDTA is inadvisable because small signals caused by EDTA could pretend potential impurities. The presence of other metal ions such as iron (Fe^{3+}) and zinc (Zn^{2+}) does not affect the signals of heparin and OSCS, and metal ions like copper (Cu^{2+}) and cobalt (Co^{2+}) only affects resonances of heparin but not the N-acetyl signal of OSCS [78]. Additionally EDTA itself may be a potential contaminant in heparin preparations [79] because it is used for the removal of heavy metal ions which could originate from permanganate oxidation [70].

Temperature dependence of contaminant detection in the fingerprint region

^{1}H NMR spectra of heparin measured at room temperature show a large signal of the applied solvent D_2O resonating at approximately δ=4.8 ppm in the middle of the heparin fingerprint region. However, characteristic signals of OSCS and DS appearing in this heparin-free region at about δ= 4.8 ppm are hidden by the water resonance. Due to the temperature dependence of the HOD signal [29], this resonance can be upfield shifted relative to the other signals of the polysaccharides from δ=4.8 to 4.2 ppm by increasing the temperature from 300 K up to 353 K (Fig. 9). In this case, the fingerprint provides a second distinguishing feature for the identification of both OSCS and DS as well as other potential contaminants which may not be identified in the aforementioned region of the ^{1}H NMR spectrum due to interference with the water resonance.

^{13}C NMR and 2D NMR experiments

One-dimensional ^{1}H NMR spectroscopy is a powerful technique which allows for identification and quantification of a variety of potential sulfated polysaccharide contaminants by focusing on the *N*-acetyl region. However, ^{1}H NMR spectroscopy does not permit to identify impurities whose signals are superimposed on those of heparin, unless of a high level of contamination is present. For example, the proton NMR method might fail to detect both non-*N*-acetylated contaminants such as polysulfated alginate or *N*-sulfated heparinoids and *N*-acetylated impurities like heparan sulfate and hyaluronic acid whose characteristic methyl resonances exhibit similar chemical shifts to that of heparin [74, 80]. Therefore, the application of ^{13}C NMR spectroscopy or 2D NMR techniques such as HSQC

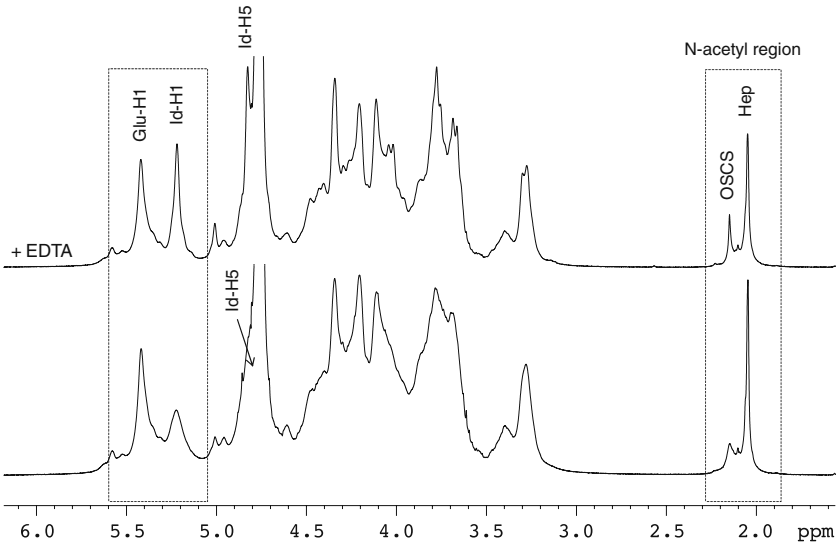

Fig. 8 ^{1}H NMR spectra (400 MHz, 300 K, D_2O) of a heparin sample containing Mn^{2+}. Addition of EDTA to the heparin sample results in sharpened signals of the iduronic acid moiety (*Id-H1*, *Id-H5*) and the *N*-acetyl resonance of OSCS

provides an opportunity to distinguish pure heparin from contaminated samples.

Due to a spectral width 20 times larger compared with ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy can often yield separated carbon signals for molecules with nearly identical chemical structures. For example, not only the *N*-acetyl resonances of heparin, OSCS, and DS are completely resolved but also other carbon signals of the contaminants can easily be identified in the spectrum (Fig. 10). The PhEur has implemented ^{13}C NMR spectroscopy as a mandatory test for the identification of low molecular weight heparin [81]; however, due to its low sensitivity the application of ^{13}C NMR spectroscopy for the investigation of contaminated heparin samples is used rarely.

The application of two-dimensional NMR techniques like HSQC mostly enables the detection of signals hidden in the 1D spectra, because the resonances are spread into two axes, ^{1}H and ^{13}C, and generally there is a much higher chance to find crosspeaks which do not coincide with those of heparin (Fig. 11). Additionally, when unknown impurities are detected, multidimensional NMR techniques provide information for the structural elucidation of those molecules; for example, OSCS was identified as a contaminant in heparin using 2D NMR techniques [50].

The group of Torri routinely differentiates pure heparin from contaminated heparin for a wide variety of potential contaminants using at least a 500 MHz spectrometer when the level of the potential contaminants is greater than 2–4% [80]. The measurement time for this purity test takes only a few hours; the application of instruments with weaker magnetic field strength is not recommended since this time has to be increased considerably in order to obtain

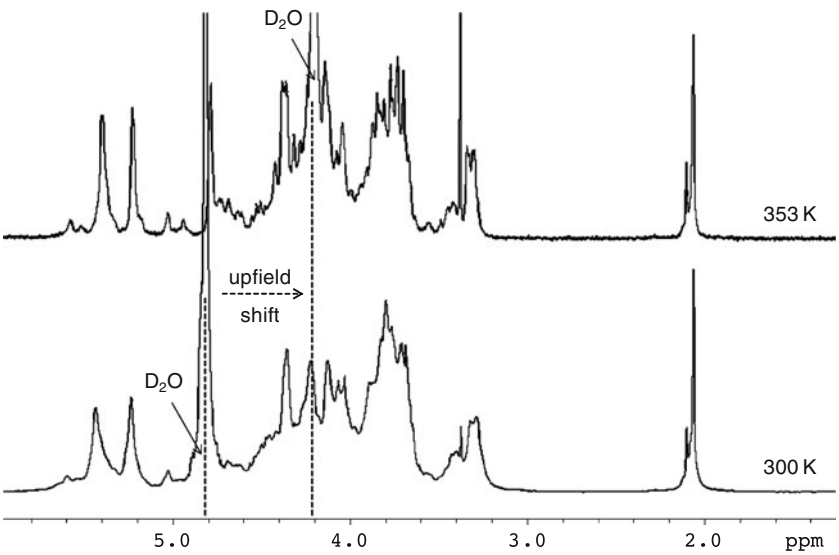

Fig. 9 ^{1}H NMR spectra (300 MHz, D_2O) of heparin sodium at temperatures of 300 K and 353 K

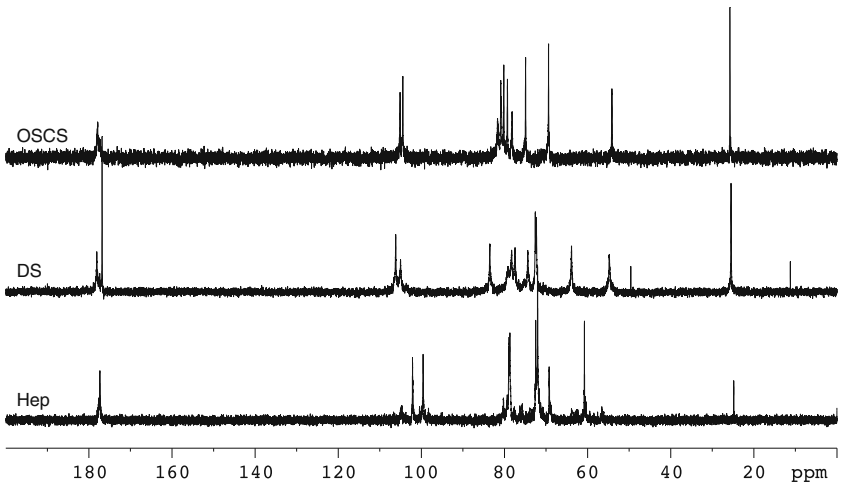

Fig. 10 ^{13}C NMR spectra (400 MHz, 300 K, D_2O) of heparin, OSCS, and DS

comparable results. However, 2D NMR spectroscopy is recommended when it comes to suspicious heparin samples.

Quantitative analysis of OSCS and DS in heparin

Glycosaminoglycans such as heparin and its potential impurities are complex mixtures of heterogeneous polysaccharides differing in chain length, sulfation pattern, and repeating disaccharide units. Therefore, conventional quantification of the expected contaminants using the internal standard method is not feasible.

In a collaborative NMR study on the detection and quantification of potential contaminants in heparin, the quantitative analysis of OSCS and DS was carried out by both the signal integration and the measurement of the peak heights using the corresponding *N*-acetyl resonances [51].

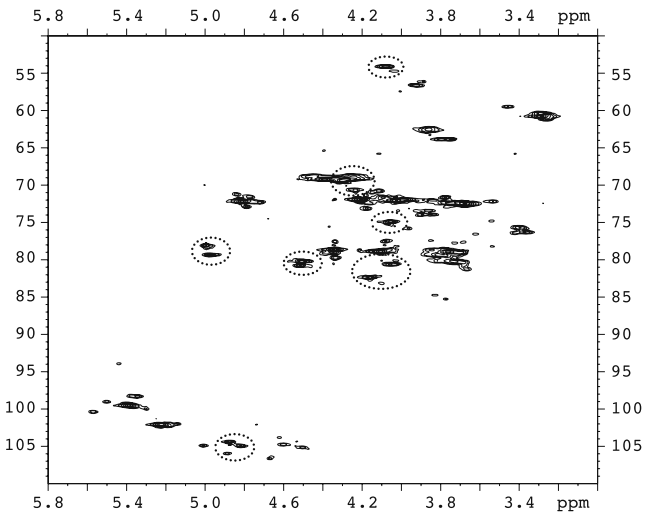

Fig. 11 HSQC spectrum (600 MHz, 315 K, D_2O) of heparin contaminated with OSCS. Characteristic contaminant crosspeaks are indicated

The comparison of both methods shows that more reproducible quantitative results for OSCS are obtained when using the peak height method since it is less complex and therefore less error-prone. Since the signals of OSCS and DS are located on the slope of the large *N*-acetyl heparin resonance the accuracy of the peak integrals depends considerably on post-acquisition processing parameters, i.e. baseline correction and integration. Additionally, the variable degree of *N*-acetylation in heparin (roughly 20% of the amino groups in heparin are acetylated) affects the accuracy of the quantitative analysis of OSCS and DS in heparin because the corresponding correction factor underlies statistical fluctuation. Using the standard addition method more accurate results can be obtained [28, 51].

The analysis of heparin model mixtures spiked with known amounts of OSCS of DS by means of ^1H NMR spectroscopy is consistent when evaluating the peak height of the *N*-acetyl resonances. However, it must be pointed out that more accurate results can be achieved when the calibration of the response factor is performed with the local NMR instrument applied because the response factor varies depending on the kind of heparin used for the titration, the concentration of the heparin test solution, the temperature, and the magnetic field strength of the NMR spectrometer [51].

Purity control of pharmaceutical grade L-alanine

Nowadays, amino acids are one of the most widespread biological compounds; besides their main field of application as food and feeding stuff additives, amino acids are applicable in the field of nutrition, medicine, cosmetics, and, agriculture [82]. Due to their chirality amino acids are favoured starting materials and intermediates for the chiral synthesis [83]; e.g. L-proline is used as a starting compound

for the synthesis of angiotensin converting enzyme inhibitors [84], L-tyrosine is applied for the production of levodopa [84], and thalidomide can be synthesized starting from L-glutamine [85]. In the pharmaceutical domain, the application of amino acids is part of the parenteral nutrition therapy of patients with insufficient renal clearance or liver insufficiency and in the paediatrics; furthermore, amino acids such as L-tryptophan or L-ornithine–L-aspartate are used in medicinal therapy because of their specific pharmacological effects [86, 87]. Owing to the widespread medical care of amino acids their quality control is of crucial importance. However, due to the absence of the essential chromophor required for an UV-detection in most of the amino acids, the determination of low level impurities is an analytical challenge [19, 88]. Since amino acids are produced by various manufacturing processes, i.e. chemical and enzymatic synthesis, extraction of protein hydrolysates, and fermentation, it has to be considered that each production pathway exhibits a different impurity profile [82, 89]. Whereas starting products, intermediates and synthesis by-products are potential impurities for amino acids produced by chemical synthesis, other amino acids have to be expected as related substances for protein hydrolysation. Fermentative production exhibits a more complex impurity profile particularly dependent on the biochemical pathway utilized for production and on the purification process [90]. Thus, for an optimal development of analytical methods it is essential to know the appropriate production pathway(s).

In a comparative case study using L-alanine (Ala) as a representative amino acid a quantitative analysis of potential impurities was carried out using HPLC equipped with different evaporation based detectors and, as an orthogonal method, the non-separating technique ^{1}H qNMR spectroscopy [19]. The industrial scale production of Ala utilizes chemical and enzymatic synthesis as well as extraction of protein hydrolysates [90]; thereby, fumaric acid (FA), maleic acid (MA), aspartic acid (Asp), and glutamic acid (Glu) were identified as potential impurities in Ala.

^{1}H NMR spectroscopy yields at least one baseline separated signal for each impurity except for Asp. The CH_2-resonances of Asp and MA overlap and the CH-signal of Asp coincides with a ^{13}C satellite of Ala (Fig. 12). However, the percentage of Asp can be determined by both integration of the CH-resonance after removal of all ^{13}C satellites using a ^{13}C decoupling pulse sequence and by subtracting the peak area of the CH-signal of MA from the signal intensity of the combined CH_2-resonances of MA and Asp, respectively.

Variations of the exact chemicals shifts can be observed in samples such as Ala when the level of acidic impurities is changing. This is due to a corresponding variation of the pH value of the test solution. It is well known that the proton chemical shifts of an analyte containing acidic and/or basic groups are sensitive to the pH value of the sample since the chemical shift of an atom is related to the amount of charge present in the molecule [15]. The pH-dependency of chemical shifts complicates both the identification and the quantitative analysis of the potential impurities in Ala when utilizing D_2O as solvent. In this case a shifting of resonances can be observed due to varying pH values, which adversely affects the signal separation of the CH_2-resonances of Asp, MA, and Glu. The less the amount of acidic related substances, the more distinctive is the upfield shift (Fig. 13).

However, the effect of the pH value on the chemical shift of hydrogen atoms may be utilized to achieve an optimal signal separation. As mentioned before both resonances of Asp overlap either with the methylene resonance of MA or with one of the ^{13}C satellites of Ala. Increasing the pH value of the sample by adding NaOD, an upfield shift of the resonances is observed in the ^{1}H NMR spectrum. Using alkaline solutions (pH>10) the CH_2-resonances of Asp and MA still overlap, however, due to varying shifts of the CH-resonances of Ala and Asp, signal separation is achieved [30]. Decreasing the pH-value of the sample solution by adding DCl, the resonances are strongly downfield shifted in comparison to the measurements using D_2O. Utilizing acidic solutions (pH<1.5) the CH_2-resonances of Asp and MA as well as the CH-signal of Asp are completely separated (Fig. 14). A comparison of alkaline and acidic solutions reveals perfect agreement of the quantitative results with experiments in D_2O.

The qNMR analysis of the impurities present in the model mixture was in perfect agreement with both nominal and HPLC values with a recovery rate of more than 95% [19]. Interestingly, a very sensitive determination of the impurities was possible utilizing ^{1}H NMR spectroscopy and chromatography. The limit of quantification obtained by using a 400 MHz NMR spectrometer was in the same range as for HPLC utilizing the nano quality analyte detector, the charged aerosol detector, and even better than the evaporative light scattering detector (ELSD). The ICH impurity level of 0.03% corresponding to drug substances with an average daily dose above 2 g was met in all of the mentioned methods except ELSD. If necessary, the detection sensitivity of NMR spectroscopy may be improved by increasing the number of scans and/or the concentration of the sample solution as well as by applying spectrometers with higher magnetic field strength or modern probes, e.g. cryoprobes [29]. With regard to sensitivity and accuracy the quantitative results obtained by NMR spectroscopy reflect in a very impressive manner the suitability of qNMR for the control of low level impurities.

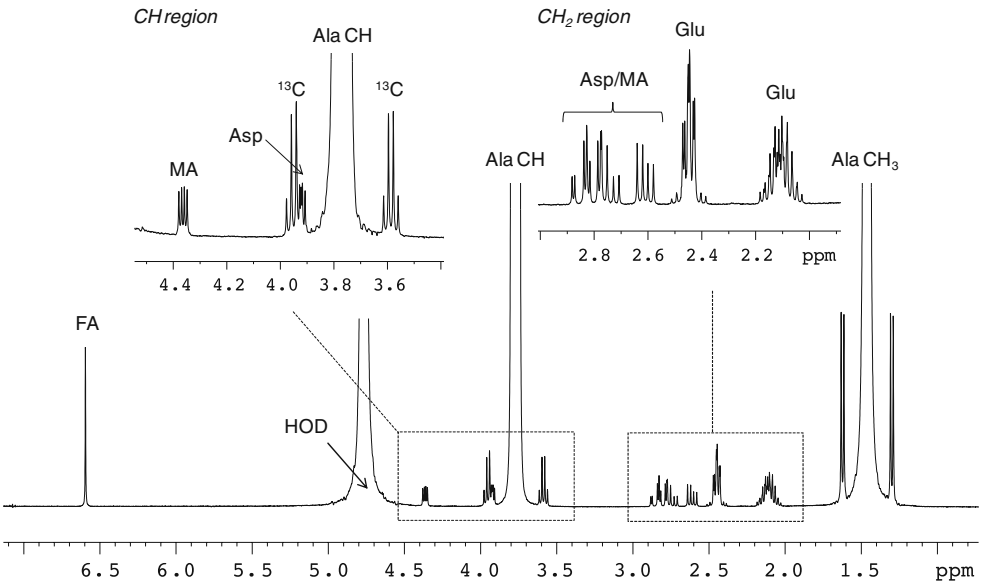

Fig. 12 ^{1}H NMR spectrum of the alanine batch analyzed in D_2O (400 MHz, 300 K, pH value=4.3). The individual components are alanine (*Ala*), aspartic acid (*Asp*), glutamic acid (*Glu*), malic acid (*MA*) and fumaric acid (*FA*)

Lipid characterization

Lipids are a prime example for classical multi-component analysis. The test item "lipid" is of central importance for the analysis of food and feed but also for drugs and technical chemicals. The combination of neutral and polar lipids, sterols and other biomolecules from animal and vegetable sources demands a comprehensive regulation opus of official methods, e.g. by the Deutsche Gesellschaft für Fettforschung (DGF) or the American Oil Chemist Society (AOCS). This collection of official methods has the advantage of a worldwide comparable analysis of oils and lipids. On the other hand the commitment to this selection of methods can be a disadvantage. Technical and scientific scopes in lipid analysis had changed during the time and new and more effective methods became available. They also have to be accepted and implemented as official methods in the future.

Initially, lipid analysis focused on the generation of key parameters, e.g. via titration of chemical reaction products

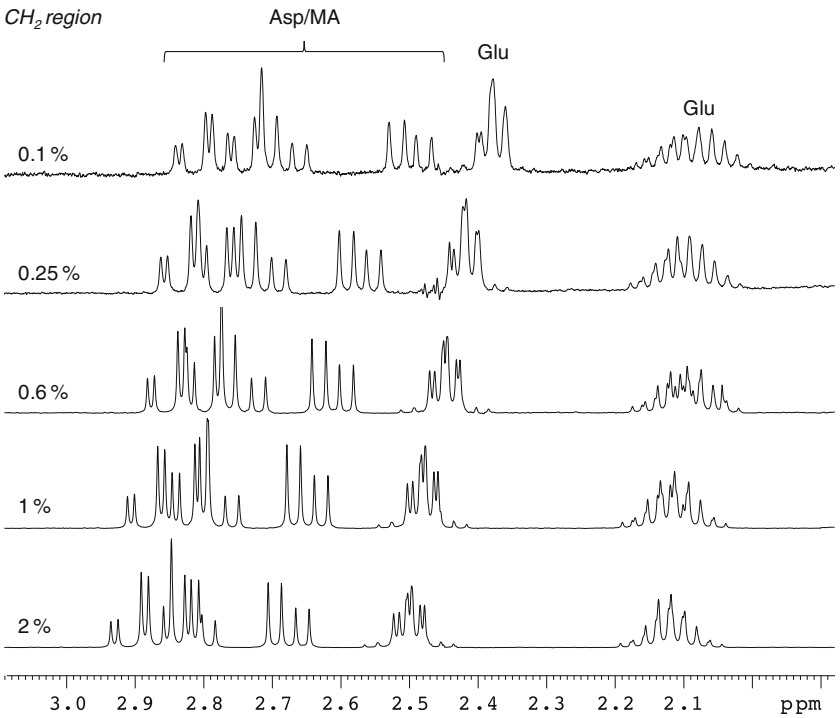

Fig. 13 CH_2 region (400 MHz, 300 K) of potential impurities of alanine (*Ala*) in D_2O for different contaminant concentrations (2%, 1%, 0.6%, 0.25%, and 0.1%) exhibiting specific chemical shifts for aspartic acid (*Asp*), malic acid (*MA*), and glutamic acid (*Glu*). The pH value ranges between 3.8 and 4.9 for impurity concentrations between 2% and 0.1%

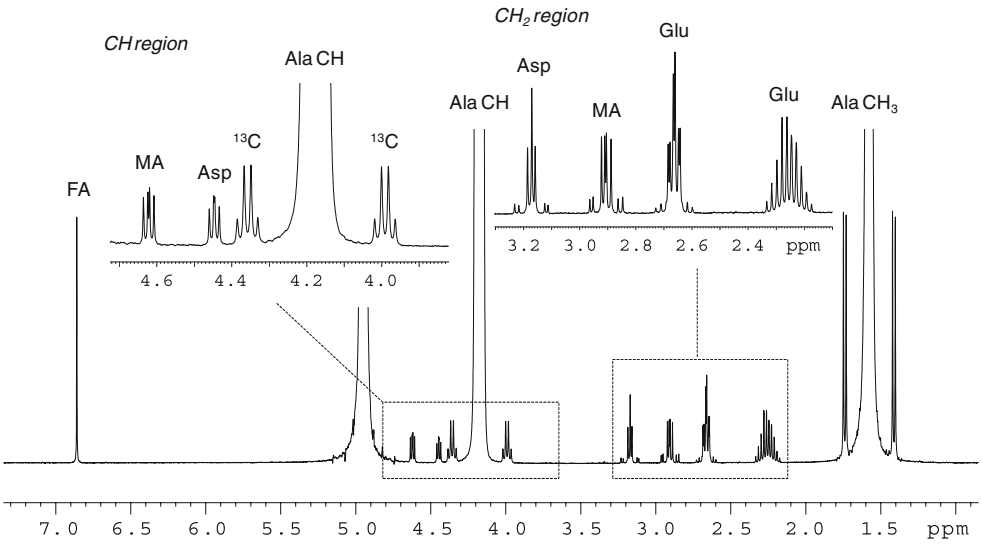

Fig. 14 ^{1}H NMR spectrum of the alanine batch analyzed in a mixture of D_2O and DCl (35% (*w*/*w*)) in a ratio of 9:1 *v*/*v* (400 MHz, 300 K, pH-value <1). The individual components are alanine (*Ala*), aspartic acid (*Asp*), glutamic acid (*Glu*), malic acid (*MA*), and fumaric acid (*FA*). In contrast to pure D_2O, each impurity exhibits a baseline separated signal

(peroxide value, iodine value, acid value, etc.) or photometric determination (molybdatophosphate for total phosphorous as a dimension for phospholipids). These methods produce data without a detailed statement on the individual composition in terms of single molecules or groups of lipid families.

The next step in the evolution of lipid analysis was the introduction of chromatography (TLC, HPTLC, GC, HPLC, and CE) combined with different kinds of detector systems (UV/VIS, FID, ELSD, MS). In any case, the separation of lipid components is the first step followed by the detection. Both separation and detection often are not universal, and so the chromatographic methods are mostly designated to analyze parts of similar components from a more complex mixture. Gas chromatography plays one of the most important roles in lipid analysis especially for the qualitative and quantitative analysis of fatty acid distribution. However, the method is destructive and cannot distinguish between fatty acids from different classes, e.g. triglycerides and phospholipids.

Nowadays a third analytical principle became of interest, i.e. spectroscopy. Three methods are playing a major role. The optical method (UV/VIS) is mostly used for preparing key parameter (e.g. peroxide value by the thiocyanate method) or is used as a detector, e.g. for HPLC. Vibration spectroscopy (IR, NIR, Raman) shows a low dispersion and is only useful for very special applications. In contrast, the NMR spectroscopy is able to fully characterize oils and fats.

As early as 1962, Johnsthon and Shoolery [91] have shown that commercial vegetable oils can be analyzed regarding the amount of double bonds by means of ^{1}H NMR spectroscopy. This led to the first evaluation of the key parameter iodine value. The results could be confirmed in the following years [92, 93]. Several groups developed qualitative and quantitative applications of NMR spectroscopy for lipid analysis [92, 94–99]. One of the domains of the NMR spectroscopy is the assignment of the stereochemistry and regio-selectivity in fatty acid distributions [100–107]. Analysis of sterols [108, 109] mono- und diacylglycerides [110–112] as well as other minor components of natural origin like phenols, squalene, alcohols, chlorophyll, terpenes, and phospholipids [108, 113] was proved to be possible and effective by NMR methods. Important parameters like the cis/trans-ratio of fatty acids, oxidation states, etc. were developed [92, 114–117]. Hydrolysis by deterioration, mono- and diacylglyceride and free fatty acids are described [115, 118]. Today the latest techniques of high field instruments equipped with the high sensitive cryo-technology enable the appliance in lipid routine analysis. The NMR provides fast and precise results in analogy to the common sum parameters and key values, but simultaneously the qualitative and quantitative determination of single molecules and lipid families.

Three nuclei are playing an important role in the structure relevant parts of lipids: hydrogen, carbon and phosphorous. Some representative NMR methods in lipid analysis are summarized in Table 3. Three different approaches of quantitative NMR will be exemplified in the following.

Composition of vegetable oils by internal calibration

The first approach is the characterisation of the composition of a vegetable oil by internal calibration. Triolein consists of nine hydrogen atoms in methyl position (δ=0.9–1 ppm), 6 as double bonds (δ=5.35 ppm), five of the glycerol backbone (splitted into 1 CH- and 2 CH_2-groups), six methylene α (δ=2.3 ppm) and β, respectively, to the carbonyl group (δ=1.6 ppm).

Table 3 List of lipid analysis parameter and proposed methods

Assay	Common analysis	NMR type
Iodine value	Titration	^{1}H NMR
Peroxide value	Titration and Photometric	^{1}H NMR
Free fatty acids	Titration	^{1}H NMR, ^{13}C NMR
Fatty acid distribution	GC/FID, GC/MS	^{1}H NMR, ^{13}C NMR
Regio-selectivity of fatty acids	Enzymatic GC/FID, GC/MS	^{13}C NMR
Mono- and diglyceride	HPLC	^{1}H NMR, ^{13}C NMR
Phospholipids	HPLC, HPTLC,	^{31}P NMR
Glycolipids	HPLC, HPTLC	^{1}H NMR, ^{13}C NMR
Sterols	GC, HPLC	^{1}H NMR, ^{13}C NMR
Origin,		^{1}H NMR, ^{13}C NMR, ^{31}P NMR
Degradation		^{1}H NMR, ^{13}C NMR, ^{31}P NMR
Mixtures, test on frauds		^{1}H NMR, ^{13}C NMR, ^{31}P NMR
Bleaching, raffination		^{1}H NMR

Each double bond causes two allylic methylene groups (δ=2.0 ppm), etc. The integral values of triolein so far can be used for internal calibration and validation of a NMR experiment. Saturated fatty acids do not contain double bonds, but each fatty acid (except some exotic fatty acid types) has 3 methyl and 6 α and β-methylene hydrogens, respectively. Higher unsaturated fatty acids show additional double allylic methylene groups (δ=2.8 ppm). However, chain end methyl and α-methylene groups can be used as a fixed molar quantity for the internal calibration. It is common to calibrate the integral of the α-methylene group at δ=2.3 ppm to 200 units. This way, in an intact triacylglycerid the glycerol signals must be 33.3 for each CH (δ=5.25 ppm) and 66.6 for each CH_2-signal (δ=4.3 resp. 4.1 ppm). Deviations of these integer integral values can be caused by degradation to mono- and diacylglycerides.

A typical ^{1}H NMR spectrum of a real vegetable oil is shown in Fig. 15. The detailed integrated regions of interest are shown in Fig. 16. Using of the integral values the key data given can easily be calculated. The iodine value is a direct function of the number of double bonds (δ= 5.35 ppm) in a lipid. The NMR value gives the theoretical iodine value of a real triacylglyceride. In contrast to the titration the chemical shift of a ^{1}H NMR spectrum distinguishes between double bonds of different origin, e.g. in the case of olive oil a separate detection of the triglycerides and the squalene part is possible.

The peroxide value is calculated using the normalized areas of the OOH proton signals (δ=11–12 ppm in Fig. 17) according to the linear regression vs. the titration method of Wheeler Fig. 18.

The sum of total unsaturated fatty acids can be calculated from the sum of allylic methylene protons taken into account that each unsaturated fatty acid consists of four hydrogens of this type per mol fatty acid. The methyl groups are shifted by the anisotropy of the double bonds depending on the distance of the methyl group to the double bond. This way, saturated, ω-3, ω-6, and ω-9 fatty acids can be distinguished. A baseline separation of ω-3 type methyl groups is possible. The integral values of all methyl groups are normalised to 100, so the integral value of the ω-3 triplet directly gives the molar amount of all ω-3 fatty acids as a sum parameter including not only linoleic acid but also EPA and DHA if present. In the case of simple vegetable oils, the double allylic methylene groups belong to linolic (δ=2.75 ppm) and linoleic acid (δ=2.80 ppm). At 600 MHz the signals of both groups are baseline separated and can be used for the calculation of 18:2 and 18:3.

The integrals of the ω-3 methyl signals at δ=0.95 ppm groups should be in a good consistence to the double allylic methylene signal at δ=2.80 ppm. The next parameter is the molar amount of free fatty acids. Depending on the sample preparation either the COOH (δ=1–12 ppm) or the α-CH_2 (δ=2.70 ppm) can be used. Finally, a lot of other signals in the ^{1}H NMR spectrum give information about the presence and amount of minor components like mono- and diglycerides, phosphatidylcholine, squalene, and sterols.

The total sterol content can be estimated using the methyl signal, e.g. of sitosterol at δ=0.7 ppm. 10 min of measuring time are sufficient to characterize a vegetable oil, to enable the determination of all important key numbers, to proof the identity and quality by the internal calibration method. In Table 4 the lipid key data are summarized.

Quantification using an internal standard

The second quantitative approach is the use of an internal standard. ^{31}P NMR spectroscopy has become the universal method in phospholipid analysis [119–121] and is reference method of the ILPS (International Lecithin and Phospholipid Society). Especially the analyses of phospholipids,

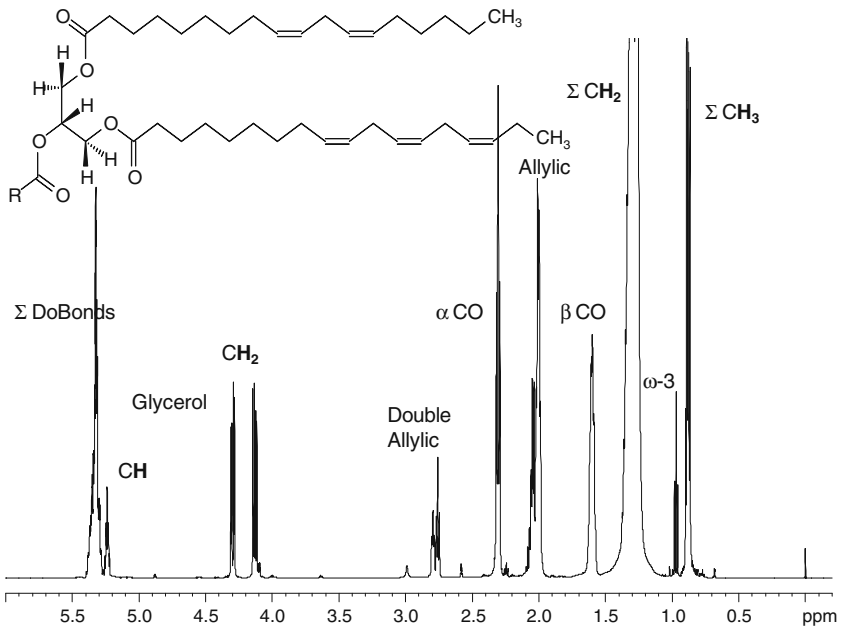

Fig. 15 Chemical structure and ^{1}H NMR spectrum (600 MHz) of a vegetable oil

which normally are obtained as complex mixtures, e.g. lecithin, give problems to HPLC chromatographic methods. ^{31}P NMR spectroscopy has some obvious advantages. First, the 100% natural abundance of ^{31}P nuclei makes ^{31}P NMR spectroscopy a selective and sensitive method. Second, NMR is a non-destructive ab initio method. Thus, no specific standards are necessary for quantification. The selectivity makes the ^{31}P NMR approach universal.

The lecithin analysis of different origin demonstrates its usefulness. One important source for lecithin is milk (Fig. 19). All ^{31}PNMR experiments can easily be performed quantitatively to give phospholipid values in % *w*/*w*. In quantitative ^{31}P NMR the most common standard is triphenylphosphate (TPP). It is used for both calibration and quantification purposes as an internal standard. Different nuclear Overhouser enhancement (NOE) effects and relaxation times have to be taken into account. The inverse gated decoupling technique and adequate relaxation delays allow a processing of signals without response factors. TPP normally is used for analyzing complex mixtures because its chemical shift ($\delta = -17.8$ ppm) differs from that of natural phospholipids ($\delta = 5$ to -1 ppm).

Alternatively, artificial phospholipids, e.g. distearoylphosphatidylglycerol are also useful as reference material. The internal standard method had been fully validated with

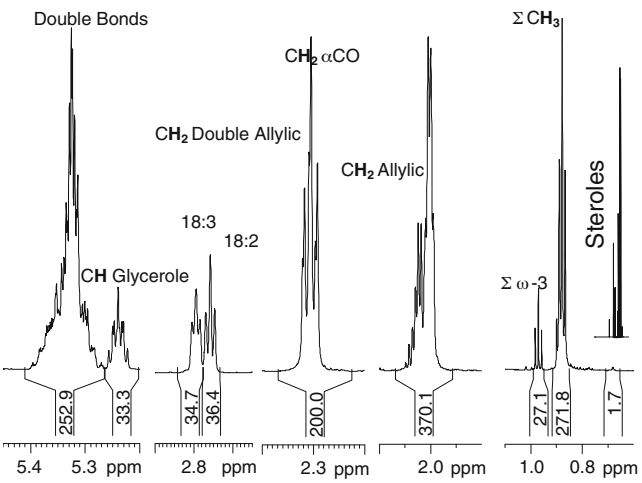

Fig. 16 Expansions and assignments of the ^{1}H NMR spectrum (600 MHz) of a vegetable oil

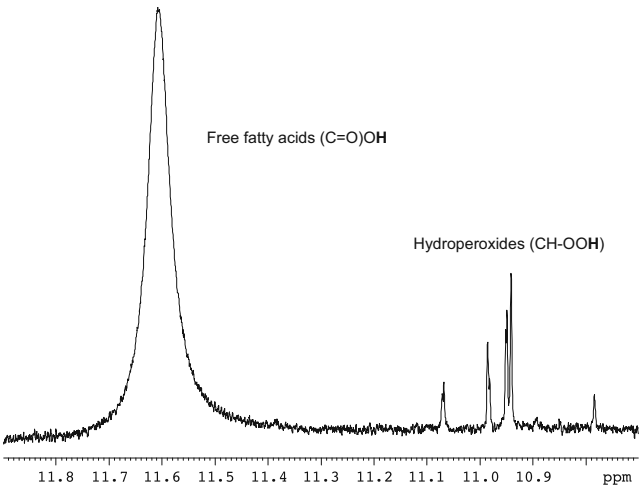

Fig. 17 Expansion and assignments of the downfield area of the ^{1}H NMR spectrum (600 MHz) of a vegetable oil

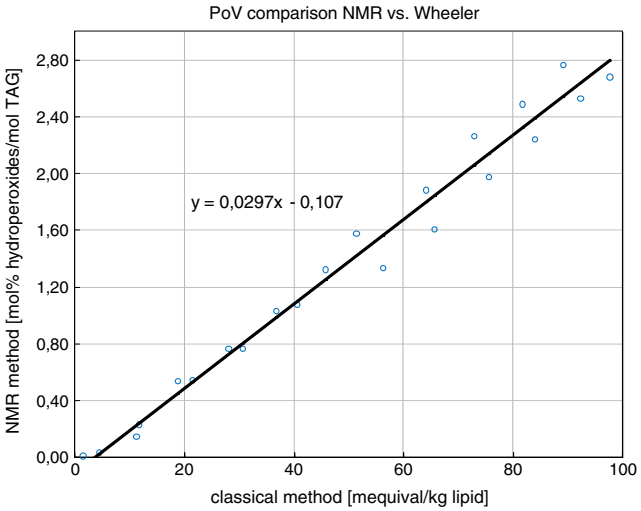

Fig. 18 Cross-validation of the PoV determination by ^{1}H NMR and Wheeler

respect to selectivity, recovery, reproducibility, instrument precision and robustness.

Between 1992 and 2000 the ILPS carried out trials to establish HPLC methods for phospholipid analysis. The recommended detector was a light scattering system. For native soy lecithin this method is the official DGF and AOCS meanwhile. However, because all reference materials used in these round robin tests using the HPLC/LSD technique were calibrated by ^{31}P NMR, the ^{31}P NMR spectroscopy is the real base of the AOCS method Ja 7c-07.

By contrast to hydrogen or phosphorous, carbon consists of only 1.1% of the NMR active nucleus ^{13}C. The proton decoupling techniques and the varying relaxation times for carbons in different positions in the same molecule causes larger effects in the comparison to the integrals. Internal referencing and the internal standard methods must be used very carefully. It is critical to use the integral values, e.g. of methyl, methylene and carbonyl groups without the knowledge of response factors. A carbonyl bonded carbon shows significant lower intensities compared with methylene bonded ones. However, within a group of similar surrounded carbon atoms the direct comparison of integrals is allowed. The region of the glycerol backbone carbon atoms of tri-, di-, and monoacylglyceride demonstrates these applications and is shown in Figs. 20 and 21. Mogosa oil, originated from the Neem tree, is a complex mixture of glycerides and terpenes. However, a quantification of mono- and diacylglycerol is possible even in presence of terpenes.

Table 4 ^{1}H-NMR data evaluation for lipid key data

	Assay	Integral	Chemical shift δ [ppm]
Iodine value	172.4	252.9	5.35
Peroxide value	7.5	0.04	10.7–11.2
Sum unsaturated	92.5 (% mol)	370.1	2.0
Sum saturated	7.5 (% mol)		
w-3	9.1 (% mol)	27.1	0.95
Methyl		271.8	0.90
Oleic acid	65.7 (% mol)		
Linolic acid	18.2 (% mol)	36.4	2.75
Linoleic acid	8.7 (% mol)	34.7	2.80
Free fatty acids	0.24 (% mol)	0.24	12.0
Sterol	0.8 (% *w*/*w*)	1.7	

Quantification by using the standard addition method

The third principle method to obtain absolute quantitative data is the use of the standard addition method. Defined amounts of the component to be analyzed is added to the test item. The increasing characteristic signals are integrated and its value correlated to the percentage addition of the component. The initial amount of the component is calculated from the linear regression y=mx+n. By dividing the intercept n by the slope m the total amount of the component can be calculated, independently whether the component is a defined chemical substance or a complex mixture. An example is the determination of butter in butter cookies. Even in mixtures with vegetable oil the quantification is possible due to the specific fatty acid profile of milk fat which contains butyric acid (see Fig. 22), CLA and several other markers like cholesterol.

In Fig. 23, the effect of increasing signals of the ^{1}H NMR spectrum after the addition of real butter to a butter cookie sample is shown. The initial weight of 10% butter could be confirmed within the limits of the analytical procedure and the allowed tolerances in the cookies.

Conclusions

In this work, we reviewed the application of qNMR to the field of pharmaceutical analysis by focusing on a selection of classes of biologically relevant substances. We demonstrated that qNMR spectroscopy may be an orthogonal method or even a substitute, offering many advantages over conventional techniques, which often fail to sufficiently characterize substances of interest. Therefore, we adjudge this powerful tool to deserve far more attention that it has received until today.

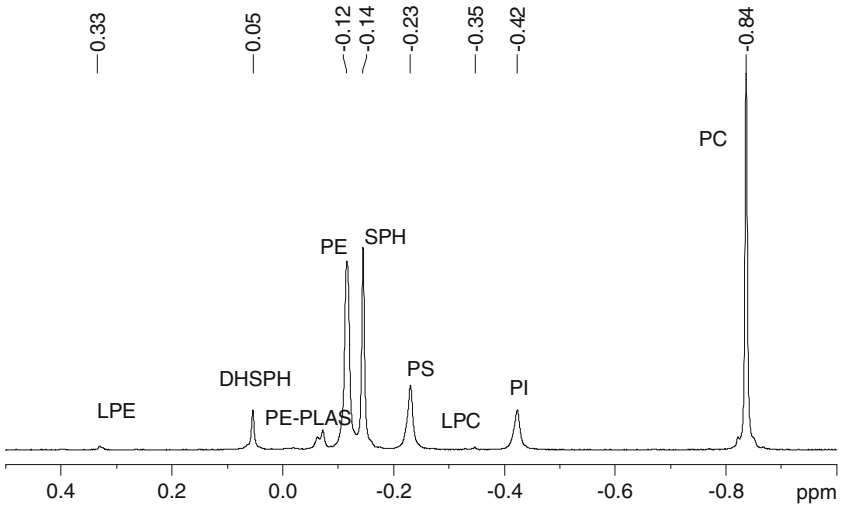

Fig. 19 Expansions and assignments of the ^{31}P NMR spectrum (600 MHz) of milk lecithin. *PC* phosphatidylcholin, *P* phosphatidylinositol, *LPC* lysophosphatidylcholine, *PS* phosphatidylserin, *PE* phosphatidylethanolamin, *Plas* plasmalogen, *SPH* sphingomyelin, *DHSPH* dihydrosphingomyelin, *LPE* lysophosphatidylethanolamine

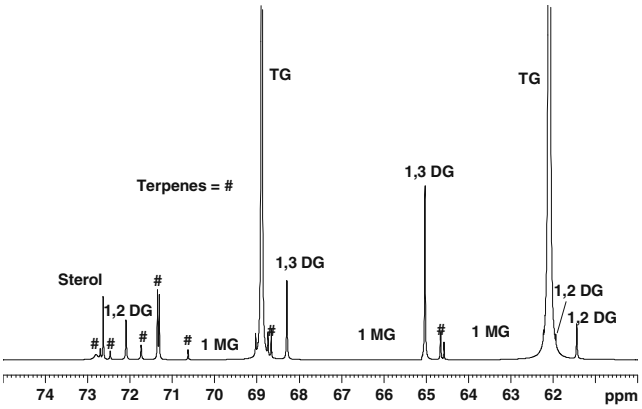

Fig. 20 Expansion and assignment of the ^{13}C NMR spectrum (600 MHz) of Magosa oil. *1,2 DG* 1,2 diacylglycerol, *1 MG* 1 monoacylglycerol, *TG* triacylglycerol, *1,3 DG* 1,3 diacylglycerol

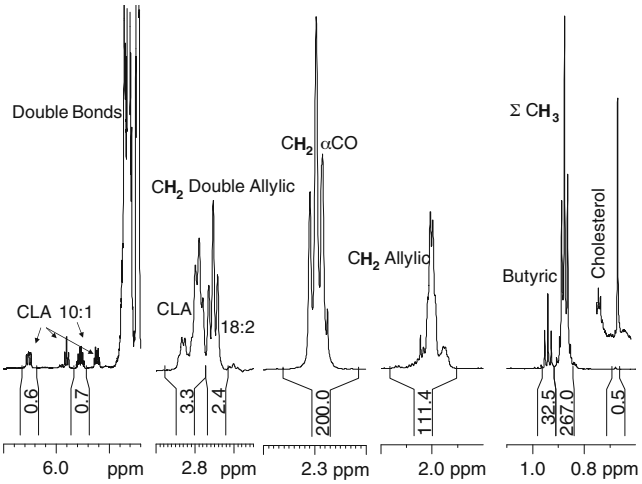

Fig. 22 Expansion and assignment of the ^{1}H NMR spectrum (600 MHz) of butter fat. *CLA* conjugated linoleic acid, *10:1* caproleic acid

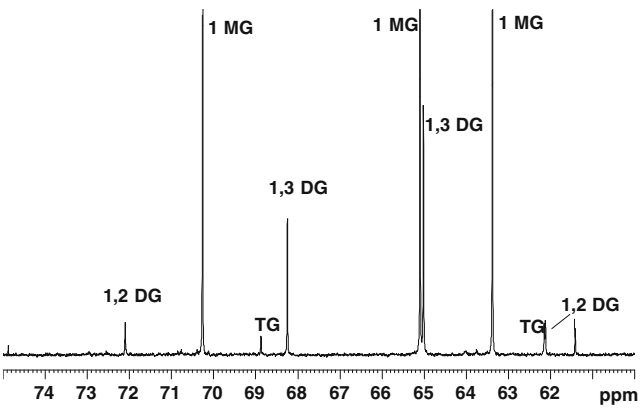

Fig. 21 Expansion and assignment of the ^{13}C NMR spectrum (600 MHz) of commercial available 1-monoacylglycerol

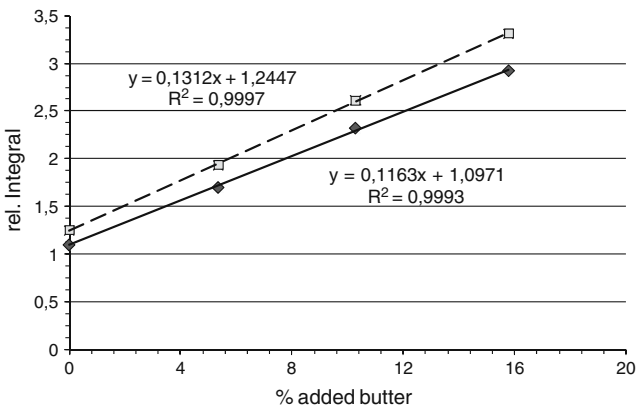

Fig. 23 Linear regression of the signal enhancement of CLA and caproleic acid during standard addition of butter to butter cookies

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Conflicts of interest The authors state that they have no conflict of interest

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