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Comprehensive Quantitative Nmr Analysis of Galantamine

Kalpana Bandla
Vignan's Foundation for Science, Technology and Research
Sibbala Subramanyam
sibbalaphd@gmail.com
Vignan's Foundation for Science, Technology and Research
Jithendra Chimakurthy
Vignan's Foundation for Science, Technology and Research
Penke Vijaya Babu
Vignan's Foundation for Science, Technology and Research

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Abstract

Galantamine is a clinically important Amaryllidaceae alkaloid commonly used in the treatment of Alzheimer's disease, where accurate structural confirmation and purity determination are critical due to its narrow therapeutic index. Conventional chromatographic methods used for galantamine analysis often require extensive calibration, reference standards, and complex sample preparation, which may limit analytical efficiency and traceability. In the present study, a comprehensive nuclear magnetic resonance (NMR) based analytical strategy was developed for both structural elucidation and quantitative determination of galantamine. Complete structural confirmation was achieved using one-dimensional (^1H, ^{13}C, and DEPT) and two-dimensional (HSQC, HMBC, and COSY) NMR techniques. Quantitative analysis was performed using quantitative ^1H NMR (qNMR) with imidazole as an internal standard. The developed qNMR method exhibited excellent linearity over the concentration range of 0.5–3.0 mg/mL ($R^2 > 0.997$), high accuracy with recoveries between 97–100%, and good intra- and inter-day precision (%RSD < 1%). Robustness studies demonstrated that minor variations in acquisition parameters had no significant impact on quantification. The combined qualitative and quantitative NMR results confirm the structural integrity, chemical purity, and analytical reliability of galantamine. This study highlights qNMR as a non-destructive, reproducible, and traceable analytical platform suitable for routine pharmaceutical quality control, and regulatory applications of galantamine and related alkaloids.

1. INTRODUCTION

Galantamine is a benzazepine alkaloid characteristic of several *Amaryllidaceae* species, including *Galanthus nivalis* and *Leucojum aestivum*, and is a well-established therapeutic agent for the symptomatic treatment of Alzheimer's disease. Its clinical efficacy arises from a dual mechanism of action involving reversible inhibition of acetylcholinesterase and positive allosteric modulation of nicotinic acetylcholine receptors, resulting in enhanced cholinergic neurotransmission and cognitive function [1,2]. Owing to its narrow therapeutic index and increasing global demand, stringent quality control of galantamine is essential to ensure patient safety and therapeutic consistency, particularly for pharmaceutical-grade active ingredients and plant-derived raw materials [3,4].

Reliable analysis of galantamine remains analytically challenging, especially in botanical matrices. The alkaloid content in natural sources is highly variable and strongly influenced by genetic diversity, geographical origin, cultivation practices, harvesting period, and environmental conditions, leading to significant batch-to-batch variability [5–7]. Recent studies have emphasized that such intrinsic variability necessitates analytical methodologies capable of delivering high accuracy, reproducibility, and robustness across diverse matrices to ensure consistent pharmaceutical quality [8,9].

Currently, chromatographic techniques such as high-performance liquid chromatography (HPLC) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) are widely employed for galantamine determination [10]. Although these methods offer high sensitivity, they are inherently dependent on external reference standards, extensive calibration procedures, and complex sample preparation.

Moreover, matrix effects and co-eluting structurally related alkaloids can compromise specificity, method transferability, and long-term reproducibility, particularly in plant-derived samples [11,12].

In contrast, quantitative proton nuclear magnetic resonance (^{1}H qNMR) has emerged as a robust and increasingly accepted analytical technique for pharmaceutical analysis. qNMR is based on the fundamental principle that NMR signal integrals are directly proportional to the number of resonating nuclei, enabling absolute quantification using a single internal standard of known purity [13–15]. Recent collaborative and regulatory-oriented studies have demonstrated that qNMR provides traceable, reproducible, and calibration-independent quantification when appropriately validated, making it highly suitable for assay determination and purity evaluation of active pharmaceutical ingredients [16–18]. Accordingly, qNMR methodologies are well aligned with the principles of ICH Q2(R2) for analytical method validation, particularly with respect to linearity, accuracy, precision, and robustness [19].

Galantamine is particularly amenable to qNMR analysis due to its chemically distinctive and well-resolved proton resonances, which can be selectively integrated even in the presence of complex matrices or structurally related alkaloids [20,21]. Importantly, qNMR uniquely enables simultaneous qualitative structural verification and quantitative determination within a single experiment, thereby strengthening identity assurance and analytical system suitability [22,23]. Despite these advantages, recent literature indicates that most reported analytical methods for galantamine remain predominantly chromatographic, with limited emphasis on comprehensive NMR-based validation strategies that integrate full multidimensional structural elucidation with quantitative analysis [24,25].

Therefore, a clear analytical gap exists for a systematic approach that combines complete one- and two-dimensional NMR-based structural characterization with a rigorously validated qNMR method for galantamine. The present study addresses this gap by developing and validating an ICH-aligned ^{1}H qNMR method, supported by extensive ^{1}H, ^{13}C, DEPT, HSQC, HMBC, and COSY analyses, for accurate, precise, and robust quantification of galantamine. The proposed analytical strategy is intended to support pharmaceutical quality control, reference standard qualification, and regulatory applications for galantamine and related alkaloid-based products.

2. MATERIALS AND METHODS:

2.1 Chemicals and Reagents

Galantamine reference standard (purity ≥ 99%) and imidazole (purity ≥ 99%) were obtained from M/s Thota Natural Solutions, Visakhapatnam, India. Deuterated chloroform ($CDCl_3$, 99.8% D), used as the NMR solvent, was procured from Sigma-Aldrich, Mumbai, India. All chemicals were of analytical grade and were used as received without further purification.

2.2 NMR Instrumentation

All NMR experiments were performed on a Bruker Ascend 400 MHz spectrometer equipped with a broadband observe probe and z-axis gradients, operated using Bruker TopSpin software. Prior to data acquisition, automated tuning, locking, shimming, and receiver gain optimization were carried out to ensure optimal magnetic field homogeneity, spectral reproducibility, and instrumental stability.

Quantitative ^{1}H NMR spectra were acquired at a controlled probe temperature of 25 °C using a 90° pulse angle, a spectral width of 20 ppm, and a digital resolution corresponding to 64K data points. The relaxation delay was selected based on the experimentally determined longest spin–lattice relaxation time (T_1) of galantamine protons to ensure complete relaxation between scans and to avoid signal saturation. All quantitative spectra were accumulated to achieve a signal-to-noise ratio (S/N) greater than 150 for the selected analytical resonance, consistent with established qNMR best practices and analytical precision requirements.

2.3 qNMR acquisition and optimization

Quantitative NMR data were acquired using manually optimized acquisition parameters. During data collection, the receiver gain, lock, and shim settings were automatically adjusted to maintain spectral consistency. Standard ^{1}H NMR acquisition parameters included a spectral width of 20 ppm, a relaxation delay of 5 s, 16–32 scans, and 32K data points.

Broadband proton decoupling was applied for the acquisition of ^{13}C and DEPT spectra. Two-dimensional NMR experiments, including HSQC, HMBC, and COSY, were recorded using standard Bruker pulse sequences optimized for sensitivity and resolution, enabling unambiguous proton–carbon and proton–proton correlation assignments.

2.4 Preparation of solutions

For qualitative structural analysis, galantamine was dissolved in $CDCl_3$ at a concentration of approximately 10 mg/mL. For quantitative analysis, 0.25 mL of galantamine solution (10 mg/mL) was mixed directly with 0.25 mL of imidazole solution (1 mg/mL) in an NMR tube. The mixture was homogenized thoroughly prior to spectral acquisition to ensure uniform distribution of analyte and internal standard.

2.5 NMR Method development for quantitative analysis of Galantamine

The resulting qNMR procedure was qualified in compliance with ICH Q2(R2) protocols on analytical procedures that should be used in assay determination. Specificity was also presented in the fact that galantamine signals were distinctly resolved without being affected by solvent, internal standard, and possible impurities.

Linearity was evaluated over a defined concentration range by plotting normalized integral ratios of galantamine to the internal standard against concentration, with acceptability assessed using the correlation coefficient (R^2). Accuracy was determined through spike-recovery experiments conducted at

multiple concentration levels (typically 50%, 100%, and 150% of the target concentration), and results were expressed as percentage recovery.

Precision was assessed in terms of repeatability (intra-day precision) and intermediate precision (inter-day and inter-analyst precision), with relative standard deviation (%RSD) values used as acceptance criteria. Method robustness was evaluated by introducing small, deliberate variations in key acquisition parameters, including the number of scans, relaxation delay, and temperature, and assessing their impact on quantitative performance.

3. RESULTS & DISCUSSION

3.1 Structural Elucidation of Galantamine by 1D and 2D NMR

Galantamine structural elucidation, purity determination, and quantitative validation were thoroughly done with a combination of a nuclear magnetic resonance (NMR) spectroscopic approach [7]. One-dimensional (^{1}H, ^{13}C, and DEPT) and two-dimensional (HSQC, HMBC, COSY) NMR analyses obtained a comprehensive picture of the structure of the compound on a molecular level, and quantitative NMR (qNMR) with an internal standard determined the analytical validity of the technique in giving accurate quantitative results. Such an integrated NMR strategy is widely recognized as the most reliable approach for confirming the molecular framework and stereochemical integrity of complex alkaloids prior to quantitative analysis [19,20].

The NMR experiments utilized $CDCl_3$ as the solvent because Imidazole and Galantamine have the best possible solubility with it [18]. Imidazole was the internal standard due to its high chemical purity, chemical stability, and the presence of non-overlapping aromatic proton resonances in $CDCl_3$. The NMR spectrum of Galantamine (Fig. 1), Imidazole (Fig. 2), and their mixture (Fig. 3) in $CDCl_3$ (400 MHz) was used in the selection of the proton signal to be quantitatively analyzed. The ^{1}H NMR spectrum of galantamine recorded in $CDCl_3$ displayed well-resolved resonances corresponding to aliphatic methylene protons (δ 2.2–3.2 ppm), methoxy protons (δ ~3.74 ppm), oxygenated methine protons (δ 4.75 ppm), and aromatic protons (δ 6.6–7.0 ppm). The singlet observed at δ 4.75 ppm was assigned to the oxygenated methine proton (H-8), a structurally and pharmacologically critical feature of galantamine. Preservation of this resonance without chemical shift distortion or signal splitting confirms the absence of degradation, epimerization, or chemical modification during analysis, which is essential for pharmaceutical quality control [18,24].

The proximity of these data to previously reported spectra supports the structure and electronic state of the molecule[4]. The lack of artifactual resonances, as well as the narrow chemical shift dispersion (±0.3 ppm) suggest that the isolated sample is chemically pure and that its conformations are relatively stable. Being chemically inert, it does not perturb the analyte, and as its number of protons is discrete, quantification and comparison between spectra are possible. Thus, the ^{1}H NMR spectrum of imidazole in $CDCl_3$ can be used as a reliable internal reference for checking chemical shifts and thereby confirming

completeness of spectra and promoting quantitative analysis in both routine and advanced NMR investigations [26].

On the other hand, the ¹H NMR spectrum of Imidazole in $CDCl_3$ (Fig. 2) contains proton resonances corresponding to its aromatic protons in the heterocyclic ring. Two separate peaks are observed at δ 7.75 ppm and δ 7.162 ppm, signifying the C4–H and C5–H protons, respectively, indicating that the imidazole ring is aromatic. The broad singlet at δ 12.9 ppm is assigned to the N–H proton, according to the literature reports for imidazole in non-polar solvents [27]. The chemical shifts and coupling patterns are in good agreement with the literature's NMR spectra, confirming the structure identity of the compound. The overlay spectrum is shown in Fig. 3 with the reference within the red trace and Test containing sample in blue. The overlay shows large spectral overlap, especially in the aromatic and downfield regions, which suggests the absence of strongly chemically shifted or impurity peaks. Only small variations in intensity level are attributed to solvent effects or concentration, rather than a change in structure. In general, from the spectras it is seen that the imidazole standard and the test sample had the same proton environments, thus confirming the reproducibility of the method and also the chemical stability of IMZ under experimental conditions.

Analysis of the ^{1}H and ^{13}C NMR spectra of Galantamine corroborated both structural integrity and resonance pattern typical for Galantamine. ^{13}C NMR spectrum showed 17 different carbon signals, which were in agreement with the proposed carbon skeleton of Galantamine (Table 1). The oxygenated methine carbon was found at δ 87.9 ppm, and aromatic carbons were observed in the region of δ 111.9–146.9 ppm, indicating the presence of a substituted benzene moiety as a certain structural feature commonly found in *Amaryllidaceae* alkaloids (Fig. 4). The comparable signals in the ^{1}H NMR spectra were also found as multiplets and doublets for aliphatic protons and aromatic protons indicating the presence of methylene, methoxy, and aromatic units. These features of the EEM spectra are well in accordance with earlier reported NMR data for Galantamine in *Sternbergia candida* and related species [6]. The overall data obtained from these analyses prove that Galantamine did indeed maintain its native, natural configuration, and no degradation, isomerization, or purity-induced shifts were caused either by extraction techniques or the estimation methods. The high matchings observed in spectrum data against a literature database indicate the reproducibility of NMR spectroscopy as validation tool for the primary structure of complex alkaloids.

Table 1: 1 NMR and 13C analysis of Galantamine.

ATOM ID	CHEMICAL SHIFTS	
	13C	1H
1	33.2	2.27(d,1H), 1.86(d,1H)
2	37.3	3.16(1H, m), 2.75(d,1H)
3	**42.4**	**2.46(3H, s)**
4	48.9	-
5	54.1	1.7(2H, s)
6	56.0	3.8(3H, s)
7	60.6	4.09(1H, d), 3.74(1H, d)
8	87.9	4.75(1H, s)
9	111.9	6.66(1H, q)
10	122.0	3.2(1H, d)
11	122.0	3.2(1H, d)
12	127.1	6.05(1H, d)
13	129.4	-
14	130.5	-
15	144.0	-
16	144.3	6.96(1H, d)
17	146.9	-

3.2 DEPT Analysis and Carbon Type Assignment

The validation of a ¹³C-DEPT NMR spectrum is essential for confirming both the structural integrity and purity of Galantamine. In addition to increasing the sensitivity of the signal, ¹³C-DEPT distinguishes between different types of carbons — CH_3, CH_2, and CH — through characteristic phase patterns that it produces. The result is that you can then identify functional groups and precisely confirm the compound's framework. Using $CDCl_3$ as the solvent, the ^{13}C-DEPT spectrum of Galantamine peaks are sharp and well-resolved and fall within expected chemical shift ranges (δ 33–146 ppm). That's the normal range for aliphatic carbons. Oxygenated methine (δ 88 ppm), corresponding to the oxygenated methine (C-8), serves as a marker of Galantamine's pharmacologically active conformation [18]; methoxy carbon (δ 56 ppm) confirms the 3-O-methoxy substitution, in line with the natural alkaloid configuration of isoquinoline derivatives seen by other authors [28]; and aromatic carbons (δ 112–144 ppm) showed multiple peaks consistent with a 3,4-dimethoxybenzene substitution pattern (Fig. 5). The two-

dimensional NMR analyses succeeded in fully confirming intra- and interatomic bond connectivity, offering a three-dimensional understanding of the Galantamine molecule.

HSQC to directly correlate each proton to its attached carbon (deconvolutes ambiguous CH/CH_2/CH_3 assignments). The 2D ^{1}H-^{13}C HSQC NMR spectrum of Galantamine (in $CDCl_3$) has the characteristic cross-peaks that directly relate proton and carbon chemical shifts, confirming protonated carbon environments and helping in precise signal assignment. The well-dispersed cross-signals in the range from the δC 42-88 ppm and δH 1.8-4.8 ppm range match the aliphatic and oxygenated methine/methylene carbons of the Galantamine scaffold. Strong correlations at δH 4.75/ δC 88.0 ppm and δH 3.74 - 4.09/ δC 60.6 ppm confirm the existence of the oxygenated methine (C-8) and methoxy/methine groups, respectively, in agreement with the benzazepine-oxazolidine ring system. The aromatic area, correlated with δH 6.66-7.29 ppm and δC 111.9-144.4 ppm, clearly indicates the 3,4-dimethoxyphenyl substitution characteristic of *Amaryllidaceae* alkaloids [29]. The absence of spurious cross-peaks demonstrated sample homogeneity and the absence of sample degradation (Fig. 6).

HMBC to link methyls, methoxy, and oxygenated proton(s) to quaternary carbons. The 2D HMBC spectrum of Galantamine measured in $CDCl_3$ showed sharp long-range (2J and 3J) heteronuclear correlations of protons and carbons, which verified the structural relationship of the molecule. Correlations between δH 6.68-6.99 ppm and δC 129-147 ppm were used to prove the aromatic substitution pattern, and δH 3.74-4.09 ppm and δC 87.9 ppb were used to prove the oxygen bridge system between azepine and oxazolidine rings [17]. Long-range correlations between δH 4.75 ppm (H-8) and δC 87.9 ppm were used to confirm the oxygenated methine linkage that is characteristic of the Galantamine skeleton. Additional cross-peaks between δH 2.2-3.2 ppm and δC 37.3-60.6 ppm were aliphatic carbon-proton couplings in the azepine and oxazolidine rings. The lack of non-assigned and extraneous correlations was another confirmation of the compound's structural purity and the integrity of the spectra (Fig. 7). The 2D COSY spectrum of Galantamine in $CDCl_3$ showed well-defined scalar couplings with well-understood spin-spin connectivity between neighbouring protons in the molecule: The COSY spectrum showed organizations of scalar coupling networks of neighboring protons, connecting intra-ring connectivity. Cross-peaks between δH 2.16-3.26 ppm were assigned to aliphatic chain coupling, and δH 6.66, 6.71, and 6.99 ppm correlations determined ortho and meta-aromatic couplings between the benzene ring. The pattern of connectivity between aliphatic and aromatic regions is related to the maintenance of the molecular framework (Fig. 8). Collectively, the HSQC, HMBC, and COSY analyses gave an unequivocal confirmation of all the proton-carbon and proton-proton relationships, proving the resonance assignment to be complete and the structural authenticity of Galantamine.

3.3 Quantitative ¹H NMR Analysis of Galantamine

A mixture containing 0.25 mL of galantamine standard solution (10 mg/mL) and 0.25 mL of imidazole solution (1 mg/mL) was transferred into an NMR tube, and the ¹H NMR spectrum of the mixture was recorded under optimized quantitative conditions. In the resulting spectrum, the methyl proton signal of

galantamine at δ 2.40 ppm and the aromatic proton signal of imidazole at δ 7.10 ppm were well resolved, free from overlap, and exhibited stable integration behavior. These resonances were therefore selected for quantitative evaluation, in accordance with recommended qNMR practices for internal standard–based quantification [4,26,27]. Using the established qNMR equation, the calculated purity of galantamine was found to be 97.996%, confirming the suitability of the selected signals and experimental conditions for subsequent method validation.

3.4 Signal to Noise Ratio

Signal-to-noise ratio (S/N) studies were conducted to assess the sensitivity and quantitative reliability of the NMR system across different galantamine concentrations (0.5–3.0 mg/mL) and numbers of scans (8, 16, and 32). The S/N ratio increased proportionally with increasing galantamine concentration, demonstrating a direct relationship between analyte concentration and NMR signal intensity, which is a fundamental requirement for quantitative NMR analysis [11–16].

At the lowest tested concentration (0.5 mg/mL), S/N values ranged from approximately 211 to 274, while at the highest concentration (3.0 mg/mL), S/N values exceeded 1100. These results indicate a substantial enhancement in signal detectability with increasing concentration and confirm that all measurements were performed well above the generally accepted S/N threshold of 150 required to achieve a quantification uncertainty below 1% in qNMR [11–13].

An increase in S/N was also observed with increasing scan numbers from 8 to 32, consistent with the theoretical √N relationship between signal-to-noise ratio and the number of scans (Table 2). However, the effect of concentration on S/N was more pronounced than that of scan number, indicating that analyte concentration plays a dominant role in determining spectral detectability under the selected acquisition conditions. Overall, these findings demonstrate the reproducibility, sensitivity, and robustness of the NMR system for galantamine quantification within the investigated concentration range.

Table 2: Signal-to-noise ratio under various acquisitions and Galantamine concentrations.

Galantamine Concentration (mg/mL)	Scan Number	Signal to Noise Ratio
0.5	8	213.93
	16	211.22
	32	274.21
1.0	8	469.23
	16	501.34
	32	527.87
1.5	8	598.81
	16	547.67
	32	598.59
2.0	8	769.14
	16	708.68
	32	715.42
2.5	8	911.40
	16	902.5
	32	954.71
3.0	8	1123.37
	16	1098.84
	32	1053.01

3.5 Linearity of the qNMR method

Based on the satisfactory S/N performance, linearity of the qNMR method was evaluated over a concentration range of 0.5–3.0 mg/mL using a five-point calibration model. The normalized integral ratios of galantamine (δ 2.40 ppm) to imidazole (δ 7.10 ppm) were plotted against concentration. A strong linear relationship was observed, with correlation coefficients (R^2) consistently greater than 0.9975 (Fig. 9), confirming excellent linearity across the evaluated range.

Quantitative NMR analysis was performed for Galantamine with Imidazole (internal standard), in which the aromatic proton resonances of Imidazole at δ 7.10 ppm were used as a reference, and the methyl protons of Galantamine at δ 2.40 ppm were analyzed to determine the concentration. Test concentration of 0.5 mg/mL to 3.0 mg/mL, with actual analyte masses (0.2–3.0 mg) were assessed in the study. The PCC (peak integral) value of Galantamine improved successively and proportionately with

the increasing concentration, thereby achieving a high correlation between signal intensity and content. The mean integral values increased from 1.074 (0.5 mg/mL) to 4.422 (3.0 mg/mL), demonstrating the reliability of measurements through three independent experiments. The near-linear pattern indicates that the NMR procedure is appropriate for Galantamine quantification with an Imidazole as stable reference compound. The results demonstrate that the integral ratio of internal standard to analyte could reflect the concentration variations well, and further verify the linearity and precision of developed qNMR method for Galantamine in $CDCl_3$. The peak integrals of Galantamine linearly increased from 1.074 at 0.5 mg/mL to 4.422 at 3.0 mg/mL, which matched with the expected direct proportion between signal area and number of nuclei. Overall, the linearity results validate the reliability and precision of the developed qNMR method and further support the applicability of NMR spectroscopy as a reproducible and sensitive tool for quantitative analysis of alkaloid-based pharmaceutical compounds [14–20].

3.6 Limit of Quantification (LOQ) and Limit of Detection

In quantitative NMR (qNMR), detection and quantification capabilities are governed primarily by signal-to-noise ratio (S/N) rather than by detector response limits, as the technique is inherently linear and based on direct proportionality between signal integral and molar concentration [10–12,15]. Therefore, conventional chromatographic concepts of LOD and LOQ should be interpreted with caution when applied to qNMR methods.

In the present study, method sensitivity was primarily evaluated using signal-to-noise ratio criteria. All quantitative measurements were performed at galantamine concentrations yielding S/N values greater than 150 for the selected analytical resonance, a threshold widely accepted in qNMR to ensure quantification uncertainty below 1% [11,16]. Under the optimized experimental conditions, the lowest evaluated concentration of galantamine (0.5 mg/mL) consistently met this criterion and was therefore considered the practical lower limit of quantification for the method.

For completeness and comparative purposes, apparent LOD and LOQ values were estimated using linear regression of the calibration data according to the equations:

$$LOD = (3.3 \times \sigma)/ S$$

$$LOQ = (10 \times \sigma)/ S$$

σ: Standard error of the predicted y-value for each x in the linear regression.

S: Slope of the linear regression.

Using this statistical approach, apparent LOD and LOQ values were estimated to be approximately 1.32 mg/mL and 0.44 mg/mL, respectively. However, it should be noted that these values are derived from statistical regression and do not define the true operational limits of the qNMR method.

From a practical analytical standpoint, the validated quantitative range of the method was established between 0.5 and 3.0 mg/mL, within which the method demonstrated excellent linearity ($R^2 > 0.997$), accuracy, precision, and robustness. Concentrations below 0.5 mg/mL were not included in routine quantification, as they approach the lower boundary where uncertainty may increase despite statistical detectability.

To estimate the mass –

$$\frac{np}{nis} = \frac{\frac{Ip}{Np}}{\frac{Iis}{Nis}} \times milli\ moles\ of\ internal\ standard$$

Ip = Integration value of product; Np = no of protons corresponding to the chosen peak of the product; I = Integration value of internal standard; N = no of protons corresponding to the internal standard

3.7 Quantitative Performance and validation summary

Quantitative ^{1}H NMR analysis of galantamine was performed using imidazole (0.2 mg/mL) as an internal standard. Over the validated concentration range of 0.5–3.0 mg/mL, percentage recovery values ranged from 97.0% to 100.4%, indicating excellent accuracy within the generally accepted ±2% range for pharmaceutical assay methods (Table 3). %RSD values were below 0.02 for all measurements, indicating excellent precision and low variability among triplicates. The minimal concentration (0.5 mg/mL) yielded 97.17% recovery, and the maximal concentration (3.0 mg/mL) provided a 100.38%, indicating that the signal integral ratio was positively proportional to the analyte concentration. Reference signals were those of the Imidazole aromatic proton (δ 6.1 ppm) and the Galantamine methyl signal at δ 2.4 ppm in order that the considered/quantified signals were integrated consistently.

Collectively, these results confirm that the developed qNMR method provides reliable, accurate, and precise quantification of galantamine within the defined working range and that imidazole serves as a suitable and stable internal standard under the applied experimental conditions.

3.7 Precision of the qNMR method

Precision of the quantitative ^{1}H NMR (qNMR) method was evaluated in terms of system precision (instrument repeatability), intra-day precision (repeatability), and intermediate precision (inter-day and inter-analyst variability), in accordance with the principles outlined in ICH Q2(R2) for assay determination.

The system precision (instrument repeatability) of the qNMR method was examined based on a relative standard deviation (RSD) obtained from five consecutive measurements. This was done by determining the ratio of the peak integral area of Galantamine at δ 2.4 ppm to the Imidazole peak at δ 7.1 ppm with a sample down at a concentration of 2.0 mg/ml for one Galantamine sample only. Intra-day precision (or repeatability) was determined from the RSD of five sample preparations at 2.0 mg/ml, consecutively

analysed. Intermediate precision was assessed by variation of results generated from two analysts for preparing and assaying five samples at 2.0 mg/ml on different days.

Intra-day precision of the quantitative ^{1}H NMR assay method on Galantamine was carried out at a fixed concentration of 2 mg/mL (Imidazole 0.2 mg/mL). Acquisitions of the proton integral values (δ 2.4) of Galantamine methyl protons was performed on different times (0–8 h) to check instrument stability and analytical reproducibility (Table 4). The mean of the recovery at the tested levels was 98.06 %. The small SD of 0.0237 with a low %RSD of 0.00024 is reflective of good intraday precision, and it appears that there were no large differences over the 8-hour analytical working time. In conclusion, the robustness of Galantamine and internal standard under NMR measurement was validated by these results, which indicate that the presented qNMR method is reliable for routine quantification within a single day.

Inter-day precision of the quantitative ^{1}H NMR method for Galantamine was investigated over four days with the same concentration (2 mg/mL) employing Imidazole (0.2 mg/mL) as internal standard. The Galantamine peak at δ 7.87 ppm and the Imidazole aromatic signal were used as a reference pair for quantification. The determined assay values varied from 98.02% to 98.10% (mean: 98.07%), demonstrating a high reproducibility over the days (Table 5). The low %RSD of 0.00036 also indicates the outstanding inter-day precision and analytical reproducibility (0.0353). These findings prove that the qNMR developed is stable and reproducible over different experimental days, as no evident drift or deterioration was detected for the Galantamine nor for the internal standard lines throughout time.

3.8 Robustness of the qNMR method

Robustness of the quantitative ¹H NMR (qNMR) method for galantamine was evaluated by examining the influence of deliberate and minor variations in experimental conditions on quantitative performance, in line with ICH Q2(R2) principles for method validation. Temperature robustness was assessed by recording ¹H NMR spectra over a temperature range of 20–35 °C. Across this range, all proton resonances of galantamine exhibited negligible chemical shift variation (≤ 0.05 ppm), indicating thermal stability of the analyte and confirming that moderate temperature fluctuations do not affect spectral integrity or quantitative reliability under routine operating conditions [11–16].

The reproducibility of quantitative measurements was further assessed by varying key instrumental acquisition parameters, including the number of scans, relaxation delay, spectral width, number of data points, and receiver gain, while maintaining constant sample composition and internal standard concentration. Under all tested conditions, recovery values remained within the range of approximately 97.9% to 100.8% (Table 6), demonstrating that small variations in acquisition parameters had minimal impact on quantification accuracy.

Table 6: Robustness Studies

Parameters	Modification	Recovery (%)
Number of Scans (16)	8	97.91
	16	98.38
	32	100.03
Delay Time (1)	2	98.90
	5	99.1
Spectral Width (20 ppm)	15 ppm	99.2
	25 ppm	100.8
Data points (32K)	16 K	98.0
	64 K	99.32
Receiver Gain (400s)	206	98.19
	64	98.06

Specifically, increasing the number of scans from 8 to 32 resulted in an improvement in recovery from 97.91% to 100.03%, consistent with enhanced signal averaging and reduced noise, without introducing systematic bias. Variation of relaxation delay between 1 and 5 s and spectral width between 15 and 25 ppm produced stable recovery values close to 99–100%, indicating sufficient relaxation and appropriate spectral coverage. Similarly, consistent recoveries (approximately 98–99%) were obtained when the number of data points was varied from 16K to 64K and receiver gain was adjusted within the tested range, confirming robustness against instrumental drift and acquisition variability [20].

Collectively, these results demonstrate that the developed qNMR method for galantamine is robust, reproducible, and resilient to moderate changes in instrumental and experimental conditions. The consistent agreement between quantitative results and the structural information obtained from 1D and 2D NMR experiments further confirms preservation of structural identity, stereochemical integrity, and the characteristic electronic environment of galantamine throughout the analysis. These findings support the suitability of the method for routine quantitative applications in pharmaceutical quality control and analytical laboratories.

The robustness evaluation presented in this study is novel in that it systematically demonstrates the stability of a qNMR-based assay for galantamine under multiple, deliberately varied instrumental and environmental conditions, while simultaneously preserving structural integrity as confirmed by complementary 1D and 2D NMR analyses. Unlike many reported qNMR applications that focus primarily on linearity and precision, the present work establishes robustness by correlating quantitative performance with chemical shift stability, relaxation adequacy, and acquisition parameter tolerance

across a realistic operational range. This integrated assessment provides strong evidence that the method is not only analytically reliable but also practically resilient, a critical requirement for routine pharmaceutical quality control and inter-laboratory transfer. The demonstrated insensitivity of quantitative results to moderate variations in temperature and acquisition settings highlights the suitability of the method for real-world analytical environments, where minor instrumental fluctuations are unavoidable.

3.9 Method Accuracy, Precision, and Validation

These findings are consistent with previously reported qNMR validation studies, which demonstrate that quantitative NMR signal integration is fundamentally governed by physical constants and the number of resonating nuclei, rather than by detector-dependent calibration factors or instrumental response functions [19]. This intrinsic characteristic contributes to the high reproducibility and traceability of qNMR measurements. The observed signal stability throughout the analytical period further indicates the absence of sample degradation and negligible solvent interference under the applied experimental conditions, supporting the suitability of the method for routine and longer-term analytical applications. The use of imidazole as an internal standard enhanced normalization precision due to its chemical stability and the presence of a well-resolved, non-overlapping proton resonance, making it appropriate for reliable quantitative comparison in the developed qNMR method [20].

4. Industrial Applicability

The present study makes a significant contribution by integrating complete multidimensional NMR-based structural elucidation with a rigorously validated quantitative ^{1}H NMR method for galantamine. While previous studies have largely focused on chromatographic quantification or partial spectroscopic characterization [5–9,24], this work demonstrates that qNMR can simultaneously ensure identity confirmation, structural integrity, and accurate quantification within a single analytical platform.

From an industrial perspective, the developed method offers substantial advantages for pharmaceutical and herbal drug quality control, including reduced analytical complexity, elimination of extensive calibration procedures, non-destructive analysis, and improved traceability. The approach is particularly suitable for batch release testing, stability studies, reference standard qualification, and regulatory submissions for galantamine and related alkaloid-based products [11,15,21,23]. Moreover, the analytical framework presented here can be readily extended to other structurally complex natural products, reinforcing the broader applicability of qNMR in modern pharmaceutical analysis.

5. Conclusion

This study presents a comprehensive and rigorously validated NMR-based analytical strategy for galantamine that integrates complete multidimensional structural elucidation with quantitative ^{1}H NMR analysis. The novelty of the work lies in the combined application of 1D and 2D NMR techniques to unequivocally confirm structural identity and stereochemical integrity, followed by a systematically

validated qNMR method capable of providing accurate, precise, and robust quantification within a single analytical platform.

The analytical performance of the developed qNMR method for galantamine was compared with previously reported chromatographic and spectroscopic methods to evaluate its relative efficiency and reliability. In contrast to conventional chromatographic methods that rely on extensive calibration, matrix-dependent corrections, and multiple reference standards, the developed qNMR approach enables absolute quantification using a single internal standard, while simultaneously ensuring identity assurance. Conventional chromatographic techniques, including HPLC and LC–MS/MS, have been widely used for galantamine quantification and typically demonstrate recovery values ranging from approximately 95% to 102% and precision values between 1% and 2% RSD, depending on sample matrix complexity and calibration strategy [7,8]. While these methods provide high sensitivity, they rely heavily on external calibration curves and may be affected by matrix interferences and reference standard variability.

The present qNMR method demonstrated recovery values between 97% and 100% with precision well below 1% RSD, indicating excellent reproducibility and analytical reliability. These performance characteristics are comparable to or superior to many chromatographic approaches reported for galantamine and related alkaloids [14]. Furthermore, unlike calibration-dependent chromatographic techniques, qNMR enables absolute quantification based on the proportional relationship between signal integral and molar concentration, thereby improving traceability and reducing systematic calibration bias [10,15].

From a scientific perspective, this work advances current knowledge by providing a validated framework that links structural verification and quantitative analysis through NMR, addressing an important analytical gap in galantamine analysis. From an industrial and regulatory standpoint, the method offers significant advantages for pharmaceutical quality control, including reduced analytical complexity, non-destructive testing, enhanced traceability, and improved method transferability. The analytical strategy described herein is readily extendable to other alkaloid-based and structurally complex pharmaceutical compounds, reinforcing the broader applicability and relevance of qNMR in modern pharmaceutical analysis.

Declarations

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Conflict of Interest:

The authors declare that there is no conflict of interest.

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Author Contribution:

Kalpana Bandla: Conceptualization, Methodology, Investigation, writing – original draft, **Sibbala Subramanyam:** Conceptulization, supervision, Writing – review and editing, **Jithendra Chimakurthy:** Methodology, formal analysis,

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Tables 3, 4, and 5

Tables 3, 4, and 5 are available in the Supplementary Files section.

Figures

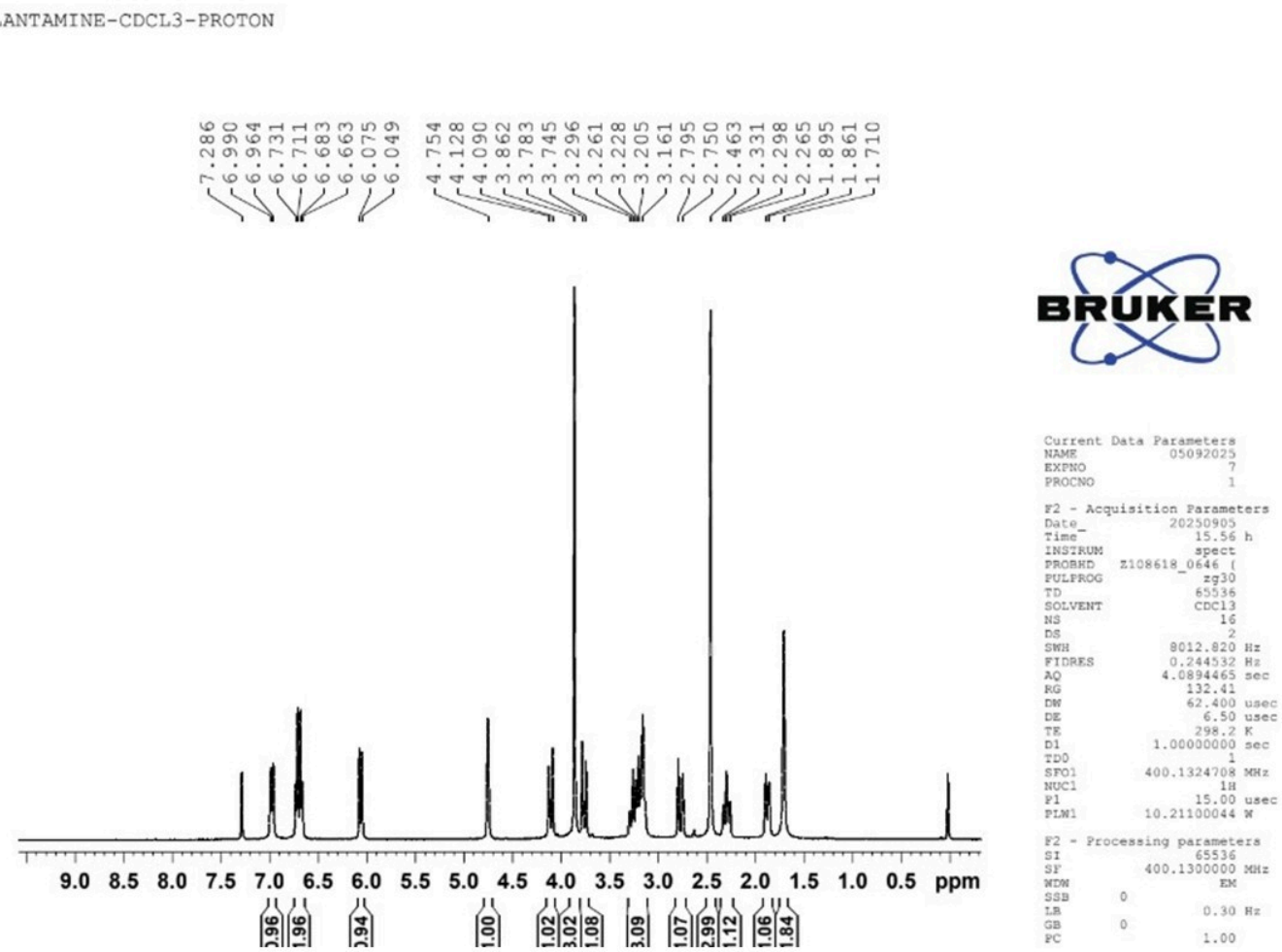


Figure 1

The 1H NMR spectrum of Galantamine obtained in $CDCl_3$

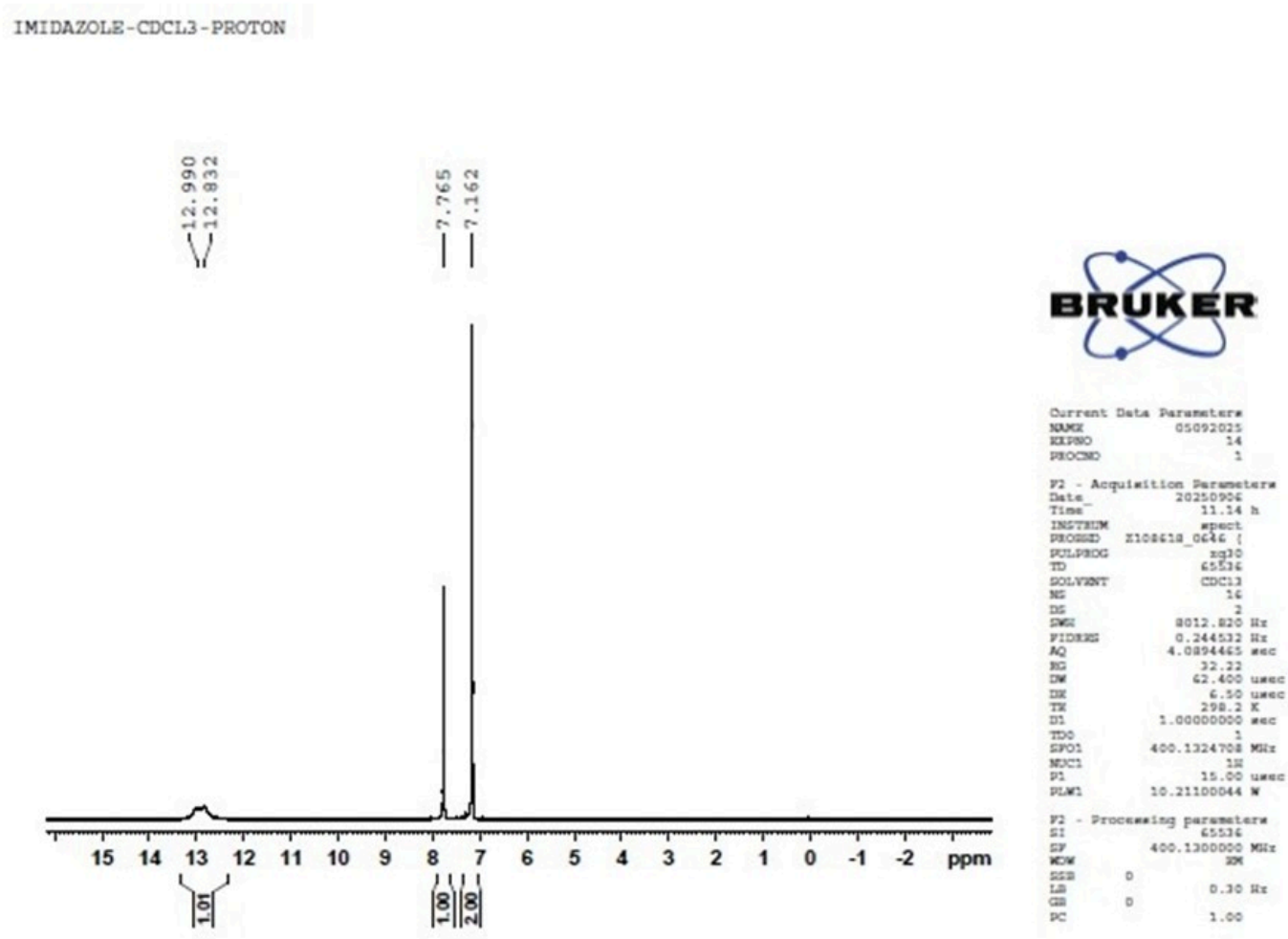


Figure 2

1H Proton spectrum of imidazole in $CDCl_3$

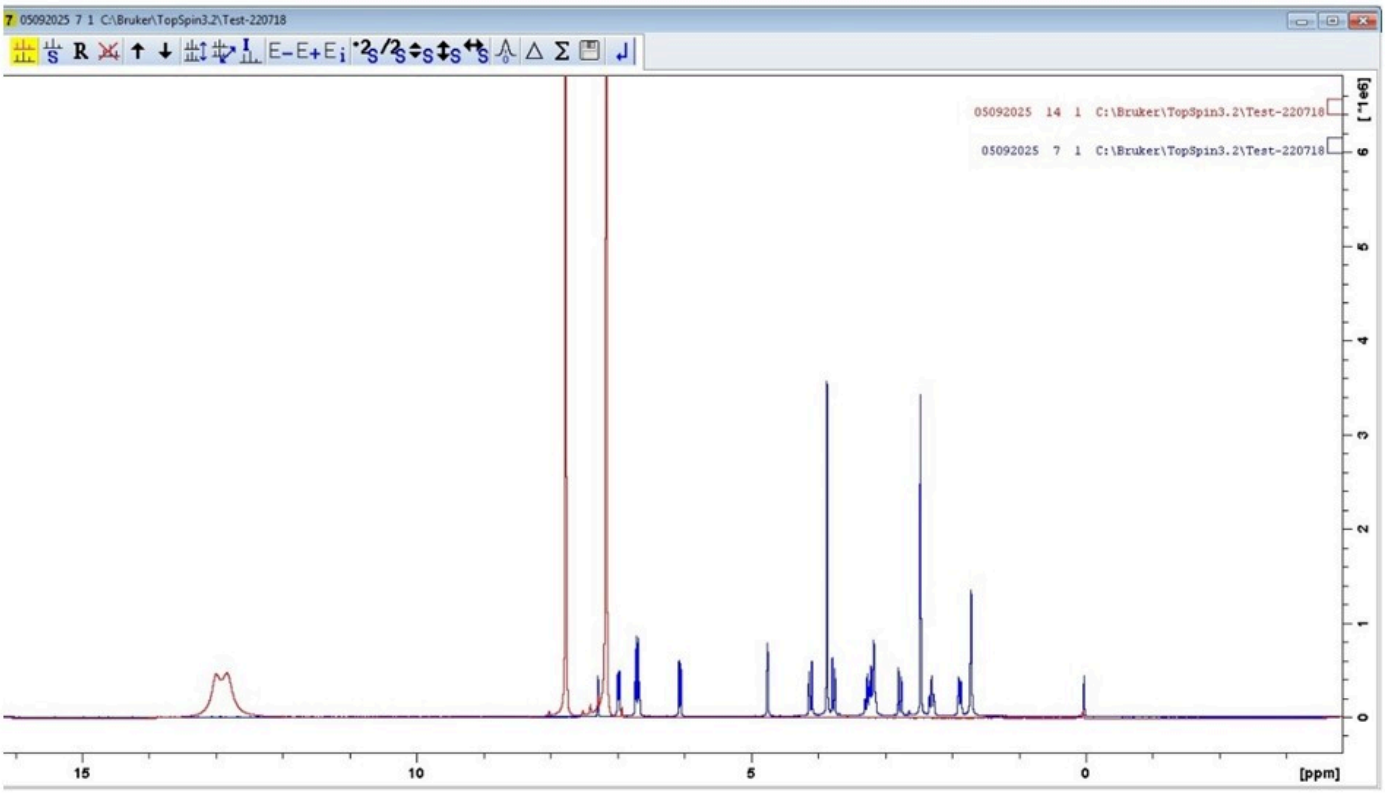


Figure 3

Comparision of proton spectra of Galantamine (Blue) and Imidazole (Red)

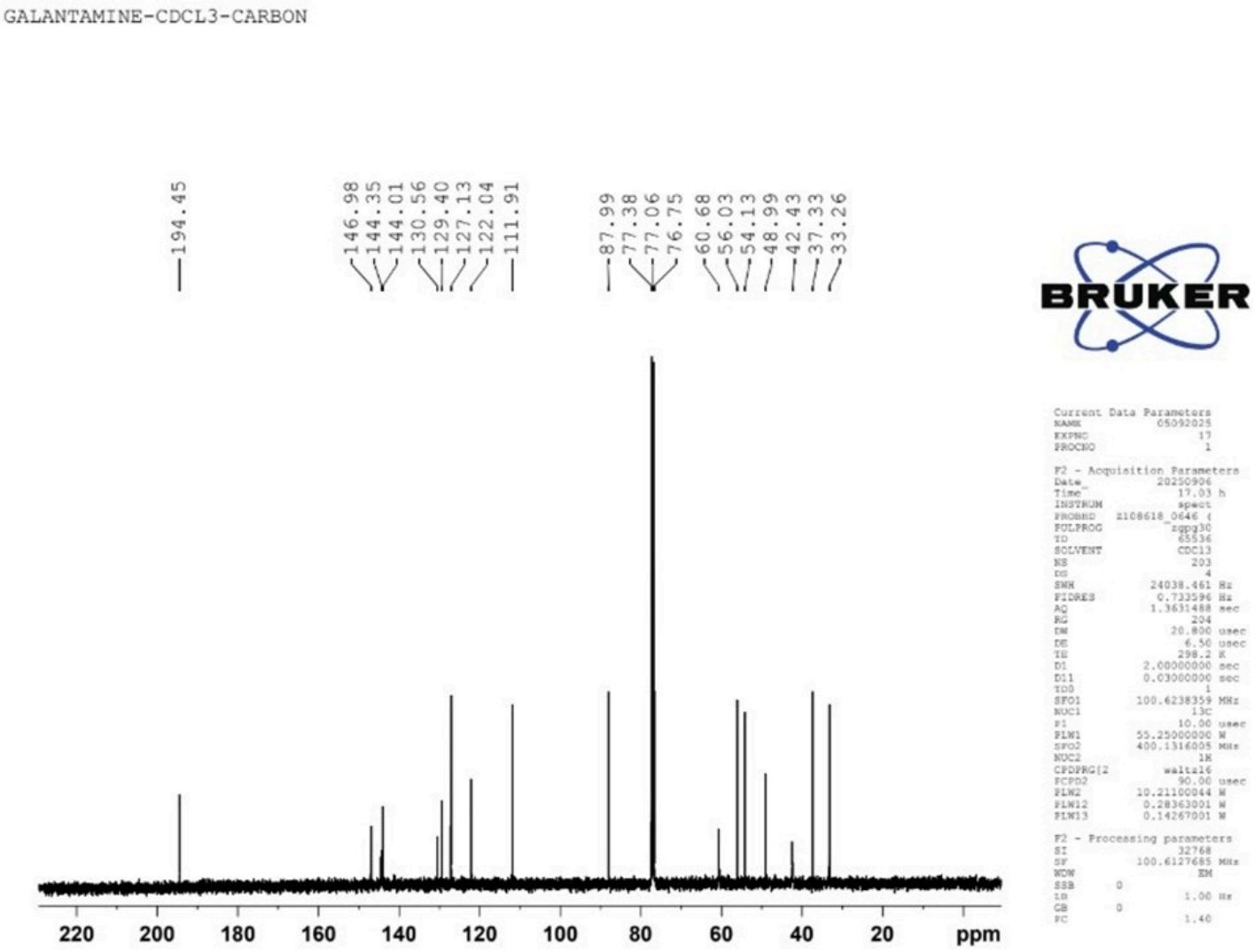


Figure 4

13C NMR spectrum of Galantamine obtained in $CDCl_3$

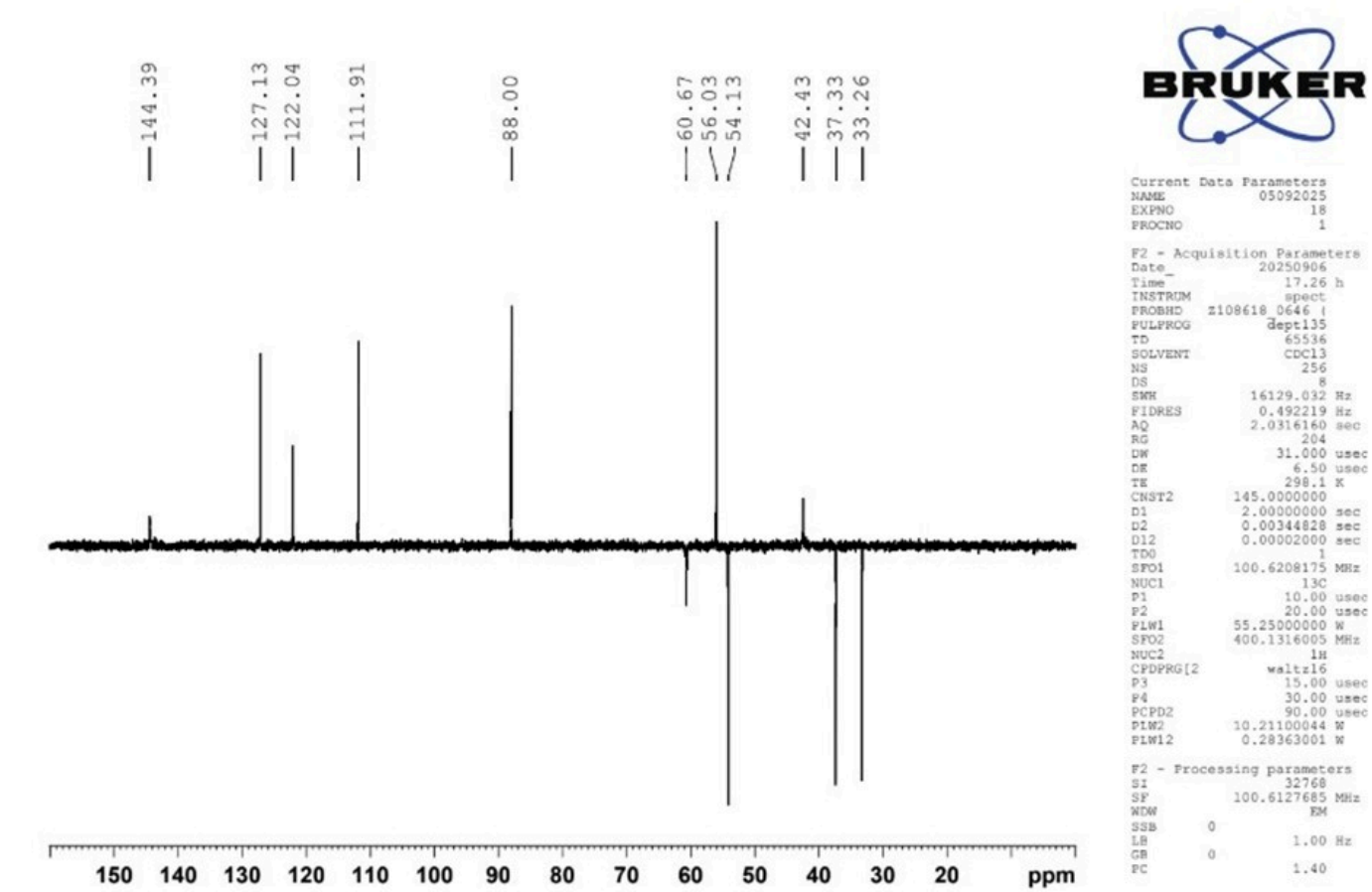


Figure 5

13C DEPT NMR spectrum of Galantamine in $CDCl_3$

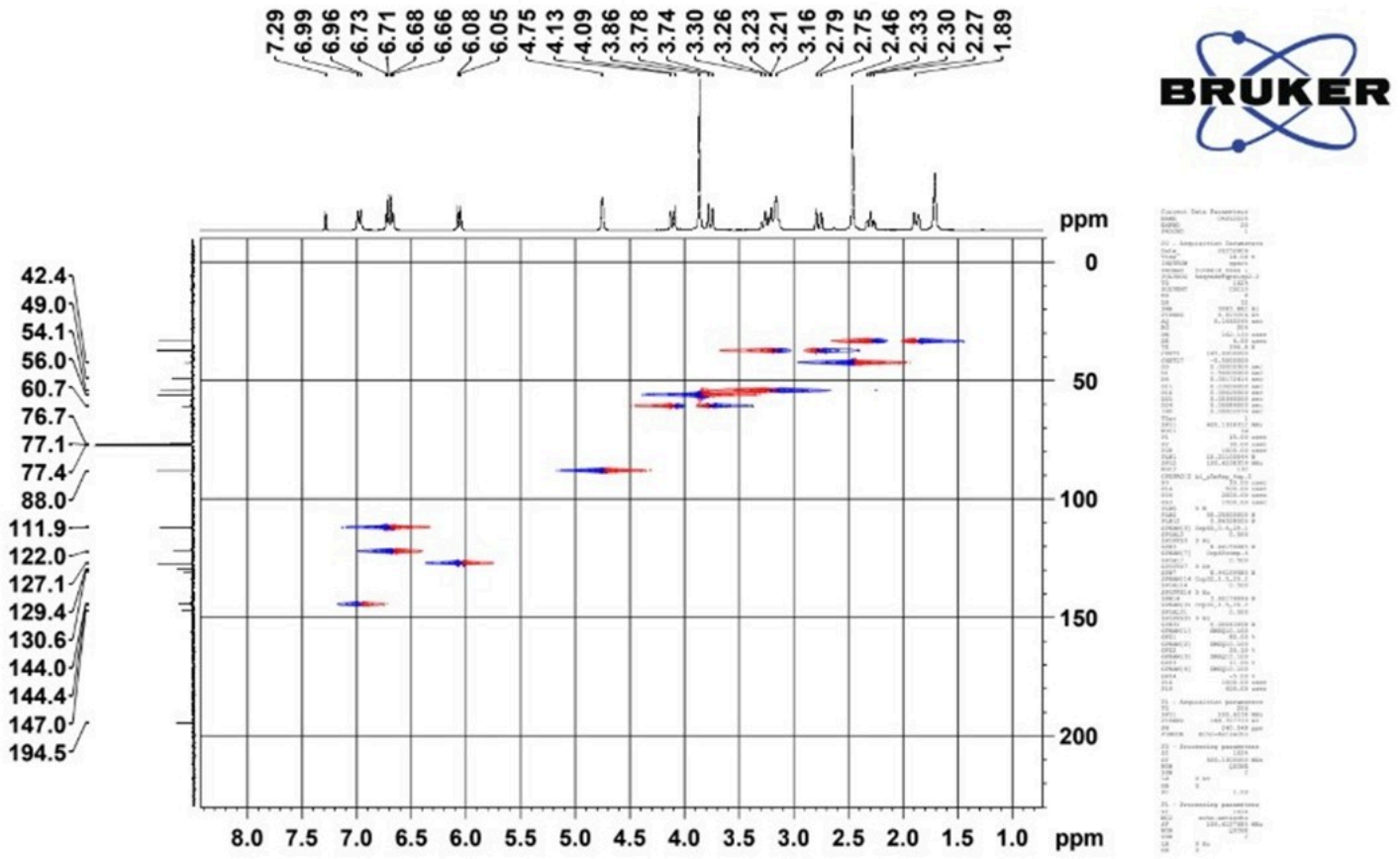


Figure 6

1H-13C HSQC spectrum of Galantamine in $CDCl_3$

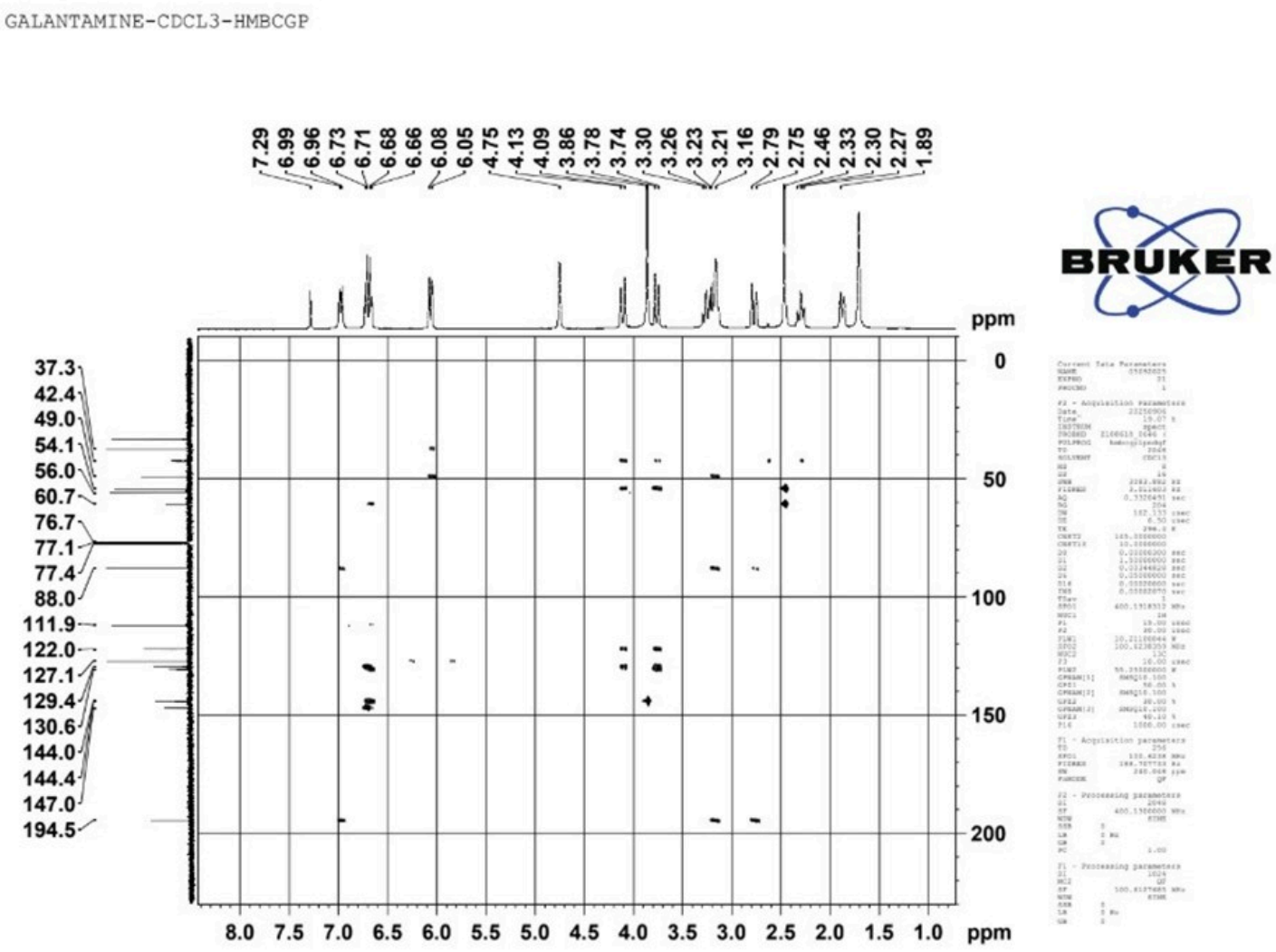


Figure 7

1H-13C HMBC spectrum of Galantamine in $CDCl_3$

GALANTAMINE-CDCL3-COSY

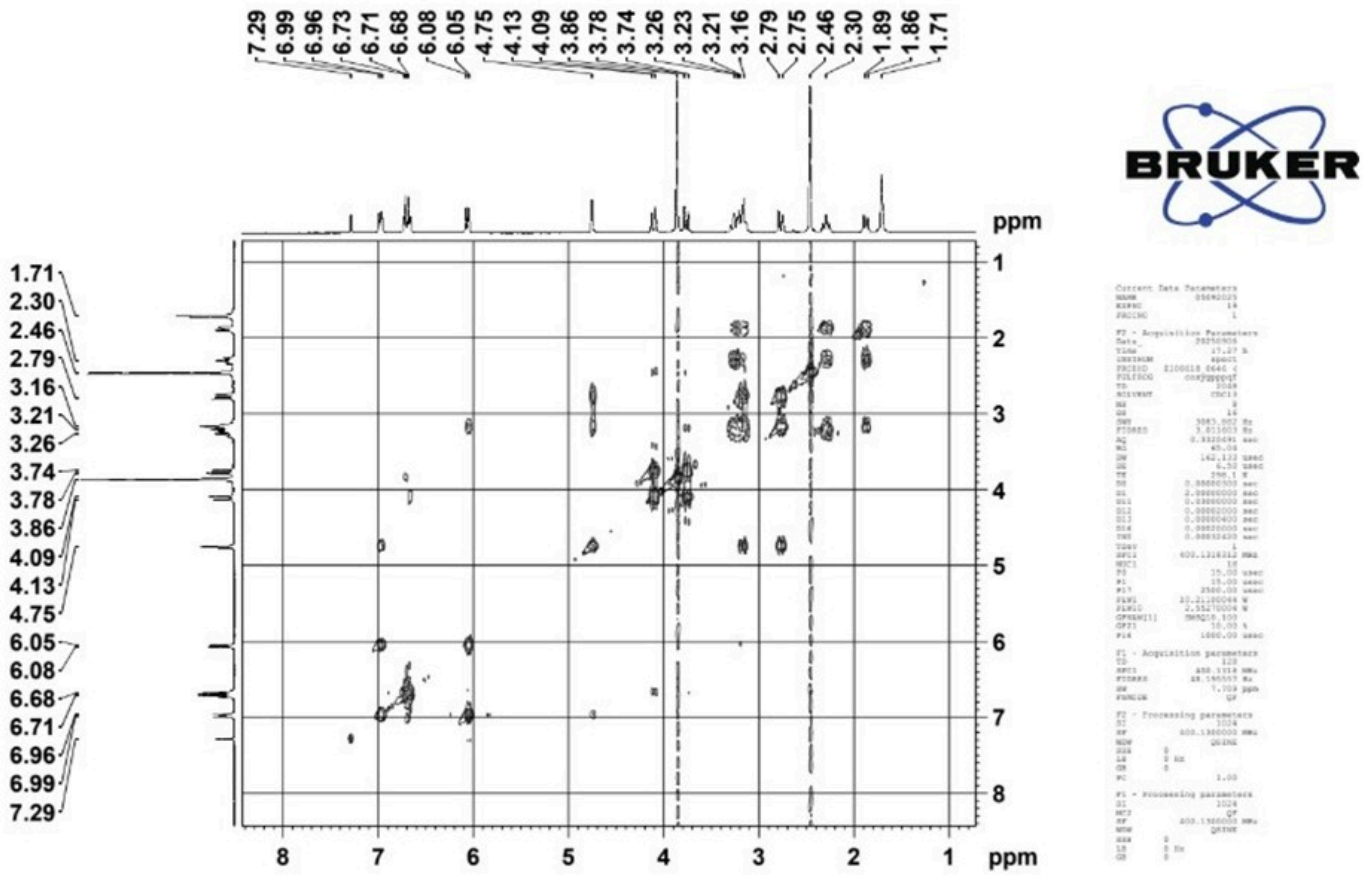


Figure 8

1H-1H COSY spectrum of Galantamine in $CDCl_3$

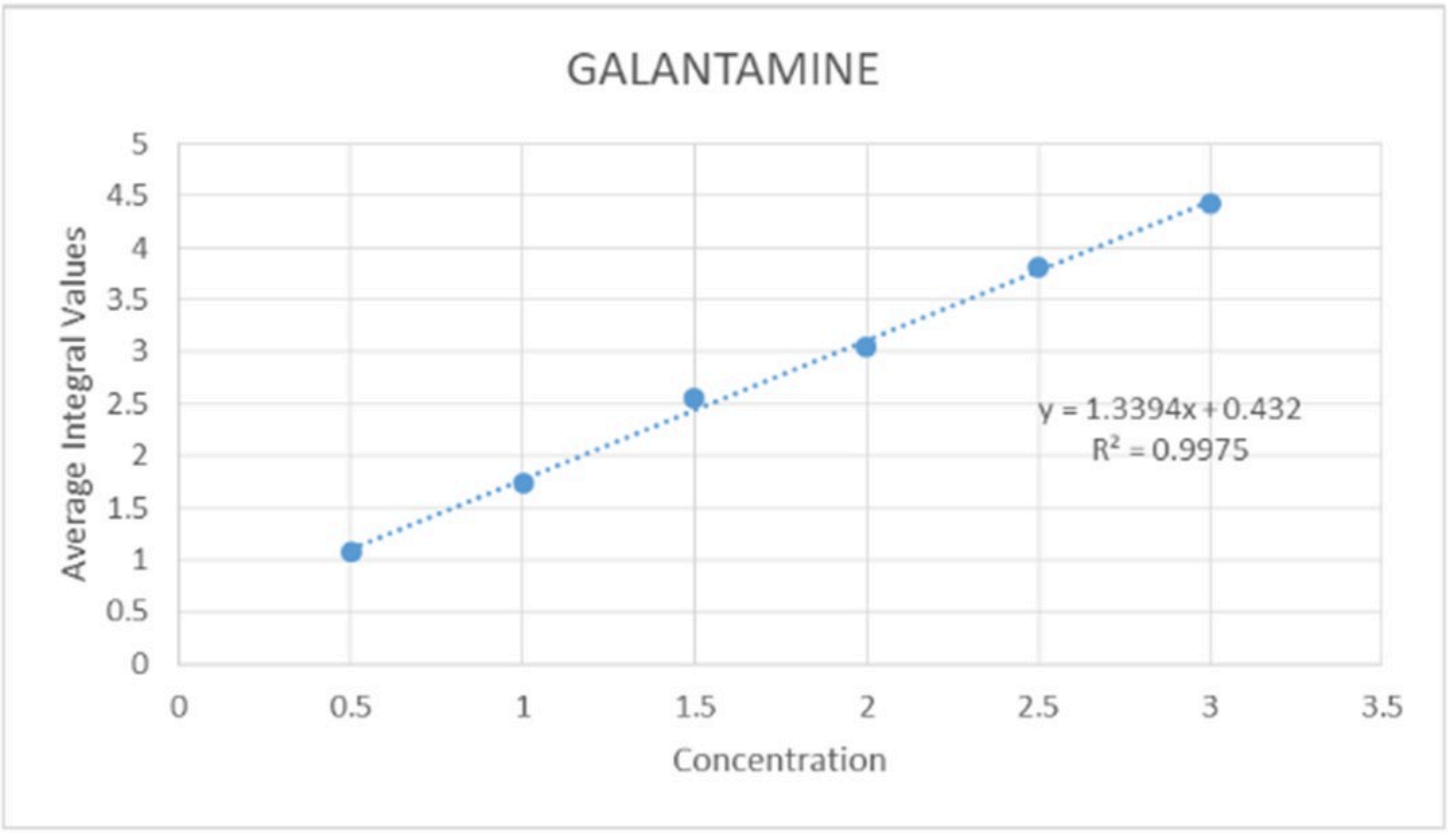


Figure 9

Linearity of samples.

Supplementary Files

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- graphicalabstract.jpg