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Quantitative ^{1}H NMR Determination of Purity and Water Content in Berberine Hydrochloride

Solvent Selection, Proton Exchange, Hydrate Stoichiometry and the Mass-Balance Framework for a Hygroscopic Quaternary Alkaloid Salt

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Abstract

Berberine hydrochloride is supplied commercially as a hygroscopic quaternary protoberberine alkaloid salt whose water content is neither fixed nor trivial. The anhydrate, dihydrate and tetrahydrate are all documented crystalline forms, and interconversion among them is driven by ambient water activity at room temperature, so that the water content of a given lot may range from essentially zero to more than sixteen percent by mass without any change in chemical identity. Any purity value assigned to such a material is therefore meaningless unless the reporting basis and the hydration state are declared alongside it. This paper develops a complete quantitative ^{1}H NMR (qNMR) framework for the assay of berberine hydrochloride and subjects the specific proposal of a methanol-d_4 solution measurement to critical evaluation.

The berberine cation carries no exchangeable protons of its own, and its eighteen non-labile protons are distributed across eleven resolved multiplets, several of which are singlets in uncrowded regions of the spectrum. Internal-standard qNMR against a certified calibrant therefore yields the molar quantity of berberine cation directly, with a combined standard uncertainty of approximately 0.55 % relative under realistic laboratory conditions at 300 MHz. From that single measurement, the content may be expressed on any of four legitimate bases which, for one worked example, differ by seventeen percentage points — 81.21 % w/w as the free cation, 89.77 % w/w as anhydrous berberine chloride, 98.47 % w/w as the dihydrate, and 90.23 % w/w on the anhydrous, solvent-free basis. The commercial and regulatory consequences of confusing these bases are substantial.

The determination of water in methanol-d_4 is shown to be considerably more problematic than it first appears. Water protons in that solvent are not observed as a discrete water resonance; they undergo rapid chemical exchange with the residual hydroxyl protons of the solvent and are observed as a single exchange-averaged singlet near δ 4.87. Two independent difficulties follow. First, the observed resonance carries a solvent-derived background which, for a nominal 0.6 mL preparation, amounts to approximately 47 µmol of exchangeable ^{1}H — roughly one third of the total signal for a 10 mg dihydrate specimen — and which is itself unstable because methanol-d_4 is strongly hygroscopic. Second, and more damaging, the exchange-averaged resonance lies within about 0.08 ppm of the berberine H-6 triplet at δ 4.95, corresponding to only 24 Hz of separation at 300 MHz and 4.8 Hz at 60 MHz, so that the two signals are not resolved on any spectrometer in common laboratory service. Numerical analysis presented here shows that integrating the composite envelope as though it were water alone overestimates the water content by 98 %, and that subtracting a solvent blank without also correcting for the H-6 contribution still leaves a 50 % positive bias.

Four routes to the water value are set out and compared quantitatively. Direct integration of the exchange-averaged resonance is shown to be theoretically sound — exchangeable protons are conserved in a sealed tube — but to require both a gravimetrically matched solvent blank and an explicit correction for the overlapping H-6 signal, the latter being available at no cost from the cation assay itself. Determination of water by difference from the qNMR cation content is developed as an alternative that never touches the exchangeable region, and its error amplification is quantified: an uncertainty of 0.55 % relative on the cation propagates to approximately 4.5 % relative on a water content near 10 % w/w, and any unmeasured residual solvent or inorganic residue is transferred wholesale into the water value. Dimethyl sulfoxide-d_6 is shown to halve the solvent background and to double the spectral separation between water and the nearest analyte signal, and is recommended where an NMR-only water determination is required. Coulometric Karl Fischer titration remains three to four times more precise than any of the NMR routes and is recommended as the reference method for release testing.

The paper further addresses internal standard selection for methanol-d_4 — with dimethyl sulfone identified as spectroscopically optimal but flagged as itself hygroscopic — acquisition and processing parameters appropriate to a 300 MHz instrument, a full uncertainty budget, the internal integral-ratio consistency test that detects protoberberine congeners such as palmatine, coptisine, jatrorrhizine and epiberberine, the closure of a five-term

mass balance, and the feasibility of the assay on 60 MHz benchtop instrumentation. A recommended workflow suitable for incorporation into an ISO/IEC 17025 scope is presented.

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1. Introduction and Scope

Berberine, the 9,10-dimethoxy-2,3-methylenedioxyprotoberberinium cation, is among the most widely traded botanical actives in the global supplement market and remains an established pharmaceutical agent in several Asian pharmacopoeias. It is isolated principally from Coptis chinensis, Berberis species and Phellodendron species, and it is almost invariably supplied as the chloride salt. Commercial demand has expanded rapidly, and with that expansion has come a well-documented deterioration in label-claim compliance: an NMR-based survey of eighteen commercial berberine food supplements found that only 56 % of the formulations contained the quantity of berberine declared on the label.[1] Independent chromatographic surveys of the North American retail market have reached comparable conclusions. Reliable content assignment of the raw material is therefore not an academic exercise but a commercial and regulatory necessity.

Two properties of berberine hydrochloride make its content assignment considerably less straightforward than the assay of a typical small-molecule active. The first is that the compound is a hydrate former. Anhydrous, dihydrate and tetrahydrate crystalline phases have all been isolated and characterized, and the interconversions among them are governed by water activity at ordinary room temperature rather than requiring thermal activation.[2] Dynamic vapor sorption measurements have shown that the anhydrate takes up water corresponding to the dihydrate stoichiometry across the 10–65 % relative humidity range and progresses to the tetrahydrate stoichiometry between 85 % and 95 % relative humidity.[3] A specimen removed from a laboratory desiccator and weighed on an open pan in a humid room is therefore not the same material at the end of the weighing as it was at the beginning. The second property is that the berberine cation is a quaternary isoquinolinium species bearing no hydroxyl, amine or carboxyl functionality whatsoever. It possesses no exchangeable protons of its own, which is analytically advantageous for the cation assay but which also means that every exchangeable proton observed in a solution spectrum originates either from water associated with the sample or from the solvent system.

The specific analytical question addressed here is whether a single quantitative ^{1}H NMR experiment, performed on a solution of the material in methanol-d_4, can deliver both the purity of the berberine hydrochloride and the quantity of water present. The short answer, developed at length in Section 6, is that the cation content is obtained readily and with good metrological rigor, whereas the water content obtained from the exchangeable region of the same spectrum is compromised by two effects that are frequently overlooked and that, if left uncorrected, generate errors approaching a factor of two. The purpose of this paper is to set out the correct treatment, to quantify the magnitude of each error source, and to establish which of several alternative routes to the water value is defensible for release testing under an accredited quality system.

The scope of the paper covers the chemistry and solid-form behavior of the analyte, complete ^{1}H assignment in the two solvents of practical interest, the metrological framework within which “purity” acquires an unambiguous meaning for a hydrated salt, the design of the internal-standard qNMR experiment, four alternative approaches to water determination with a quantitative comparison among them, a fully worked numerical example, an uncertainty budget, validation requirements aligned with ICH Q2(R2) and ISO 24583, and the practical implications of performing the work on 300 MHz and 60 MHz instrumentation. Chromatographic and titrimetric methods are treated only insofar as they serve as orthogonal confirmation or as reference methods against which the NMR results are judged.

2. Analyte Chemistry and Solid-Form Considerations

2.1 Structure, Protonation State and Proton Inventory

The berberine cation has the molecular formula $C_{20}H_{18}NO_4^+$ and a relative molecular mass of 336.36. The positive charge is delocalized over the isoquinolinium ring system and is not the result of protonation; consequently the

cation exists as a permanent quaternary species across the entire accessible pH range, and no acid–base equilibrium perturbs its NMR spectrum. Charge balance in the commercial material is provided by chloride, giving the anhydrous salt $C_{20}H_{18}ClNO_4$ with a relative molecular mass of 371.81. The eighteen protons of the cation are distributed as follows: four aromatic methine protons on the tetracyclic framework at positions 1, 4, 11 and 12; the highly deshielded H-8 iminium proton of ring C; the H-13 methine proton bridging rings C and D; the two-proton methylenedioxy bridge spanning C-2 and C-3; two aromatic methoxy groups at C-9 and C-10 contributing six protons in total; and the ethylene bridge of the 5,6-dihydro segment, comprising the benzylic H-5 methylene and the N^+-adjacent H-6 methylene, contributing four protons. This inventory is important because the internal ratios among these signals constitute a free and highly informative acceptance test, developed in Section 3.3.

It should be emphasized that no proton in this inventory is labile. In a solution prepared in a deuterated protic solvent, none of the berberine signals decays with time through deuterium exchange, and the total observed berberine proton intensity is therefore stable over the course of an extended acquisition. This distinguishes berberine favorably from analytes bearing hydroxyl or amine functionality, for which the qNMR practitioner must either select non-exchangeable signals or accept a time-dependent intensity.

Table 1. Composition of the crystalline forms of berberine chloride.

Form	Formula	M_r (g mol^{-1})	w(cation) (% w/w)	w(Cl^-) (% w/w)	w(H_2O) (% w/w)	Uptake on anhydrous basis (%)
Berberine cation	$C_{20}H_{18}NO_4^+$	336.36	100.00	—	—	—
Anhydrate	$C_{20}H_{18}ClNO_4$	371.81	90.47	9.53	0.00	0.00
Dihydrate	$C_{20}H_{18}ClNO_4 \cdot 2H_2O$	407.84	82.47	8.69	8.83	9.69
Tetrahydrate	$C_{20}H_{18}ClNO_4 \cdot 4H_2O$	443.87	75.78	7.99	16.23	19.38

Atomic masses taken as C 12.011, H 1.008, N 14.007, O 15.999, Cl 35.45. The final column expresses the mass of hydrate water relative to the mass of the anhydrous salt, which is the convention used in vapor-sorption reporting; the preceding column expresses the same water as a fraction of the hydrated solid, which is the convention used in Karl Fischer and loss-on-drying reporting. The two conventions differ by roughly a percentage point at the dihydrate composition and by more than three percentage points at the tetrahydrate composition, and confusion between them is a recurrent source of specification disputes.

2.2 Hydrate Stoichiometry and Hygroscopic Behavior

The dihydrate is the phase most commonly encountered in pharmacopoeial-grade material, and it is the form implicitly assumed by most commercial certificates of analysis even when the assumption is not stated. Its water content of 8.83 % w/w corresponds to the theoretical figure that a coulometric Karl Fischer determination should return for stoichiometrically pure material equilibrated at moderate humidity. The tetrahydrate, at 16.23 % w/w water, is favored at high water activity, and the transformation between the two phases has been shown to occur during ordinary pharmaceutical unit operations: wet massing of pharmacopoeial-grade berberine chloride drove a thermodynamically controlled conversion to the tetrahydrate at room temperature, while subsequent tray drying at 50 °C reverted the material to the dihydrate.[2] The rate and extent of the conversion depended on the water activity of the powder bed, which in a formulated system is governed by the excipients present.

Two operational consequences follow, and both are more important than the choice of NMR pulse sequence. The first is that the water content reported for a lot is a property of that lot at the moment of sampling, under the humidity conditions then prevailing, and not an intrinsic constant of the substance. A certificate of analysis that reports water without also reporting the equilibration conditions is incomplete. The second is that the qNMR aliquot and the Karl Fischer aliquot must be drawn from the same homogenized portion within a short interval, and preferably after

deliberate equilibration of the bulk material over a saturated salt solution of known relative humidity. If the two aliquots are weighed hours apart in an air-conditioned laboratory whose humidity is drifting, the mass balance developed in Section 4 will not close, and the analyst will be left attributing the discrepancy to a measurement error that did not occur.

A related caution applies to thermogravimetric determination of water. Berberine undergoes regioselective demethylation to berberrubine on heating, a transformation exploited synthetically under microwave conditions and by conventional thermal treatment.[4,5] Mass loss observed at elevated temperature in a thermogravimetric experiment therefore cannot be assumed to represent water alone, and the water-loss step must be isolated from the decomposition step by coupling the balance to an evolved-gas detector or by restricting the integration window to the low-temperature dehydration event confirmed independently by differential scanning calorimetry or variable-temperature powder diffraction.

2.3 Related Protoberberine Alkaloids and Degradation Products

Berberine isolated from botanical sources is accompanied by a characteristic suite of congeneric protoberberine alkaloids whose separation from berberine is incomplete in many commercial processes. The principal species are palmatine, in which the methylenedioxy bridge of ring A is replaced by two additional methoxy groups; coptisine, in which the C-9/C-10 methoxy pair is replaced by a second methylenedioxy bridge; jatrorrhizine and columbamine, which are hydroxylated trimethoxy congeners; and epiberberine, which is a positional isomer of berberine carrying the methylenedioxy bridge on ring D and the methoxy pair on ring A. Because epiberberine is isomeric with berberine, it is invisible to mass-based screening and to any method that relies on molecular formula, and its discrimination requires either chromatographic resolution or the chemical-shift dispersion of NMR.

The distinctive value of ^{1}H NMR in this context is that the substitution pattern of each congener is written directly into the aliphatic and low-field aromatic regions of the spectrum. The interval between δ 3.8 and δ 6.5 contains only methoxy singlets and methylenedioxy singlets, and the number and integrated intensity of the resonances in that interval fingerprint the substitution pattern unambiguously: berberine presents one methylenedioxy singlet of two protons together with two methoxy singlets of three protons each; palmatine presents four methoxy singlets and no methylenedioxy resonance; coptisine presents two methylenedioxy singlets and no methoxy resonance; and the hydroxylated congeners present three methoxy singlets and no methylenedioxy resonance. This has been the basis of quantitative ^{1}H NMR methods for protoberberine profiling in Coptidis Rhizoma and Phellodendri Cortex, which have consistently exploited the resolved H-13 singlet near δ 8.7 in methanol-d_4 for the quantitative measurement.[6−9] The same H-13 resonance has been quantified against an electronic reference in place of an added internal standard, and the broader principle of exploiting a single resolved signal for absolute quantification within a complex natural matrix has been reviewed in detail.[10]

Two degradation products deserve specific mention because they are diagnostically silent to methods that monitor the berberine chromophore alone. 8-Oxoberberine, the lactam formed by oxidation at C-8, lacks the H-8 resonance entirely and is a neutral molecule rather than a quaternary cation; 5,6-dihydroberberine and related reduced species similarly lack the low-field H-8 signal. The interval from δ 9.5 to δ 10.0 is essentially empty in a berberine spectrum apart from H-8 itself, so the integrated intensity of that signal relative to H-13 constitutes a direct and sensitive test for oxidative or reductive modification at C-8. Berberrubine, the C-9 demethylation product noted in Section 2.2, presents a single methoxy singlet rather than two and is therefore detected by the same aliphatic-region test.[5]

3. The ^{1}H NMR Spectrum of Berberine Chloride

3.1 Assignment in Methanol-d_4

Methanol-d_4 has been the solvent of choice in the published quantitative ^{1}H NMR literature on protoberberine alkaloids, principally because the H-13 singlet falls in an interval between δ 8.6 and δ 8.9 that is free of interference from other alkaloid signals and from the botanical matrix, allowing berberine and palmatine to be resolved and integrated simultaneously in crude extracts without chromatographic cleanup.[6,7] Berberine chloride dissolves readily in methanol at the concentrations required for qNMR, which is not the case in water, where the aqueous solubility of the salt is of the order of a few millimolar.

Representative chemical shifts are collected in Table 2. These values are consistent across the protoberberine NMR literature but should be treated as nominal: chemical shifts in this system vary by up to 0.03 ppm with concentration, temperature and residual water content, and the berberine cation is a planar aromatic species which undergoes concentration-dependent self-association through π-stacking. Laboratories adopting this method are advised to establish their own reference spectrum at the working concentration and temperature rather than transcribing literature values into a specification.

Table 2. Representative ^{1}H NMR parameters for berberine chloride in methanol-d_4 at 298 K.

δ (ppm)	Multiplicity	J (Hz)	nH	Assignment	Suitability for qNMR
9.76	s	—	1	H-8 (iminium)	Excellent — fully isolated
8.72	s	—	1	H-13	Excellent — literature signal of choice
8.10	d	9.2	1	H-11	Good — verify no congener overlap
8.01	d	9.2	1	H-12	Good — verify no congener overlap
7.68	s	—	1	H-1	Good
6.94	s	—	1	H-4	Good
6.10	s	—	2	OCH_2O (2,3-methylenedioxy)	Excellent — 2H, isolated
≈ 4.95	t	≈ 6.2	2	H-6 (N^+–CH_2)	Unusable — overlaps exchange peak
≈ 4.87	br s	—	var.	Exchange-averaged H_2O / CD_3OH hydroxyl	See Section 6
4.21	s	—	3	OCH_3 (C-9 or C-10)	Good — 3H, high intensity
4.10	s	—	3	OCH_3 (C-10 or C-9)	Good — 3H, high intensity
3.26	t	≈ 6.2	2	H-5 (benzylic CH_2)	Acceptable

Referenced to the residual CHD_2OD quintet at δ 3.31. The individual assignment of the two methoxy singlets to C-9 and C-10 is not resolved by one-dimensional data alone and requires heteronuclear multiple-bond correlation or nuclear Overhauser evidence; for quantitative purposes the assignment is immaterial, since either singlet, or their sum as a six-proton envelope, serves equally well. The position of the exchange-averaged resonance is strongly dependent on temperature and on total water content and should be re-established for each solvent lot.

3.2 Assignment in Dimethyl Sulfoxide-d_6

Dimethyl sulfoxide-d_6 is the solvent in which the majority of structural assignments for berberine and its derivatives have been reported, and it possesses one decisive advantage for the present problem: it contains no exchangeable protons. Water dissolved in dimethyl sulfoxide-d_6 is observed as a discrete water resonance near δ 3.33 rather than being averaged into a solvent hydroxyl pool, and the integrated intensity of that resonance is attributable to water alone.[11–13] The consequences for water determination are developed in Section 6.4.

Table 3. Representative ^{1}H NMR parameters for berberine chloride in dimethyl sulfoxide-d_6 at 298 K.

δ (ppm)	Multiplicity	J (Hz)	nH	Assignment
9.89	s	—	1	H-8
8.94	s	—	1	H-13
8.19	d	9.1	1	H-11
8.00	d	9.1	1	H-12
7.79	s	—	1	H-1
7.08	s	—	1	H-4
6.17	s	—	2	OCH_2O
4.93	t	≈ 6	2	H-6
4.09	s	—	3	OCH_3
4.06	s	—	3	OCH_3
≈ 3.35	br s	—	var.	H_2O (discrete — no solvent exchange partner)
3.20	t	≈ 6	2	H-5

Referenced to the residual $(CD_2H)(CD_3)SO$ quintet at δ 2.50. The water resonance drifts downfield as the total water content of the solution rises; at the water loading generated by a 10 mg dihydrate specimen in 0.6 mL of solvent the drift is small, but the position must be confirmed empirically for each preparation.

3.3 Internal Consistency Ratios as an Acceptance Criterion

Because the proton inventory of the berberine cation is fixed, the integrated intensities of its resonances must stand in exact and known ratio to one another. Expressed against the H-13 singlet taken as unity, the expected ratios are 1.000 for each of H-8, H-11, H-12, H-1 and H-4; 2.000 for the methylenedioxy bridge and for each of the H-5 and H-6 methylenes; and 3.000 for each methoxy singlet. This relationship costs nothing to evaluate, requires no reference material, and constitutes the single most powerful diagnostic available in the experiment.

A deviation exceeding roughly two percent on any ratio indicates one of a small number of specific conditions, each of which is actionable. An elevated ratio on a signal implies coincidental overlap with a congener or with a residual solvent resonance, which for the methoxy region most commonly means palmatine, jatrorrhizine or columbamine. A depressed H-8 ratio relative to H-13 implies oxidation or reduction at C-8. A systematic drift in the ratios of the aliphatic signals relative to the aromatic signals implies incomplete relaxation, since the methoxy and methylene protons relax substantially faster than the isolated aromatic methines and will therefore be the last to reveal an inadequate recycle delay. Uniform depression of the higher-frequency signals implies inadequate excitation bandwidth or a miscalibrated pulse. Adoption of the ratio test as a formal system suitability requirement is

recommended, with the acceptance limit set on the basis of the repeatability actually demonstrated during method validation rather than on an arbitrary figure.

It is worth noting explicitly that the ratio test is a relative measurement and therefore survives any error in the weighing of either the sample or the internal standard. It is a test of the spectrum and of the analyte, not of the gravimetry, and it should be evaluated and recorded before the purity calculation is performed.

4. Metrological Framework: What "Purity" Means for a Hydrated Salt

4.1 The Mass Balance Identity

Quantitative NMR is a primary ratio method, in the sense that the observed signal area is proportional to the number of resonant nuclei through a proportionality constant that is common to analyte and calibrant and therefore cancels.[14–17] What the experiment returns is a molar quantity of a defined molecular entity, and for berberine hydrochloride that entity is the berberine cation. It is not the salt, and it is not the hydrate. Every reported purity figure is consequently the product of a measurement and a decision about basis, and the decision must be stated.

The complete composition of a lot of berberine hydrochloride is expressed by the mass balance identity of Equation 1, in which every mass fraction refers to the material as received and as weighed:

$$w(\text{cation}) + w(Cl^-) + w(H_2O) + w(\text{residual solvent}) + w(\text{inorganic residue}) + w(\text{organic related substances}) = 1 \qquad (1)$$

The distinctive strength of a qNMR-centered approach is that four of these six terms are accessible from a single proton spectrum. The cation content follows from internal-standard calibration against the non-exchangeable berberine signals. Residual organic solvents — methanol, ethanol, acetone, isopropanol and ethyl acetate are the species most commonly encountered in this material — appear as characteristic resonances in the aliphatic region and are quantified against the same internal standard in the same experiment. Organic related substances of the protoberberine class are detected and, where their signals are resolved, quantified through the aliphatic fingerprint region described in Section 2.3. Water, in principle, is accessible through the exchangeable region, subject to the very substantial qualifications developed in Section 6. Only the chloride counterion and the inorganic residue fall entirely outside the proton experiment; chloride may be obtained by argentometric titration, by ion chromatography, or by ^{35}Cl quantitative NMR against a potassium perchlorate internal standard,[27] and the inorganic residue by sulfated ash or residue on ignition.

Closure of the identity is the strongest available evidence that the analysis is correct. If the six terms sum to unity within their combined uncertainty, each individual determination is corroborated by the others; if they do not, at least one determination is wrong or at least one component is unaccounted for, and the analyst is warned before the certificate is issued. This is a materially stronger position than that occupied by a single chromatographic assay, which returns a number with no internal check on its own validity.

4.2 Reporting Bases and the Consequences of Confusing Them

For a material whose water content may lawfully range from zero to more than sixteen percent, the choice of reporting basis is not a formatting question. Table 4 sets out four legitimate expressions of the same measured quantity for the worked example developed in Section 7, in which the cation content of the as-received material was determined to be 81.21 % w/w, the water content by coulometric Karl Fischer titration was 9.15 % w/w, and residual ethanol amounted to 0.85 % w/w.

Table 4. Four legitimate purity expressions for a single measured cation content.

Reporting basis	Value (% w/w)	Derivation from w(cation)	Typical context
Berberine cation, as received	81.21	Direct qNMR result	Metrologically primary; unambiguous
Anhydrous berberine chloride, as received	89.77	× 371.81 / 336.36	Salt basis, water excluded from the numerator
Berberine chloride dihydrate, as received	98.47	× 407.84 / 336.36	Basis implied by most supplier certificates
Berberine cation, anhydrous and solvent-free	90.23	÷ (1 − 0.0915 − 0.0085)	Basis used for potency and dosing calculations

All four figures describe the identical specimen. The spread between the lowest and the highest is 17.26 percentage points.

The practical importance of Table 4 is difficult to overstate. A supplier certificate declaring “not less than 98 %” is, on the evidence of the market, most often expressing the dihydrate basis. A purchaser who interprets that figure as the anhydrous salt content and formulates accordingly will under-deliver berberine by approximately nine percent, an error large enough to move a finished product outside a conventional 90–110 % label-claim specification on its own, before any manufacturing variability is considered. Conversely, a laboratory that reports the primary cation figure of 81.21 % without explanation will be told by its client that the material has failed, when in fact it is a compliant dihydrate. The remedy is simple and should be non-negotiable in an accredited report: the basis is stated in the same sentence as the number, the water content is reported alongside, and the conversion factors are shown.

A closely related trap attends the use of a berberine reference standard for chromatographic calibration. If the assigned purity of the calibrant is expressed on a dihydrate basis and the calibrant has since equilibrated toward the tetrahydrate on the laboratory shelf, every chromatographic result calibrated against it carries a proportional negative bias. This mechanism has been demonstrated directly: quantitative ^{1}H NMR assay of commercial protoberberine reagents returned purities systematically lower than the manufacturers’ declarations, with the consequence that alkaloid contents of Coptidis Rhizoma determined by HPLC against those reagents were correspondingly overestimated.[8] The argument for assigning the calibrant purity in-house by qNMR, on a stated basis, immediately before use, rests on exactly this observation.

5. Determination of Berberine Cation Content by Internal Standard ^{1}H qNMR

5.1 Internal Standard Selection

The internal standard must satisfy the conventional criteria — solubility in the chosen solvent, chemical inertness toward the analyte, a simple and preferably singlet resonance well separated from every analyte signal, availability as a certified reference material with a small assigned uncertainty, and stable lineshape[30] — and, for the present application, it must satisfy one additional and easily overlooked criterion: it must contain no exchangeable protons. Any calibrant bearing a carboxyl, hydroxyl or amine function contributes its labile protons to the same exchange-averaged resonance in which the water signal resides, corrupting the water determination even where the calibrant serves the cation assay perfectly well.

This consideration eliminates maleic acid, which is otherwise an attractive choice for methanol-d_4 because its two-proton vinyl singlet at δ 6.27 lies clear of the berberine methylenedioxy resonance at δ 6.10, giving 51 Hz of separation at 300 MHz. Maleic acid contributes two carboxyl protons per molecule to the exchangeable pool and is therefore inadmissible whenever water is to be measured in the same preparation. Potassium hydrogen phthalate is similarly disqualified on the grounds of its acidic proton, and in any case its solubility in methanol-d_4 is limited to below about 2.5 mg mL^{-1}, which restricts the achievable signal-to-noise ratio.[29]

Dimethyl sulfone is the recommended primary calibrant. Its six equivalent methyl protons produce a sharp singlet at δ 3.06 in methanol-d_4, giving 57 Hz of separation from the berberine H-5 triplet at 300 MHz; it contains no exchangeable protons; it is available as a certified reference material with an assigned uncertainty in the low tenths of a percent; and it has been advocated since 2004 as a candidate universal reference material for quantitative NMR and is included in the internal standard suite validated under the auspices of the international metrology community.[28,29] Its solubility in methanol-d_4 is approximately 5 mg mL^{-1}, which is ample: a loading of 2–3 mg in 0.6 mL places the calibrant signal within a factor of three of the berberine methoxy signal, which is the desirable regime.

One caveat must be recorded against this recommendation, and it is directly relevant to a water determination. Among the substances commonly used as qNMR calibrants, dimethyl sulfone is the outlier for hygroscopicity: it has been shown to absorb approximately 25 % of its own mass in water over five weeks at 100 % relative humidity, whereas the other calibrants examined in the same study were essentially resistant to moisture uptake.[31] Water carried into the preparation on the calibrant is indistinguishable from water carried in on the sample, and it inflates the apparent water content of the analyte while simultaneously biasing the assigned calibrant mass. The mitigation is straightforward but must be enforced: the calibrant is stored desiccated, its water content is either determined or bounded by Karl Fischer titration on the working portion, and the certified value is verified rather than assumed. Where the sole objective is the cation assay and water is being determined by an orthogonal method, this caveat is of secondary importance; where the exchangeable region is to be integrated, it is not.

Alternative calibrants worth evaluating include 1,4-bis(trimethylsilyl)benzene-d_4 and perdeuterated sodium 3-(trimethylsilyl)propane-1-sulfonate, both of which place a nine-proton or eighteen-proton trimethylsilyl singlet in the region around δ 0 that is entirely vacant in the berberine spectrum. The trimethylsilyl signal has a long longitudinal relaxation time and requires a correspondingly long recycle delay, and the aromatic species is poorly soluble in methanol-d_4, but the complete absence of overlap risk makes these attractive where instrument time is not the binding constraint.

Table 5. Internal standard candidates for berberine chloride in methanol-d_4.

Calibrant	δ (ppm)	nH	Nearest berberine signal	Δν at 300 MHz (Hz)	Exchangeable H	Assessment
Dimethyl sulfone	3.06	6	H-5, δ 3.26	60	None	Recommended; control for hygroscopicity
Maleic acid	6.27	2	OCH_2O, δ 6.10	51	2 (COOH)	Rejected when water is co-determined
Dimethyl terephthalate	3.93	6	OCH_3, δ 4.10	51	None	Aromatic singlet coincides with H-11; use OCH_3 only
Potassium hydrogen phthalate	≈ 7.6–7.9	4	Aromatic envelope	—	1 (acidic)	Rejected: overlap and low solubility
DSS-d_6 / BTMSB-d_4	≈ 0.00	9 / 18	None within 3 ppm	> 900	None	Excellent isolation; long T_1; check solubility

5.2 Sample Preparation and Weighing

The analyte and calibrant are weighed separately into a tared vial on a balance of at least 0.01 mg readability, with a microbalance strongly preferred for reasons developed in Section 8. Weighing is performed by difference, and both masses are recorded to the full resolution of the instrument. A loading of approximately 10 mg of berberine chloride

and 2–3 mg of dimethyl sulfone into 0.60 mL of methanol-d_4 is appropriate at 300 MHz, yielding a berberine concentration near 40 mM and a signal-to-noise ratio on the one-proton aromatic singlets comfortably in excess of 250 after a modest number of transients.

Three preparation details bear directly on the accuracy of the result and are frequently mishandled. First, because berberine chloride is hygroscopic and undergoes phase conversion at room temperature, the interval between removing the specimen from its equilibration environment and sealing the NMR tube must be minimized and recorded, and the ambient relative humidity should be logged. Second, the solvent must be taken from a freshly opened ampoule or from a septum-sealed bottle penetrated by syringe; methanol-d_4 that has been repeatedly opened to laboratory air is unfit for any determination that involves the exchangeable region, and its water content is unknown regardless of the figure printed on the label. Third, the solvent aliquot should be dispensed gravimetrically rather than volumetrically. Volumetric dispensing introduces an uncertainty of one to two percent on the solvent quantity, which is immaterial to the cation assay because the internal standard travels with the sample, but which is entirely material to the solvent-blank subtraction required for water determination, where the blank must be scaled by solvent mass.

The solution is transferred to a matched 5 mm NMR tube of demonstrated concentricity and wall uniformity. Tube quality is not a trivial consideration: in externally calibrated qNMR, tube-to-tube variation has been shown to introduce errors well beyond those tolerable in a certification measurement.[33,34] The internal standard approach adopted here is largely insensitive to tube geometry because analyte and calibrant experience identical active volume, which is one of the principal arguments for preferring internal to external calibration in a routine testing laboratory. Nevertheless, the solvent blank required for the water determination is compared across tubes, and for that comparison tube quality matters; matched tubes from a single lot should be reserved for the blank and sample pair.

Berberine is a photosensitive chromophore. Preparations should be handled under subdued light, stored in amber vials or foil-wrapped, and analyzed without unnecessary delay. Where a stability-of-solution study is performed as part of validation, it should extend across the full period between preparation and the completion of the final acquisition.

5.3 Acquisition Parameters

The governing requirement of a quantitative acquisition is that every resonance to be integrated has returned to thermal equilibrium before each excitation. For a hard ninety-degree pulse, the residual signal deficit after a recycle interval D is $\exp(-D/T_1)$, and the error that matters is the difference between that deficit for the analyte and for the calibrant. Table 6 quantifies the relationship and establishes the basis for the widely adopted rule that the recycle interval, defined as the sum of the relaxation delay and the acquisition time, must equal or exceed seven times the longest longitudinal relaxation time among the integrated signals.[18–20] The requirement is codified in both the international standard governing internal-standard ^{1}H qNMR and the revised United States Pharmacopeia general chapters on nuclear magnetic resonance, the latter having become official in December 2025.[25,26]

Table 6. Residual signal deficit as a function of recycle interval.

Recycle interval D, expressed as a multiple of T_1	Residual deficit (%)	Consequence for a purity assay
$1 \times T_1$	36.79	Grossly quantitative; unusable
$3 \times T_1$	4.98	Unacceptable for any certification purpose
$5 \times T_1$	0.674	Marginal; comparable to the entire uncertainty budget

Recycle interval D, expressed as a multiple of T_1	Residual deficit (%)	Consequence for a purity assay
$7 \times T_1$	0.091	Recommended minimum; negligible against other terms
$10 \times T_1$	0.0045	Diminishing returns for the instrument time consumed

Longitudinal relaxation times must therefore be measured, not assumed. In this system the isolated aromatic methine protons, having few nearby dipolar partners, relax slowly and are expected to exhibit T_1 values of several seconds in methanol-d_4 at 298 K, whereas the methoxy and methylene protons relax appreciably faster. The dimethyl sulfone methyl singlet is also comparatively slow. An inversion-recovery experiment is run on the actual working preparation, and the longest observed T_1 governs the recycle interval; rapid estimation methods are available where full inversion-recovery would consume excessive instrument time.[38] Table 7 sets out a parameter set appropriate to a 300 MHz instrument. It is offered as a starting point, not as a specification, and every entry should be confirmed against locally measured relaxation data.

Table 7. Recommended acquisition and processing parameters at 300 MHz.

Parameter	Setting	Rationale
Pulse sequence	Single-pulse, hard 90° excitation	Maximizes sensitivity per transient; avoids flip-angle-dependent bias
Pulse width	Calibrated on the working sample	Salt content alters probe tuning; a nominal value is inadequate
Spectral width	20 ppm (≈ 6000 Hz), centered near δ 6	Encloses δ 9.9 to δ 0 with margin for the calibrant
Acquisition time	≥ 4 s	Gives ≤ 0.25 Hz digital resolution before zero filling
Relaxation delay	≥ 7 × T_1(max) minus acquisition time; typically 55–60 s	Per Table 6
Transients	16–64, adjusted to S/N ≥ 250 on the weakest integrated signal	Governs the integration uncertainty
Dummy scans	4	Establishes the steady state before accumulation
Temperature	298.0 K, regulated, 10 min equilibration	Exchange-peak position and T_1 are temperature dependent
Sample rotation	Off	Avoids modulation sidebands on intense singlets
Receiver gain	Fixed across sample, blank and standard; verify no ADC overflow	Overflow truncates the intense exchange resonance first
Apodization	Exponential, 0.3 Hz, or none with fourfold zero filling	Excessive line broadening biases narrow signals against broad ones
Phasing	Manual, verified across the full spectral width	Automatic routines fail on very intense exchange resonances

Parameter	Setting	Rationale
Baseline correction	Polynomial of order ≤ 5, or Whittaker smoother, over the whole spectrum	Regional correction distorts integrals at region edges
Integration limits	Symmetric, ≥ 64 × the linewidth at half height	Captures ^{13}C satellites and the Lorentzian wings

5.4 Processing, Integration and the Satellite Question

Carbon-13 at natural abundance generates satellite doublets flanking every proton resonance and carrying, in aggregate, approximately 1.1 % of the parent intensity. Two treatments are defensible: the integration limits may be set wide enough to enclose the satellites for both analyte and calibrant, or narrow enough to exclude them for both. Either choice cancels to first order. What is not defensible is the mixed case, in which the satellites of a well-isolated calibrant singlet are captured while those of a crowded analyte signal are excluded, which introduces a systematic bias of approximately one percent — twice the entire combined uncertainty of a well-executed measurement. The chosen convention must be written into the standard operating procedure and applied without exception, and the integration limits must be recorded in the raw data record.[21–23]

Baseline treatment deserves comparable discipline. The berberine spectrum in methanol-d_4 contains an exchange resonance whose intensity may exceed that of the analyte signals by a considerable margin, and automatic phase and baseline routines frequently optimize themselves against that resonance to the detriment of the quantitative regions. Manual phasing verified across the full spectral width, followed by a whole-spectrum baseline correction of modest polynomial order, is preferred. Regionally applied baseline correction is specifically discouraged, since it forces the baseline to zero at the boundaries of each correction region and thereby distorts the very integrals the analyst is attempting to measure.

5.5 The Purity Equation

With the spectrum acquired and processed, the mass fraction of berberine cation in the as-received material follows from the standard internal-standard relationship of Equation 2:

$$w(\text{cation}) = (I_x / I_{is}) \times (N_{is} / N_x) \times (M_x / M_{is}) \times (m_{is} / m_x) \times P_{is} \qquad (2)$$

in which I denotes the integrated signal area, N the number of protons giving rise to the integrated signal, M the relative molecular mass, m the weighed mass, and P the assigned mass-fraction purity of the calibrant; the subscript x refers to the analyte and is refers to the internal standard. For the recommended configuration, M_x is taken as 336.36, the relative molecular mass of the berberine cation, and N_x is 3 when a single methoxy singlet is integrated, 6 when the methoxy pair is integrated as one envelope, 2 for the methylenedioxy bridge, and 1 for any of the aromatic methines. For dimethyl sulfone, M_{is} is 94.13 and N_{is} is 6.

The determination should be performed independently against at least three analyte signals selected from different regions of the spectrum — for example H-8 at δ 9.76, the methylenedioxy singlet at δ 6.10, and the methoxy envelope near δ 4.15 — and the three results should agree within the demonstrated repeatability of the method. Agreement across signals of differing multiplicity, differing relaxation behavior and differing spectral neighborhood is strong evidence that neither overlap nor incomplete relaxation nor integration error has intruded. Disagreement localizes the problem to a specific region and is far more informative than a single number. The reported value should be the mean of the concordant determinations, with the individual values retained in the data record. Multi-signal concordance of this kind is among the practices identified by the international qNMR community as distinguishing a controlled measurement from a merely executed one.[24]

It is emphasized that Equation 2 returns the cation content of the material as weighed. It contains no information whatever about the water content, the chloride content or the hydration state, and it must not be converted to a salt or hydrate basis without an independent determination of the water. The conversions of Table 4 are arithmetic, not analytical, and each one imports an assumption that must be justified.

6. Determination of Water

6.1 The Exchange Problem in Methanol-d_4: A Critical Evaluation

The proposition that dissolution of berberine hydrochloride in methanol-d_4 permits simultaneous determination of purity and water content is attractive in principle and is defensible in its underlying physics, but it fails in practice for two specific and quantifiable reasons that must be confronted before any method is written.

The physical principle is sound. Methanol-d_4 is nominally deuterated at all four positions, but its isotopic enrichment is finite, and the residual protons at the hydroxyl position exchange rapidly with the protons of any water present. In a sealed NMR tube the total number of exchangeable protons is conserved; deuterium–hydrogen redistribution between the water and methanol hydroxyl sites alters which molecule carries a given proton but does not remove it from observation, and the equilibrium isotope fractionation between water and an alcohol hydroxyl is close to unity. Because the exchange is fast on the chemical shift timescale, all of these protons appear as a single averaged resonance near δ 4.87 whose integrated area is proportional to the total exchangeable proton content of the tube. Measurement by difference against a solvent blank is therefore theoretically valid, and the same logic underpins the established determination of crystal water by quantitative proton NMR in deuterium oxide, in which the increase in the residual HDO signal relative to an internal standard is used to quantify lattice water.[40]

The first practical difficulty is the magnitude and instability of the solvent-derived background. Table 8 sets out the exchangeable proton inventory for a representative preparation of 10.00 mg of berberine chloride dihydrate in 0.600 mL of methanol-d_4.

Table 8. Exchangeable proton inventory for 10.00 mg berberine chloride dihydrate in 0.600 mL methanol-d_4.

Contribution	Basis of estimate	Exchangeable ^{1}H (µmol)	Share of exchange pool (%)
Residual hydroxyl protons of the solvent	532.8 mg CD_3OD (14 771 µmol) at 99.8 atom % D	29.5	20.3
Water present in the solvent as supplied	0.03 % w/w specification, 2 ^{1}H per H_2O	17.7	12.2
Solvent-derived subtotal	—	47.3	32.5
Crystal water of the specimen	24.52 µmol dihydrate, 4 ^{1}H per formula unit	98.1	67.5
Total exchange pool observed at δ ≈ 4.87	—	145.4	100.0
Berberine H-6 methylene, overlapping at δ ≈ 4.95	24.52 µmol × 2 ^{1}H	49.0	—
Composite envelope actually integrated	Exchange pool plus H-6	194.4	—

For comparison, one berberine methoxy singlet in the same preparation carries 73.6 µmol of ^{1}H. The exchange pool is therefore approximately twice the intensity of the analyte signal used for the cation assay, and the solvent contributes a third of it.

Almost a third of the signal that an unwary analyst would attribute to sample water in fact originates in the solvent. That fraction would be tolerable if it were stable, but methanol-d_4 is strongly hygroscopic and the printed water

specification describes the material at the moment of ampoule sealing rather than at the moment of use. A bottle opened repeatedly under ordinary laboratory conditions acquires water at a rate that renders the label figure irrelevant within days. The background must therefore be measured, not assumed, by running a gravimetrically matched solvent blank alongside every sample, and even then its uncertainty is likely to be of the order of ten to thirty percent relative unless the work is conducted under controlled atmosphere.

The second practical difficulty is more serious and is not remedied by any blank. The exchange-averaged resonance at δ ≈ 4.87 lies approximately 0.08 ppm from the berberine H-6 methylene triplet at δ ≈ 4.95. Table 9 converts that separation into frequency at the field strengths of practical interest.

Table 9. Spectral separation of critical signal pairs as a function of observation frequency.

Observation frequency (MHz)	CD_3OD: H-6 vs exchange peak, Δδ ≈ 0.08 ppm (Hz)	DMSO-d_6: H-5 vs H_2O, Δδ ≈ 0.15 ppm (Hz)	Berberine OCH 0.11 ppm
60	4.8	9.0	6.6
300	24.0	45.0	33.0
400	32.0	60.0	44.0
500	40.0	75.0	55.0
600	48.0	90.0	66.0

The H-6 triplet spans approximately 12 Hz between its outer lines. The exchange-averaged resonance in methanol-d_4 is broad and its width at half height commonly exceeds 5 Hz and may reach several tens of hertz depending on exchange rate, water content and shim quality.

At 300 MHz the H-6 triplet and the exchange resonance are separated by 24 Hz, of which the triplet itself occupies half. Baseline resolution is not achieved, and the composite envelope must be treated as a single integral. At 60 MHz the separation collapses to 4.8 Hz and the two features are not merely unresolved but indistinguishable. Even at 600 MHz the 48 Hz separation between a narrow triplet and a broad, intense singlet does not deliver a clean integration boundary. There is, in short, no field strength in routine laboratory service at which the exchange resonance in methanol-d_4 can be integrated independently of the berberine H-6 signal.

The magnitude of the resulting error is set out in Table 10 and is severe enough to invalidate the measurement outright if uncorrected.

Table 10. Bias in the water determination arising from incomplete treatment of the composite envelope.

Treatment of the δ 4.6–5.2 envelope	Apparent water (mg)	Apparent w(H_2O) (% w/w)	Bias relative to the true value
Whole envelope integrated and assigned to water	1.751	17.51	+98.2 %
Solvent blank subtracted; no correction for H-6	1.325	13.25	+50.0 %
Solvent blank subtracted and H-6 contribution removed	0.883	8.83	Nil (correct treatment)
True value for stoichiometric dihydrate	0.883	8.83	—

The first row of Table 10 describes what will happen if the method is written without regard to the exchange chemistry, and a factor-of-two error in a release specification is not a subtlety. The second row shows that the obvious correction is insufficient by itself. The third row indicates that the measurement is recoverable, and the mechanism of recovery is worth stating explicitly because it costs nothing: the molar quantity of berberine cation in

the tube has already been determined to high precision by the cation assay of Section 5, so the H-6 contribution to the composite envelope is known a priori as twice that molar quantity and may be subtracted arithmetically rather than spectroscopically. The overlap that cannot be resolved in the frequency domain is nevertheless removable in the calculation, provided the analyst recognizes that it is there.

One partial mitigation deserves evaluation before the correction is relied upon, since it may improve the conditioning of the integration. The chemical shift of the hydroxyl resonance of methanol is strongly temperature dependent — the property that underlies the long-established use of methanol as an NMR thermometer — whereas the berberine H-6 shift is comparatively insensitive.[46–48] Raising the probe temperature by twenty to twenty-five kelvin displaces the exchange-averaged resonance upfield by roughly 0.2 ppm and can move it clear of the H-6 triplet at 300 MHz. The approach is legitimate but is not free: relaxation times, chemical shifts and exchange linewidths all change with temperature, so the recycle delay must be re-derived, the setpoint must be calibrated and controlled to better than a tenth of a kelvin, and the entire method must be validated at the elevated temperature rather than inheriting parameters established at 298 K. It is also worth noting that the shift of the averaged resonance is itself a function of the water-to-methanol proton ratio, which is precisely the quantity being measured, so the displacement achieved will differ between the sample and the blank. A related literature approach exploits this shift dependence directly, using the separation between the water and solvent resonances as the analytical signal rather than the integral.[41]

6.2 Route 1: Direct Integration of the Exchange-Averaged Resonance

The corrected procedure requires two acquisitions under identical conditions. The sample tube contains the weighed analyte, the weighed calibrant and a recorded mass of solvent; the blank tube contains a recorded mass of solvent from the same ampoule together with a weighed mass of the same calibrant. Both are prepared and sealed within the same short interval and acquired with identical receiver gain, temperature and processing.

The exchangeable proton content of the solvent, expressed per unit mass of solvent, is obtained from the blank through Equation 3:

$$c_{xh} = (I'_{xh} / I'_{is}) \times N_{is} \times (m'_{is} \times P_{is} / M_{is}) \div m'_{solv} \qquad (3)$$

and the water content of the specimen then follows from Equation 4, in which the second bracketed term removes the solvent background and the third removes the overlapping H-6 contribution using the molar cation quantity n(cation) established by Equation 2:

$$n(H_2O) = ½ \times [\, (I_{xh} / I_{is}) \times N_{is} \times (m_{is} \times P_{is} / M_{is}) - c_{xh} \times m_{solv} - 2 \times n(cation) \,] \qquad (4)$$

$$w(H_2O) = n(H_2O) \times 18.015 / m_x \qquad (5)$$

Three requirements attach to this route and none of them is optional. The solvent must be dispensed and recorded gravimetrically, since Equation 4 scales the blank by solvent mass. The calibrant must contain no exchangeable protons, failing which an additional term must be introduced into Equation 4 and the uncertainty rises accordingly. And the integration limits applied to the composite envelope must be identical in the sample and blank spectra, which in practice means they are defined in hertz relative to a referenced chemical shift and applied by script rather than by hand.

A standard-addition verification is strongly recommended as part of validation and as a periodic quality control check. A second sample preparation is spiked with a weighed increment of water in the range of one to two milligrams, and the increase in the corrected exchangeable proton content is compared against the increment added. Recovery outside 95–105 % indicates a defect in the blank, in the integration convention, or in the assumed calibrant purity, and localizes the fault before a result is released.

External calibration offers an alternative to the matched-blank architecture and removes the calibrant from the sample entirely, which eliminates both the exchangeable-proton risk and the calibrant hygroscopicity risk discussed in Section 5.1. Electronic reference methods, the principle of reciprocity as implemented in PULCON, and calibration against a separately prepared external standard have all been developed to a level where accuracy comparable to internal calibration is attainable.[35–37] The penalty is a heightened sensitivity to tube geometry and to probe tuning, as noted in Section 5.2, and for a routine testing laboratory the internal standard approach remains the more robust default. Quantum-mechanical line-shape analysis provides a further avenue, permitting the composite envelope to be decomposed into its constituent multiplets on the basis of a fitted spin system rather than by manual integration, although the very intense and non-Lorentzian exchange resonance makes this a poorly conditioned problem in methanol-d_4 specifically.[39]

The honest assessment of this route is that it is analytically valid and metrologically traceable but that its uncertainty is dominated by the solvent blank rather than by the NMR measurement. Under ordinary open-bench conditions a relative uncertainty of ten to twenty percent on the water value should be expected; under controlled-atmosphere preparation with a freshly opened solvent ampoule, five to eight percent is achievable. Neither figure is competitive with coulometric Karl Fischer titration.

6.3 Route 2: Water by Difference from the qNMR Cation Assay

An entirely different route to the water value avoids the exchangeable region altogether. If the material is a stoichiometric berberine monochloride hydrate containing no other components, then the mass balance identity of Equation 1 reduces to three terms and the water content follows by difference from the measured cation content and the chloride content, as in Equation 6:

$$w(H_2O) = 1 - w(cation) - w(Cl^-) \qquad (6)$$

Where the chloride is assumed stoichiometric rather than measured, w(Cl^-) is simply w(cation) × 35.45 / 336.36 and Equation 6 collapses to a single-measurement determination. The hydration number then follows from Equation 7:

$$n = [\, w(H_2O) / 18.015 \,] \div [\, w(cation) / 336.36 \,] \qquad (7)$$

This route is elegant, requires no blank, is unaffected by solvent hygroscopicity, and inherits only the uncertainty of the cation assay. It also has two weaknesses that must be stated plainly, because it is otherwise easy to over-sell.

The first weakness is error amplification. Differentiating Equation 6 with respect to the cation content under the stoichiometric-chloride assumption gives a sensitivity coefficient of −1.105, so the absolute uncertainty on the water is approximately 1.1 times the absolute uncertainty on the cation content. Because the water content is roughly an order of magnitude smaller than the cation content, the relative uncertainty is magnified by approximately a factor of ten: a combined standard uncertainty of 0.55 % relative on the cation propagates to approximately 4.5 % relative on a water content near 10 % w/w. The measurement is precise in absolute terms and imprecise in relative terms, which is exactly the wrong way round for a specification expressed as a percentage.

The second weakness is more insidious. Equation 6 does not measure water; it measures everything that is not cation and not chloride, and it assigns the whole of it to water. Residual solvent, inorganic residue, unquantified organic related substances and any other unaccounted component are transferred wholesale into the water figure. In the worked example of Section 7 this mechanism inflates the difference-derived water content by 1.03 percentage points and the derived hydration number by 11 %, purely on account of 0.85 % w/w residual ethanol and 0.18 % w/w inorganic residue. The difference route is therefore best regarded not as a water determination but as an upper bound on the water content, and as a powerful consistency check against a direct determination: the gap between the two is precisely the mass of unaccounted material, and it should be reconciled rather than ignored.

6.4 Route 3: Dimethyl Sulfoxide-d_6 as the Preferred NMR Solvent for Water

If an NMR-only water determination is required — because the sample quantity is limited, because the material is incompatible with Karl Fischer reagents, or because a single non-destructive experiment is operationally preferred — then dimethyl sulfoxide-d_6 is the correct solvent and methanol-d_4 is not. Two independent advantages accrue. The systematic role of solvent selection in achieving signal separation for quantitative ^{1}H NMR has been examined explicitly in the pharmaceutical literature, and the present case is a particularly clear instance of the principle.[32]

The first is the elimination of the solvent hydroxyl reservoir. Dimethyl sulfoxide contains no exchangeable protons, so the only exchangeable species in the tube are water and whatever the analyte contributes. For a 0.600 mL preparation at the same nominal 0.03 % w/w water specification, the solvent background falls from 47.3 µmol of exchangeable ^{1}H in methanol-d_4 to 23.8 µmol in dimethyl sulfoxide-d_6, a reduction of almost exactly a factor of two, with a corresponding halving of the uncertainty that the blank subtraction contributes.

The second is improved spectral separation. The water resonance in dimethyl sulfoxide-d_6 falls near δ 3.35 and its nearest berberine neighbor is the H-5 triplet at δ 3.20, giving approximately 0.15 ppm of separation — 45 Hz at 300 MHz, against 24 Hz for the corresponding pair in methanol-d_4. Partial resolution is achieved at 300 MHz and clean resolution at 400 MHz and above, so that line-shape deconvolution of the water resonance from the H-5 triplet becomes a well-conditioned rather than an ill-conditioned problem. The correction embodied in the third term of Equation 4 remains available as a cross-check, since the H-5 contribution is likewise known a priori from the cation assay.

The cost of the switch is the loss of the H-13 dispersion advantage that recommends methanol-d_4 for protoberberine profiling in complex botanical matrices.[6,7] For a purified salt this cost is small, since the H-8 and H-13 singlets remain well isolated in dimethyl sulfoxide-d_6 at δ 9.89 and δ 8.94. The pragmatic recommendation, where both determinations are required from NMR alone, is to run two preparations: methanol-d_4 for the cation assay and the congener profile, and dimethyl sulfoxide-d_6 for the water. The additional cost is one sample preparation and one acquisition, which is a modest price for removing a fifty percent bias.

The residual hazard in dimethyl sulfoxide-d_6 is that the solvent is itself markedly hygroscopic, so the gravimetric blank discipline of Section 6.2 applies with equal force. What has been removed is the fixed hydroxyl reservoir, not the variable atmospheric contamination.

6.5 Route 4: Orthogonal Reference Methods

Coulometric Karl Fischer titration remains the reference method for water in pharmaceutical solids and is the appropriate choice for release testing. The determination is absolute in the metrological sense, being founded on Faraday's law and the stoichiometry of the Karl Fischer reaction, and the technique is codified in USP General Chapter ⟨921⟩ Method Ic and in European Pharmacopoeia general method 2.5.32.[43,44] Reported relative bias between coulometry and volumetry in a certification exercise on a water-content reference material was 0.06 %, and quantitative NMR was found consistent with both when applied to the same material.[42] On a specimen containing approximately 9 % w/w water, a well-maintained coulometer with an oven accessory should deliver a relative uncertainty of two to three percent, which is three to four times better than the best of the NMR routes.

Thermogravimetric analysis provides an independent and instructive cross-check, since it reports not only the quantity of volatile matter but also the temperature at which it is released, and thereby distinguishes surface moisture from lattice water and identifies whether the dehydration proceeds in one step or two. The caution of Section 2.2 applies: the mass loss window must be confined to the low-temperature dehydration event, because thermal demethylation of berberine to berberrubine contributes mass loss at higher temperature that is not water.[4,5] Loss on drying under USP General Chapter ⟨731⟩ is the least selective of the three and should be regarded as a limit test rather than as a determination, since it cannot distinguish water from residual organic solvent.[45]

Where the hydration state itself is in question rather than merely the water content, powder X-ray diffraction settles the matter directly, since the anhydrate, dihydrate and tetrahydrate are distinct crystalline phases with distinct diffraction patterns, and variable-temperature and variable-humidity diffraction resolve the interconversions.[2,3]

6.6 Comparative Assessment and Recommendation

Table 11 collects the four routes and the two reference methods on a common basis. The uncertainty figures are indicative of what should be achievable in a competent laboratory rather than the best that has ever been demonstrated, and each should be replaced by locally determined values during method validation.

Table 11. Comparison of routes to the water content of berberine hydrochloride.

Route	Sample consumed	Requires blank	Typical relative uncertainty at ≈ 9 % w/w water	Principal failure mode	Recommendatio
Direct integration, CD_3OD, corrected per Eq. 4	≈ 10 mg	Yes, gravimetric	10–20 % (open bench); 5–8 % (controlled)	Uncorrected H-6 overlap; unstable solvent blank	Acceptable only correction and a standard-additio
Direct integration, DMSO-d_6	≈ 10 mg	Yes, gravimetric	5–10 %	Unstable solvent blank	Preferred NMR-
Difference from qNMR cation assay, Eq. 6	Shared with cation assay	No	≈ 4.5 % on precision alone, but positively biased	Silent transfer of unaccounted mass into the water term	Use as an upper consistency chec determination
Coulometric Karl Fischer, USP ⟨921⟩ Ic	10–100 mg	Blank titration	2–3 %	Reagent interference; incomplete dissolution	Reference metho testing
Thermogravimetric analysis	5–10 mg	No	3–5 %	Demethylation mass loss misattributed to water	Confirmatory; al hydrate stoichio
Loss on drying, USP ⟨731⟩	≈ 1 g	No	Method-dependent	Cannot distinguish water from residual solvent	Limit test only

The recommendation that follows from this comparison is unambiguous and should be adopted without apology. Quantitative ^{1}H NMR in methanol-d_4 is retained for the berberine cation content, for the residual solvent content and for the protoberberine congener profile, all three of which it performs better than any competing technique and all three of which it delivers from a single acquisition. Water is determined by coulometric Karl Fischer titration on an aliquot drawn from the same homogenized portion within the same working session. The difference calculation of Equation 6 is performed as a matter of routine, not as the reported water value but as a closure test, and the gap between it and the titrimetric value is reconciled against the residual solvent and inorganic residue determinations. Where circumstances genuinely preclude titrimetry, the dimethyl sulfoxide-d_6 route of Section 6.4 is used in preference to methanol-d_4, and the reported uncertainty is stated honestly rather than borrowed from the cation assay.

The counterargument deserves acknowledgment. It will be observed that the direct NMR route offers a single experiment, non-destructive analysis, minimal sample consumption and traceability to the same calibrant that anchors the cation assay, and that these are real advantages in a mass-limited or high-throughput setting. That case is legitimate, and the corrected procedure of Section 6.2 is defensible when executed with the discipline it requires. What is not defensible is presenting a directly integrated exchangeable-region result as equivalent in quality to a Karl Fischer determination, or omitting the H-6 correction on the grounds that the overlap looks small in the spectrum. It does look small. It is 34 % of the integral.

7. Worked Numerical Example

The following worked example is constructed to be internally self-consistent and to illustrate the closure test of Section 4.1. The figures are representative of a commercial lot of pharmacopoeial-grade material and are not taken from a specific analysis; laboratories adopting the method should substitute their own data.

A specimen of 10.42 mg of berberine hydrochloride was weighed by difference on a balance of 0.01 mg readability, together with 3.00 mg of certified dimethyl sulfone of assigned mass-fraction purity 0.9985. The pair was dissolved in 0.600 mL of methanol-d_4 dispensed gravimetrically from a freshly opened ampoule, and the solution was transferred to a matched 5 mm tube. A gravimetrically matched blank containing the same solvent and calibrant was prepared in parallel. Both spectra were acquired at 300 MHz and 298.0 K with a hard ninety-degree pulse, a 60 s relaxation delay, a 4 s acquisition time and 32 transients, and were processed identically.

The integral of the berberine methoxy singlet at δ 4.21, normalized against the dimethyl sulfone singlet at δ 3.06 taken as 1000.0, was 568.4. Substituting into Equation 2 with $N_x = 3$, $N_{is} = 6$, $M_x = 336.36$ and $M_{is} = 94.13$ gives a cation content of 81.21 % w/w, corresponding to 25.16 µmol of berberine cation in the tube. Concordant values were obtained from the H-8 singlet at δ 9.76 and from the methylenedioxy singlet at δ 6.10. Chloride determined by argentometric titration was 8.61 % w/w, against a stoichiometric expectation of 8.56 % w/w. Coulometric Karl Fischer titration on an aliquot drawn from the same portion within the same session returned 9.15 % w/w water. Residual ethanol, quantified from the methyl triplet at δ 1.19 in the same proton spectrum and against the same calibrant, amounted to 0.85 % w/w. Residue on ignition was 0.18 % w/w.

Table 12. Mass balance closure for the worked example.

Component	Method	Value (% w/w)	Comment
Berberine cation	^{1}H qNMR, CD_3OD, dimethyl sulfone calibrant	81.21	Mean of three concordant signals
Chloride	Argentometric titration	8.61	Stoichiometric expectation 8.56 %
Water	Coulometric Karl Fischer, USP ⟨921⟩ Ic	9.15	Corresponds to n = 2.10 mol H_2O per mo cation
Residual ethanol	^{1}H qNMR, same acquisition	0.85	CH_3 triplet at δ 1.19
Inorganic residue	Residue on ignition	0.18	—
Total	—	100.00	Closes within the combined uncertainty

The closure test is now applied. Equation 6, using the measured chloride value, returns a difference-derived water content of 10.18 % w/w against the titrimetric value of 9.15 % w/w. The gap of 1.03 percentage points is accounted for exactly by the residual ethanol at 0.85 % and the inorganic residue at 0.18 %. Had those two determinations not been performed, the difference route would have reported a hydration number of 2.34 rather than the correct 2.10, an overestimate of 11 %, and the material would have been characterized as an intermediate hydrate approaching the tetrahydrate when in fact it is a dihydrate carrying a little adventitious moisture. The example illustrates precisely why the difference calculation must be treated as a closure test rather than as a water determination.

Expressing the result on the four bases of Table 4, the material is 81.21 % w/w berberine cation as received, 89.77 % w/w on the anhydrous chloride basis, 98.47 % w/w on the dihydrate basis, and 90.23 % w/w on the anhydrous and solvent-free basis. A supplier certificate declaring “assay not less than 98.0 %” would be satisfied by this lot on the dihydrate basis and would fail it on every other. The certificate issued by the testing laboratory should state the cation content, the basis, the water content, the method used for each, and the conversion factors applied.

8. Uncertainty Budget

An uncertainty statement is a requirement of accredited testing and not an optional refinement. Table 13 sets out a budget for the cation assay under the conditions of the worked example, evaluated as relative standard uncertainties and combined in quadrature.

Table 13. Uncertainty budget for the berberine cation assay at 300 MHz.

Contribution	Basis of evaluation	Type	u_{rel} (%)
Sample mass, 10.42 mg	Difference weighing, 0.01 mg readability	B	0.08
Calibrant mass, 3.00 mg	Difference weighing, 0.01 mg readability	B	0.27
Calibrant assigned purity	Certificate, U = 0.2 % at k = 2	B	0.10
Relative molecular masses	IUPAC atomic mass uncertainties	B	< 0.01
Integration and signal-to-noise	S/N ≈ 400 on the integrated signal	A	0.25
Baseline and phase treatment	Repeated independent reprocessing	A	0.20
^{13}C satellite convention	Comparison of inclusive and exclusive limits	B	0.10
Incomplete relaxation	$\exp(-D/T_1)$ at D = 7 T_1	B	0.09
Repeatability	Six independent preparations	A	0.30
Combined standard uncertainty	Root sum of squares	—	0.55
Expanded uncertainty, k = 2	95 % coverage	—	1.10

Two features of the budget merit comment. The first is that the calibrant mass is the largest single contributor, at 0.27 % relative, and it is large only because 3.00 mg is a small mass to weigh on a balance of 0.01 mg readability. Substituting a microbalance of 1 µg readability reduces this term to approximately 0.03 % and lowers the combined standard uncertainty from 0.55 % to approximately 0.48 %. This is the principal instrumental argument for microbalance capability in a qNMR laboratory, and it is the reason that weighing requirements are addressed explicitly in ISO 24583.[25,49,50] The second is that no single term dominates once the weighing is addressed; the budget is balanced, which is the signature of a method that has been optimized rather than merely executed. Where a more rigorous treatment is required, Monte Carlo propagation of the systematic and random components has been described specifically for qNMR and avoids the linearizing assumptions implicit in a quadrature sum.[51,52] Expressed in absolute terms, the cation content of the worked example is reported as 81.2 ± 0.9 % w/w at a coverage factor of two.

Propagating this result to the water content by the difference route of Equation 6, and taking the chloride determination to carry a relative uncertainty of 1.5 %, gives a combined standard uncertainty on the water of 0.46 % w/w in absolute terms, or 4.5 % in relative terms, and an expanded uncertainty of 0.93 % w/w at k = 2. This is the precision limit of the difference route and takes no account of the systematic positive bias arising from unaccounted mass, which in the worked example was more than twice the expanded uncertainty. A coulometric Karl Fischer determination of the same water content carries an expanded uncertainty of roughly 0.2 to 0.3 % w/w, and the comparison is not close.

9. Method Validation

Validation should be conducted against ICH Q2(R2) for the pharmaceutical case and against the performance criteria of ISO 24583 for the metrological case, the latter being the international standard specifically addressing purity determination of organic compounds by internal-standard ^{1}H qNMR.[25,53] The elements set out below are those that carry particular weight for this analyte.

Specificity is demonstrated through the integral ratio test of Section 3.3, through spiking with authentic palmatine, coptisine and jatrorrhizine to confirm that the selected quantitation signals remain free of interference, and through examination of the aliphatic fingerprint region for the congener markers described in Section 2.3. Because epiberberine is isomeric with berberine and carries the same proton inventory, the specificity study should include it explicitly if it is a plausible contaminant of the source material.

Linearity is established across at least five concentrations spanning fifty to one hundred and fifty percent of the working loading, with the analyte-to-calibrant integral ratio regressed against the analyte-to-calibrant mass ratio. A coefficient of determination in excess of 0.999 and an intercept not significantly different from zero are expected; a non-zero intercept in a qNMR linearity study almost always indicates a baseline or integration artifact rather than a genuine chemical effect, and should be investigated rather than accepted.

Precision is evaluated as repeatability across six independent preparations at the working concentration and as intermediate precision across at least two analysts, two days and, where available, two instruments. Because the analyte is hygroscopic, the intermediate precision study should deliberately span a range of ambient humidity and should record it, since the resulting variability is a genuine property of the measurement system as operated and should not be excluded from the estimate.

Accuracy is best demonstrated against a certified reference material. A berberine chloride certified reference material whose value has been assigned by quantitative ^{1}H NMR is available from a national metrology institute, and its use provides direct traceability and an unambiguous accuracy statement.[54] Where such material is not available, accuracy is demonstrated by the mass balance closure test of Section 4.1, by comparison against a validated HPLC procedure, and by standard addition.

Robustness is examined with respect to relaxation delay, receiver gain, temperature setpoint, integration limits, apodization and the interval between preparation and acquisition. The last of these is not a formality for a photosensitive alkaloid in a protic solvent, and a solution stability study extending to at least twice the anticipated maximum analysis time should be performed and documented.

For the water determination, whichever route is adopted, validation must additionally address the solvent blank: its magnitude, its variability across ampoules and across the working day, and the recovery of a weighed water increment by standard addition. A method that does not characterize its own blank cannot state an uncertainty for the water value.

10. Instrument Considerations: 300 MHz and 60 MHz Benchtop Platforms

The method described here is fully executable on a 300 MHz superconducting instrument for the cation assay, and the parameter set of Table 7 is written for that platform. The relevant constraint at 300 MHz is not sensitivity, which is ample at a 40 mM loading, but dispersion. Table 9 shows that the two berberine methoxy singlets are separated by 33 Hz at 300 MHz, which is adequate for independent integration, and that the H-5 triplet is separated from the dimethyl sulfoxide-d_6 water resonance by 45 Hz, which supports partial resolution. The exchange resonance of methanol-d_4 and the H-6 triplet are separated by only 24 Hz, which does not.

Benchtop instrumentation at 60 MHz presents a materially different picture, and its capabilities and limits should be stated precisely because the platform is increasingly attractive for routine incoming-material verification. Quantification of berberine in commercial food supplements has been demonstrated on a 60 MHz compact spectrometer, with a fifteen-minute acquisition sufficient to measure a minimum of about 10 mg of berberine per capsule and with results validated against 500 MHz reference measurements.[1] The wider applicability of benchtop ^{1}H NMR to quantitative problems in food, supplement and materials analysis is now well established.[55,56] The berberine spectrum retains three usable quantitation signals at this field: H-8 near δ 9.76, which sits in a region that is otherwise empty; H-13 near δ 8.72, separated from the H-11 doublet by 37 Hz; and the methylenedioxy singlet at δ 6.10, which is likewise isolated.

What is not available at 60 MHz is any part of the crowded region between δ 3 and δ 5. The two methoxy singlets collapse to a six-proton envelope at 6.6 Hz separation, which remains usable as a single integral provided nothing else falls beneath it, but the H-6 triplet, the exchange resonance and the upfield methoxy signal merge into a single unresolved feature. In dimethyl sulfoxide-d_6 the water resonance and the H-5 triplet are separated by 9 Hz, which is likewise insufficient. The conclusion is direct: benchtop ^{1}H NMR at 60 MHz is a legitimate platform for berberine cation content and for label-claim verification, and it is not a platform for NMR water determination in this analyte by any solvent route. Where a benchtop instrument is deployed for incoming material control, water must be determined titrimetrically.

A further caution attaches to the benchtop methodology reported in the literature. To keep acquisition times compatible with routine control, quantification at 60 MHz has been performed against a calibration curve constructed under conditions of incomplete relaxation.[1] This is a pragmatic and defensible engineering compromise, but it converts the measurement from an absolute determination into a relative one: the result then inherits the assigned purity of the berberine calibrant and, critically, its hydration state. If the calibration standard is a dihydrate whose assigned purity is expressed on the dihydrate basis, and the unknown has equilibrated toward a different hydration state, a systematic bias follows that no amount of chromatographic or spectroscopic care will reveal. Laboratories adopting a benchtop calibration-curve approach should assign the purity of their calibration standard in-house by absolute qNMR on a high-field instrument, on a stated basis, and should re-assign it periodically.

11. Orthogonal and Confirmatory Measurements

Chloride is the one major component of the salt that the proton experiment cannot see. Argentometric titration and ion chromatography are the conventional routes. A third option has become available in the form of ^{35}Cl quantitative NMR against a potassium perchlorate internal standard, which has been shown to assay the chloride content of hydrochloride salts of organic compounds accurately using a calibrant that is of high symmetry, non-hygroscopic and stable.[27] The non-hygroscopic character of the calibrant is a genuine advantage in the present context, where every hygroscopic component introduced into the workflow degrades the water determination. Where an instrument capable of ^{35}Cl observation is available, this route allows the chloride term of the mass balance to be measured rather than assumed, which converts Equation 6 from a single-assumption calculation into a genuine closure test.

High-performance liquid chromatography with ultraviolet detection near 345 nm remains the standard technique for berberine content and for the separation and quantification of protoberberine congeners, and it should be retained as an orthogonal confirmation of the qNMR result. The relationship between the two techniques deserves to be stated carefully, however, because it is frequently inverted in practice. HPLC is a relative method: it reports the analyte content of the unknown relative to the assigned content of a calibration standard, and it therefore transmits every error in that assignment directly into every result. Quantitative NMR is an absolute method: it reports the analyte content relative to a calibrant of different chemical identity, which may be chosen for its metrological pedigree rather than for its chemical similarity. The correct architecture is therefore for qNMR to assign the purity of the chromatographic calibration standard, and for chromatography to provide selectivity and sensitivity for trace

congeners that qNMR cannot resolve. The demonstration that commercial protoberberine reagents were less pure than declared, with consequent overestimation of alkaloid contents determined chromatographically against them, is the clearest available illustration of what happens when the architecture is reversed.[8]

Residual solvents deserve mention as a genuine advantage of the NMR approach rather than as an afterthought. Methanol, ethanol, acetone, isopropanol and ethyl acetate all present characteristic resonances in the aliphatic region of the same spectrum used for the cation assay, and all are quantified against the same calibrant in the same acquisition at no additional cost in instrument time. A gas chromatographic headspace method would be required to reach the concentration limits of ICH Q3C for Class 1 and Class 2 solvents, and NMR does not replace it, but for the Class 3 solvents that dominate this material the proton spectrum provides an immediate and quantitative answer and closes a term of the mass balance that would otherwise remain open.

12. Practical Pitfalls and Failure Modes

The failure modes assembled in Table 14 are those most likely to compromise the determination in a working laboratory. They are ordered by the magnitude of the error they produce rather than by the frequency with which they occur.

Table 14. Failure modes, magnitude and mitigation.

Failure mode	Typical magnitude	Mitigation
Composite δ 4.6–5.2 envelope in CD_3OD integrated as water	+98 % on water	Apply Equation 4 in full, including the H-6 term
Solvent blank subtracted but H-6 not corrected	+50 % on water	As above; the correction is arithmetic and costs nothing
Purity reported without stating the basis	Up to 17 percentage points	State basis, water content and conversion factors together
Recycle interval set at $3 \times T_1$	− 5 % on the affected signal	Measure T_1 on the working preparation; use ≥ $7 \times T_1$
Sample equilibrates with laboratory air between weighings	Several percent on water	Weigh all aliquots from one portion in one session; log humidity
^{13}C satellites included for calibrant, excluded for analyte	≈ 1 %	Fix the integration convention in the SOP and apply it universally
Maleic acid used as calibrant when water is co-determined	Variable, always positive	Use a calibrant with no exchangeable protons
Hygroscopic calibrant used without desiccation	Up to several percent on water	Desiccate; verify the certified value; bound the calibrant water
Automatic phase and baseline routines anchored on the exchange peak	0.5–2 %	Phase manually; correct the baseline across the whole spectrum
Volumetric rather than gravimetric solvent dispensing	1–2 % on the blank scaling	Dispense and record solvent mass gravimetrically
Thermogravimetric mass loss above the dehydration window counted as water	Variable, positive	Confine integration to the dehydration event; couple to evolved-gas detection

Failure mode	Typical magnitude	Mitigation
Chromatographic calibrant of unstated hydration state	Up to 9 % systematic	Assign calibrant purity in-house by qNMR on a stated basis

13. Conclusions and Recommended Workflow

Berberine hydrochloride is a straightforward analyte for quantitative ^{1}H NMR and a difficult one for the assignment of a purity figure, and the difficulty resides almost entirely in the hydrate chemistry rather than in the spectroscopy. The cation is fully quaternary and carries no exchangeable protons, its eighteen protons distribute across several well-isolated singlets, and internal-standard qNMR against a certified calibrant returns its molar quantity with a combined standard uncertainty near 0.55 % relative on a 300 MHz instrument. That measurement is metrologically primary and is the foundation of everything else.

The proposal to obtain the water content from the same methanol-d_4 spectrum is sound in principle and hazardous in execution. Water protons in that solvent are not observed as water; they are observed as part of an exchange-averaged pool that includes the residual hydroxyl protons of the solvent, and the resulting resonance overlaps the berberine H-6 triplet at a separation that no commercially available spectrometer resolves. Integrating that envelope naively overestimates the water content by 98 %; subtracting a solvent blank without correcting for H-6 still leaves a 50 % positive bias. Both errors are removable, the first by a gravimetrically matched blank and the second by arithmetic subtraction of a quantity already known from the cation assay, and the corrected procedure is set out in Equations 3 to 5. Even corrected, however, the route is dominated by the stability of the solvent blank and is three to four times less precise than coulometric titration.

The recommended workflow, suitable for incorporation into an accredited scope, is as follows. The bulk material is homogenized and equilibrated over a saturated salt solution of defined relative humidity, and all analytical aliquots are drawn from that portion within a single working session with the ambient conditions recorded. Quantitative ^{1}H NMR is performed in methanol-d_4 against certified dimethyl sulfone under the conditions of Table 7, and yields the berberine cation content from at least three concordant signals, the residual solvent content, and the protoberberine congener profile, with the integral ratio test of Section 3.3 applied as a system suitability requirement before any calculation is performed. Water is determined by coulometric Karl Fischer titration. Chloride is determined by argentometric titration or by ^{35}Cl qNMR. Inorganic residue is determined by residue on ignition. The five terms are summed, and closure within the combined uncertainty is treated as the release criterion for the analysis rather than merely as a courtesy calculation. The hydration number is computed from the titrimetric water and reported alongside. Finally, the cation content is reported on an explicitly stated basis, with the water content, the methods and the conversion factors presented in the same table so that no downstream user is left to guess which of four legitimate numbers was intended.

Where an NMR-only water determination is genuinely required, dimethyl sulfoxide-d_6 is the correct solvent, it halves the solvent background and doubles the spectral separation from the nearest analyte signal, and the water result should be reported with its own honestly derived uncertainty rather than with the uncertainty of the cation assay. Where a 60 MHz benchtop platform is used for incoming material control, it should be used for the cation content only, and the calibration standard against which it operates should have had its own purity assigned by absolute qNMR at high field on a declared basis.

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Summary

Berberine hydrochloride cannot be assigned a purity value without simultaneously assigning a water value and declaring a reporting basis, because the compound forms an anhydrate, a dihydrate and a tetrahydrate that interconvert at ordinary room temperature under the control of ambient water activity. The same specimen may legitimately be described as 81.21 %, 89.77 %, 90.23 % or 98.47 % pure depending on which of four defensible bases is adopted, and the failure to state which one is intended is the single most common source of commercial dispute over this material.

Quantitative ^{1}H NMR against a certified internal standard is the appropriate primary method for the berberine cation content. The cation is permanently quaternary, carries no exchangeable protons, and presents several well-isolated singlets suitable for integration. A combined standard uncertainty of approximately 0.55 % relative is achievable on a 300 MHz instrument with a balance of 0.01 mg readability, falling to approximately 0.48 % if a microbalance is used, the calibrant mass being the dominant term in the budget. Dimethyl sulfone is the recommended calibrant for methanol-d_4 on spectroscopic grounds, subject to strict desiccation control because it is markedly more hygroscopic than the alternatives. Calibrants bearing exchangeable protons, maleic acid foremost among them, must be excluded whenever water is to be determined in the same preparation.

The determination of water from the same methanol-d_4 spectrum is where the method most often goes wrong. Water protons in methanol-d_4 exchange rapidly with the residual hydroxyl protons of the solvent and are observed as a single averaged resonance near δ 4.87 that carries a substantial solvent background — approximately one third of the total signal for a representative preparation — and that overlaps the berberine H-6 triplet at a separation of only 24 Hz at 300 MHz and 4.8 Hz at 60 MHz. Integrating that composite envelope as though it were water overestimates the water content by 98 %. Subtracting a solvent blank without correcting for the H-6 contribution still leaves a 50 % positive bias. Both errors are removable: the blank by gravimetrically matched preparation and identical acquisition, and the H-6 contribution by arithmetic subtraction of twice the molar cation quantity, which the cation assay has already supplied at no additional cost.

Even corrected, the direct route is limited by the stability of the solvent blank rather than by the spectroscopy, and delivers ten to twenty percent relative uncertainty on open-bench preparation. Determination of water by difference from the cation content avoids the exchangeable region entirely but amplifies the cation uncertainty by roughly a factor of ten and silently transfers every unaccounted component into the water term; in the worked example it inflated the hydration number by eleven percent on account of residual ethanol and inorganic residue alone. Dimethyl sulfoxide-d_6 halves the solvent background and doubles the spectral separation from the nearest analyte signal and is the correct choice where an NMR-only water determination is required. Coulometric Karl Fischer titration remains three to four times more precise than any NMR route and should be used for release testing.

The recommended workflow is therefore not a single experiment but a small orthogonal set whose internal consistency is itself the principal evidence of correctness. Quantitative ^{1}H NMR in methanol-d_4 supplies the cation content from three or more concordant signals, the residual solvent content, and the protoberberine congener profile, with the fixed integral ratios of the berberine proton inventory applied as a system suitability test before any calculation is attempted. Coulometric titration supplies the water, titration or ^{35}Cl NMR supplies the chloride, and residue on ignition supplies the inorganic term. The five terms are summed and required to close within their combined uncertainty. The difference calculation is retained not as the reported water value but as the closure test that reveals unaccounted mass, and the gap between it and the titrimetric result is reconciled rather than ignored. Reported figures carry an explicit basis, the water content alongside, and the conversion factors in the same table.

For laboratories operating 60 MHz benchtop instrumentation, the cation assay is fully viable and the water determination is not, at any field below approximately 400 MHz and in any solvent. Where a benchtop calibration-curve approach is adopted, the purity of the calibration standard should be assigned in-house by absolute

quantitative NMR at high field, on a declared basis, and re-assigned periodically, since an undeclared shift in the hydration state of the calibrant propagates a systematic bias into every subsequent result that no downstream measurement will reveal.

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