Quantification of Cathinone Analogues without Reference Standard using $^1$H quantitative NMR

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Abstract

Synthetic cathinones are a type of new psychoactive substances (NPS) that have been seriously abused. Owing to the rapid variation in their structures, the absence of reference standards poses a challenge in quantitative investigations. In this study, a \(^1\)H quantitative nuclear magnetic resonance (\(^1\)H qNMR) method was established using maleic acid as the internal standard and the shared signal (i.e., the methylidyne hydrogen) on the parent synthetic cathinones structure as the quantitative peak. Taking 3-methoxy-2-(methylamino)-1-(4-methylphenyl)propan-1-one (mexedrone) as an example, this study optimized the acquisition parameters and conducted method validation, including evaluation of the specificity, linearity, accuracy, precision, and robustness. Using this \(^1\)H qNMR method, the contents of mexedrone and its analogues, including 1-(3-chlorophenyl)-2-(ethylamino)-propan-1-one (3-CEC), 4-chloro-α-pyrroli-dinopropiophenone (4-Cl-α-PVP), 1-(3, 4-methylenedioxy-phenyl)-2-propylamino-propan-1-one (propylone), and methcathinone, were obtained. The obtained results showed that the method was accurate, rapid, versatile, and can be used to address the qualitative and quantitative issues related to similar substances.

Keywords: quantitative nuclear magnetic resonance, assay, synthetic cathinones, structure identification, quantitation
**Introduction**

By modifying the chemical structures of controlled drugs, new psychoactive substances (NPS) have been produced by criminals to circumvent legal restrictions. The *World Drug Report 2020* published by the United Nations Office on Drugs and Crime (UNODC) revealed that NPS are rapidly evolving and spreading. Synthetic cathinones are the second most abused NPS monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Synthetic cathinones are phenethylamine derivatives bearing a β-keto group on the side chain (Fig. 1), and their chemical syntheses are relatively straightforward. Owing to the variety in the types and positions of the substituent groups, slight changes in these aspects can produce novel cathinones, such as 4-methylmethcathinone (4-MMC), 3, 4-methylenedioxypyrovalerone (MDPV), α-pyrrolidinopropiophenone (α-PPP), 4-methoxymethcathinone (βkPMMA), and flephedrone (4-FMC). The main pharmacological mechanism of synthetic cathinones is their interaction with monoamine neurons in the central nervous system (CNS). Long-term abuse therefore leads to dependence and addiction, resulting in both physical and psychological damage, and even death, in some cases.

In the judicial theory and practice of drug-related crime, the conviction and sentencing of the defendant depend on the type (identification) and quantity (quantification) of illicit drugs; hence, appropriate analytical techniques are required. Conventional methods for quantifying synthetic cathinones include liquid chromatography (LC), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS), among others, and for all such techniques, reference standards are indispensable for quantitation. However, it is difficult to obtain reference standards for all target NPS owing to their rapidly changing structures; hence, this issue should be urgently addressed to allow their detection. In this
context, quantitative nuclear magnetic resonance (qNMR) spectroscopy can be used for quantitative analysis through the application of a suitable and readily available internal standard, which is independent of the target component structure. For example, Ameline et al. quantitated seven small bags of NPS using the qNMR method with maleic acid as an internal standard; purities in the range 51-89 % were obtained. In addition, Gaspar et al. investigated qNMR as a tool for the quantification of a new cathinone derivative seized by the Portuguese police. Importantly, NMR analysis is advantageous due to its high reproducibility, lack of destruction of the target component, and convenient sample handling; hence, it has great potential for application in the pharmaceutical, biological, and food industries, among others. However, to date, reports on the determination of synthetic cathinones in seized samples by qNMR analysis are scarce.

Thus, we herein report the identification of four seized compounds, namely 1-(3-chlorophenyl)-2-(ethylamino)-propan-1-one (3-CEC), mexedrone, propylone, and 4-chloro-α-pyrrolidinopropiophenone (4-Cl-α-PVP) (Fig. 2). Mexedrone is a novel substance of the cathinone family, and was released in 2015 as an alternative to mephedrone. Its pharmacological data suggest that mexedrone exhibits a lower pharmacological activity than many other cathinones, and so higher doses are required to achieve noticeable effects. Due to its classification as a controlled substance in China, the possession and distribution of mexedrone must be carefully regulated. Thus, based on mexedrone as a target compound, we establish a \(^1\)H qNMR method for the quantification of cathinone derivatives using maleic acid as the internal standard. Systematic studies are carried out for the first time, and the contents of other cathinone analogues are determined. Ultimately, we wish to establish whether the reported method can be applied to the accurate determination of synthetic cathinones.
Experimental

Reagents and chemicals

The four seized samples (numbered No.1, 2, 3 and 4) analyzed in this work were provided by the Narcotic Control Division of Nanjing Public Security Bureau (Nanjing, China); the methcathinone hydrochloride reference standard (2013-201201, 92.6%) was provided by the Key Laboratory of Drug Monitoring and Control of the Ministry of Public Security (Beijing, China); DMSO-d$_6$ (99.9%, containing 0.03% TMS) was purchased from Cambridge Isotope Laboratories (MA, USA); D$_2$O (99.9%) was obtained from Asfierst Science (Qingdao, China); maleic acid (190015-201302, 99.7%) was purchased from the National Institutes for Food and Drug Control (Beijing, China); methanol and acetonitrile were purchased from Tedia (Ohio, USA); formic acid was purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China).

NMR analysis

NMR experiments were carried out on a Bruker AV500 MHz spectrometer equipped with a Quattro Nucleus Probe (QNP). The NMR probe was maintained at a temperature of 303 K. The optimized acquisition parameters used for $^1$H qNMR analysis were as follows: pulse angle, 30°; number of scans, 16; relaxation delay, 30 s; spectral width, 14.6 ppm; data points, 21879; pulse program, s1pul; window function, exponential; line broadening, 0.3 Hz; and acquisition time, 3 s. All data were processed using TopSpin 2.1 software with a manual baseline, phase correction, and integration. Data analyses were performed with Microsoft Excel software using a data analysis tool pack.

For structural analysis, a portion (3–5 mg) of the seized sample was accurately weighed and transferred to Eppendorf (EP) tubes. DMSO-d$_6$ (0.5 mL) was then added to ensure the complete dissolution of the powder. After transferring each solution to a 5
mm NMR tube, the 1D (1H, 13C, DEPT90, and DEPT135) and 2D (1H/1H COSY, 1H/13C HSQC, and 1H/13C HMBC) NMR spectra were recorded.

For quantification, a portion (10.0 mg) of each sample and maleic acid (2.4 mg) were accurately weighed and transferred to EP tubes. After the addition of DMSO-d_6 (0.5 mL) and D_2O (0.1 mL), the mixture was mixed thoroughly to ensure dissolution of the powders. Subsequently, each solution (0.5 mL) was transferred to a 5 mm NMR tube.

The quantitative principle of 1H qNMR spectroscopy is that the intensity of each signal in the spectrum (i.e., the integrated signal area) is directly proportional to the number of hydrogen atoms that generate the signal. The quantitative result of the seized sample was therefore calculated according to the following equation 21:

\[
C_x = \frac{I_x \times N_x \times M_x \times m_x}{I_s \times N_s \times M_s \times m_s} \times C_s
\]

where I_x and I_s are the mean integrated areas of the selected signals for the sample and the internal standard (IS), respectively; N_x and N_s correspond to the number of protons generating the signal for the seized sample and the IS, respectively; M_x and m_x are the molar mass and the weighed mass of the sample, respectively; M_s and m_s define the molar mass and the weighed mass of the IS, respectively; and C_x and C_s represent the contents of the sample and the IS, respectively.

**HPLC-MS analysis**

An Agilent Technologies series 1290 UHPLC instrument coupled with an Agilent Ultivo triple quadrupole mass spectrometer was used for analysis. The separation was performed at 35 °C with a CAPCELL PAK C18 MG column (150 mm × 3 mm, 3 μm, SHISEIDO). For gradient elution, 0.1% formic acid in water (A) and acetonitrile (B) were used as the mobile phases, and were mixed according to the following conditions:
0–1.5 min, 2% B; 1.5–6.5 min, 2–90% B; 6.5–9.4 min, 90% B; 9.4–9.5 min, 90–2% B; 9.5–12 min, 2% B. The flow rate was 0.3 mL/min and the injection volume was 5 μL. The conditions for carrying out the MS measurements were as follows: ion mode, positive; gas temperature, 300 °C; gas flow, 5 L/min; nebulizer pressure, 45 psi; sheath gas heater, 250 °C; sheath gas flow, 11 L/min; and capillary voltage, 3.5 kV. Data acquisition was performed using Mass Hunter Qualitative Analysis software, version B.08.00.

After confirming the precursor ion over a full scan, collision-induced dissociation (CID) of the protonated molecular ion $[M+H]^+$ was carried out by applying collision energies of 15, 25, 35 and 45 eV. The full scan and the product-ion scan were operated in the mass ranges of $m/z$ 100–800 and 50–800, respectively.

For preparation of the sample solution for HPLC-MS analysis, an aliquot (~3 mg) of the desired seized sample was accurately weighed and transferred to an EP tube, where it was dissolved in methanol (3 mL). Subsequently, 10 μL of the solution was diluted to 1 μg/mL with methanol.

**IR analysis**

The Fourier transform infrared (FTIR) spectra of the seized samples were recorded on a JASCO 4100 FTIR spectrometer in the 4000–400 cm$^{-1}$ range. Data acquisition was managed using Spectra Manager software.

For sample preparation, the desired amount of each seized sample was triturated thoroughly with dry potassium chloride (w/w, 1:100), and a transparent slice prepared using a moderate amount of the mixed powder was used for IR analysis.


**Results and Discussion**

*Identification*

The HPLC-MS spectra of Full Scan are shown in Fig. S1, and the data for the product ions obtained at a collision energy of 15 eV are summarized in Table S1.

The parent structure of the synthetic cathinones is composed of a phenethylamine unit bearing a β-keto group on the side chain. Hence, the $^1$H NMR signals of the synthetic cathinones can be divided into three parts, namely the benzene ring, the methyldiyne group, and the alkyl chain. Among them, the signals of the benzene ring and the methyldiyne group, which are affected by the appended carbonyl and amino groups, can be considered as specific features of the synthetic cathinones. In addition, a single signal above 160 ppm in the $^{13}$C NMR spectrum indicates the presence of a carbonyl group in the structure.

Thus, the signals observed in the $^1$H and $^{13}$C NMR spectra at $\delta_H$ 6.5–8.0, $\delta_H$ 5.0–5.5, and $\delta_C$ 185 ppm, suggested that the four seized compounds were cathinone analogues. In addition, the characteristic fragmentation pathways of synthetic cathinones were observed in the mass spectra of the seized samples, with example fragmentation pathways including the elimination of water, the cleavage of the C–N bond, and cleavage of the CO–CN bond, thereby also indicating the presence of this structure.

To determine the number and positions of the substituents on the benzene moiety, the chemical shifts and integrations of the signals were examined. More specifically, the presence of four signals with different chemical shifts for No.1 suggested that this structure contained an $m$-substituted ethcathinone.

The ratio between the protonated molecular ion [M+H]$^+$ at $m/z$ 212.1 and the isotopic ion signal [M+2+H]$^+$ at 214.1 was 3:1, which implied the presence of a chlorine substituent. Based on the 1D and 2D NMR spectra and MS results, No.1 was believed
to be 3-chloroethecathinone (3-CEC). The $^1$H and $^{13}$C NMR signal assignments for this compound are given in Fig. S2 and Table S2, respectively. In addition, we note that the $^1$H NMR spectrum of this proposed compound matched with the reported data 22, although this was the first report of the corresponding 2D NMR data.

In the case of No.2, substitution at the para position of the benzene moiety was confirmed by the presence of two signals (i.e., $\delta_H$ 7.42 (2H) and 7.94 (2H)) (Table S3). In the mass spectra, the loss of CH$_3$OH ($m/z$ 32) and the presence of a product ion at $m/z$ 88 revealed that the side chain contained a methoxy group (Fig. S3), thereby indicating that No.2 was mexedrone. The MS and $^1$H and $^{13}$C NMR results were consistent with the published data 20, 22, 21.

In the case of No.3, the $^1$H NMR spectra indicated that two aromatic substituents were present. In terms of the MS results, the primary product ion formed by the dissociation of CH$_4$O$_2$ indicated that No.3 contained the 3,4-methylenedioxy-substituted cathinone structure 24. In addition, the signals observed by 1D and 2D NMR spectroscopy confirmed the framework of the side chain. Thus, the experimental results and previously published data 25 indicated that No.3 was propylone. The dissociation pathways and the $^1$H and $^{13}$C NMR signal assignments for this compound are shown in Fig. S4 and Table S4, respectively.

Similar to the case of No.2, the presence of a single chlorine substituent at the para position of No.4 was inferred from the $^1$H NMR and MS spectra. In addition, the observation of product ions at $m/z$ 70, 84, 126, 139, and 195 suggested that this compound was a pyrrolidinyl-substituted cathinone 26, 27 (Fig. S5). The structure of No.4 was further elucidated using the 2D NMR spectra (Table S5), and overall the obtained data indicated that No.4 was 4-Cl-$\alpha$-PVP, which is consistent with existing literature data 22, 28, 29.
In terms of the IR spectra (see Table S6), absorption bands at approximately 3000–3100, 1600, and 1500 cm\(^{-1}\) indicated the presence of an aromatic ring in the structures of all compounds, whereas strong absorptions at \(\sim 1650–1700\) cm\(^{-1}\) were attributed to the presence of a carbonyl group. Furthermore, the broad and strong absorption signals detected at 2500–2850 cm\(^{-1}\) for No.1, 2, and 3, and at 2250–2700 cm\(^{-1}\) for No.4 indicated that the samples existed in their secondary amine salt and tertiary amine salt forms, respectively (Fig. S6 and Table S6). This is the first report of an IR spectrum for propylone, whereas the spectra for the other compounds corresponded to previous literature reports\(^{22,30}\).

**Quantitative method development**

As cathinone analogues are soluble in DMSO and the addition of D\(_2\)O can result in exchange of the dissociative protons, to avoid interference in purity determinations, a mixed solvent system of 0.5 mL DMSO-d\(_6\) and 0.1 mL D\(_2\)O was used for all samples.

Previous studies have shown that eight compounds exist that are suitable for use in conventional \(^1\)H qNMR applications\(^ {31}\). In this case, maleic acid was selected as the internal standard because of its unique chemical shift (\(\delta_H\) 6.30 ppm), which does not interfere with the shared signal of the cathinones. Moreover, the excellent solubility of maleic acid in DMSO, in addition to the presence of a single sharp singlet, met the requirements for this method.

**Optimization of acquisition parameters**

The sample was measured at a pulse angle of 30° to obtain a suitable signal-to-noise (S/N) ratio in addition to appropriately stable experimental conditions in a relatively short acquisition time\(^{32}\).
In $^1$H qNMR analysis, the relaxation delay time is a crucial measurement parameter to ensure the full relaxation of protons. To get suitable the relaxation delay time (d1), the same sample was measured by changing the d1 (Fig.3). More specifically, the S/N ratio of the quantitative signal showed no apparent change from 30 to 40 s. To avoid selective saturation effects, a d1 of 30 s was therefore selected. The longest spin-lattice relaxation time (T1) values of the quantified protons of the analytes and the internal standard were then measured to confirm the appropriate selection of the d1 time. Thus, the T1 values of the quantified protons of compounds 1, 2, 3, 4, and methcathinone, in addition to that of the internal standard were 0.73, 0.64, 0.62, 0.46, 0.83, and 3.02 s respectively. Therefore, a relaxation delay of 30 s, which is more than five times the longest T1, can guarantee the reliability of the obtained experimental data.

Detailed studies previously showed that a minimum S/N of 150 is required to satisfy the uncertainty of 1% in qNMR analysis. Although an increased number of scans (NS) results in a higher S/N ratio, it also lengthened the experimental time. Therefore, the selection of an appropriate NS is critical for achieving a good response and saving analysis time. As shown in Fig. 4, experiments were carried out using 2, 4, 8, 16, 24, and 32 scans, and through data comparison, it was found that the S/N ratios of all conditions met the quantitative requirements; beyond an NS of 16, the S/N ratio did not increase significantly. Therefore, the study was performed using the parameter of NS = 16 to ensure both reliable results and an optimized testing time.

Method validation

Specificity. The $^1$H NMR spectra of the sample solution, the mexedrone solution, and the internal standard solution were measured to ensure that there was no overlap between mexedrone and the internal standard signals. A comparison of the spectra
shown in Fig. 5 confirmed that the selected peaks were suitable for quantitative analysis.

**Linearity.** Based on the hydrogen atom selected in the method development, a series of linear solutions were prepared with different molar ratios of mexedrone to maleic acid. The regression curve was plotted using $n_{\text{SH}}/n_{\text{RH}}$ as the abscissa and $A_S/A_R$ as the ordinate, as shown in Fig. 6, wherein $n_{\text{SH}}$ and $n_{\text{RH}}$ are the molar concentrations of the quantitative hydrogen atoms of mexedrone and maleic acid, respectively, and $A_S$ and $A_R$ are the areas of the quantitative signals of mexedrone and maleic acid, respectively. The regression equation was $y = 0.9824x + 0.0021$, and the correlation coefficient $R$ was 1.000.

**Accuracy.** To verify the accuracy of the developed method, samples covering three levels were prepared in triplicate, wherein the molar ratios of mexedrone to maleic acid was 1:1, 2:1, and 4:1; the corresponding hydrogen atom ratios were 0.5:1, 1:1, and 2:1, respectively. As shown in Table 1, the method can be considered accurate within the range of investigation.

**Precision.** By measuring the $^1$H NMR signals of six solutions prepared in parallel, the repeatability of the method was investigated. The same solutions were then measured the following day to evaluate the intermediate precision. The results presented in Table 2 demonstrate that this method exhibits a good precision.

**Stability.** The $^1$H NMR spectra were recorded at 0, 2, 4, 6, 8, 12, 24, and 48 h after solution preparation. During the experiment, the sample solution was maintained at room temperature, and as indicated by the results presented in Table 3, the sample solutions were stable over 48 h at room temperature.

**Robustness.** The robustness of the method was tested by varying the temperature of the probe (298–308 K). The results listed in Table 4 and demonstrate that minor changes in
the temperature do not influence the test results for these samples.

*Quantification of the seized compounds and the methcathinone hydrochloride reference standard.* The contents of the four seized compounds were then determined using the proposed and optimized method. To ensure the reliability and accuracy of the established method, a commercial methcathinone hydrochloride reference standard was also measured using this method. The results are summarized in Table 5, where it is apparent that the measured content of methcathinone was consistent with its labeled amount (i.e., 92.6%), thereby indicating the reliability of this method for the determination of cathinone derivatives.

**Conclusions**

Owing to the rapid emergence of new psychoactive substances (NPS), drug control is becoming an even more significant challenge. As a result, novel analytical methods are required to determine such NPS, often in the absence of an internal reference or standard where the NPS structure may be unknown. We therefore proposed the application of quantitative nuclear magnetic resonance (qNMR) spectroscopy for the determination of NPS, wherein an appropriate internal standard can be selected to provide reliable content data, and ultimately offer support for drug control. Thus, using maleic acid as the internal standard along with a mixture of DMSO-d$_6$ and D$_2$O as the solvent, a $^1$H qNMR method was developed to measure the absolute contents of synthetic cathinones. The specificity, linearity, accuracy, precision, and robustness of this method were found to meet all necessary requirements. Importantly, due to the fact that the selected quantitative peak is a common signal of the core synthetic cathinone structure, and suffers no interference from the internal standard, the proposed method can be considered a suitable tool for the determination of this type of compound.
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Supporting Information: Related data about seized samples identification. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.
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| No. | Taken/mg | Found/mg | Assay, % | Mean, % | RSD, % |
|-----|----------|----------|----------|---------|--------|
| 1   | 5.142    | 5.056    | 98.33    |         |        |
| 2   | 5.221    | 5.128    | 98.22    |         |        |
| 3   | 5.335    | 5.250    | 98.41    |         |        |
| 4   | 10.443   | 10.251   | 98.16    |         |        |
| 5   | 10.708   | 10.512   | 98.17    | 98.20   | 0.13   |
| 6   | 10.267   | 10.086   | 98.24    |         |        |
| 7   | 15.103   | 14.825   | 98.16    |         |        |
| 8   | 15.180   | 14.878   | 98.01    |         |        |
| 9   | 15.356   | 15.061   | 98.08    |         |        |
| No. | Taken/mg | Found/mg | Assay, % | Mean, % | RSD, % |
|-----|----------|----------|----------|---------|--------|
|     |          |          |          |         |        |
| repeatability |          |          |          |         |        |
| 1   | 10.266   | 10.091   | 98.30    |         |        |
| 2   | 10.260   | 10.112   | 98.56    |         |        |
| 3   | 10.201   | 10.017   | 98.20    |         |        |
| 4   | 10.096   | 9.899    | 98.04    |         |        |
| 5   | 10.312   | 10.140   | 98.33    |         |        |
| 6   | 10.679   | 10.473   | 98.08    |         |        |
| Intermediate precision |          |          |          |         |        |
| 7   | 10.266   | 10.052   | 97.92    |         |        |
| 8   | 10.260   | 10.087   | 98.32    |         |        |
| 9   | 10.201   | 9.993    | 97.96    |         |        |
| 10  | 10.096   | 9.880    | 97.86    |         |        |
| 11  | 10.312   | 10.132   | 98.25    |         |        |
| 12  | 10.679   | 10.468   | 98.03    |         |        |
| Time interval/h | Taken/mg | Found/mg | Assay, % | Mean, % | RSD, % |
|----------------|----------|----------|----------|---------|--------|
| 0              | 10.266   | 10.089   | 98.28    |         |        |
| 2              | 10.266   | 10.089   | 98.28    |         |        |
| 4              | 10.266   | 10.079   | 98.17    |         |        |
| 6              | 10.266   | 10.070   | 98.09    |         |        |
| 8              | 10.266   | 10.072   | 98.11    | 98.11   | 0.14   |
| 12             | 10.266   | 10.069   | 98.08    |         |        |
| 24             | 10.266   | 10.055   | 97.95    |         |        |
| 48             | 10.266   | 10.052   | 97.91    |         |        |
Table 4  Robustness tests of mexedrone

| Parameters       | Changes | Taken/mg | Found/mg | Assay, % | Mean, % | RSD, % |
|------------------|---------|----------|----------|----------|---------|--------|
| Probe temperature/K | 298     | 10.708   | 10.503   | 98.09    |         |        |
|                  | 303     | 10.708   | 10.510   | 98.15    | 98.1    | 0.10   |
|                  | 308     | 10.708   | 10.514   | 98.19    |         |        |
Table 5  Quantitative analytical results of methcathinone hydrochloride reference standard and seized samples

| No.       | Assay, %      | Mean, %  |
|-----------|---------------|----------|
|           | 1  | 2    |          |
| Methcathinone (HCl) | 93.02 | 92.61 | 92.82 |
| No.1      | 97.55 | 97.56 | 97.56 |
| No.2      | 98.19 | 98.14 | 98.16 |
| No.3      | 98.66 | 98.51 | 98.58 |
| No.4      | 87.94 | 88.05 | 87.99 |
Figure Captions

Fig. 1  Structures of substituted cathinones

Fig. 2  Chemical structures of seized samples

Fig. 3  Effects of d1 on S/N of selected signals of mexedrone

Fig. 4  Effects of scan number on S/N of selected signals of mexedrone

Fig. 5  $^1$H NMR spectra of (A) mexedrone, (B) maleic acid, and (C) their mixed solutions in 0.6 mL DMSO-d$_6$+D$_2$O (5:1, v/v) (marked signals were selected for quantitative analysis)

Fig. 6  Linearity curve of mexedrone of molar ratio (quantitative hydrogen atom) $n_{SH}/n_{RH}$ versus integration ratio $A_S/A_R$
Fig. 1  Structures of substituted cathinones

$R_1 = \text{H, 4-Methyl, 3, 4-Methyleneedioxy, etc.}$

$R_2 = \text{Methyl, Ethyl, etc.}$

$R_3 = \text{Methyl, Ethyl, etc.}$
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