Analytical Methods

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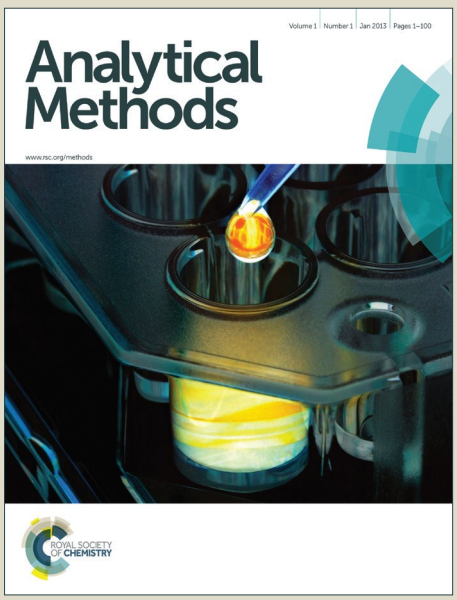

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ARTICLE

High Performance Liquid Chromatography - Quantitative Nuclear Magnetic Resonance (HPLC-qNMR) with Two-Signal Suppression Method for Purity Assessment of Avermectin B1a

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HPLC-qNMR (high performance liquid chromatography – quantitative nuclear magnetic resonance) is a technology with great potential, which combines the high separation capability of HPLC and the high qualitative/quantitative analysis capability of NMR. It overcomes the problem of overestimation in the qNMR, and the problem of unavailable reference material in the HPLC. But its current application is narrow due to lack of a good method to eliminate the vast amount of signals from the protons of mobile phase. This study proposed a new HPLC-qNMR method for a natural complex pesticide (avermectin B1a). Common solvents (CH_3CN and H_2O = 70:30) are introduced as the HPLC mobile phase, which reduce the cost to make it possible for wider application. The mixture solution of the sample and the internal standard (benzoic acid) were separated by HPLC. Only the eluent of target analyte (avermectin B1a) and the eluent of the internal standard were collected in one vial, and then were determined by the qNMR with a new signal suppression method to suppress two signals from CH_3CN and H_2O simultaneously. After the optimization of key parameters for the qNMR, the mean and uncertainty (94.63% ± 1.97%) is consistent with that from the mass balance method based on many instruments (93.70% ± 0.46%). The bias is no more than 1%. This method can be widely applied in the future due to its efficient separation capability, high qualitative/quantitative capability, very low cost, rapid operation and good accuracy.

Keywords: high performance liquid chromatography; quantitative nuclear magnetic resonance; two-signal suppression, solvent suppression; purity assessment; avermectin B1a.

1. Introduction

Quantitative nuclear magnetic resonance (qNMR) is a promising primary assessment method. It is non-destructive and requires minimal sample preparation. It can establish the traceability of the purity value for the analyte without the same pure reference material as the analyte, if the purity value of the internal standard is traceable to the International Standard Units. It was widely used in chemical purity assessments [1]. However, without separating organic impurities from the analyte, direct qNMR is at high risk of overestimating the purity when some impurity peaks overlap with the peak of the analyte. High performance liquid chromatography (HPLC) is one of the most important approaches for separation of organic compounds. Therefore, the hyphenation of HPLC and qNMR is a promising technology.

HPLC-NMR is a new technique in mixture analysis which combines the separation efficiency of HPLC with the specificity of NMR [2, 3]. Since the mobile phase of HPLC contains vast ^1H (proton), there are three modes of HPLC-NMR to eliminate the influence of these protons : (1) continuous LC-NMR: expensive deuterated solvents are used as the HPLC mobile phases such as D_2O and CD_3CN; (2) LC-SPE-NMR: after HPLC with common solvents as the mobile phases, the analyte is trapped in some solid phase extraction (SPE) columns, and the analyte is dried by nitrogen and then eluted out by deuterated solvents; (3) single deuterated mobile phase LC-NMR method: After HPLC with one common solvent (often CH_3CN) and one deuterated solvent (often D_2O) as the mobile phases, NMR with solvent suppression method was carried out to eliminate the huge signal from the protons of the common mobile phase. HPLC-NMR is often used in qualitative analysis, especially the elucidation of unknown compounds from natural products. [3, 4].

Quantitative HPLC-NMR (or HPLC-qNMR) is reported less frequently than qualitative HPLC-NMR, because the mode (1) costs too much;

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the mode (2) is at a risk of loss of sample during trapping, drying and re-elution; the mode (3) costs much and it is at a risk of distortion of signal thus inaccuracy quantitative result.

Saito [2] applied the continuous mode with CD_3CN and D_2O as mobile phases to determine the purity of *o*-xylene, but it is too expensive to become a routine application. Godejohann [5] applied the signal suppression method by 1-D version of the noesyprtp (Bruker, Rheinstetten, Germany) with CH_3OH and D_2O as mobile phases to determine several nitroaromatic compounds, and the deviations between the injected and calculated amounts of analytes are usually below 10%. But the high cost of D_2O is still limiting it from wider application. Therefore, these HPLC-qNMR studies are rarely reported.

Avermectins, macrolytic lactones produced by the fungus *Streptomyces avermitilis*, have found wide application as pesticides and antiparasitic drugs for humans and animals. Natural avermectins have 8 components: A1a, A2a, B1a, B2a, A1b, A2b, B1b and B2b. The most extensively used compound of this class is avermectin B1a. [6] The avermectin B1a without any other 7 components as impurities is not commercially available. Moreover, the ^{1}H-NMR peaks of the 7 components overlap with almost peaks from avermectin B1a in NMR. Therefore, it is difficult to determine the purity of avermectin B1a by HPLC or direct NMR, hence, avermectin B1a is chosen as the analyte in this HPLC-qNMR study.

This study proposed a new HPLC-qNMR method. Common solvents (CH_3CN and H_2O) are used as the HPLC mobile phases to reduce the cost for making it possible of wider application of this method. The sample and the internal standard (benzoic acid) were dissolved in one solvent, and separated by HPLC. Only the eluent of target analyte (Avermectin B1a) and the eluent of the internal standard were collected into one vial, and then were determined by a qNMR with a new signal suppression method to suppress two signals from these two mobile phases simultaneously. The accuracy of quantitative result after suppressions was studied.

Before this study, the purity of the sample of avermectin B1a was previous determined by the mass balance method [7], using HPLC, LC-MS, Karl Fisher titration, head-space gas chromatography, inductively coupled plasma - mass spectrometry, etc. It involved many instruments and spent many days. The new HPLC-qNMR method is promising to reduce the time and cost. Based on the advantage of qNMR, it can also establish the traceability of determination of avermectin B1a to another pure compound, since pure avermectin B1a is unavailable. This low-cost, high specific, rapid and traceable method is a promising method to be widely used.

2. Experimental

2.1. Materials and reagent

Avermectin sample was provided by NIM, determined by the mass balance method, with a purity value of 93.70% and an expanded uncertainty of 0.46%. Benzoic acid was a standard reference material (SRM 350b) of NIST, with a purity value of 99.9978% and an expanded uncertainty of 0.0044%. Dimethyl sulfoxide-d_6 (DMSO-d_6) was purchased from Sigma-Aldrich (US).

2.2. Apparatus

Measurements were carried out on a Bruker Ascend 800 spectrometer with a 5 mm CPQCI cryoprobe at 800 MHz (^{1}H). The TopSpin 3.1 Bruker NMR software was used for data processing. Liquid chromatography was carried out by an Agilent 1260 system with a DAD detector, equipped with an Inertsil ODS-3 column (250 mm × 4.6 mm × 3 μm). The weighing was carried out on a Sartorius SE 2 balance (d=0.1 μg).

2.3. LC

The sample solution of valine was prepared as following steps: avermectin sample (~20 mg) and benzoic acid (~2.3 mg) were accurately weighed and were dissolved in DMSO-d_6 (1 mL). The solution was injected into the LC-DAD system with 100 μL. The mobile phase was acetonitrile: water = 70:30, with a flow rate of 1.0 mL/min. By experiments under this flow rate, the time for solution from the DAD detector to the outlet is determined (13 s). Only the eluent with the avermectin B1a and the eluent with the benzoic acid were collected (~5 mL) into a 10-mL glass tube. Then, 500 μL collected solution were transferred into a NMR tube, added by 50 μL D_2O (for lock of the field at NMR), and were determined by NMR. The chromatography at wavelength of 244 nm was shown in **Figure 1**.

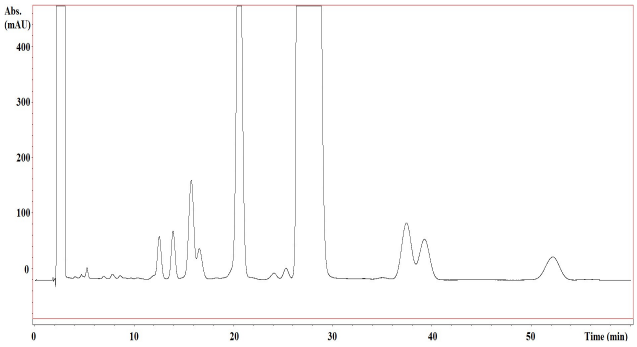

Fig. 1 chromatogram for the solution of avermectin B1a and benzoic acid (Benzoic acid: peak at 2~3 min; avermectin B1a: peak at 26~30 min; impurities in avermectin B1a sample: other peaks)

2.3. NMR

The experiments were carried out using the following parameters for qNMR: 30° pulse, 65536 data points. Fourier transformation was done with exponential filtering of zero after zero filling the data to 131072 time domain points. Automatic receiver gain was performed to obtain a suitable receiver gain (RG) before each determination.

(1) A basic ^{1}H-NMR spectrum was acquired with NS (number of scan) as 1 time and D1 (relaxation delay) as 1 s, to obtain the chemical shifts of two solvents. Only two solvent peaks of water and acetonitrile were showed (**Figure 2**). The automatic receiver gain was very small (e. g. RG: 1.12), so the peaks of analytes are undetectable.

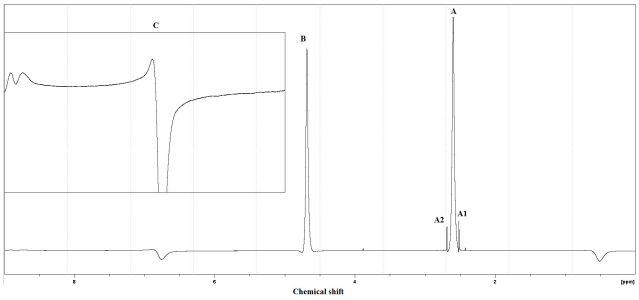

Fig. 2 NMR spectroscopy without signal suppression for the eluent of avermectin B1a and benzoic acid after HPLC separation.

(A: acetonitrile, A1 and A2: satellite peaks of acetonitrile; B: water; C: enlarged view of the part below using the same vertical axis)

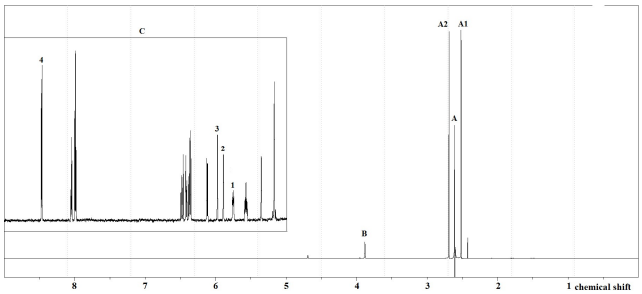

Fig. 3 NMR spectroscopy with two-signal suppression for the eluent of avermectin B1a and benzoic acid after HPLC separation.

(A: acetonitrile, A1 and A2: satellite peaks of acetonitrile; B: water; C: enlarged view of the part below using the same vertical axis; 1~3: the three quantitative peaks from avermectin B1a, 4: the quantitative peak from benzoic acid).

(2) The pulse program was set to noesygpps1d.comp1.d1 (Bruker, Rheinstetten, Germany). The O1 (irradiation frequency offset) was set to the centre of acetonitrile peak (~2080 Hz). Set D1 to 5 s, L6 (loop counter) to 1, and NS to 1.

(3) Using the GS (Interactive parameter optimization during acquisition) command to tune the O1 slightly, to make the sum of FID to be as small as possible. This can set the O1 accurately at the centre of the acetonitrile peak.

(4) Using "st generate Rectangle 50000 100 filename=H2O_ACN_SHAPE1; st manipulate H2O_ACN_SHAPE1 offs b s 1000000 2 0 100 1672.8 100" command to set a macro for suppression and apply the macro with parameters. The last five numbers of the command represent: (a) the number of suppressions; (b) the offset of 1st suppression from the O1 (the 1st suppression is for acetonitrile, so this value is always 0); (c) the strength for 1st suppression (100 is recommended); (d) the offset of 2nd suppression from the O1 (the 2nd suppression is for water, so this value is the offset of water minus that of acetonitrile); (e) the strength for 2nd suppression (100 is recommended).

(5) Perform a preliminary scan, and check the peak heights of acetonitrile and water. If the height of water is still rather high, change the offset of 2nd suppression from the O1 at step (4) and perform step (5) again, until the heights of both solvents are small. Then, the automatic receiver gain increases (e. g. RG: 16) to let the peaks from analytes detectable. A good suppressed NMR spectroscopy is showed in **Figure 3**. The two satellite peaks of acetonitrile (A1 and A2 in **Figure 2** and **Figure 3**) are not changed since they are not suppressed. Compared with **Figure 2** and **Figure 3**, since peaks of A1 and A2 are unchanged, it is showed that both peaks from water and acetonitrile are suppressed to very low.

(6) Set NS to 160, D1 to 32 s, and perform quantitative scan.

(7) After manual phasing, automatic baseline correction, and manual integration, the peak at chemical shift at 8.5 from benzoic acid was used as the quantitative peaks of internal standard. The peaks at chemical shift of 6.0, 5.9 and 5.8 from avermectin were used as quantitative peaks and the average result from these three peaks was regarded as the result of this determination. Since the uncertainty from other sources (mass weighing, purity of internal standard, molecular weight) are much less than the SD, the expanded uncertainty is equal to 2 (coverage factor) times of SD.

3. Results and discussion

3.1. Loop counter (L6)

The results of experiments and expected values with each expanded uncertainty (by traditional mass balance method) were shown in **Table 1**. The pulse program suppresses the signal of two solvents simultaneously by pre-saturating signals with selective shaped pulses. Loop counter is the number of times of the pre-saturation shape pulse imposed before one scan. While the loop counter decreases, lesser suppression was imposed to the solvents and lesser distortion of peak areas was observed. While the loop counter increases, more suppression was imposed, and then higher receiver gain and higher signal-to-noise was obtained. Experiment A, B, C and D (**Table 1** and **Figure 4**) were tests based on L6 as 4, 3, 2 and 1, respectively. Lower L6 showed less biases, so the least value of loop counter (L6=1) was chosen.

3.2. Suppression strength

With lesser suppression strength imposed to the solvents, lesser distortion of peak areas may be observed. However, comparison between strengths of 100 (C1 and D1) and lesser strengths (C2, C3, C4 and D2) at **Table 1** and **Figure 4** showed that lesser suppression strength did not improve the accuracy significantly. Therefore, the recommended strengths (100 for each solvent) were used.

3.3 Number of scan

Since lesser loop counter leads to lower receiver gain and lower signal-to-noise, higher number of scan was tried to improve the signal-to-noise. By setting L6 to 1, and setting two suppression strengths to 100, the number of scan was increased from 64 to 1024 (D1 and D3 at **Table 1** and **Figure 4**), higher signal-to-noise was obtained, the bias decreased slightly. Therefore, the number of scan as 160, between 64 and 1024, was applied for lesser bias and shorter time. (Since D1 = 32 s, about 0.5 min for a scan)

3.4. Repeatability and bias

Based on these experiments, the optimized condition is that with loop counter as 1, two suppression strengths as 100, and number of

Table 1 Parameters and results of experiments

No.	*L6*	S_{ACN}	S_{D2O}	*RG*	*NS*	*P*	*U*
A	4	100	100	16	32	94.7%	2.3%
B	3	100	100	16	32	97.5%	2.0%
C1	2	100	100	8	64	92.5%	1.6%
C2	2	100	50	8	64	95.3%	3.4%
C2	2	100	20	8	32	96.1%	2.1%
C4	2	90	50	4	32	92.3%	5.9%
D1	1	100	100	5.6	64	95.5%	3.9%
D2	1	100	50	5.6	64	92.5%	4.0%
D3	1	100	100	4	1024	94.8%	3.7%
E1	1	100	100	12.7	160	94.5%	1.5%
E2	1	100	100	14.2	160	94.3%	2.8%
E3	1	100	100	8	160	94.7%	1.6%
E4	1	100	100	10	160	93.1%	1.5%
E5	1	100	100	8	160	96.2%	2.1%
E6	1	100	100	8	160	95.0%	0.4%

L6: the loop counter of suppression method; S_{ACN}: the suppression strength for acetonitrile; S_{D2O}: the suppression strength for acetonitrile; *RG*: automatic receiver gain; *NS*: number of scans; *P*: purity result of avermectin B1a; *U*: uncertainty (*k*=2) of the purity.

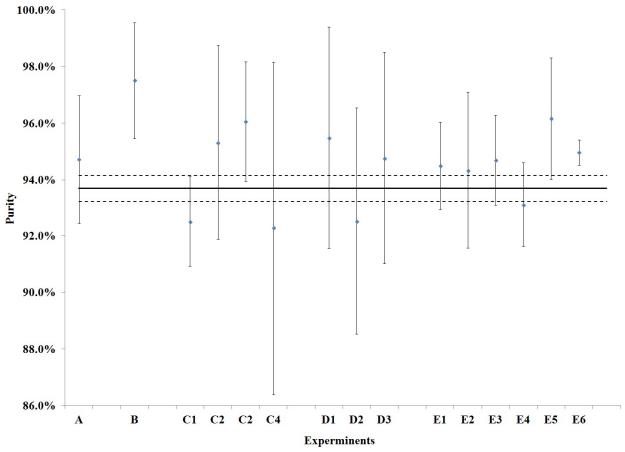

Fig. 4 Results of experiments

See the conditions in **Table 1**. The purity results with each uncertainty were plotted. The vertical solid line and two dash lines: the result with its uncertainty (*k*=2) of the mass balance method.

scan as 160. Six determinations were performed and the results are shown in E1~E6 at **Table 1** and **Figure 4**. The mean and uncertainty (94.63% ± 1.97%, RSD=0.985%) is consistent with that from mass balance method (93.70% ± 0.46%), because the difference between two methods (0.93%) is smaller than the square root of sum of two uncertainties (2.02%).

3.4. Comparison with traditional method

A rough estimation for comparison among these methods was listed in **Table 2**. For the mass balance method, HPLC, LC-MS, gas chromatography, gas chromatography-mass spectrometry, inductively coupled plasma mass spectrometry and Karl Fischer titration was involved, then its cost and time for each determination is high, but the RSD is low. For the continuous LC-NMR using two deuterated mobile phases (CD_3CN and D_2O), its cost for mobile phase is extremely high. For the Single deuterated mobile phase LC-NMR using one deuterated mobile phases of (D_2O), its cost for mobile phase is very high. For the LC-SPE-NMR using SPE instrument, the bias and RSD is limited by the recovery rate of SPE procedure. For the two-signal suppression LC-qNMR in this study using common mobile phases, its cost is low, moreover, the bias and RSD is low after optimization of key parameters.

Table 2 Rough estimation for comparison of methods

	$Cost_{(mp)}$	$Cost_{(Ins)}$	Mass	Time	Bias	RSD
Mass balance method	10	5000	30	2 days	-	0.5%
Continuous LC-NMR	14000	300	10	1 hour	1%	0.7%
Single deuterated mobile phase LC-NMR	4000	300	10	1 hour	10%	3%
LC-SPE-NMR	10	1000	10	3 hours	5%	3%
Two-signal suppression LC-qNMR	10	300	20	2 hours	1%	1%

$Cost_{(mp)}$: cost of mobile phase (RMB) per determination; $Cost_{(ins)}$: cost of instrument (RMB) per determination; Mass: mass (mg) of sample per determination; Time: time per determination; Bias: bias from mass balance method; RSD: relative standard deviation of determinations.

4. Conclusions

A new HPLC-qNMR method using common HPLC mobile phases (CH_3CN and H_2O) and a new two-signal suppression method was proposed and verified. It has the advantage of lower cost, compared to the continuous LC-NMR and the single deuterated mobile phase LC-NMR method. Its operation is simpler and more rapid, compared to LC-SPE-NMR and the mass balance method. After several time of short scans, the optimized condition can yield an accurate result with bias no more than 1%. Therefore, it will be widely applied in the future, since it combines the efficient separation capability of HPLC and high qualitative/quantitative capability of NMR, with very low cost, rapid operation and good accuracy.

Abbreviations

D1 relaxation delay
DAD diode array detector
DMSO dimethyl sulfoxide
FID free induction decay
GS Interactive parameter optimization during acquisition
HPLC high performance liquid chromatography
L6 loop counter
LC-MS liquid chromatography - mass spectrometry
NIM National Institute of Metrology, China
NIST National Institute of Standard and Technology, USA
NMR nuclear magnetic resonance
NS number of scan
O1 irradiation frequency offset
qNMR quantitative nuclear magnetic resonance

RG	receiver gain
RMB	currency of China
SD	standard deviation
SPE	solid phase extraction
SRM	Standard Reference Material®

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